**MC1R:** three novel variants identified in a malignant melanoma association study in the Spanish population


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The human melanocortin-1 receptor (MC1R) gene, which plays a crucial role in pigmentation, also appears to be important in malignant melanoma (MM). This case–control study in the Spanish population included 116 consecutive MM patients and 188 controls frequency matched for sex and age. Sequence analysis of the entire coding region of MC1R was performed, identifying 21 variants, all of them previously reported except for three novel non-synonymous changes: Ser41Phe, Met128Thr and Asn281Ser. Simulated structural analyses suggested disruption of the local structure around Phenylalanine 41, possible destabilization of the hydrophobic interior of the molecule in Threonine 128 and that Asparagine 281 could be in a region of functional importance. The fact that these three novel variants were not present in 1,000 healthy individuals tested adds further weight to them having putative adverse effects on the functional protein. Six variants, all non-synonymous changes, were individually associated with MM risk (odds ratios: 10.44, 95% confidence interval = 4.48–24.33, P = 5 × 10-4) and haplotype analysis, verified by cloning, confirmed that this is predominantly due to varying each on a different chromosome. Our results suggest that both red hair colour (RHC) and non-red hair colour variants, and possibly other rare non-synonymous variants, in MC1R are implicated in the development of MM. In addition to carrying MC1R variant alleles, having blond/red hair and childhood sunburns were independent risk factors for MM.

Introduction

Malignant melanoma (MM) is a malignancy of pigment-producing cells (melanocytes) located predominantly along the basal layer of the epidermis, but also found in mucous membranes. The most relevant epidemiologic characteristic of MM is its increasing incidence among Caucasian populations in the USA, Australia and Europe. While Spain has some of Europe’s lowest melanoma incidence and mortality rates, the incidence of this disease is currently increasing faster than that of any other malignancy (1). MM accounts for only 4% of all skin cancers; however, it causes the greatest number of skin-cancer-related deaths worldwide. Early detection of thin cutaneous melanoma is the best means of reducing mortality.

The aetiology of MM remains unclear but it is known that both genetic and environmental factors influence the development of sporadic disease (2). The main reason for the increasing incidence of MM is greater sun exposure. Epidemiologic studies confirm that ultraviolet radiation is the main factor involved in the pathogenesis of the disease. Among phenotypic factors, fair pigmentation, low tanning ability and multiple benign or atypical nevi are the most important risk factors for developing MM. Melanoma predisposition is highest in fair skin, blond or red-haired individuals who never tan and always burn (Fitzpatrick’s phototypes I and II). The presence of MM that occur in kindreds (3–15%) suggests a hereditary predisposition. Nevertheless, this predisposition is genetically heterogeneous. Until now, two high-penetration genes have been described: CDKN2A and CDK4. However, these mutations are found in only 30–40% of kindreds, indicating that other genes may also predispose to MM (2).

Human pigmentation and consequently sun sensitivity are complex characteristics. In mice, >50 loci are implicated in coat colour (3). In humans, there is a long list of genes known to be involved in rare pigmentation syndromes such as albinism (4). The melanocortin-1 receptor (MC1R) gene (MIM#155555) plays a crucial role in human pigmentation and also appears to be important in determining MM risk. MC1R is located on 16q24.3 and encodes a G-protein-coupled receptor with seven transmembrane domains. The MC1R protein interacts with the natural pro-opiomelanocortin and adrenocorticotropin hormone peptide ligands. The binding of these ligands leads to the activation of tyrosine kinase enzyme transduction which in turn promotes photoprotective eumelanin production.

MC1R is considered important in determining MM risk because its function has been implicated in melanin production in response to ultraviolet exposure. MC1R is highly polymorphic in Caucasian populations with >70 variants having been reported (5). Specific MC1R variants such as R151C, R160W and D294E have been associated with the red hair colour (RHC) phenotype and have been referred to as RHC alleles or ‘R’ alleles (6). The RHC phenotype is characterized by fair skin, red hair and freckles and high sun sensitivity (poor tanning and solar lentigines). On the other hand, the association of the V60L, V92M and R163Q variants with the RHC phenotype has not been clearly established and they have been considered non-red colour NRHC alleles or ‘r’ alleles (6). Wong et al. (5) classified MC1R variants according to their functional implications into two categories: ‘M’ major functional alleles which include the ‘RHC’ alleles as well as D64E and R142H and ‘m’ minor functional alleles which include ‘NRHC’ alleles. Nevertheless, it has been suggested that other non-synonymous MC1R changes also affect receptor functionality (7,8). These other non-synonymous MC1R changes are infrequent and their frequency varies among populations (4). Some studies have demonstrated the functional consequences of some of these changes (7,9) whereas predictive models suggest putative functional implications for others (10).

