

Genetic 3'UTR variation is associated with human pigmentation characteristics and sensitivity to sunlight

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TITLE PAGE

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KEY WORDS:

3' untranslated region, microRNA, SNP, naevus, solar lentigines

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ABSTRACT

Sunlight exposure induces signalling pathways leading to the activation of melanin synthesis and tanning response. MicroRNAs (miRNAs) can regulate the expression of genes involved in pigmentation pathways by binding to the complementary sequence in their 3'-untranslated regions (3'UTRs). Therefore, 3'UTR SNPs are predicted to modify the ability of miRNAs to target genes, resulting in differential gene expression. In this study, we investigated the role in pigmentation and sun-sensitivity traits, as well as in melanoma susceptibility, of 38 different 3'UTR SNPs from 38 pigmentation-related genes. A total of 869 individuals of Spanish origin (526 melanoma cases and 343 controls) were analysed. The association of genotypic data with pigmentation traits was analysed via logistic regression. Web-based tools for predicting the effect of genetics variants in microRNA-binding sites in 3'UTR gene regions were also used. Seven 3'UTR SNPs showed a potential implication in melanoma-risk phenotypes. This association is especially noticeable for two of them, rs2325813 in the *MLPH* gene and rs752107 in the *WNT3A* gene. These two SNPs were predicted to disrupt a miRNA-binding site and to impact on miRNA-mRNA interaction. To our knowledge, this is the first time that these two 3'UTR SNPs have been associated with sun-sensitivity traits. We state the potential implication of these SNPs in human pigmentation and sensitivity to sunlight, possibly as a result of changes in the level of gene expression through the disruption of putative miRNA-binding sites.

INTRODUCTION

Cutaneous melanoma incidence is increasing rapidly among white-skinned populations (1). Melanoma incidence reveals a clear relationship between pigmentation traits and sunlight damage, with individuals with fair skin, green and blue eyes, red and blond hair, high naevus count, freckles, and inability to tan showing greater melanoma susceptibility (2). These phenotypic traits has been shown to be genetically determined by genes implicated in pigmentation and tanning ability (3,4), and genetic variations in these genes have been associated with the susceptibility to melanoma (5–11). Factors that are mainly involved in the aetiology of melanoma are not only of pigmentary/genetic nature, but also of environmental nature (12). Chronic sun exposure thus plays a key role in causing melanoma through DNA damage (13).

Ultraviolet (UV) exposure stimulates the synthesis of melanin in melanosomes via activation of human pigmentation pathways, with the aim of protecting skin from the harmful effects of sunlight (14). Gene expression can be regulated by a wide range of mechanisms. Recently, posttranscriptional regulatory processes – specifically controlled by mRNA-binding factors – have emerged as a fundamental and effective cellular mechanism to regulate gene expression, and alterations in these processes can cause numerous pathologies including immunological disease (15), neurodegeneration (16), and tumour development (17,18). Therefore, differential gene expression may be as important for disease susceptibility as non-synonymous coding changes.

Among the mRNA-binding factors, microRNAs (miRNAs) – short non-coding RNA molecules (22-24 nt) encoded by intronic or intergenic sequences – act as key gene

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3 regulators by repressing mRNA translation or by destabilizing/degrading mRNAs in the
4 cytoplasm, via perfect or imperfect binding to their complementary base pair sequence
5 in the 3'untranslated region (3'UTR) of the mRNA target (19). Therefore, the 3'UTR
6 region is emerging as critically important in regulating gene expression (17), and
7 polymorphisms in the miRNA-binding sites of the 3'UTR of genes may alter the
8 binding efficiency and miRNA-mRNA gene expression regulation. In support of this
9 hypothesis, recent studies have identified variants in the 3'UTR of genes that increase
10 the susceptibility for melanoma (20), lung (21), colorectal (22) and ovarian cancer (23)
11 by affecting the ability of miRNAs to bind. In particular, two sequence changes in the
12 3'UTR of the *CDKN2A* gene have been significantly correlated with melanoma risk
13 (24), but also with a shorter progression time from primary to metastatic melanoma
14 (25).
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32 Here, we hypothesise that differences identified in nucleotide composition of 3'UTRs
33 SNP sites of genes previously associated with pigmentation and/or skin cancer can be a
34 reason for causing differences in human pigmentation, sensitivity to sunlight, and thus
35 in melanoma susceptibility. In the current study, we describe the role of 38 different
36 3'UTR polymorphisms from 38 different candidate pigmentation and melanoma
37 susceptibility genes in a population of Spanish origin. Additionally, we use miRNA
38 binding prediction tools to identify variants affecting putative miRNA-binding sites, and
39 to predict their impact on miRNA-mRNA interaction.
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METHODS

Study subjects and data collection

A total of 526 melanoma cases and 343 cancer-free controls were included in this study. Melanoma cases were recruited at the Departments of Dermatology of four Spanish hospitals: Gregorio Marañón General University Hospital (Madrid), La Paz University Hospital (Madrid), Ramon y Cajal University Hospital (Madrid) and Castellon Province Hospital (Castellon). Volunteer cancer-free control samples were recruited from the Madrid College of Lawyers, Gregorio Marañón Hospital, Valencia Clinic Hospital and Castellon Province Hospital. We carefully selected all cases and controls included in the current study to account for confounding variables. As far as it was possible, controls were frequency-matched to the cases by age, sex and place of birth. All individuals were Caucasians of Spanish origin with the same genetic background, since there is evidence of high genetic homogeneity within different Spanish geographical regions (26).

Each participant completed a standardised questionnaire to collect information on sex, age, pigmentation characteristics (eye colour, hair colour, skin colour, number of naevi and presence of solar lentigines), history of childhood sunburns, and personal and family cancer history.

Genomic DNA from cases and controls was isolated from peripheral blood lymphocytes using the traditional saline method or the DNAzol procedure (Invitrogen, Eugene, OR, USA) or the MagNA Pure LC Instrument according to the manufacturer's protocol (Roche Molecular Biochemicals AQ2, Mannheim, Germany). DNA concentration was quantified in samples before genotyping by using a Nanodrop 2000 spectrophotometer

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2
3 or Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Eugene, OR, USA). Genomic
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5 DNA was amplified using the GenomiPhi DNA Amplification Kit (GE Healthcare Bio-
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7 Sciences AB, Uppsala, Sweden). Samples were diluted to a final solution of 50 ng/ml
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9 and stored at -20°C.
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14 The study was approved by the Ethics Committee of the Biomedical Research Institute -
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16 INCLIVA (Valencia, Spain). Written informed consent was obtained from all
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18 participants.
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20 21 22 23 **SNP Selection**

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25 Previous literature and information of public databases were used to perform our
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27 candidate gene list. We selected genes previously associated with pigmentation
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29 pathways and/or melanoma risk (7–9,27,28), preferably including direct targets of
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31 functional miRNA that happen to be deregulated in melanoma. Ensembl BioMart
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33 (<http://www.ensembl.org/biomart/martview>) was used to retrieve germline variants
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35 from all genes selected. Filters were used to ensure that all SNPs were located within
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37 the 3'UTRs. SNP codes, locations, minor and ancestral alleles and their frequencies,
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39 were obtained from the NCBI (www.ncbi.nlm.nih.gov/SNP), HapMap
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41 (www.hapmap.org) and Ensembl Variation (www.ensembl.org/info/genome/variation)
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43 databases. From the data retrieved, Haploview v4.2 was used to identify tag-SNPs that
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45 optimally capture allelic variation among SNPs, using a pairwise SNP approach with a
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47 minimum r^2 threshold of 0.8 (29). To ensure a high genotyping success rate, a minor
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49 allele frequency (MAF) threshold of 0.1 in the Caucasian population from the
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51 International 1000 Genomes Project (<http://www.1000genomes.org/>) was established in
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53 the SNP selection process. Forty-five tag-SNPs were finally selected.
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Genotyping

SNPs genotyping was conducted by the Spanish National Genotyping Centre (CeGen-PRB2, Santiago de Compostela) as a contract service using the iPLEX Gold MassARRAY technology, according to manufacturer's protocol (Sequenom, San Diego, CA, USA). All assays were performed in 384-well plates, including a negative control and a trio of Coriell samples (Na10860, Na10861 and Na11984) for quality control. Genotyping specificity was assessed by adding three DNA duplicates (two intra-assays and one inter-assay) per plate, yielding 100% consistent replication results. In addition, cases and control samples were always included in the same run. SNPs with a genotyping rate lower than 90% (10% missing data) were excluded for further analysis.

Identification of potential microRNA binding sites

The potential effect of 3'UTR polymorphisms on miRNA binding was examined using MirSNP (<http://cmbi.bjmu.edu.cn/mirsnp>) (30) and miRNASNP (<http://www.bioguo.org/miRNASNP/>) (31).

MirSNP employs the miRanda target prediction algorithm (<http://www.microrna.org>)(32), with stringent 7-nt seed site pairing as major criteria for prediction consistency. To increase precision, we only considered target sites with an alignment score cutoff ≥ 140 , energy cutoff ≤ -10 kcal/mol, and miRSVR score ≤ -0.1 .

