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1. Introduction

Although malignant melanoma (MM) is mainly a sporadic disease, about 3 to 15% of the cases may show familial aggregation [1, 2]. The diagnosis of melanoma in different members of the same families does indeed suggest there is a genetically-based hereditary predisposition in a significant percentage of the cases. However, this predisposition has proven to be genetically heterogeneous. Only two high-penetrance genes had been described so far: \textit{CDKN2A} and \textit{CDK4} [1]. Yet mutations in these genes are found in only 30–40% of melanoma kindreds, indicating the existence of additional genes involved in melanoma predisposition [1]. Also, common low-penetrance alleles of the human pigmentation \textit{MC1R} gene have been implicated in melanoma predisposition as well [3-13]. More recently, several other pigmentation genes, such as \textit{ASIP}, \textit{TYR}, \textit{TYRP1}, \textit{SLC45A2} and \textit{OCA2} have also emerged as being potentially important in both normal human pigmentation variation and in melanoma susceptibility [14-17].

Other putative low-penetrance genes involved in melanoma predisposition are DNA repair genes belonging to the base excision repair (BER) and the nucleotide excision repair (NER) mechanisms. BER and NER pathways eliminate a wide variety of DNA damage, including ultraviolet (UV) photoproducts. Therefore, the ability of each individual to repair DNA damage following different causes might explain at least in part the variability in melanoma susceptibility. Although several studies have investigated the association between polymorphisms in NER genes and risk of melanoma, most of the study sizes were relatively small, and the results were not consistent [18-24]. On the other hand, genetic polymorphisms have been identified in several BER genes and studies suggest that some of these polymorphisms may be associated with cancer risk [25-29].

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Further candidate low-penetrance genes allegedly linked to melanoma predisposition are members of the glutathione S-transferase (GST) gene family. GSTs are multifunctional enzymes involved in the detoxification of a wide range of reactive oxygen species (ROS) which, together with inflammatory response to UV exposure, contribute to skin carcinogenesis by oxidative stress mechanisms [30-32]. Since UV radiation can also indirectly induce oxidative stress via ROS, several GST genes have been considered as possible low-penetrance melanoma predisposition genes. Among the GST genes, GSTM1, GSTT1 and GSTP1 commonly harbor functional polymorphisms in the general population [33]. The frequencies of variants in the GSTM1 and GSTT1 genes have been studied in relation to susceptibility to melanoma; however, conflicting results have been reported [34-39].

In recent years, several genome-wide association studies (GWAS) have identified novel genomic loci associated with pigmentation and skin cancer [40-44]. GWAS are the ideal strategy to identify common, low-penetrance susceptibility loci without prior hypothesis bias due to gene role knowledge. Some of the associations detected were already known, such as MC1R with pigmentation and skin cancer, ASIP, TYR, OCA2, etc. However, several novel chromosomal regions and genes have been revealed using large cohorts of samples, such as IRF4, PARP1, CASP8, CCND1 and others.

What follows is a summary of the results obtained in our laboratory after the screening of genes belonging to three different genetic pathways: pigmentation, DNA repair and oxidative stress. For the past few years, our group has been studying melanoma candidate genes in Spain, a southern European population, displaying a considerably darker skin than most of the other well-studied Caucasian populations, including Australian, North American and Northern Europe populations.

2. Research methods

2.1. Study subjects and data collection

A case-control collection of 946 non-related MM cases from several Spanish Hospitals and 353 volunteer cancer-free controls were recruited from 1 September 2004 to January 2011. All participants were Caucasians of Spanish origin. A standardized questionnaire was used to collect information on pigmentation characteristics (eye, hair and skin color, number of nevi, presence of solar lentigines, sun exposure habits and presence of childhood sunburns), Fitzpatrick’s classification of skin type (extracted from the medical record of cases only), tumor localization, Breslow index (depth index), and personal or family history of cancer. All study subjects gave informed consent and the study was approved by the Ethics Committee of Gregorio Marañón General University hospital and Clínico University Hospital.

Genomic DNA from cases and controls was isolated from peripheral blood lymphocytes and diluted to a final solution of 50ng/ml. MagNA Pure LC Instrument DNA extracción was used according to the manufacturer’s protocol (Roche Applied Science, Mannheim, Germany); the DNAzol procedure (Invitrogen, Eugene, OR, USA) or traditional saline method was
used. DNA concentration was quantified in samples prior to genotyping by using Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Eugene, OR, USA).