Association studies of MC1R and MM have traditionally been carried out in Caucasian populations from Northern Europe and Australia (11–13). More recently, the effect of MC1R variants have been reported in four studies of Mediterranean populations (14,15). In this study, Spanish MC1R variability and its contribution to MM risk is analysed for the first time.

Materials and methods

**Study subjects and data collection**

A total of 116 consecutive and non-related MM cases were recruited from the Department of Dermatology at the Gregorio Marañón General University Hospital (Madrid) from 1 September 2004 to 30 September 2005. A total of 188 volunteer cancer-free controls, frequency matched to cases by sex and age in 10-year categories, were recruited from the Madrid College of Lawyers (Supplementary Table 1A, available at Carcinogenesis Online). All participants were Caucasians of Spanish origin living in Madrid.

A standardized questionnaire was used to collect information on pigmentation characteristics (eye colour, hair colour, skin colour, number of nevi, presence
of solar lentigines, sun exposure habits and presence of childhood sunburns) (see supplementary Table IA, available at Carcinogenesis Online), Fitzpatrick’s classification of skin type, tumour localization, Breslow index (deep index) (see supplementary Table IB, available at Carcinogenesis Online) and personal or family history of cancer. Fitzpatrick’s classification of skin type was extracted from the medical record of cases only. All study subjects gave informed consent and the study was approved by the Ethics Committee of Gregorio Marañon General University Hospital.

**Sequencing of MC1R**

Genomic DNA from cases and controls was isolated from peripheral blood lymphocytes and diluted to a final solution of 50 ng/ml using the MagNA Pure LC Instrument. This was done according to the manufacturer’s protocol (Roche Molecular Biochemicals AQ2, Mannheim, Germany) for controls and using the traditional saline method for cases.

The MC1R coding region was amplified by polymerase chain reaction (PCR) using two overlapping pairs of primers which have been described previously (14). PCR products were 671 and 610 bp in length, respectively, and they overlapped by 104 bp.

PCR amplification was performed in a total volume of 15 μl containing 50 ng of DNA, a final primer concentration of 0.2 μM, 0.2 mM of deoxynucleosides triphosphate, 1 U/μl of Eco Taq polymerase (Eppendorf AG, Hamburg, Germany) and its buffer 1×. The PCR cycling conditions were as follows: 95°C for 2 min followed by 35 cycles of 15 s of denaturation at 95°C, 15 s of annealing at 60°C and 45 s of elongation at 72°C and finally, an extension of 72°C for 10 min. PCR products were purified using exonuclease I and alkaline phosphatase.

Sequence analysis was performed on the ABI Prism system (Applied Biosystems, Foster city, CA) using the BigDye Terminator Cycle Sequencing Kit and the ABI 3730 automated DNA sequencer according to the manufacturer’s instructions. The sequence results were analysed using Polyphred (5.03), Phred (0.020425.4), Phrap (0.990329) and Consed (15.0) software (16–18) in order to detect all possible changes. All detected changes were confirmed manually.

**Statistical analyses**

Associations between MC1R variants and risk of MM were initially assessed individually using Fisher’s exact test. Variants were grouped according to their functional importance into two categories: functional and ‘other’. Functional variants were defined as: changes that have been strongly associated with RHC (D164E, R142H, R151C, R160W and D294H) (5); changes that have been weakly associated with RHC (V60L, V92M and R163Q) (5); and non-synonymous changes that have either either been described as affecting the receptor function or predicted by sorting intolerant from tolerant to be intolerant (C35Y, F45L, S83P, T95M, V122M, Y152X and N155T) (7–11). Other variants were defined as synonymous changes or other variants that have not previously been studied (S41F, M128T and N281S). The total number of each type of variant carried was then calculated. For any particular variant, the contribution of a homozygote to the total number of variants was two.