MiRNASNP uses two miRNA target prediction tools: TargetScanHuman (<http://www.targetscan.org/>) (33) and miRanda (32). MiRNASNP also incorporates RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid>) (34) to quantify the

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3 binding energy changes in the interaction of miRNAs with the wild-type target
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5 sequence compared to the derived 3'UTR sequence. Only the duplexes with
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7 hybridization free energy ≤ -20 kcal/mol were chosen (35).
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11 **Identification of validated pathways targeted by *in silico* predicted microRNAs**

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13 In order to further investigate the miRNAs predicted to bind to the two 3'UTR SNPs
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15 highly associated with phenotypic traits (hsa-miR-149-5p, hsa-miR-892b, hsa-miR-185-
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17 3p and hsa-miR-762), we used DIANA-miRPath v2.0
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19 (<http://www.microna.gr/miRPathv2>) to identify the miRNA targeted pathways. The
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21 output provides intuitive heat maps and enriched KEGG pathway visualizations for
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23 easier inspection (36).
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30 ***In silico* quantitative analysis of tissue-specific expression**

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32 Data from the Genotype-Tissue Expression (GTEx) project (dbGaP accession No.
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34 phs000424.v6.p1) was used for external validation and to evaluate differential tissue-
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36 specific gene expression regarding 3'UTR SNP genotypes
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38 (<http://www.gtexportal.org/home/>).
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43 **Statistical Analysis**

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45 For each polymorphism studied, Fisher's exact test was used both to check for
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47 deviations from Hardy-Weinberg equilibrium (HWE) among controls and to compare
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49 differences in allele counts between cases and controls. In order to account for
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51 differences between populations, allele frequencies of our Spanish population were
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53 compared to those of both a North European population (CEU) and a Southern one from
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55 Tuscany (TSI) using Fisher's exact test.
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5 Associations between the genotyped genes and various pigmentation characteristics
6 were assessed via logistic regression. Association analyses were done for all samples
7 pooled, with eye colour (blue/green *versus* brown/black), hair colour (brown/black
8 *versus* blond/red), skin colour (fair *versus* brown), number of naevi (≥ 50 *versus* < 50),
9 presence of lentigines (yes *versus* no), and childhood sunburns (yes *versus* no) as the
10 outcome variables. This was performed for four different patterns of inheritance:
11 dominant (major homozygotes *versus* heterozygotes plus minor homozygotes), over-
12 dominant (major homozygotes plus minor homozygotes *versus* heterozygotes),
13 recessive (major homozygotes plus heterozygotes *versus* minor homozygotes), and
14 additive (counting additively for each copy of minor allele). Genotype-related odds
15 ratios (ORs), their corresponding 95% confidence intervals (CIs) and associated *P*-
16 values were estimated. Association analyses with phenotypic traits were adjusted by
17 sex, since sex-differentiated allelic effects for pigmentation traits, sensitivity to sunlight
18 and melanoma have been previously shown (38–40).
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38 In order to assess associations among genotypes and melanoma risk, genotype-related
39 ORs, their corresponding 95% CIs and associated *P*-values were estimated via
40 unconditional logistic regression. Multivariate logistic regression was also carried out
41 combining sex and all significant risk factors revealed in Table S1. This was also done
42 for all four patterns of inheritance.
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51 Statistical analyses and plots were conducted using R statistical framework
52 (<http://www.R-project.org>). All genetic analyses were performed estimating the effect
53 of the minor allele in the Spanish population. Unknown and missing values were
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3 excluded at each specific analysis. All P -values were two-sided, and those less than 0.05
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5 were considered statistically significant.
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RESULTS

The role of 38 polymorphisms in as many pigmentation and melanoma susceptibility genes was initially investigated. No evidence of departure from HWE for any of the 38 SNPs was found. Two 3'UTR polymorphisms revealed differences in minor allele frequencies (MAFs) between cases and controls: *ADAMTS20* rs6582463 and *HOXB7* rs15689. We did not observe differences in MAFs between cases and controls for any other SNP (Table S2).

We compared Spanish allele frequencies to those of CEU and TSI subjects, using the 1000 Genomes Project (phase 3) allele counts as the reference (Table S2). Spanish MAFs differed significantly from CEU frequencies in three SNPs (7.89%): rs4733967 (*ADAM9*), rs3212369 (*MC1R*), and rs1690916 (*MDM2*). Seven SNPs presented different allele frequencies from those reported in TSI population data: rs6582463 (*ADAMTS20*), rs742106 (*DTNBPI*), rs12952 (*EXOC2*), rs8022 (*KIT*), rs995030 (*KITLG*), rs14983 (*MMP7*), and rs1551306 (*TPCN2*). In spite of these differences, allele frequencies in Spain were very similar to those from both a North European population (CEU) and a Southern one (TSI), with a high correlation (R^2) of 0.916 and 0.913, respectively (Figure S1).

Association analysis

Evidence of association with phenotypic characteristics for the thirty-eight 3'UTR SNPs was assessed. Considering a P -value threshold of 0.05, 17 SNPs were associated with at least one sun response trait, and 11 SNPs showed association with at least one pigmentation trait (Figure 1). Among them, we further investigated the 7 SNPs that

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3 presented the most potential allelic effects for phenotypic traits in the Spanish
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5 population (P -value < 0.01). The rs2325813 SNP, located in the *MLPH* gene, was
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7 correlated with the presence of more than 50 naevi ($P=8.97 \times 10^{-4}$). Two SNPs, *HOXC8*
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9 rs4142680 and *WNT3A* rs752107, correlated with the presence of lentigines
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11 ($P=6.57 \times 10^{-3}$ and $P=4.53 \times 10^{-4}$, respectively); while *LYST* rs6696123 showed
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13 association with an absence of lentigines ($P=2.56 \times 10^{-3}$). Two more SNPs, rs10270 in
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15 the *CLIP1* gene and rs4980113 in the *KCNMA1* gene, were associated with dark hair
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17 colour ($P=1.44 \times 10^{-3}$ and $P=2.67 \times 10^{-3}$, respectively). Finally, *KIT* rs8022 was correlated
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19 with light eye colour ($P=8.88 \times 10^{-3}$) (Table 1).
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26 Likewise, we carried out an association analysis between genotypes and melanoma risk.
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28 Five SNPs showed a tendency to correlate with melanoma susceptibility in the Spanish
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30 population. Among them, three SNPs (*HOXB7* rs1589, *MARCKS* rs28558559 and
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32 *ADAM9* rs4733967) showed a melanoma protective effect ($OR < 1$). On the other hand,
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34 *PTCH2* rs41269085 and *ADAMTS20* rs6582463 displayed a melanoma risk effect
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36 ($OR > 1$) (Table S3).
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43 For the association results to be adjusted by the confounding variables, we performed a
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45 multivariate analysis including phenotypic risk factors (hair colour, solar lentigines and
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47 the presence of childhood sunburn) and sex as covariates. Polymorphisms located in
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49 *HOXB7*, *MARCKS*, *ADAM9* and *PTCH2* remained significant after the adjustment, with
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51 no substantial changes in allelic effects, confirming the putative role of these variants in
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53 melanoma susceptibility. Additionally, *KCNMA1* rs4980113 and *IRF4* rs9391997 were
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55 marginally associated with melanoma protection (Table S3).
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Variants affecting microRNA binding sites in human pigmentation

All 3'UTR polymorphisms that presented association with phenotypic characteristics and/or melanoma were analysed by two specialized web-based programmes for predicting miRNA-binding sites in the 3'UTR.

Cross-prediction was required for verifying the predicted target sites. After applying all sequential filtering steps, eight of all 3'UTR polymorphisms evaluated had at least one miRNA predicted to bind (Table 2). Three 3'UTR variants interrupted miRNA-mRNA interaction or reduced miRNA-mRNA interaction by increasing the free energy of the corresponding duplexes after the minor allele introduction in the target sequence.

Conversely, three variants created new miRNA target sequences or enhanced miRNA binding efficiency by decreasing hybridization free energy. Two variants both disrupted/decreased and created/enhanced multiple miRNA target sequences in the sequences studied (Table 2).

Once miRNAs of interest were identified using binding prediction tools, we used an *in silico* approach to identify pathways that are under the regulation of the predicted miRNA signature. The four selected miRNAs and the targeted KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways are displayed in Figure 2. Among all the significant targeted KEGG pathways, we identified three of them involved in pigmentation and skin cancer: “Wnt signalling pathway-hsa04310” ($P=4.24 \times 10^{-5}$), “MAPK signalling pathway-hsa04010” ($P=1.07 \times 10^{-4}$) and “Basal cell carcinoma-

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3 hsa05217” ($P=2.52 \times 10^{-3}$). Figure S2 represents in detail these three KEGG pathways,
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5 highlighting the specific target genes of the selected miRNAs.
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8 We further evaluated the association between the genotype of both *MLPH* rs2325813
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10 and *WNT3A* rs752107 and the gene expression levels in sun-exposed skin by using the
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12 GTEEx portal. Individuals carrying rs752107*T allele, which was predicted to decrease
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14 miRNA-mRNA binding efficiency, seem to present increased expression of *WNT3A* in
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16 sun-exposed tissue (Figure S3). No changes in *MLPH* expression regarding genotype
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18 were observed.
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DISCUSSION

In the current study, 38 tag-SNPs located in the 3'UTRs of pigmentation-related genes were successfully genotyped in 869 individuals from Spain, with the intention of detecting novel genetic variants with putative phenotypic implications. Since 3'UTRs are critical regulatory elements in gene expression (41), polymorphisms located in this region of genes associated with pigmentation pathways may contribute to pigmentation characteristics and sensitivity to sunlight, as well as to melanoma susceptibility.