2.2. SNP genotyping

2.2.1. MC1R sequencing

MC1R variants were detected by automated gene sequencing. The MC1R coding region was amplified by PCR using two overlapping pairs of primers previously described [9]. PCR products were 671 and 610 bp in length, respectively, and they overlapped by 104 bp. PCR amplification was performed according to Matichard and cols [9]. Sequence analysis was performed on the ABI Prism system (Life Technology, Foster city, CA) using the BigDye Terminator Cycle Sequencing kit and the ABI 3700 automated DNA sequencer according to the manufacturer’s instructions. The sequence results were analyzed using Polyphred, Phred Phrap and Consed software [45-47] or SeqScape in order to detect all possible changes. All detected changes were confirmed manually.

2.2.2. Gene and SNP selection

The rest of the genes in the study were analyzed by genotyping selected SNPs. Public databases were used to collect information about single nucleotide polymorphisms (SNPs): NCBI (http://www.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org/index.html) and HapMap (http://www.hapmap.org). SNPs selected were located in exons, in putative promoter regions or had been reported to be associated with cancer in previous studies. All SNPs had a minor allele frequency (MAF) greater than or equal to 5%. As a quality control measure we included two sample duplicates and a non-template sample per 96-well plate. For some high-throughput platforms three DNA duplicates (two intra-assays and one inter-assay) were added. For all the studies performed genotypes were scored by two different personnel in the laboratory and no discrepancies were observed.

2.2.3. Taqman and kaspar assays

The PCR primers and probes were designed by Life Technology (Foster City, CA) using their Custom Taqman SNP genotyping assays or KASPAR SNP Genotyping System KBiosciences (Hoddesdon, Herts UK). The primer and allele-specific probe sequences for Taqman as well as those used for Kaspar assays are detailed elsewhere [6, 7, 14, 16, 17, 27].

PCR conditions used were according to the manufacturer’s protocol (Life technology, Foster City, CA). After PCR, the genotype of each sample was automatically determined by measuring allele-specific final fluorescence in the ABI Prism 7900HT Detection System, using the SDS 2.1 software for allele discrimination (Life technology, Foster City, CA).

2.2.4. Sequenom

Genotyping assays were designed according to the Sequenom MassARRAY Assay Design software (version 3.0.0; Sequenom Inc., San Diego, CA, USA). Assay primers are
detailed elsewhere [15, 27]. One duplicate sample, one father–mother–child trio and two negative controls were included across the plates to assess the accuracy of genotyping. SNPs were genotyped using iPLEX™ chemistry on a MALDI-TOF Mass Spectrometer (Sequenom Inc, San Diego, CA, USA). PCR reactions were carried out according to their own instructions (Sequenom Inc.).

2.2.5. Illumina

A total of 384 SNPs were genotyped using the GoldenGate Genotyping Assay system according to the manufacturer’s protocol (Illumina, San Diego, CA, USA) [16]. Genotyping was carried out using 350 ng of DNA per reaction. In addition, cases and control samples were always included in the same run. Genotypes were called using the proprietary software supplied by Illumina (BeadStudio, version 3.1.3.).

2.2.6. Taqman quantitative real-time PCR

The GSTM1 and GSTT1 copy number polymorphisms were determined using the TaqMan Quantitative real-time PCR. Assay designs were Hs02575461_cn (GSTM1) and Hs000100004_cn (GSTT1) and were used according to Life Technology instructions. After PCR, the genotype of each sample was automatically determined by measuring allele-specific final fluorescence in the ABI Prism 7900HT Detection System, using the SDS 2.3 software for allele discrimination (Life Technology, Foster City, Ca, USA). Analysis of PCR products was done using the COPY CALLER Software v1.0 (Life Technology, Foster City, USA) that allowed the classification of unambiguous homozygous (zero copies), heterozygous (one copy) and homozygous (two copies) GSTM1 or GSTT1 carrier individuals.

2.3. Statistical analysis

Associations between MC1R variants and melanoma risk were initially assessed individually using Fisher’s exact test. Associations with melanoma were assessed using logistic regression. Estimating odds ratios (ORs), their associated 95% confidence intervals (CIs) and P-values were obtained using SPSS v19. Multivariate logistic regression was also applied, including age, sex, hair color, skin color, solar lentigines and childhood sunburn as covariates. Associations between the number of variants carried and various individual and tumor characteristics were assessed via logistic regression.