Associations with MM were assessed for each of these two aggregate variables and combinations of them using logistic regression, estimating odds ratios (ORs) and associated 95% confidence intervals (CIs) and P-values: for carrying at least one variant (dichotomous), for carrying one and carrying two variants (multinomial) and for variant carriage (continuous, assuming a multiplicative effect per copy). Departure from multiplicative effects was tested for by comparing the latter two models via the likelihood ratio test on 1 degree of freedom. These analyses were repeated, stratifying on age (<50 and ≥50 years) and sex. Multivariate logistic regression was also applied, including age (categorical: <30, 30–39, 40–49, 50–59, 60–69, 70–79 and ≥80), sex (male, female), hair colour (brown/black, blond/red), skin colour (fair, brown), lentigines (yes, no) and childhood sunburn (yes, no) as covariates. Interactions with age (<50 and ≥50 years), sex, lentigines (yes, no) and childhood sunburn (yes, no) were tested for via the likelihood ratio test on 1 degree of freedom, comparing the sum of the likelihoods from the stratified models to that from the unstratified model. Associations between the number of variants carried and various individual and tumour characteristics were assessed via logistic regression. This was done for cases and controls pooled for each of eye colour (blue/green versus brown), hair colour (brown/black versus blond/red), skin colour (fair versus brown), number of nevi (>50 versus ≤50), presence of lentigines (yes versus no) and childhood sunburn (yes versus no) as the outcome variable and among cases only for each of phototype (II versus III/IV), tumour location (head/neck/trunk versus extremities) and tumour depth (T1 versus T2/T3/T4) as the outcome variable. Stata v8.2 was used to carry out these analyses.

PHASE v2.0 was used to infer MC1R haplotypes and to compare haplotype distributions in cases and controls. Haplotype-associated ORs, 95% CIs and P-values were estimated via unconditional logistic regression using Stata v8.2 by treating each chromosome (two per study subject) as a study unit, assuming PHASE-inferred haplotypes were observed and taking the most common haplotype as reference. These results were confirmed using the haplo.stats library implemented in R which includes haplotype uncertainty in the estimation of ORs.

**Cloning PCR products to confirm haplotype phase**

The PCR product of infrequent (frequency < 0.004) or statistically significant (P < 0.05) haplotypes detected by PHASE was purified with the High Pure PCR Product Purification kit from Roche Molecular Biochemicals and cloned into the pGEM-T Easy cloning vector (Promega, Madison, WI), according to the manufacturer’s protocol. The cloned product was sequenced by primer walking using the BigDye Terminator Cycle Sequencing kit and the ABI 3700 automated DNA sequencer according to the manufacturer’s instructions.

**Structural and functional evaluation of novel changes**

**Estimation of the frequency of novel changes in a control sample.** The frequency of the three novel variants detected in our study (S41F, M128T and N281S) was estimated in an independent sample of 1,000 healthy Caucasian Spanish subjects available in the laboratory. Genotyping assays were carried out using Taqman, Amplifluor probes or denaturing high performance liquid chromatography (DHPLC) depending on design conditions. Assay priors, probes and PCR conditions can be given upon request.

**Structural evaluation of novel changes.** The three-dimensional structural prediction model of the protein, and local regions in which the three novel variants are located, was based on a modification of Zhang et al., 2006 (19). The protein consensus sequence used in the model was Q01756. Structures and rotamers of the residue variants were displayed with the program Coot (20).

**Results**

**Distribution of MC1R variants in the Spanish population and their association with MM risk**

Of the 304 individuals studied, 186 (61.2%) carried at least one MC1R variant including 86 (74.3%) of 116 cases and 100 (53.2%) of 188 controls. The 21 MC1R variants identified are listed in Table I. Among these, 18 variants were non-synonymous changes, 15 of which had been described previously (5) and 3 were identified for first time: S41F, M128T and N281S. The other three variants detected were a nonsense change (Y152X) and two additional synonymous changes [Q233Q (G > A) and T314T (A > G)]. The estimated frequency of each MC1R variant, as well as the estimated OR for MM and associated P-value, is shown in Table I. Among the 21 detected changes, six were individually associated with melanoma risk: V60L, V92M, I115T, R160W, D294H and T314T (all P < 0.05). The highest OR was estimated for I115T (OR: 7.82, 95% CI: 1.57–75.2, P = 0.004).