This study allowed us to observe interesting associations between genotypic and phenotypic traits in our population. Despite detecting several candidate 3'UTR SNPs with a potential implication in pigmentation and sensitivity to sunlight, we could not validate them since associations did not reach genome-wide nor candidate gene levels of statistical significance. Perhaps our restricted sample size resulted in limited statistical power to detect unequivocal associations for these SNPs. Replication of our findings in a larger study is therefore essential before drawing any firm conclusion. It is noted that adjusting analyses by sex has conferred strength to our results, excluding bias from the sexual disparity in pigmentation and melanoma incidence and outcome observed in previous studies (38–40,42,43).

The first interesting finding was the reasonably strong association of rs2325813, located in the 3'UTR of the *MLPH* gene, with high naevus count. The human *MLPH* gene (OMIM #606526) has been shown to be involved in mature melanosome transport within melanocyte before being transferred to keratinocytes. *MLPH* gene encodes a

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2
3 member of the exophilin subfamily of Rab effector proteins known as melanophilin,
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5 which acts as a link between the small GTPase melanosome-bound RAB27A and the
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7 actin-associated motor protein MYO5A (44). This protein complex plays a crucial role
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9 in the melanosome motility in melanocytes, and aberrations in any of the complex
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11 components has been shown to result in perinuclear localization of melanosomes and
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13 therefore failure to transfer mature melanosomes to adjacent keratinocytes, eventually
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15 causing hypopigmentation (45). Human individuals homozygous for a pathogenic
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17 *MLPH* mutation (c.102C>T; p.R35W) display Griscelli syndrome type 3, a pigmentary
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19 disorder characterized by a hypopigmented phenotype (45–47). The naevus-associated
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21 SNP in this work, rs2325813, is predicted to disrupt a binding site of two miRNAs (hsa-
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23 miR-185-3p and hsa-miR-762). The presence of the minor allele in the target sequence
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25 enhances miRNA binding efficiency, repressing mRNA translation of *MLPH*, and
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27 ultimately limiting the formation of RAB27A/Melanophilin/Myosin-5a complex. Thus,
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29 reduction of *MLPH* gene expression may cause an abnormal accumulation of mature
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31 melanosomes around the nucleus of melanocytes, resulting in light pigmentation and
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33 poor tolerance to sunlight. Interestingly, our results are consistent with the well-known
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35 correlation between melanocytic naevus number, a main risk-prediction factor for
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37 melanoma incidence, and the propensity to burn, rather than tan, of light-skinned
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39 individuals (48). Therefore, genes implicated in functions related with melanosome
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41 trafficking, especially the RAB27A/Melanophilin/Myosin-5a membrane transport
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43 pathway, would be relevant candidates for additional investigation in further
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45 pigmentation and melanoma studies.
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55 WNT/ β -catenin signalling has a pivotal role in the formation of melanocytes, since this
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57 pathway has been implicated in promoting the development of neural crest-derived
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3 melanocytes (49,50). In humans, the WNT pathway is significantly up-regulated in
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5 solar lentigines, suggesting that overstimulation of melanocytes proliferation and
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7 differentiation play a crucial role in the pathogenic mechanism of solar lentigines (51).
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9 Interestingly, in this work we identify a polymorphism, rs7352107, located in the
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11 3'UTR of the *WNT3A* gene that is strongly associated with the presence of solar
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13 lentigines. *WNT3A* (OMIM #606359) encodes a WNT ligand that acts through the
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15 WNT/ β -catenin pathway promoting melanocyte differentiation, and may promote
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17 melanoma differentiation as well (49). Furthermore, the minor allele of rs7352107 is
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19 predicted to decrease the binding efficiency to the 3'UTR gene region of two
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21 microRNAs (hsa-miR-149-5p and hsa-miR-892b), leading to a weaker miRNA-mRNA
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23 interaction and therefore a higher level of secreted WNT3A ligand. This probably
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25 enhances the activation of the WNT/ β -catenin signalling and subsequently the
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27 proliferation of melanocytes. These observations, together with the results from
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29 Yamada and cols. (2014) (51), suggest that abnormal regulation of melanogenesis via
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31 gene expression changes is expected to be involved in several pigmentary disorders and
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33 in melanoma risk phenotypes. Thus, studies focusing on the regulation of WNT/ β -
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35 catenin signalling could potentially clarify the causal mechanisms of pathogenic
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37 hyperpigmentation and hypopigmentation conditions.
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47 The miRNAs predicted to bind to *MLPH* rs2325813 (hsa-miR-185-3p and hsa-miR-
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49 762) and to *WNT3A* rs7352107 (hsa-miR-149-5p and hsa-miR-892b) seem to target
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51 genes involved in pigmentation mechanisms and skin cancer. Remarkably, out of all
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53 significant pathways, “Wnt signalling pathway” and “MAPK signalling pathway” were
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55 the only ones targeted by three of the four miRNAs. Furthermore, “Basal cell
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57 carcinoma” pathway was also targeted by hsa-miR-185-3p and hsa-miR-762. These
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3 observations may corroborate the importance of these miRNAs in both human
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5 pigmentation and skin cancer pathways. Based on GTEx project data, genes encoding
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7 for these miRNAs, except for hsa-miR-892b, are expressed in sun-exposed skin (Figure
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9 S3), confirming the expression of these miRNAs in skin tissue, and suggesting a
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11 possible role of these miRNAs in skin regulation and function.
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17 Additionally, five polymorphisms displayed a notable statistical association with
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19 phenotypic characteristics. Among these SNPs, we would like to highlight that the
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21 variant rs4142680, located in the 3'UTR of *HOXC8*, displays an interesting
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23 predisposition tendency towards sun-damaged phenotypes. The *HOXC8* gene has been
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25 shown to be massively up-regulated in melanoma cancerous cells as a consequence of
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27 diminished miR-196a levels, leading to an aggressive melanoma phenotype via the
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29 overexpression of several tumorigenic target genes (52). Curiously, the web-based
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31 miRNA binding prediction analysis in this work showed an intermediate free energy (-
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33 16.60 kcal/mol) for binding hsa-miR-4509 to the 3'UTR sequence containing the
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35 rs4142680*T allele, and predicted that presence of the C allele may break the putative
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37 binding site. Thus, the association between rs4142680*C and the presence of solar
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39 lentiginos may be the result of increased *HOXC8* expression that could be possibly
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41 promoting melanocyte proliferation.
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50 In summary, we analysed the potential implications of 3'UTR polymorphisms in
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52 pigmentation, sensitivity to sunlight and skin cancer. A plausible cause of the action of
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54 these 3'UTR SNPs in the appearance of different sun-related benign pigmented skin
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56 lesions might be the differential gene expression attained by disrupting putative
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3 miRNA-binding sites. Specifically, we detected two potential associations with well-
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5 recognised skin cancer risk traits that modify miRNA-mRNA interactions: rs2325814 in
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7 the 3'UTR of the *MLPH* gene and rs752107 in the 3'UTR of the *WNT3A* gene. Future
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9 functional studies will be needed to determine the exact implications of these
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11 polymorphisms. In addition, we detected five genes that might contribute to
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13 pigmentation variation in our population. The fact that *MLPH*, *LYST* and *CLIP1*
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15 functions have been related to intracellular membrane trafficking and pigment disorders
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17 reinforces the need to explore more deeply the role of melanosome transport pathways
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19 in pigmentation and tanning ability. Similarly, the study of genes that are at least
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21 partially involved in melanocyte proliferation and differentiation, such as *WNT3A*,
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23 *KCNMA1*, *KIT* and *HOXC8*, may allow for the detection of novel low-penetrance genes
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25 involved in human pigmentation and in susceptibility to skin cancer.
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BH performed the research; BH, MI-V and MP-C analysed the data; MM-G, CG-F, GR and CM-C contributed essential samples; BH, MP-C, MI-V, GR and CM-C wrote the paper; GR and CM-C conceived and designed the research study; all authors reviewed the manuscript.

CONFLICTS OF INTEREST

The authors state that there are no conflicts of interest to declare.