To study the effect of combined protective and risk genotypes, we reduced the sample set to 528 samples successfully genotyped for all the associated SNPs. We used a 2x2 contingency table and a t-student test between SLC45A2 (rs35414) and both SILV (rs2069398) and NOS1 (rs2682826), as well as with all three genes together. In addition, we studied the results between the risk alleles, TYR (rs17793678), ADAMTS20 (rs1510521), GSTP1 (rs1695) and OCA2/HERC2 (rs12913832). Finally, we analyzed possible interactions between MC1R (0, 1 or 2 variants) and all previous risk and protective alleles.
3. Results

3.1. MC1R

Of the 946 individuals studied, 559 (59.15%) carried at least one MC1R variant, including 388 (65.43%) of 593 cases and 171 (48.4%) of the 353 controls. A total of 36 MC1R variants were identified.

Among these, 25 variants were non-synonymous changes, 20 of which had been described previously [5] and 5 were identified for the first time: S41F, M128T, P268R, A285V and N281S. Six variants of the receptor have been traditionally associated with red hair color (RHC): D84E, R142H, R151C, R160W, I155T and D294H.

Similarly, another three variants have not been associated with RHC phenotype and have been designated as NRHC (V60L, V92M and R163Q). These amino acid changes have been studied in different populations because their frequency is greater than 1%.

The other variants detected in the MCIR gene have frequencies lower than 1% in control samples and included C35Y, F45L, a trinucleotide deletion that results in a new amino acid in position 54 (c.161delTGG, V54E), S83P, G89R, V92L, T95M, a nonsense change (Y152X), two nucleotide insertions (c.537insC, p.ins179C; and c.537insT, p.ins179T), R213W, R272M, K278E, and T308M, plus all novel ones described before. These rare variants have been designated as “ns_rare SNPs” in Figure 1. The synonymous variants included the most common change, T314T (A>G), and the rare changes I63I, Q233Q, I264I, F300F as well as I180I and S316S, the last two described for the first time. These synonymous variants are called “s_rare” with or without T314T in Figure 1. The estimated frequency of common MC1R variants and some combinations such as all synonymous changes with and without the common T314T and all non-synonymous rare variants, as well as the corresponding estimated OR for MM and associated P-values are shown in Figure 1.

Among the 36 changes detected, five were individually associated with melanoma risk: V60L, R151C, I155T, R160W and D294H (P< 0.05). The highest OR was estimated for I155T (OR 3.65, 95% CI: 1.40–9.52; P=0.006). The estimated OR associated with carrying one non-synonymous variant was 1.58 (95% CI: 1.19–2.09; P =0.0013); however, the OR for carrying two non-synonymous variants was 4.38 (95% CI: 2.72–7.05; P =1.33x 10^-9). The MM associated OR among those Spanish patients carrying one RHC variant was 2.36 (95% CI: 1.71–3.26; P =1.86 x10^-5). However if we consider individuals homozygous or compound heterozygous for two RHC variants, the OR increased to 12.76 (95% CI: 3.06–53.29; P =1.9 x10^-5).

We considered blue/green eye color, blond/red hair color, solar lentigines and childhood sunburns as confounders in a multivariate model. MC1R variant analysis retained statistically significant results when adjusted for all potential confounders (OR: 1.77, 95% CI: 1.37–2.27; P =9.57x 10^-5). Hair color, solar lentigines and childhood sunburns were independently associated with MM (OR: 2.11, 95% CI: 1.22–3.66, P=0.008; OR: 2.28 95% CI: 1.61–3.23, P=3.54x10^-9; OR: 4.77, 95% CI: 3.37–6.77, P=9.57x 10^-5, respectively).
3.2. Other genes from pathways associated to melanoma

Several studies have been performed in order to evaluate other pigmentation-related genes and their relationship to MM susceptibility. The first results generated by Fernandez and cols. [4] analyzed the oculocutaneous albinism (OCA) genes: TYR (MIM#606933), OCA2 (MIM#611409), TYRP1 (MIM#155501) and SLC45A2 (MIM#606202); the melanocyte protein SILV (MIM#155550) and MCIR inverse agonist ASP (MIM#600201).