**Haplotype analysis and association between number of MC1R variants carried and melanoma risk**

Haplotype analysis was carried out including all variants identified across the MC1R gene. Estimated OR, 95% CI and P-values were very similar regardless of whether haplotype uncertainty was taking into account in the analysis (data not shown) and so results from PHASE are presented. The majority of the haplotypes inferred (apart from that with no changes) consisted of a single change only (see supplementary Table II, available at Carcinogenesis Online). Four of the haplotypes with individual changes were associated with MM, relative to most frequent haplotype with no changes. Three of the changes represented were V60L [OR: 2.16 (1.33–3.50), P = 0.002], R160W [OR: 8.04 (1.49–80.0), P = 0.005] and D294H [OR: 4.98 (1.71–16.3), P = 0.002], confirming the results obtained in the individual analyses. The fourth haplotype contained the r variant R163Q [OR: 4.14 (1.36–12.62), P = 0.013]. Although PHASE software inferred a low number of frequent haplotypes with two changes, cloning these haplotypes revealed that only three combinations were real: V92M/T314T, I115T/T314T and K278E/T314T. The first two conferred significantly increased MM risk (OR: 3.34, 95% CI: 1.40–8.21, P = 0.005 and OR: 10.34, 95% CI: 2.08–99.2, P = 0.00083, respectively). Seven individuals unequivocally carried the haplotype with the synonymous T314T variant alone and all of them were controls. The estimated OR associated with carrying one functional variant was 1.92 (95% CI: 1.13–3.27, P = 0.015), whereas that associated...
with carrying two functional variants was 10.44 (95% CI: 4.48–24.33, \(P = 5 \times 10^{-8}\)). The estimated OR was 2.78 per variant carried (CI: 1.91–4.05, \(P = 8 \times 10^{-8}\)) (see Table II), although there was marginal evidence (\(P = 0.06\)) that the effect of carrying two variants was more than double that of carrying one.

### Associations between phenotypic characteristics, MC1R variants and melanoma risk

Table III presents the estimated ORs for melanoma associated with various phenotypic characteristics (detailed in supplementary Table I A, available at Carcinogenesis Online) based on univariate analyses. MM risk was associated with the presence of blond or RHC (OR: 4.86, 95% CI: 2.35–10.03, \(P = 2 \times 10^{-5}\)), solar lentigines (OR: 1.71, 95% CI: 1.04–2.81, \(P = 0.032\)) and childhood sunburn (OR: 10.41, 95% CI: 5.81–18.65, \(P = 3 \times 10^{-11}\)). No association with melanoma risk was observed for eye colour, skin colour or number of nevi.

We also carried tested for associations between the number of MC1R variants and phenotype among cases only (as presented in supplementary Table IB, available at Carcinogenesis Online). We found that number of variants was associated with cases having fair skin and poor capacity for tanning (phototypes I and II) (OR: 1.92, 95% CI: 1.04–3.53, \(P = 0.036\)). We found no evidence of an association with tumour location or Breslow index.

### Multivariate analyses

We considered the number of MC1R variants (per copy), blond/RHC, fair/pale skin colour, solar lentigines and the presence of childhood sunburn as potential risk factors for MM in a multivariate model. The number of MC1R variants (OR: 2.47, 95% CI: 1.54–3.95, \(P = 0.0002\)), blond/RHC (OR: 2.9, 95% CI: 1.07–7.83, \(P = 0.036\)) and childhood sunburns (OR: 7.95, 95% CI: 4.12–15.32, \(P = 6 \times 10^{-10}\)) were independently associated with MM (Table V). These associations were consistently observed when stratifying by age (<50 and ≥50) and sex.

There was marginal evidence (\(P = 0.07\)) of an interaction between the number of functional variants and childhood sunburns, with sunburn having increasing effects on risk of melanoma for individuals with increasing number of variants (OR associated with childhood sunburn having increasing effects on risk of melanoma for individuals with increasing number of variants (OR associated with childhood

<table>
<thead>
<tr>
<th>MC1R variant</th>
<th>Nucleotide change</th>
<th>Cases ((N = 116))</th>
<th>Controls ((N = 188))</th>
<th>Dominant model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Homozygotes</td>
<td>Minor allele homozygotes</td>
<td>MAF (%)</td>
</tr>
<tr>
<td>C35Y</td>
<td>G &gt; A</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S41F</td>
<td>C &gt; T</td>
<td>1 (0.9)</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>F45L</td>
<td>T &gt; C</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>V60L</td>
<td>G &gt; T</td>
<td>40 (34.5)</td>
<td>3 (2.6)</td>
<td>19.8</td>
</tr>
<tr>
<td>S83P</td>
<td>T &gt; C</td>
<td>1 (0.9)</td>
<td>—</td>
<td>0.4</td>
</tr>
<tr>
<td>D84E</td>
<td>C &gt; A</td>
<td>1 (0.9)</td>
<td>—</td>
<td>0.4</td>
</tr>
<tr>
<td>V92M</td>
<td>G &gt; A</td>
<td>16 (13.8)</td>
<td>—</td>
<td>6.9</td>
</tr>
<tr>
<td>T95M</td>
<td>C &gt; T</td>
<td>2 (1.7)</td>
<td>—</td>
<td>0.9</td>
</tr>
<tr>
<td>V122M</td>
<td>G &gt; A</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
| M12T         | T > C            | 1 (0.9)   | —                      | 0.4     | —          | —                      | —       | 1.09–2.3, \(P = 0.014\) | for fair skin and 2.35 (95% CI: 1.6–3.45, \(P = 10^{-5}\)) for the presence of childhood sunburn.