REFERENCES

- 1 Usher-Smith J A, Emery J, Kassianos A P *et al.* Risk prediction models for melanoma: a systematic review. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 1450–1463.
- 2 Scherer D, Kumar R. Genetics of pigmentation in skin cancer--a review. *Mutat Res* 2010; **705**: 141–153.
- 3 Sulem P, Gudbjartsson D F, Stacey S N *et al.* Genetic determinants of hair, eye and skin pigmentation in Europeans. *Nat Genet* 2007; **39**: 1443–1452.
- 4 Liu F, Wen B, Kayser M. Colorful DNA polymorphisms in humans. *Semin Cell Dev Biol* 2013; **24**: 562–575.
- 5 Fernandez L p., Milne R I., Pita G *et al.* SLC45A2: a novel malignant melanoma-associated gene. *Hum Mutat* 2008; **29**: 1161–1167.
- 6 Ibarrola-Villava M, Fernandez L P, Pita G *et al.* Genetic analysis of three important genes in pigmentation and melanoma susceptibility: CDKN2A, MC1R and HERC2/OCA2. *Exp Dermatol* 2010; **19**: 836–844.
- 7 Ibarrola-Villava M, Fernandez L P, Alonso S *et al.* A Customized Pigmentation SNP Array Identifies a Novel SNP Associated with Melanoma Predisposition in the SLC45A2 Gene. *PLoS ONE* 2011; **6**: e19271.
- 8 Ibarrola-Villava M, Kumar R, Nagore E *et al.* Genes involved in the WNT and vesicular trafficking pathways are associated with melanoma predisposition. *Int J Cancer* 2015; **136**: 2109–2119.
- 9 Peña-Chilet M, Blanquer-Maceiras M, Ibarrola-Villava M *et al.* Genetic variants in PARP1 (rs3219090) and IRF4 (rs12203592) genes associated with melanoma susceptibility in a Spanish population. *BMC Cancer* 2013; **13**: 160.
- 10 Duffy D L, Zhao Z Z, Sturm R A *et al.* Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma. *J Invest Dermatol* 2010; **130**: 520–528.
- 11 Fernandez L P, Milne R L, Pita G *et al.* Pigmentation-related genes and their implication in malignant melanoma susceptibility. *Exp Dermatol* 2009; **18**: 634–642.
- 12 Bataille V. Genetic epidemiology of melanoma. *Eur J Cancer* 2003; **39**: 1341–1347.
- 13 Narayanan D L, Saladi R N, Fox J L. Review: Ultraviolet radiation and skin cancer. *Int J Dermatol* 2010; **49**: 978–986.
- 14 Gilchrist B A, Eller M S. DNA photodamage stimulates melanogenesis and other photoprotective responses. *J Invest Dermatol* 1999; **4**: 35–40.
- 15 Khabar K S A. The AU-rich transcriptome: more than interferons and cytokines, and its role in disease. *J Interferon Cytokine Res* 2005; **25**: 1–10.

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2
3 **16** Musunuru K, Darnell R B. Paraneoplastic neurologic disease antigens: RNA-binding
4 proteins and signaling proteins in neuronal degeneration. *Annu Rev Neurosci* 2001; **24**:
5 239–262.
- 6
7 **17** Vislovukh A, Vargas T R, Poleskaya A *et al.* Role of 3'-untranslated region translational
8 control in cancer development, diagnostics and treatment. *World J Biol Chem* 2014; **5**: 40–
9 57.
- 10
11 **18** López de Silanes I, Quesada M P, Esteller M. Aberrant regulation of messenger RNA 3'-
12 untranslated region in human cancer. *Cell Oncol* 2007; **29**: 1–17.
- 13
14 **19** Filipowicz W, Bhattacharyya S N, Sonenberg N. Mechanisms of post-transcriptional
15 regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; **9**: 102–114.
- 16
17 **20** Przybyła A, Lamperska K, Mackiewicz A. Analysis of sequence variants in the 3'UTR of
18 CDKN2A gene in melanoma patients. *Contemp Oncol* 2015; **19**: 276–279.
- 19
20 **21** Chin L J, Ratner E, Leng S *et al.* A SNP in a let-7 microRNA complementary site in the KRAS
21 3' untranslated region increases non-small cell lung cancer risk. *Cancer Res* 2008; **68**:
22 8535–8540.
- 23
24 **22** Bhaumik P, Gopalakrishnan C, Kamaraj B *et al.* Single Nucleotide Polymorphisms in
25 MicroRNA Binding Sites: Implications in Colorectal Cancer, Single Nucleotide
26 Polymorphisms in MicroRNA Binding Sites: Implications in Colorectal Cancer. *Sci World J*
27 2014; : e547154.
- 28
29 **23** Ratner E, Lu L, Boeke M *et al.* A KRAS-variant in ovarian cancer acts as a genetic marker of
30 cancer risk. *Cancer Res* 2010; **70**: 6509–6515.
- 31
32 **24** Aitken J, Welch J, Duffy D *et al.* CDKN2A Variants in a Population-Based Sample of
33 Queensland Families With Melanoma. *J Natl Cancer Inst* 1999; **91**: 446–452.
- 34
35 **25** Sauroja I, Smeds J, Vlaykova T *et al.* Analysis of G1/S checkpoint regulators in metastatic
36 melanoma. *Genes Chromosomes Cancer* 2000; **28**: 404–414.
- 37
38 **26** Laayouni H, Calafell F, Bertranpetit J. A genome-wide survey does not show the genetic
39 distinctiveness of Basques. *Hum Genet* 2010; **127**: 455–458.
- 40
41 **27** Segura M F, Greenwald H S, Hanniford D *et al.* MicroRNA and cutaneous melanoma: from
42 discovery to prognosis and therapy. *Carcinogenesis* 2012; **33**: 1823–1832.
- 43
44 **28** Syed D N, Lall R K, Mukhtar H. MicroRNAs and photocarcinogenesis. *Photochem Photobiol*
45 2015; **91**: 173–187.
- 46
47 **29** Barrett J C, Fry B, Maller J *et al.* Haploview: analysis and visualization of LD and haplotype
48 maps. *Bioinformatics* 2005; **21**: 263–265.
- 49
50 **30** Liu C, Zhang F, Li T *et al.* MirSNP, a database of polymorphisms altering miRNA target sites,
51 identifies miRNA-related SNPs in GWAS SNPs and eQTLs. *BMC Genomics* 2012; **13**: 661.
- 52
53 **31** Gong J, Liu C, Liu W *et al.* An update of miRNASNP database for better SNP selection by
54 GWAS data, miRNA expression and online tools [Internet]. *Database* 2015; **2015**[cited
55 2016 Apr 21] DOI: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4397995/>
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- 32** Betel D, Wilson M, Gabow A *et al.* The microRNA.org resource: targets and expression. *Nucleic Acids Res* 2008; **36**: D149–D153.
- 33** Agarwal V, Bell G W, Nam J-W *et al.* Predicting effective microRNA target sites in mammalian mRNAs. *eLife* 2015; **4**: e05005.
- 34** Krüger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. *Nucleic Acids Res* 2006; **34**: W451–W454.
- 35** Marín R M, Vaniček J. Efficient use of accessibility in microRNA target prediction. *Nucleic Acids Res* 2011; **39**: 19–29.
- 36** Vlachos I S, Kostoulas N, Vergoulis T *et al.* DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. *Nucleic Acids Res* 2012; **40**: W498–W504.
- 37** Lonsdale J, Thomas J, Salvatore M *et al.* The Genotype-Tissue Expression (GTEx) project. *Nat Genet* 2013; **45**: 580–585.
- 38** Hernando B, Ibarrola-Villava M, Fernandez L P *et al.* Sex-specific genetic effects associated with pigmentation, sensitivity to sunlight, and melanoma in a population of Spanish origin. *Biol Sex Differ* 2016; **7**: 1–9.
- 39** Martinez-Cadenas C, Peña-Chilet M, Ibarrola-Villava M *et al.* Gender is a major factor explaining discrepancies in eye colour prediction based on HERC2/OCA2 genotype and the IrisPlex model. *Forensic Sci Int Genet* 2013; **7**: 453–460.
- 40** Martinez-Cadenas C, Peña-Chilet M, Llorca-Cardenosa M J *et al.* Gender and eye colour prediction discrepancies: A reply to criticisms. *Forensic Sci Int Genet* 2014; **9**: e7-9.
- 41** Mignone F, Gissi C, Liuni S *et al.* Untranslated regions of mRNAs. *Genome Biol* 2002; **3**: reviews0004.1-reviews0004.10.
- 42** Candille S I, Absher D M, Beleza S *et al.* Genome-wide association studies of quantitatively measured skin, hair, and eye pigmentation in four European populations. *PLoS One* 2012; **7**: e48294.
- 43** Roh M R, Eliades P, Gupta S *et al.* Cutaneous melanoma in women. *Int J Womens Dermatol* 2015; **1**: 21–25.
- 44** Matesic L E, Yip R, Reuss A E *et al.* Mutations in *Mlph*, encoding a member of the Rab effector family, cause the melanosome transport defects observed in leaden mice. *Proc Natl Acad Sci U S A* 2001; **98**: 10238–10243.
- 45** Westbroek W, Klar A, Cullinane A R *et al.* Cellular and clinical report of new Griscelli syndrome type III cases. *Pigment Cell Melanoma Res* 2012; **25**: 47–56.
- 46** Ménasché G, Ho C H, Sanal O *et al.* Griscelli syndrome restricted to hypopigmentation results from a melanophilin defect (GS3) or a MYO5A F-exon deletion (GS1). *J Clin Invest* 2003; **112**: 450–456.
- 47** Tomita Y, Suzuki T. Genetics of pigmentary disorders. *Am J Med Genet* 2004; **131C**: 75–81.