Figure 1. a) Summary of all MC1R variants associated with MM. Odds Ratios (OR), 95% confidence interval (95% CI) and P-values. b) Minor Allele Frequencies (MAF) of MC1R genetic variants. ns: non-synonymous variants V60L, V92M, S83P, D84E, V122M, R142H, I155T, R160W, R163Q and D294H; s_rare SNPs: synonymous variants with MAFs in controls lower than 1%: I63I, I180I, Q233Q, I264I, F300F and S316S; s_rare +T314T: same variants as before plus the addition of T314T; ns_rare SNPs: non-synonymous variants with less than 1% frequency in controls: C35Y, S41F, F45L, 54delTGG, G89R, V92L, T95M, M128T, Y152X, 179insTor C, R213W, P268R, T272M, K278E, N281S, A285V and T308M. Grey squares represent control data whereas black bars represent MM cases. Dark circles denote statistically significant values.
Allele frequencies for each SNP and the P-value for their comparisons between case and control subjects are detailed in Figure 2. After discarding two of the selected SNPs, one in the OCA2 gene due to its monomorphic nature in our sample collection, and one in the SLC45A2 gene due to its departure from HWE, we observed evidence of differences in allele frequency for one SNP in the SLC45A2 gene, corresponding to F374L (NCBI dbSNP rs16891982). The estimated OR per minor allele copy was 0.41 (95% CI, 0.24–0.70; P = 0.001) with the minor allele being more frequent in controls than cases (16% vs. 7%; adjusted P = 0.001).

In a second study we genotyped 384 SNPs from 65 genes belonging mainly to the pigmentation pathway [16]. Ten SNPs located on six individual chromosomes (one in each of ADAMTS20, TYR and SILV/CDK2; two in each of KIT and MYO7A; and three in SLC45A2) constituted the top ten MM phase I associated SNPs in our sample after establishing a re-

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**Figure 2.** Odds ratios (OR), 95% confidence interval (95% CI), P-values and Minor Allele Frequency (MAF) for the best seven genetic variants in genes belonging to pigmentation, DNA repair and oxidative stress pathways. Grey bars represent control data while black bars represent MM cases. Dark circles denote statistically significant values. Exact P-values are indicated in number.
strictive P-value threshold of 0.01. A phase II validation study was conducted to analyze the most significant SNP of each of the 6 candidate genes. One SNP, rs35414 in the SLC45A2 gene, had an unadjusted P=0.002 in this phase II and overall OR of 0.75 (95% CI 0.67–0.84, P=0.0001) (Figure 2). None of the other five SNPs tested in phase II reached statistical significance at this stage. However, three of them, located in TYR, SILV/CDK2 and ADAMTS20 had an overall P<0.05 when phase I and II were considered together.

After analysis of genes in the pigmentation pathway, we conducted two studies where 16 genes belonging to both base excision repair (BER) and nucleotide excision repair (NER) pathways, as well as 14 genes involved in oxidative stress, including GSTTI and GSTM1, were screened for copy number variation [17, 27]. Two statistically significant results suggested a putative role of oxidative stress processes in the genetic predisposition to melanoma.

First of all, a novel variant in the NOSI oxidative stress gene (rs2682826) was detected (P=0.01). A second association pointed to GSTP1 polymorphism rs1695, encoding the amino acid change I105V, and individually associated with MM (OR: 1.32, 95%CI: 1.06–1.63, P=0.01) found associated with melanoma for the first time. The best seven SNPs associated with MM in our population are shown in Figure 2. We could not detect any association between GSTM1 or GSTTI deletions and MM risk.

3.3. Phenotypic characteristics

If we take into account MC1R and the associated phenotypic characteristics we detect the estimated ORs for melanoma associated with various phenotypic characteristics 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=2x10⁻⁵), 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=3x10⁻¹³). No association with melanoma risk was observed for eye color, skin color or number of nevi.

The number of MC1R variants was statistically significantly associated with blond or RHC (OR: 1.80, 95% CI: 1.26–2.58, P=0.001), fair skin (OR: 1.42, 95% CI: 1.06–1.89, P=0.018) and with the presence of childhood sunburn (OR: 1.71, 95% CI: 1.28–2.27, P= 2x10⁻⁴). The corresponding ORs for the number of functional MC1R variants were 2.32 (95% CI: 1.42–3.78, P=0.001) for blond or RHC, 1.58 (95% CI: 1.09–2.3, P= 0.014) for fair skin and 2.35 (95% CI: 1.6–3.45, P=5 x10⁻⁵) for the presence of childhood sunburn.