| M12T         | T > C            | 1 (0.9)   | —                      | 0.4     | —          | —                      | —       | 1.09–2.3, \(P = 0.014\) | for fair skin and 2.35 (95% CI: 1.6–3.45, \(P = 10^{-5}\)) for the presence of childhood sunburn.

| M12T         | T > C            | 1 (0.9)   | —                      | 0.4     | —          | —                      | —       | 1.09–2.3, \(P = 0.014\) | for fair skin and 2.35 (95% CI: 1.6–3.45, \(P = 10^{-5}\)) for the presence of childhood sunburn.

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| M12T         | T > C            | 1 (0.9)   | —                      | 0.4     | —          | —                      | —       | 1.09–2.3, \(P = 0.014\) | for fair skin and 2.35 (95% CI: 1.6–3.45, \(P = 10^{-5}\)) for the presence of childhood sunburn.

| M12T         | T > C            | 1 (0.9)   | —                      | 0.4     | —          | —                      | —       | 1.09–2.3, \(P = 0.014\) | for fair skin and 2.35 (95% CI: 1.6–3.45, \(P = 10^{-5}\)) for the presence of childhood sunburn.

| M12T         | T > C            | 1 (0.9)   | —                      | 0.4     | —          | —                      | —       | 1.09–2.3, \(P = 0.014\) | for fair skin and 2.35 (95% CI: 1.6–3.45, \(P = 10^{-5}\)) for the presence of childhood sunburn.

Table I. Summary information on the MC1R variants detected in the Spanish population, including minor allele frequency estimates in cases and controls and assessment of individual associations with MM

Results marked in bold correspond to \(p\)-values less than 0.05.

<table>
<thead>
<tr>
<th>MC1R variants</th>
<th>Cases ((N = 116))</th>
<th>Controls ((N = 188))</th>
<th>OR (95% CI)</th>
<th>(P)-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No functional variant</td>
<td>31 (26.7)</td>
<td>94 (50.0)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>One functional variant</td>
<td>54 (46.6)</td>
<td>85 (45.2)</td>
<td>1.92 (1.13–3.27)</td>
<td>0.02</td>
</tr>
<tr>
<td>Two functional variants</td>
<td>31 (26.7)</td>
<td>85 (45.2)</td>
<td>4.04 (4.48–24.3)</td>
<td>5 \times 10^{-8}</td>
</tr>
<tr>
<td>At least one functional variant</td>
<td>54 (46.6)</td>
<td>85 (45.2)</td>
<td>2.74 (1.66–4.52)</td>
<td>8 \times 10^{-5}</td>
</tr>
<tr>
<td>Trend (per functional variant)</td>
<td>—</td>
<td>—</td>
<td>2.78 (1.91–4.05)</td>
<td>8 \times 10^{-8}</td>
</tr>
</tbody>
</table>

Results marked in bold correspond to \(p\)-values less than 0.05.

— Two-sided Fisher’s exact test \(P\)-value.

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Frequency of novel changes in a control series. Among the 21 MC1R identified variants, three non-synonymous changes were detected for the first time (S41F, M128T and N281S). We studied their frequency in a control series but none of them were detected among the 1000 controls tested.

Prediction of the structural effects of the three novel variants. The three novel variants are non-synonymous changes located in the coding region of MC1R, in the first, third and seventh domain of MC1R, respectively (S41F, M128T and N281S). The predictive structural model applied found that Serine 41 is consistently present at the N-terminal part of helix 1 that packs against helix 2 and partially to helix 7 (Figure 1A). There is insufficient space in this region to accommodate larger residues such as Phenylalanine that would mostly affect the interface between helix 1 and 2 (Figure 1B and C). We similarly found that Methionine 128 is located in a highly hydrophobic environment provided by side chains of several hydrophobic residues. Threonine is a polar residue that can therefore disturb the hydrophobic environment. Modelling the role of N281S did not show an obvious structural effect. Asparagine 281 might participate in the formation of intrahelix hydrogen bonds that could not be formed by a shorter residue such as Serine.