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- 48** Barón A E, Asdigian N L, Gonzalez V *et al.* Interactions between ultraviolet light and MC1R and OCA2 variants are determinants of childhood nevus and freckle phenotypes. *Cancer Epidemiol Biomarkers Prev* 2014; **23**: 2829–2839.
- 49** Dunn K J, Brady M, Ochsenbauer-Jambor C *et al.* WNT1 and WNT3a promote expansion of melanocytes through distinct modes of action. *Pigment Cell Res* 2005; **18**: 167–180.
- 50** Keller J J, Moon R T, Chien A J. Wnt and Related Signaling Pathways in Melanomagenesis. *Cancers* 2010; **2**: 1000–1012.
- 51** Yamada T, Hasegawa S, Inoue Y *et al.* Accelerated differentiation of melanocyte stem cells contributes to the formation of hyperpigmented maculae. *Exp Dermatol* 2014; **23**: 652–658.
- 52** Mueller D W, Bosserhoff A-K. MicroRNA miR-196a controls melanoma-associated genes by regulating HOX-C8 expression. *Int J Cancer* 2011; **129**: 1064–1074.
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Table 1. 3'UTR variants highly associated with phenotypic traits in the Spanish population (P -value < 0.01)

Trait	Gene	SNP rs#	Genotype	Protective phenotype N (%)	Risk phenotype N (%)	Inheritance mode	OR (95% CI)	P -value
Naevi	<i>MLPH</i>	rs2325813	TT	591 (82.3)	75 (69.4)	Additive	2.03 (1.36-3.02)	8.97E-04
			CT	121 (16.9)	29 (26.9)	0 / C / CC		
			CC	6 (0.8)	4 (3.7)			
Lentigines	<i>WNT3A</i>	rs752107	CC	196 (56.2)	216 (45.3)	Over-dominant	1.66 (1.25-2.21)	4.53E-04
			CT	118 (33.8)	218 (45.7)	CC+TT / CT		
			TT	35 (10.0)	43 (9.0)			
Lentigines	<i>LYST</i>	rs6696123	TT	100 (28.6)	182 (38.1)	Additive	0.73 (0.60-0.90)	2.56E-03
			CT	184 (52.6)	231 (48.3)	0 / C / CC		
			CC	66 (18.9)	65 (13.6)			
Lentigines	<i>HOXC8</i>	rs4142680	TT	138 (39.4)	160 (33.6)	Over-dominant	1.47 (1.11-1.94)	6.57E-03
			CT	143 (40.9)	240 (50.4)	TT+CC / CT		
			CC	69 (19.7)	76 (16.0)			
Hair colour	<i>CLIP1</i>	rs10270	GG	328 (46.1)	83 (56.8)	Over-dominant	0.55 (0.37-0.80)	1.44E-03
			AG	321 (45.1)	45 (30.8)	GG+AA / AG		
			AA	63 (8.8)	18 (12.3)			
Hair colour	<i>KCNMA1</i>	rs4980113	GG	182 (25.5)	47 (32.2)	Over-dominant	0.57 (0.40-0.83)	2.67E-03
			CG	377 (52.9)	57 (39.0)	GG+CC / CG		
			CC	154 (21.6)	42 (28.8)			
Eye colour	<i>KIT</i>	rs8022	GG	416 (73.5)	229 (80.9)	Over-dominant	0.62 (0.43-0.89)	8.88E-03
			GT	139 (24.6)	48 (17.0)	GG+TT / GT		
			TT	11 (1.9)	6 (2.1)			

SNP, single nucleotide polymorphism; N, number of individuals; %, percentage of individuals per group among the total; OR, odds ratio per minor allele; CI, confidence interval

Table 2. Candidate microRNAs predicted to bind to 3'UTR SNPs showing association with pigmentation traits, sensitivity to sunlight and melanoma susceptibility

Gene	3'UTR SNP rs#	Allele change	miRNA predicted to bind to the target site ¹	Effect on miRNA binding ²	Free energy of miRNA-mRNA binding for WT (kcal/mol) ³	Free energy of miRNA-mRNA binding for MA (kcal/mol) ³	Energy change (kcal/mol) ⁴
<i>DTNBP1</i>	rs742106	G=>A	hsa-miR-1293	decrease	-26.40	-23.80	-2.60
		G=>A	hsa-miR-4782-5p	create	0.00	-21.30	21.30
<i>E2F1</i>	rs3213180	C=>G	hsa-miR-1182	break	-31.30	0.00	-31.30
<i>FOXO3</i>	rs9400241	A=>C	hsa-miR-2115-5p	break	-28.40	0.00	-28.40
		A=>C	hsa-miR-22-3p	create	0.00	-24.10	24.10
<i>KIT</i>	rs8022	G=>T	hsa-miR-548as-3p	create	0.00	-20.80	20.80
<i>MLPH</i>	rs2325813	T=>C	hsa-miR-185-3p	enhance	-29.00	-31.70	2.70
		T=>C	hsa-miR-762	enhance	-28.80	-31.50	2.70
<i>MYO5A</i>	rs7176482	A=>G	hsa-miR-198	break	-25.70	0.00	-25.70
		A=>G	hsa-miR-525-5p	break	-21.90	0.00	-21.90
<i>SOX9</i>	rs1042667	A=>C	hsa-miR-1181	create	0.00	-23.60	23.60
<i>WNT3A</i>	rs752107	C=>T	hsa-miR-149-5p	decrease	-29.90	-27.60	-2.30
		C=>T	hsa-miR-892b	decrease	-30.50	-28.20	-2.30

SNP, single nucleotide polymorphism; 3'UTR, 3'untranslated region; WT, wild-type target allele; MA, minor allele target allele

¹ The prediction of miRNA-binding sites was performed using MirSNP and miRNASNP

² The effect of the SNP on miRNA binding was given by MirSNP. These effects can be classified following four categories: a) decrease – reduction of the binding efficacy, b) enhance – increase of the binding efficacy, c) break – disruption of the binding site, or d) create – creation of a new binding site.

³ The free energy value of miRNA-mRNA binding was obtained from miRNASNP

⁴ Energy change (kcal/mol) indicates difference in minimum free energy of binding before and after introduction of the minor allele

FIGURE LEGENDS

Figure 1. Manhattan plots display the significance of associated allelic effects ($-\log_{10}$ P -values) for each phenotypic trait. (a) naevus count, (b) solar lentigines, (c) childhood sunburns, (d) skin colour, (e) hair colour, and (f) eye colour. Each dot represents one of the 38 3'UTR SNPs genotyped. Black dots indicate SNPs with a significant fold change (P -values < 0.05). All rs numbers of polymorphisms highly associated with phenotypic traits are displayed next to the corresponding dot. All values displayed are from the most significant pattern of inheritance.

Figure 2. Heat map of selected miRNAs versus pathways. Darker colours represent higher significance. The attached dendrograms on both axes represent hierarchical clustering results for miRNAs (by exhibiting similar pathway targeting patterns) and pathways (by related miRNAs). Arrows indicate pathways involved in pigmentation and skin cancer.

SUPPLEMENTARY MATERIAL

Table S1. Classification of the Spanish individuals studied by age, sex and phenotype

Table S2. Minor allele frequencies in different European populations and in Spanish cases and controls

Table S3. Association analysis between SNPs and melanoma susceptibility in the Spanish population

Figure S1. Comparison of minor allele frequencies between our Spanish sample and two different European populations

Figure S2. Enriched KEGG pathways involved in pigmentation and skin cancer risk that are targeted by miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs

Figure S3. Box plot showing WNT3A expression according to SNP rs752107 genotype

Figure S4. Expression in different tissues of the four miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs

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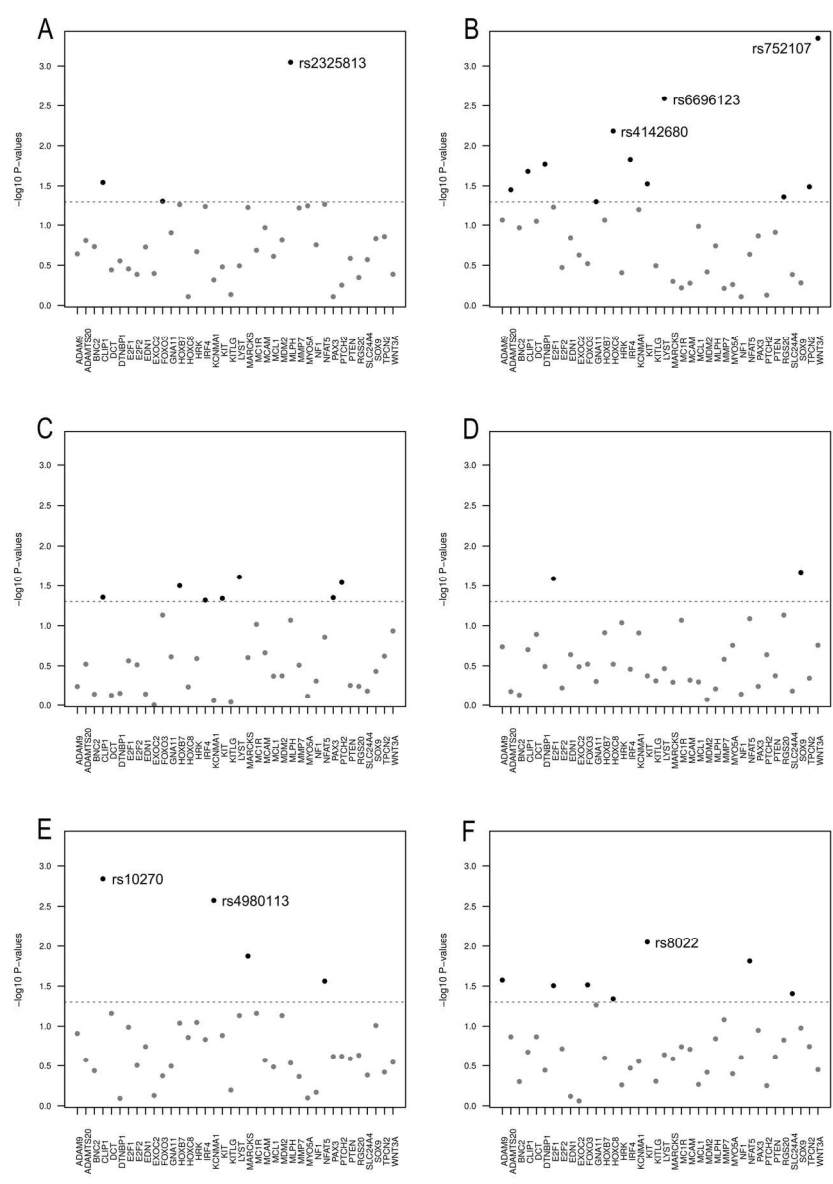