We assessed whether SLC45A2 polymorphisms were associated with various phenotypic characteristics. The F374L variant allele was associated with dark eye color, dark hair color, darker skin and absence of both solar lentigines and childhood sunburns. Finally, we tested for associations between SLC45A2 SNPs and phototype, tumor location, and tumor depth among cases only (Table 5). The minor (G) allele of the F374L variant was found to be associated with phototypes III/IV (per allele OR, 3.25; 95% CI, 1.05–10.03; P=0.04). Additionally, SLC45A2 SNPs rs35414 and rs35415 were also associated with dark skin color (P=0.028 and P=0.0485) and only rs35414 with dark hair color (P=0.0183).
Evidence of association with phenotypic characteristics for two KIT SNPs, rs759083 and rs13135792, were also present. Both SNPs appeared to be associated with both light hair color (P=0.0021 and P=0.0072) and childhood sunburns (P=0.0112 and P=0.0167).

Two different SNPs in MYO7A were associated with dark hair color (rs948970, P=0.04), and childhood sunburn (rs758708, P=0.0474). One SNP in the TYR gene (rs17793678) was associated with light eye color (P=0.0239). Likewise, the ADAMTS20 gene was associated with light eye color (P=0.0339), blond or red hair color (P=0.0353) and with number of nevi (rs1510521; P=0.0338). Finally, SILV/CDK2 SNP rs2069398 was associated with absence of childhood sunburns (P=0.0353).

3.4. Gene-gene interactions

We explored the combined effects of the individually associated SNPs located in the six relevant genes studied: SLC45A2, SILV, NOS1 (protective associated genes) and TYR, ADAMTS20 and GSTP1 (risk associated genes). Although in our series it does not show individual association with MM, we added rs12913832 from the OCA2/HERC2 gene due to its strong association with eye color [6, 48] and the fact that it has been demonstrated to have epistatic effects with the MC1R gene [49].

3.4.1. Interaction between protective alleles

Two SNPs, rs16891982 and rs35414, located in the SLC45A2 gene were associated with MM. Both of them could have been used to perform the interaction analyses; however, rs35414 was chosen due to its higher MAF. SNPs rs2069398, located in the SILV/CDK2 gene region, and rs2682826 in NOS1 were also included in the analyses. Interaction results are shown in Figure 3.

We observed some degree of epistatic protective interaction between rs35414 (SLC45A2 gene) and rs2069398 (SILV/CDK2 gene region) when considering rare allele carriers at both loci. A significant decrease in MM associated OR was observed when two (heterozygotes for both SLC45A2 and SILV or three (heterozygous for SLC45A2 and minor homozygotes for SILV) rare alleles were present at both loci (OR: 0.54, 95% CI: 0.39–0.75, P=0.0003). Similarly, when three (minor homozygotes for SLC45A2 and heterozygous for SILV) or four (minor homozygous for both SLC45A2 and SILV) rare alleles were carried, a greater decrease in MM risk was observed (OR: 0.31, 95% CI: 0.18–0.55, P=0.0001). Results including joint genotypes for rs35414 and rs2069398 SNPs, individual status (heterozygous or minor homozygous), ORs with their corresponding 95% CIs and P-values are shown in Figure 3a.

In addition, we observed some degree of epistatic protective interaction between rs35414 (SLC45A2) and rs2682826 (NOS1) when considering heterozygote alleles at both loci (OR 0.23, 95% CI 0.16–0.55, P=0.0001). However, a trend toward protective effect was detected when both homozygous rare alleles were compared (OR: 0.50, 95% CI 0.18–1.20; P=0.1). This lack of statistically significant results is most probably due to the reduced number of samples in this category (21 MM cases vs. 17 controls). Results are shown in Figure 3b.
The interaction analyses between NOS1 rs2682826 SNP and SILV rs20693989 SNP revealed a trend toward significance when both rare alleles are considered (OR: 0.19, 95% CI: 0.03–1.07, P=0.055). Results are shown in Figure 3c.

Finally, when the effect of the interaction between the three loci protectively associated with MM, rs35414 (SLC45A2), rs2682826 (NOS1) and rs2069398 (SILV) were analyzed, statistically significant results were observed. Individuals heterozygous for the three genes were more common in controls than in MM cases (OR: 0.09, 95% CI: 0.022–0.316, P=0.0001), showing a cumulative protective effect. The comparison with rare homozygous alleles in all three loci was statistically not relevant due to the small number of samples found (Figure 3d).