Discussion

Since MC1R genetic variability is strongly associated with the RHC phenotype (13), a large number of studies have investigated the implications of this gene in MM. MC1R is highly polymorphic, with >70 variants described in Caucasian populations (5). In contrast, the

**Table III.** Associations between phenotypic risk factors and MM risk

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases (N = 116)</th>
<th>Controls (N = 188)</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis ≥50</td>
<td>59 (52.2)</td>
<td>101 (54.0)</td>
<td>0.93 (0.58–1.48)</td>
<td>0.8</td>
</tr>
<tr>
<td>Sex: male</td>
<td>46 (39.7)</td>
<td>88 (46.8)</td>
<td>0.74 (0.46–1.19)</td>
<td>0.2</td>
</tr>
<tr>
<td>Blue/green eye colour</td>
<td>36 (32.1)</td>
<td>43 (23.2)</td>
<td>1.56 (0.92–2.63)</td>
<td>0.09</td>
</tr>
<tr>
<td>Blond/RHC</td>
<td>28 (25.0)</td>
<td>12 (6.4)</td>
<td>4.86 (2.35–10.03)</td>
<td>2 × 10⁻⁵</td>
</tr>
<tr>
<td>Fair/pale skin colour</td>
<td>83 (73.5)</td>
<td>114 (62.6)</td>
<td>1.65 (0.98–2.76)</td>
<td>0.06</td>
</tr>
<tr>
<td>No. of nevi ≥50</td>
<td>15 (13.3)</td>
<td>19 (12.3)</td>
<td>1.09 (0.53–2.26)</td>
<td>0.8</td>
</tr>
<tr>
<td>Lentigines</td>
<td>71 (64.6)</td>
<td>87 (51.5)</td>
<td>1.71 (1.04–2.81)</td>
<td>0.03</td>
</tr>
<tr>
<td>Childhood sunburn</td>
<td>73 (66.4)</td>
<td>25 (15.9)</td>
<td>10.41 (5.81–18.65)</td>
<td>3 × 10⁻¹³</td>
</tr>
</tbody>
</table>

Results marked in bold correspond to p-values less than 0.05.

**Table IV.** Associations between number of functional MC1R variants and various phenotypic characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue/green eye colour</td>
<td>0.96 (0.71–1.28)</td>
<td>0.8</td>
</tr>
<tr>
<td>Blond/RHC</td>
<td>1.80 (1.26–2.58)</td>
<td>0.001</td>
</tr>
<tr>
<td>Fair/pale skin colour</td>
<td>1.42 (1.06–1.89)</td>
<td>0.02</td>
</tr>
<tr>
<td>No. of nevi ≥50</td>
<td>0.88 (0.51–1.49)</td>
<td>0.6</td>
</tr>
<tr>
<td>Lentigines</td>
<td>1.14 (0.87–1.48)</td>
<td>0.3</td>
</tr>
<tr>
<td>Childhood sunburn</td>
<td>1.71 (1.28–2.27)</td>
<td>2 × 10⁻⁴</td>
</tr>
</tbody>
</table>

Results marked in bold correspond to p-values less than 0.05.

\*OR, per functional variant.

**Table V.** Multivariate analysis of MM risk factors

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1R variant (per copy)</td>
<td>2.47 (1.54–3.95)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Blond/RHC</td>
<td>2.90 (1.07–7.83)</td>
<td>0.03</td>
</tr>
<tr>
<td>Fair/pale skin colour</td>
<td>0.87 (0.44–1.71)</td>
<td>0.7</td>
</tr>
<tr>
<td>Lentigines</td>
<td>0.92 (0.48–1.76)</td>
<td>0.8</td>
</tr>
<tr>
<td>Childhood sunburn</td>
<td>7.95 (4.12–15.3)</td>
<td>6 × 10⁻¹⁰</td>
</tr>
</tbody>
</table>

Results marked in bold correspond to p-values less than 0.05.

All risk factors listed were included together in multivariate logistic regression analysis to estimate the ORs, CI and P-values.

sunburn = 5.29, 9.59 and >28 for carriers of 0, 1 and 2 functional variants, respectively).

Structural and functional evaluation of novel changes

**Frequency of novel changes in a control series.** Among the 21 MC1R identified variants, three non-synonymous changes were detected for the first time (S41F, M128T and N281S). We studied their frequency in a control series but none of them were detected among the 1000 controls tested.

**Prediction of the structural effects of the three novel variants.** The three novel variants are non-synonymous changes located in the coding region of MC1R, in the first, third and seventh domain of MC1R, respectively (S41F, M128T and N281S). The predictive structural model applied found that Serine 41 is consistently present at the N-terminal part of helix 1 that packs against helix 2 and partially to helix 7 (Figure 1A). There is insufficient space in this region to accommodate larger residues such as Phenylalanine that would mostly affect the interface between helix 1 and 2 (Figure 1B and C). We similarly found that Methionine 128 maps to the centre of helix 3 pointing directly towards the centre of the molecule. Helix 3 is buried deep in the transmembrane core establishing contacts with all other helices except helix 1. Methionine 128 is located in a highly hydrophobic environment provided by side chains of several hydrophobic residues. Threonine is a polar residue that can therefore disturb the hydrophobic environment. Modelling the role of N281S did not show an obvious structural effect. Asparagine 281 might participate in the formation of intrahelix hydrogen bonds that could not be formed by a shorter residue such as Serine.