Figure 1. Manhattan plots display the significance of associated allelic effects (-log₁₀ P-values) for each phenotypic trait. (a) naevus count, (b) solar lentigines, (c) childhood sunburns, (d) skin colour, (e) hair colour, and (f) eye colour. Each dot represents one of the 38 3'UTR SNPs genotyped. Black dots indicate SNPs with a significant fold change (P-values < 0.05). All rs numbers of polymorphisms highly associated with phenotypic traits are displayed next to the corresponding dot. All values displayed are from the most significant pattern of inheritance.

Figure 1
296x419mm (150 x 150 DPI)

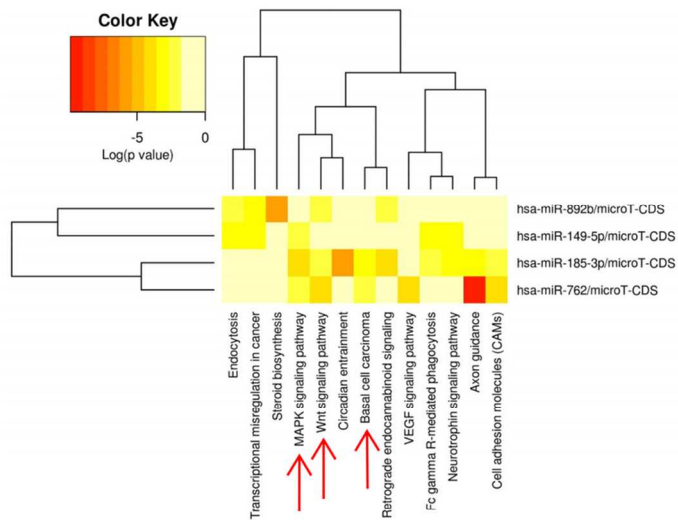


Figure 2. Heat map of selected miRNAs versus pathways. Darker colours represent higher significance. The attached dendrograms on both axes represent hierarchical clustering results for miRNAs (by exhibiting similar pathway targeting patterns) and pathways (by related miRNAs). Arrows indicate pathways involved in pigmentation and skin cancer.

Figure 2
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SUPPLEMENTARY MATERIAL

Table S1. Classification of the Spanish individuals studied by age, sex and phenotype..... 2

Table S2. Minor allele frequencies in different European populations, and in Spanish cases and controls..... 3

Table S3. Association analysis between SNPs and melanoma susceptibility in the Spanish population..... 4

For Review Only

Table S1. Classification of the Spanish individuals studied by age, sex and phenotype

		Controls (N=343)		Cases (N=526)		P-value ¹
		N	%	N	%	
Age	Mean ± SD	52.45 ± 15.95		52.63 ± 15.63		0.269
	< Mean	144	41.98	239	45.44	
	> Mean	143	41.69	280	53.23	
	Unknown	56	16.33	7	1.33	
Sex	Female	172	50.15	270	51.33	0.780
	Male	167	48.69	251	47.72	
	Unknown	4	1.17	5	0.95	
Eye Colour	Dark	239	69.68	337	64.07	0.102
	Light	101	29.45	183	34.79	
	Unknown	3	0.87	6	1.14	
Skin Colour	Dark	151	44.02	228	43.35	0.887
	Fair/Pale	185	53.94	287	54.56	
	Unknown	7	2.04	11	2.09	
Hair Colour	Dark	308	89.80	406	77.19	4.60E-06
	Light	33	9.62	113	21.48	
	Unknown	2	0.58	7	1.33	
Lentigines	No	180	52.48	170	32.32	1.76E-12
	Yes	131	38.19	347	65.97	
	Unknown	32	9.33	9	1.71	
Naevi number	≤ 50	271	79.01	447	84.98	0.395
	> 50	38	11.08	72	13.69	
	Unknown	34	9.91	7	1.33	
Childhood sunburns	No	220	64.14	170	32.32	6.64E-27
	Yes	91	26.53	347	65.97	
	Unknown	32	9.33	9	1.71	

N, number of individuals; %, percentage of individuals per group among the total

¹ Fisher's exact test P-value excluding unknown values at each specific analysis.

Significant results are presented in bold

Table S2. Minor allele frequencies in different European populations and Spanish cases and controls

Gene	SNP #rs	Chr	mA	Spanish population				CEU population		TSI population	
				HWE <i>P</i> -value	MAF Controls	MAF Cases	<i>P</i> -value ¹	MAF	<i>P</i> -value ²	MAF	<i>P</i> -value ²
<i>ADAM9</i>	rs4733967	8	T	0.364	0.234	0.209	0.234	0.146	0.017	0.262	0.164
<i>ADAMTS20</i>	rs6582463	15	C	0.411	0.270	0.317	0.038	0.389	0.155	0.243	0.006
<i>BNC2</i>	rs7035049	9	A	0.652	0.401	0.382	0.42	0.404	0.701	0.346	0.234
<i>CLIP1</i>	rs10270	12	A	0.896	0.290	0.320	0.917	0.318	0.691	0.322	0.759
<i>DCT</i>	rs17791924	14	G	0.326	0.449	0.447	0.961	0.465	0.652	0.439	0.827
<i>DTNBP1</i>	rs742106	6	A	1.000	0.359	0.389	0.222	0.354	0.536	0.299	0.024
<i>E2F1</i>	rs3213180	20	C	0.577	0.050	0.069	0.102	0.091	0.127	0.051	0.650
<i>E2F2</i>	rs3820028	4	G	0.829	0.469	0.485	0.554	0.520	0.293	0.477	1.000
<i>EDN1</i>	rs9296344	6	C	0.363	0.061	0.048	0.229	0.071	0.321	0.070	0.338
<i>EXOC2</i>	rs12952	6	G	0.414	0.273	0.292	0.384	0.273	0.803	0.383	0.004
<i>FOXO3/FKHRL2</i>	rs9400241	6	C	0.328	0.329	0.324	0.875	0.273	0.148	0.364	0.281
<i>GNA11</i>	rs397454	19	T	1.000	0.124	0.113	0.542	0.101	0.559	0.126	0.736
<i>HOXB7</i>	rs15689	17	G	1.000	0.284	0.237	0.028	0.247	0.863	0.210	0.156
<i>HOXC8</i>	rs4142680	15	C	0.658	0.426	0.395	0.21	0.394	0.190	0.425	0.482
<i>HRK</i>	rs10507275	12	A	0.102	0.159	0.163	0.841	0.136	0.412	0.131	0.275
<i>IRF4</i>	rs9391997	6	G	0.157	0.459	0.483	0.349	0.500	0.499	0.472	1.000
<i>KCNMA1</i>	rs4980113	10	C	0.589	0.496	0.533	0.128	0.490	0.454	0.490	0.169
<i>KIT</i>	rs8022	5	T	0.815	0.134	0.128	0.769	0.131	1.000	0.079	0.037
<i>KITLG</i>	rs995030	12	A	1.000	0.219	0.204	0.507	0.192	0.581	0.131	0.007
<i>LYST</i>	rs6696123	13	C	0.187	0.426	0.399	0.294	0.429	0.595	0.430	0.607
<i>MARCKS</i>	rs28558559	6	C	0.272	0.146	0.126	0.219	0.116	0.579	0.126	0.831
<i>MC1R</i>	rs3212369	16	G	1.000	0.187	0.195	0.064	0.146	0.040	0.206	0.929
<i>MCAM</i>	rs7914	11	A	1.000	0.224	0.240	0.451	0.263	0.378	0.201	0.302
<i>MCL1</i>	rs878471	22	G	0.269	0.424	0.446	0.373	0.424	0.762	0.421	0.662
<i>MDM2</i>	rs1690916	12	A	0.489	0.374	0.360	0.574	0.515	0.001	0.327	0.291
<i>MLPH</i>	rs2325813	1	C	1.000	0.093	0.110	0.295	0.131	0.225	0.117	0.555
<i>MMP7</i>	rs14983	11	A	0.640	0.224	0.227	0.525	0.212	0.593	0.168	0.037
<i>MYO5A</i>	rs7176482	9	G	0.573	0.400	0.412	0.88	0.343	0.108	0.472	0.056
<i>NF1</i>	rs1801052	17	G	0.063	0.254	0.246	0.734	0.308	0.085	0.262	0.677
<i>NFAT5</i>	rs7359387	16	G	1.000	0.150	0.137	0.513	0.101	0.391	0.140	0.143
<i>PAX3</i>	rs12620338	2	A	0.309	0.200	0.197	0.902	0.192	0.925	0.215	0.587
<i>PTCH2</i>	rs41269085	2	T	0.437	0.168	0.165	0.947	0.162	0.920	0.168	0.923
<i>PTEN</i>	rs701848	10	C	0.207	0.379	0.399	0.421	0.348	0.249	0.402	0.767
<i>RGS20</i>	rs72614663	8	G	0.269	0.140	0.143	0.888	0.101	0.127	0.187	0.082
<i>SLC24A4</i>	rs11160072	14	G	0.552	0.163	0.149	0.788	0.162	0.678	0.107	0.101
<i>SOX9</i>	rs1042667	11	C	0.653	0.394	0.386	0.801	0.394	0.939	0.336	0.137
<i>TPCN2</i>	rs1551306	11	A	0.829	0.465	0.448	0.49	0.465	0.821	0.542	0.017
<i>WNT3A</i>	rs752107	11	T	0.897	0.295	0.300	0.829	0.293	0.935	0.290	0.874