3.4.2. Interaction between risk alleles

In order to show the distinct combinations of MM risk alleles we performed two different analyses. The first comparison studied the effect in MM susceptibility by taking together four genotypes: rs17793678 (TYR), rs1510521 (ADAMTS20); rs1695 (GSTP1), and rs12913832 (OCA2/HERC2). Results are shown in Figure 4a-d.

Some degree of epistatic risk interaction was seen between rs17793678 (TYR) and rs1510521 (ADAMTS20) when considering either heterozygous or rare alleles at both loci. We observed an increased risk effect when two or three rare alleles were present at both loci (rs17793678 heterozygotes and rs1510521 heterozygous, and minor homozygous carriers; OR: 1.41, 95% CI: 1.12–1.78, P=0.004). When three or four rare alleles (rs17793678 minor homozygotes and rs1510521 heterozygotes, and minor homozygous carriers) were analyzed together, we ob-
tained an OR of 1.54 (95% CI 0.96–2.48) with a trend toward significance (P=0.088). Results are shown in Figure 4a.

Figure 4. Interactions between risk allele variants and their effect on MM susceptibility. a) TYR and ADAMTS20, b) GSTP1 and TYR, c) GSTP1 and OCA2/HERC2 and d) TYR and OCA2/HERC2. OR: odds ratios per minor allele; 95% CI: 95% confidence intervals; REF: reference value; HET: heterozygotes; H MIN: minor allele homozygotes; and MIN: only minor homozygotes for TYR and GSTP1 respectively. For genes labeled in vertical we joined heterozygous and minor homozygous status. Dark circles denote statistically significant results.

A similar effect was observed when we compared rs1695 (GSTP1) with both rs1510521 (ADAMTS20) and rs12913832 (OCA2/HERC2) polymorphisms. We detected some degree of epistatic risk interaction when considering homozygous rare alleles at both loci. Results for rs1695 and rs1510521 showed an OR of 2.53 (95% CI 1.005–6.49, P=0.04, data shown in Figure 4b), while the interaction between rs1695 and rs12913832 had an OR of 2.81 (95% CI 1.11–7.27, P=0.018, see Figure 4c).

An additional comparison with rs17793678 (TYR) and rs12913832 (HERC2/OCA2) showed increasing epistasis as the number of risk alleles augmented, showing a trend toward significance only when homozygous minor alleles were considered at both loci, OR=2.95 (95% CI 0.84–11.326] (p=0.077). See Figure 4d. All other complex comparisons did not add further information.

For the second type of analyses we included the MC1R locus together with the previous four polymorphisms. As MC1R had already been associated with MM, it seemed biologically plausible that genetic interactions would be detected between risk variants within MC1R.
and other associated genes. Indeed, increased risks appeared when heterozygotes or rare alleles at MC1R were combined with heterozygotes plus rare homozygous at GSTP1 (OR: 5.3, 95% CI: 2.80–417.42; P =1x10⁻⁴, see Figure 5a), at TYR (OR: 6.42; 95% CI 2.32–18.64; P=0.0001, see Figure 5b), and at OCA2/HERC2 (OR: 7.163; 95% CI 2.659–20.05; P=0.0001, see Figure 5c).

Figure 5. Interactions between risk allele variants on MC1R together with other risk associated genes alone or in multiple combinations, and their effect on MM susceptibility. a) MC1R and GSTP1, b) MC1R and TYR, c) MC1R and OCA2/HERC2 and d) MC1R, TYR and OCA2/HERC2, e) MC1R, GSTP1, TYR and OCA2/HERC2. OR: odds ratios per minor allele; 95% CI: 95% confidence intervals; REF: reference value; HET: heterozygotes; H MIN: minor allele homozygotes. For the genes labeled in vertical we joined heterozygotes and minor homozygotes. Dark circles denote statistically significant results.

The last group of comparisons was done taking into consideration three and four genes at the same time. Firstly, we showed that combining MC1R genotypes with both TYR and GSTP1 resulted in the highest MM associated risk (OR: 11.56, 95% CI 2.25–79.54, P=0.0001; data shown in Figure 5d). Secondly, we performed a final analysis taking into account only rare homozygotes of the four risk alleles and compared them, for MM association, with wild type genotype individuals. We obtained an OR of 4.008 (95% CI 1.25–13.2) with a P-value of 0.016 (see Figure 5e). There is not enough power to consider any other comparison with statistical significance.
3.4.3. Complex interactions

The role of SLC45A2 and the other protective genes (NOS1 and SILV) in melanoma predisposition was further analyzed in relation to MC1R, the main low-penetrance gene associated to melanoma. Since all these genes have been studied by our group, we analyzed the interaction effects between both MC1R and SLC45A2 loci (see Figure 6a) and between MC1R and all the protective alleles (see Figure 6b).