**Discussion**

Since MC1R genetic variability is strongly associated with the RHC phenotype (13), a large number of studies have investigated the implications of this gene in MM. MC1R is highly polymorphic, with >70 variants described in Caucasian populations (5). In contrast, the
variability of MC1R in African populations is very reduced, with only three synonymous changes described: T314T (A > G), F300F (C > T) and C273C (C > T) (21,22).

In this study, we have confirmed the association between six MC1R polymorphic variants and MM risk in the Spanish population. We have also described three novel MC1R variants and discussed their putative implication in functionality. We found 21 MC1R variants and this number is similar to the number found in other Mediterranean population studies (16 in France, 26–29 in Italy and 18 in Greece) (14,15). The most frequent Spanish variant is V60L with a frequency of 13.3%. This value is close to that reported in other populations (15.7% among Northern Italians, 12.4% among fair-skinned Australians and 15.0% among Northern Europeans) (4).

The RHC phenotype-associated variants (R151C, R160W and D294H) were present at frequencies of 2.4, 0.5 and 1.6%, respectively, in our population, compared with 9.9, 8.7 and 3.6%, respectively, reported in Northern European populations (4). There were no red-haired individuals among the control sample, and only 10 (8.6%) of MM cases had red hair. This finding is consistent with other results from Mediterranean populations and in contrast to Northern European populations (13). Among these 10 red-haired cases, 8 carried at least two functional MC1R variants, the 9th carried D294H and the novel Spanish variant S41F and the 10th did not carry any variants. Red-haired subjects with no MC1R variants are not uncommon and have been seen in a Northern European population as well (23). These RHC variants have been consistently associated with MM in Anglo-Saxon populations (11–13) and also in the Northern French population (14). We detected statistically significant individual associations for R160W and D294H, but not for R151C. This latter finding may be due to lack of power, since an effect of this latter change has been detected in the Northern French and Central Italian populations (14,24). We did not observe any MM risk associated with the rare RHC variant D84E (OR: 1.63, 95% CI: 0.02–128, P = 0.99), as detected in North Europeans (25–27). The I155T variant has not been associated with MM to date, but this may be due to its low frequency. However, our results clearly suggest that this rare variant increases risk of MM, at least in the Spanish population (OR: 7.82, 95% CI: 1.57–75.2, P = 0.004).

While the associations of R160W and D294H with MM were expected, those of the NRHC variants V60L, V92M and R163Q were more intriguing, because their implication in MM pathology has been generally unclear in Anglo-Saxon populations. V60L was found to be associated with MM in Mediterranean individuals for R160W and D294H, but not for R151C. This latter finding may be due to lack of power, since an effect of this latter change has been detected in the Northern French and Central Italian populations (14,24). We did not observe any MM risk associated with the rare RHC variant D84E (OR: 1.63, 95% CI: 0.02–128, P = 0.99), as detected in North Europeans (25–27). The I155T variant has not been associated with MM to date, but this may be due to its low frequency. However, our results clearly suggest that this rare variant increases risk of MM, at least in the Spanish population (OR: 7.82, 95% CI: 1.57–75.2, P = 0.004).

The T314T change (allele G), which is less often reported on in the literature, possibly because of its synonymous nature, was also associated with MM risk in our study. Nevertheless, this variant was often observed in cis with either V92M or I155T, as previously reported (10,21) suggesting that the detected risk may be due to the presence of these non-synonymous variants. This is confirmed by the observation that the T314T variant was observed alone in at least seven control individuals, but no cases. This was not entirely unexpected since the minor allele (G) is the ancestral allele (in Chimpanzee) and the most frequent in African populations, but is rarely found in Northern Europeans (14,15). We have found for the first time that these two low-penetrance alleles are important in MM in the Spanish population. That these NRHC variants are also important in MM risk is supported by our finding that risk increased with the number of non-synonymous changes carried, regardless of whether they were RHC or NRHC.