SNP, single nucleotide polymorphism; Chr, chromosome; mA, minor allele; MAF, minor allele frequency; CEU, Northern Europeans from Utah; TSI, Southern Europeans from Tuscany; HWE, Hardy-Weinberg equilibrium

¹ Fisher's exact test *P*-values for the comparison of minor allele frequencies between Spanish cases and controls

² Fisher's exact test *P*-values for the comparison of Spanish minor allele frequencies obtained from our sample to CEU and TSI frequencies

Significant results are presented in bold

Table S3. Association analysis between SNPs and melanoma susceptibility in the Spanish population

Gene	SNP rs#	mA	Non adjusted		Adjusted ¹	
			OR (%95 CI)	P-value	OR (%95 CI)	P-value
<i>ADAM9</i>	rs4733967	T	0.34 (0.17-0.70)	0.0024	0.26 (0.10-0.63)	0.0026
<i>ADAMTS20</i>	rs6582463	C	1.25 (1.01-1.54)	0.0390	1.16 (0.91-1.48)	0.2389
<i>BNC2</i>	rs7035049	A	0.77 (0.53-1.13)	0.1884	0.66 (0.42-1.04)	0.0732
<i>CLIP1</i>	rs10270	A	1.15 (0.93-1.42)	0.1857	1.18 (0.66-2.09)	0.5735
<i>DCT</i>	rs17791924	G	1.14 (0.86-1.49)	0.3618	0.74 (0.50-1.09)	0.1312
<i>DTNBP1</i>	rs742106	A	1.14 (0.93-1.39)	0.2126	1.16 (0.91-1.47)	0.2332
<i>E2F1</i>	rs3213180	C	1.42 (0.94-2.16)	0.0906	1.41 (0.86-2.32)	0.1652
<i>E2F2</i>	rs3820028	G	1.06 (0.88-1.29)	0.5283	0.99 (0.78-1.24)	0.9091
<i>EDN1</i>	rs9296344	C	0.78 (0.51-1.17)	0.2360	0.79 (0.46-1.36)	0.3961
<i>EXOC2</i>	rs12952	G	1.11 (0.89-1.38)	0.3580	1.11 (0.85-1.44)	0.4415
<i>FOXO3</i>	rs9400241	C	0.91 (0.60-1.40)	0.6783	0.84 (0.66-1.07)	0.1576
<i>GNA11</i>	rs397454	T	0.87 (0.62-1.22)	0.4240	0.68 (0.45-1.01)	0.0593
<i>HOXB7</i>	rs15689	G	0.78 (0.63-0.97)	0.0264	0.77 (0.59-1.00)	0.0483
<i>HOXC8</i>	rs4142680	C	0.83 (0.62-1.10)	0.1971	0.82 (0.65-1.04)	0.0968
<i>HRK</i>	rs10507275	A	0.44 (0.19-1.05)	0.0602	0.45 (0.18-1.12)	0.0826
<i>IRF4</i>	rs9391997	G	1.29 (0.92-1.80)	0.1422	0.67 (0.48-0.93)	0.0152
<i>KCNMA1</i>	rs4980113	C	0.86 (0.71-1.04)	0.1220	0.79 (0.62-1.00)	0.0462
<i>KIT</i>	rs8022	T	0.85 (0.62-1.19)	0.3474	1.63 (0.48-5.58)	0.4228
<i>KITLG</i>	rs995030	A	0.92 (0.72-1.16)	0.4719	0.84 (0.60-1.17)	0.3104
<i>LYST</i>	rs6696123	C	0.82 (0.61-1.09)	0.1655	0.85 (0.67-1.08)	0.1745
<i>MARCKS</i>	rs28558559	C	0.32 (0.11-0.93)	0.0300	0.23 (0.06-0.81)	0.0164
<i>MC1R</i>	rs3212369	G	1.06 (0.83-1.35)	0.6679	1.32 (0.59-2.95)	0.5016
<i>MCAM</i>	rs7914	A	1.14 (0.87-1.51)	0.3498	1.21 (0.86-1.69)	0.2695
<i>MCL1</i>	rs878471	G	1.20 (0.84-1.72)	0.3238	1.30 (0.85-2.00)	0.2261
<i>MDM2</i>	rs1690916	A	0.83 (0.56-1.24)	0.3688	0.90 (0.72-1.14)	0.4006
<i>MLPH</i>	rs2325813	C	1.20 (0.87-1.66)	0.2556	0.65 (0.15-2.85)	0.5742
<i>MMP7</i>	rs14983	A	1.37 (0.73-2.57)	0.3240	1.36 (0.64-2.88)	0.4216
<i>MYO5A</i>	rs7176482	G	1.13 (0.79-1.62)	0.5110	1.15 (0.75-1.75)	0.5168
<i>NF1</i>	rs1801052	G	0.92 (0.56-1.50)	0.7274	0.82 (0.45-1.50)	0.5239
<i>NFAT5</i>	rs7359387	G	0.87 (0.62-1.20)	0.3880	1.06 (0.72-1.55)	0.7821
<i>PAX3</i>	rs12620338	A	0.68 (0.35-1.34)	0.2656	0.47 (0.20-1.06)	0.0710
<i>PTCH2</i>	rs41269085	T	2.29 (1.00-5.39)	0.0421	0.66 (0.46-0.95)	0.0263
<i>PTEN</i>	rs701848	C	1.26 (0.96-1.65)	0.0998	1.04 (0.82-1.31)	0.7560
<i>RGS20</i>	rs72614663	G	2.15 (0.70-6.65)	0.1587	3.14 (0.79-12.46)	0.0818
<i>SLC24A4</i>	rs11160072	G	0.87 (0.65-1.17)	0.3649	0.86 (0.59-1.23)	0.4043
<i>SOX9</i>	rs1042667	C	0.96 (0.72-1.27)	0.7573	0.87 (0.69-1.10)	0.2360
<i>TPCN2</i>	rs1551306	A	0.86 (0.61-1.20)	0.3748	0.84 (0.59-1.20)	0.3419
<i>WNT3A</i>	rs752107	T	1.14 (0.71-1.82)	0.5913	0.72 (0.51-1.00)	0.0500

SNP, single nucleotide polymorphism; mA, minor allele; OR, odds ratio per minor allele; CI, confidence interval

Bold indicates significant P-values and their Odds Ratio according to the most significant model (dominant, over-dominant, recessive or additive)

¹ Adjusted for childhood sunburns, hair colour, lentiginos and sex, via multivariate logistic regression