A great reduction of risk was detected when the rare protective alleles at SLC45A2 were carried in individuals with two MC1R variants (OR: 2.20, 95% CI 1.14–4.23, P=0.02), in comparison to individuals carrying only two MC1R variants (OR: 4.64; 95% CI 1.85–11.58, P=0.001). Individuals having only one MC1R mutation plus the rare protective SLC45A2 allele also
showed a reduction in MM risk, although this decline does not seem to reach significant values (data shown in Figure 6a). These results confirmed the protective role of the rs35414 variant in SLC45A2 regarding MM risk. Similar effects are observed when we included in the calculations the protective SNPs located in NOS1 and SILV, however less statistically significant results were obtained due to the small number of samples in each class.

4. Discussion
Since MC1R genetic variability is strongly associated with the RHC phenotype [12], a large number of studies have investigated the involvement of this gene in MM susceptibility. MC1R is highly polymorphic, with more than 100 variants described in Caucasian populations [13]. Despite of its high variability, the synonymous changes are greatly reduced, with only three described in the literature: T314T (A>G), F300F (C>T) and C273C (C>T) [50, 51]. Results obtained from our laboratory confirmed the association between five MC1R polymorphic variants and MM risk in the Spanish population. We found 36 MC1R variants, this number being quite similar to the number found in other Mediterranean population studies (16 in France, 26–29 in Italy and 18 in Greece) [9, 11]. The most frequent Spanish variant is V60L with a frequency of 12.03%. 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) [52]. The RHC phenotype-associated variants (R151C, R160W and D294H) were present at frequencies of 0.71, 0.71 and 1.42%, respectively, in our population, compared to 9.9, 8.7 and 3.6%, respectively, reported in Northern European populations [52]. There were very few red-haired individuals among the control sample, and only 36 (6%) of MM cases had red hair. This finding is consistent with other results from Mediterranean populations and is at odds with red hair frequencies found in Northern European populations [12]. Red-haired subjects with no MC1R variants are not uncommon and have been seen in a Northern European population as well [53].

RHC variants have been consistently associated with MM in Northern European populations [3, 10, 12] and also in the Northern French population [9]. In Spain, we detected statistically significant individual associations for R151C, R160W and D294H. These three variants have been detected in the Northern French and Central Italian populations [9, 54]. 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 Northern Europeans [32, 55, 56], probably due to its low prevalence in Spain (0.28% in controls). The I155T variant has not been associated with MM in other populations to date, but this may also 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: 3.51, 95% CI: 1.35–9.12, P= 0.006). While the associations of RHC with MM were expected, the case of V60L (an NRHC variant) was more intriguing, since its involvement in MM pathology has been generally unclear in Caucasian populations. However, V60L could play a role in MM susceptibility only in darker skinned populations since it has been found associated with MM in other Mediterranean populations such as France and Greece [9, 11]. The fact that NRHC variants could be important in MM risk is also supported by our find-
ing that risk increased with the number of non-synonymous changes carried, regardless of whether they were RHC or NRHC. The presence of two non-synonymous changes implies that both copies of the MC1R protein are compromised. In addition, the presence of two NRHC increases by more than five times the risk of only one non-synonymous variant \( (P=1.9 \times 10^{-5}) \). All these results taken together strongly support the role of the MC1R gene as highly linked to the susceptibility of developing MM in Mediterranean countries such as Spain [3, 5, 10, 52].

In recent years, SLC45A2 has joined the group of genes (including MC1R, OCA2, and ASP) identified as being related to pigmentation, and it is now considered integral to pigmentation variation. Mutations in the SLC45A2 or MATP gene (MIM #606202), which encodes the membrane-associated transporter, have recently been associated with the OCA4 albinism subtype [57]. The non-synonymous variant F374L (rs16891982) has been reported to have a strong association with dark hair, skin and eye color in Europeans [58]. These phenotypic correlations were replicated in our analysis on the Spanish population and we also established for the first time its role in MM susceptibility [14]. We also found that other polymorphisms on SLC45A2 other than rs16891982 were also associated with dark phenotypic characteristics [16], confirming the role of SLC45A2 in pigmentation. A parallel work by Guedj and cols. [59] also detected association of this variant with melanoma in a French population, supporting our previous results. It has been proposed that the F374 allele causes a reduction of protein function that alters the intracellular trafficking of melanosomal proteins, creating an environment for decreased melanin production [58]. More than 90% of European genes carry the F374 allele, which is rare or absent in Africans. The remaining 10% of people of European descent with the Leucine ancestral allele (the most common in Africans) appear to have significantly more pigmented skin. There is a clear evidence of selective sweep of the chromosomal segment around the SLC45A2 gene in the European population, which is consistent with our data [60]. The derived L374 allele shows unusually large allele frequency differences between Europeans and other populations, and has reduced haplotype diversity. These patterns are consistent with the action of recent natural selection on these genes in Europeans.