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Our data confirm that being a carrier of two copies of non-synonymous changes is associated with a much higher risk of melanoma. Furthermore, haplotype analysis, verified by cloning, demonstrated that this is predominantly due to carrying each on a different chromosome. The presence of two non-synonymous changes implies that both copies of the protein are compromised. Moreover there was marginal evidence (P = 0.06) that the effect of carrying two variants was more than double that of carrying one.

The Spanish MC1R variability spectrum is characterized by the presence of three variants that have not been previously reported elsewhere. MC1R is a receptor with seven transmembrane domains and no experimental structure is available. However, several models have been obtained in the Q01756 initiative Zhang et al., 2006 (19). The novel Phenylalanine 41 variant showed a highly probable disruption of the three-dimensional conformation of the local region in the protein. Moreover, Serine 41 is located in the vicinity of a region reported to have functional relevance (28). M128T seems to disrupt antagonist-binding affinity (29) and the hydrophobic microenvironment of the protein could be compromised. Finally, N281T may affect stability in the local region. Position 281 maps very close to amino acid 41, suggesting that alterations in the residues towards the N-terminal part of helices 1 and 7 could have functional consequences.

The fact that the three of these novel variants were not present in any of the 1,000 healthy individuals tested adds weight to them having putative adverse effects on the functional protein. While all three variants should be further investigated, Phenylalanine 41, in particular, is a good candidate for further analysis, including loss of function assays.

In our population, the presence of blond/red hair, solar lentigines and childhood sunburns are phenotypic MM risk factors (see Table IV for full details). The proportion of fair-skinned individuals was much higher than expected. Only 36% of controls reported having dark (brown and dark brown) skin, in contrast to other Mediterranean populations such as Greeks with 55% (15) and Italians with 66% (30).

Multivariate and stratified analysis allowed us to establish carrying MC1R variants as an independent MM risk factor and risk may be even higher for variant carriers with childhood sunburns. The number of MC1R variants carried was associated with fair phenotypes, I and II, consistent with the established association of blond/red hair with MM (3), but not with tumour location or Breslow index.

Although the findings were highly internally consistent, the sample size of this study was relatively limited and additional variants in MC1R may exist in the Spanish population. Furthermore additional single nucleotide polymorphisms among those identified may be associated with MM risk but we simply lacked power to detect such associations, particularly for the rarer single nucleotide polymorphisms. Controls participated on a volunteer basis which may have introduced some selection bias, however, the fact that they were frequency matched to cases on age and sex and that the variable of primary interest was genetic would have kept such bias to a minimum.

The retrospective nature of the study raises the potential for recall bias, particularly for exposures such as childhood sunburn, although the fact that OR estimates for the number of MC1R variants carried varied little between univariate and multivariate models suggests any such bias did not confound this association.

We also recognize that there was potential for misclassification of phenotypic characteristics due to the subjective nature of the attributes considered. Furthermore, questionnaires were completed by the treating dermatologist for cases, whereas controls were assessed by a general practice nurse and so the misclassification may be differential between cases and controls, with the error being greater for controls. This may at least in part explain why the expected associations of number of nevi and skin colour with MM risk were not observed. In any case, the fact that the OR estimate for the number of MC1R variants carried varied little between univariate and multivariate models suggests any such differential misclassification bias had little impact on results. Other published studies have been very similar in terms of data collection and the number of patients analysed, and the fact that our results are concordant with those obtained in the other Mediterranean populations attests to their validity. Nevertheless, further studies are welcome to add weight to our conclusions.

In summary, we found that six MC1R variants V60L, V92M, I155T, R160W, D294H and R163Q are individual associated with MM risk in the Spanish population and identified three novel variants (S41F, M128T and N281S) with potentially functional effects. Carrying two non-synonymous variants was associated with even higher risk, with some evidence that it may be more than double the risk of carrying a single variant. These findings, with the support of predictive and functional studies (5,10,28), suggest that both RHC and NRHC variants, and possibly other rare non-synonymous variants,

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in MC1R are implicated in the development of MM, independently of the influence of environmental factors. They also point to the existence of a specific MC1R variability spectrum that includes the NHRC variants as well as I155T in the Spanish and possibly other lower latitude European populations where ultraviolet intensity is higher than for Northern Europeans.

Supplementary material
Supplementary Tables I and II can be found at http://carcin.oxfordjournals.org/

Acknowledgements
L.P.F. was funded by the Spanish Ministerio de Sanidad y Consumo under a grant from the Fondo de Investigación Sanitaria FI05/00918. We would like to thank Victoria Fernández, Emilio González, Rosario Alonso and Fátima Mercadillo for their expert technical support.

Conflict of Interest Statement: None declared.

References

Received February 1, 2007; revised March 15, 2007; accepted April 3, 2007