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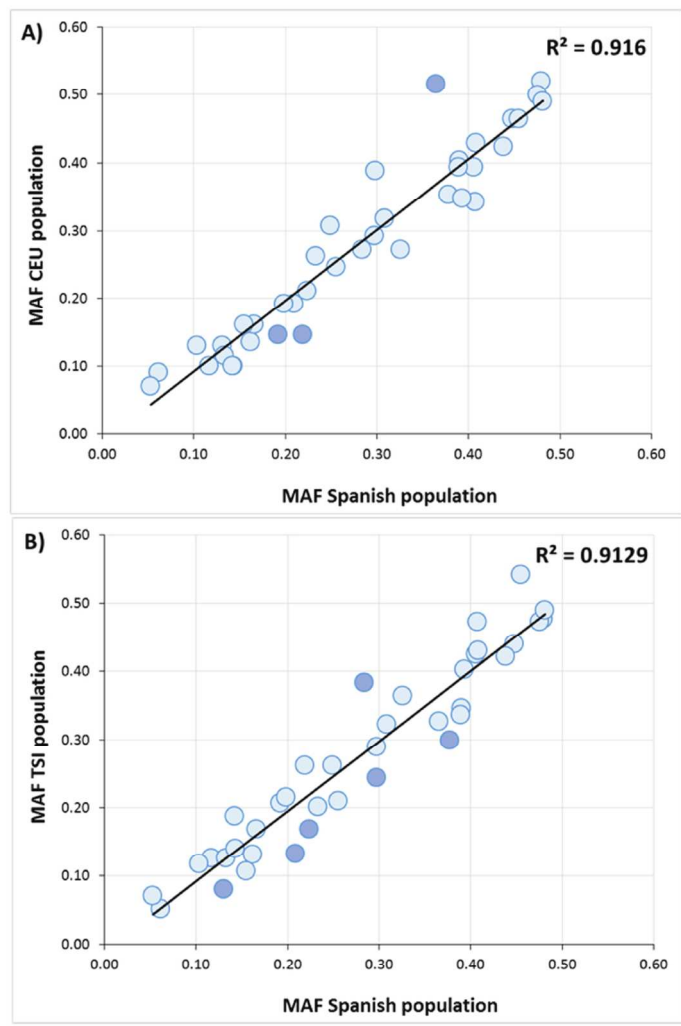


Figure S1. Comparison of minor allele frequencies between our Spanish sample and two different European populations. A) Northern Europeans from Utah (CEU), and B) Southern Europeans from Tuscany (TSI). Dark dots represent values that significantly differ from 1000 Genome Project data, when sample size was considered.

Figure S1
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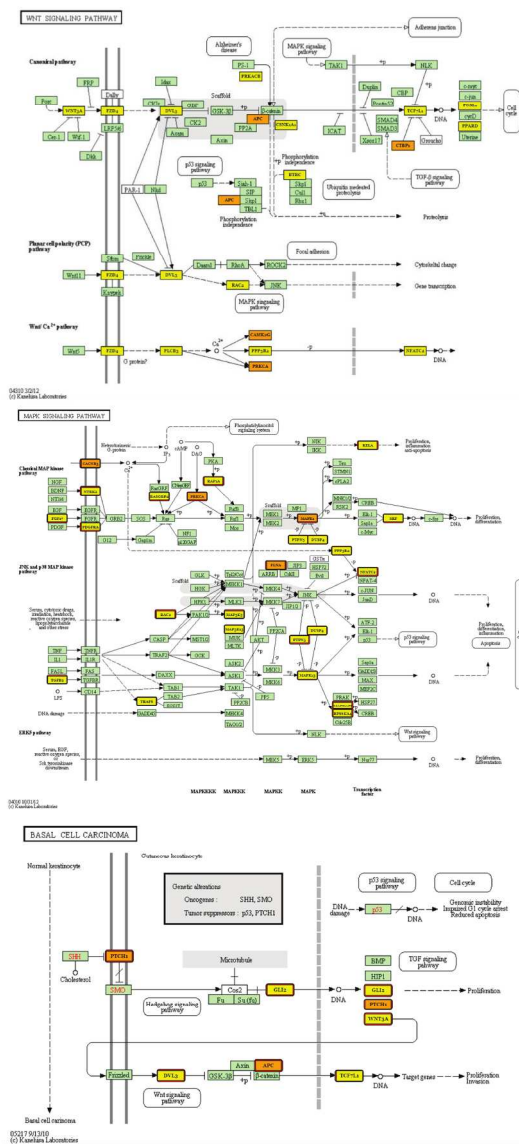


Figure S2. Enriched KEGG pathways involved in pigmentation and skin cancer that are targeted by miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs. Yellow denotes genes targeted by one miRNA of the list. Orange denotes genes targeted by more than one miRNA of the list.

Figure S2
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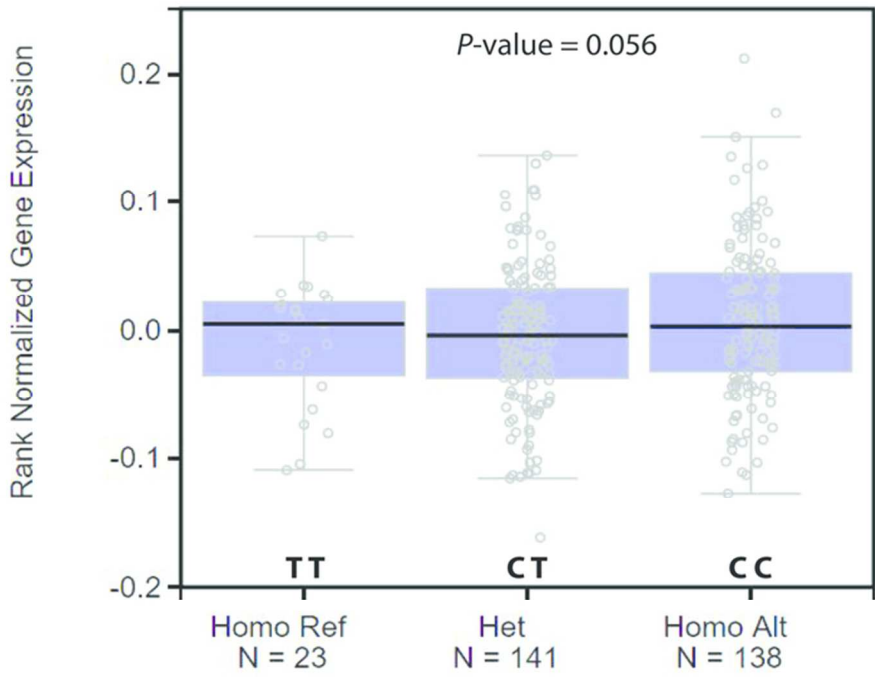


Figure S3. Box plot showing WNT3A expression according to SNP rs752107 genotype. T is the minor allele. Data taken from GTEx Portal. Figure S3 135x96mm (150 x 150 DPI)

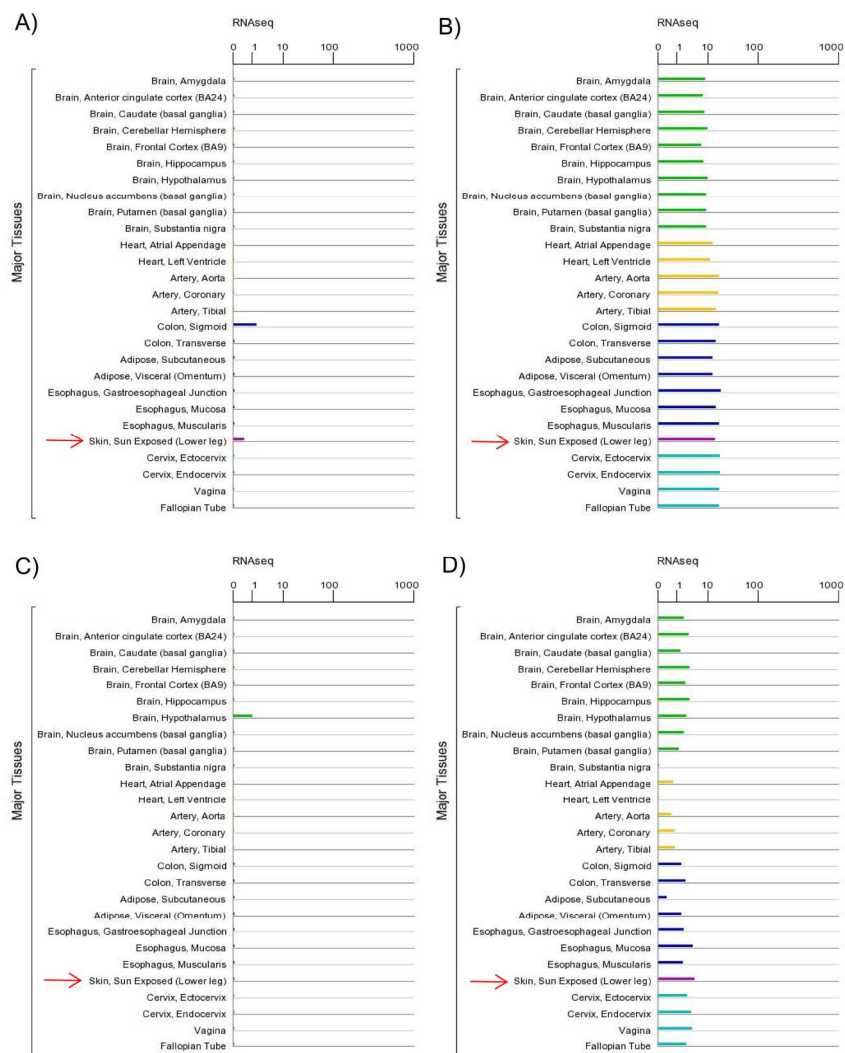


Figure S4. Expression in different tissues of the four miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs. A) hsa-miR-185-3p; B) hsa-miR-762; C) hsa-miR-892b; D) hsa-miR-149-5p. Red arrow indicates sun-exposed skin tissue. Data taken from GTEx Portal, and images downloaded from GeneCards webpage.

Figure S4
297x420mm (150 x 150 DPI)