The NOS1 gene is located on chromosome 12q24.2 and consists of 29 exons, encompassing more than 160kb of genomic DNA [61] and it is the main NO synthesizing enzyme in the central nervous system [62, 63]. The rs2682826 SNP is located in the 3' UTR of exon 29 of the NOS1 gene and was selected as the tag SNP of one of the most frequent haplotypes. However, rs2682826 seems to be the most likely functional SNP due to its location close to several miRNAs binding sites in 3'UTR region. Possibly, differences in protein translation might be elicited depending on the allele present in the mRNA of this gene. No other regulatory element close to this region seems to modulate this gene. We propose this SNP as a novel variant related to melanoma \( (P=0.01) \).

Despite not being able to detect association between MM and the absence of either GSTM1 or GSTT1 copies, minor homozygotes for rs1695 in the GSTP1 gene appeared to be strongly associated with MM. The latter SNP encodes an amino acid change, I105V, that was described for the first time associated with MM \( (P =0.01) \) in our population. This finding is consis-
tent with the hypothesis that patients with the GSTP1 V105 variant enzyme have a reduced ability to detoxify compounds, which results in lower clearance and reduced efficacy. The GSTP1 V105 variant is associated with a lower thermal stability and altered catalytic activity to a variety of substrates compared with those for GSTP1 I105 [34].

It seemed biologically plausible that genetic interactions would be detected between several of the SNPs identified in our studies, and for that reason we grouped protective alleles located in the genes SLC45A2, SILV/CDK2 and NOS1. On one hand, a great reduction of risk was detected when rare alleles at these loci were combined. The strongest protective combination was between SLC45A2 and SILV/CDK2, followed by the combination of heterozygote samples at SLC45A2 and NOS1. Other protective effects were seen, although they did not reach statistical significance due to the low number of samples in the analyzed categories.

In order to determine possible genetic interactions between MC1R and the rest of the genes studied, we compared individuals carrying risk variants in TYR, ADAMTS20 or OCA2/HERC2 with the MC1R locus. Indeed, increasing risk effects were observed in all comparisons when as more risk alleles accumulated in the same individuals. However, when MC1R was taken into consideration, the epistatic effect was far stronger, increasing from an OR of 4.38 when combined with GSTP1 up to an OR of 11.56 when MC1R, TYR and GSTP1 heterozygotes and homozygotes are considered together, and achieving risk effect values similar to having two MC1R RHC alleles. It is worth mentioning here that some genetic interactions, for instance between MC1R and OCA2, have already been described among pigmentation genes [49, 64].

Finally, we tried to see whether protective alleles were going to be able to reduce the risk induced by the accumulation of risk alleles. Some comparisons were not possible due to the absence of individuals in some of the categories. Therefore, we present only the results between MC1R risk alleles and the more robust protective gene, SLC45A2, alone or in combination with NOS1 and SILV. We could indeed observe a great reduction in risk in individuals carrying two risk alleles in MC1R when simultaneously carrying protective alleles in the other genes. These results are important because the overall risk for an individual does not rely in only one gene but in the interaction of all his/her genetic background and this should be considered in the future.

In summary, we found that five MC1R variants (V60L, R151C, I155T, R160W and D294H) are individually associated with MM risk in the Spanish population. Carrying two non-synonymous MC1R variants was associated with even higher risk, more than doubling the risk of carrying a single variant and having a five time higher risk than when carrying an NRHC variant. We described for the first time an association with the F374L variant, located on the SLC45A2 gene, which appears to be a novel protective low-penetrance melanoma susceptibility gene. We therefore propose an integral study when trying to assert the MM risk of an individual, because the combination of rare alleles at several loci modulates the final risk/protective value that predisposes him/her to MM.
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