Experimental Dermatology ADF

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

Journal:	*Experimental Dermatology*
Manuscript ID	EXD-16-0510.R1
Manuscript Type:	Regular Article
Date Submitted by the Author:	23-Jan-2017
Complete List of Authors:	Hernando, Barbara; Jaume I University of Castellon, Department of Medicine Peña-Chilet, Maria; Health Research Institute INCLIVA, Medical Oncology and Haematology Unit Ibarrola-Villava, Maider; FIHCUV, Martin-Gonzalez, Manuel; Ramon y Cajal Hospital, Dermatology Gomez-Fernandez, Cristina; La Paz University Hospital, Dermatology Ribas, Gloria; FIHCUV, Martinez-Cadenas, Conrado; Jaume I University of Castellon, Department of Medicine
Keywords:	3' untranslated region, microRNA, SNP, Naevus, Solar lentigines

TITLE PAGE

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

Authors: Barbara Hernando[1], Maria Peña-Chilet[2], Maider Ibarrola-Villava[2], Manuel Martin-Gonzalez[3], Cristina Gomez-Fernandez[4], Gloria Ribas[2]*, Conrado Martinez-Cadenas[1]*

[1]Department of Medicine, Jaume I University of Castellon, Castellon, Spain

[2]Department of Medical Oncology, Biomedical Research Institute - INCLIVA, Valencia, Spain; and Network Centre for Biomedical Cancer Research (CIBERONC)

[3]Department of Dermatology, Ramon y Cajal Hospital, Madrid, Spain

[4]Department of Dermatology, La Paz Hospital, Madrid, Spain

To whom correspondence should be addressed:

*Conrado Martinez-Cadenas. Department of Medicine, Jaume I University of Castellon, Av. Sos Baynat s/n, 12071, Castellon, Spain.

Telephone number: +34 964387607

FAX: +34 964387750

Email: ccadenas@uji.es

*Gloria Ribas. Medical Oncology Unit, Biomedical Research Institute – INCLIVA, University of Valencia, Av. Menendez Pelayo 4 accesorio, 46010, Valencia, Spain.

Telephone number: +34 963864402

FAX: +34 963987860

Email: gribas@incliva.es

Short title: Implication of 3'UTR SNPs in pigmentation

Manuscript word count: 3,469

Table count: 2

Figure count: 2

KEY WORDS:

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

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'-untrastaled 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

regulators by repressing mRNA translation or by destabilizing/degrading mRNAs in the cytoplasm, via perfect or imperfect binding to their complementary base pair sequence in the 3'untranslated region (3'UTR) of the mRNA target (19). Therefore, the 3'UTR region is emerging as critically important in regulating gene expression (17), and polymorphisms in the miRNA-binding sites of the 3'UTR of genes may alter the binding efficiency and miRNA-mRNA gene expression regulation. In support of this hypothesis, recent studies have identified variants in the 3'UTR of genes that increase the susceptibility for melanoma (20), lung (21), colorectal (22) and ovarian cancer (23) by affecting the ability of miRNAs to bind. In particular, two sequence changes in the 3'UTR of the *CDKN2A* gene have been significantly correlated with melanoma risk (24), but also with a shorter progression time from primary to metastatic melanoma (25).

Here, we hypothesise that differences identified in nucleotide composition of 3'UTRs SNP sites of genes previously associated with pigmentation and/or skin cancer can be a reason for causing differences in human pigmentation, sensitivity to sunlight, and thus in melanoma susceptibility. In the current study, we describe the role of 38 different 3'UTR polymorphisms from 38 different candidate pigmentation and melanoma susceptibility genes in a population of Spanish origin. Additionally, we use miRNA binding prediction tools to identify variants affecting putative miRNA-binding sites, and to predict their impact on miRNA-mRNA interaction.

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 Marañon 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 Marañon 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

or Quant-iT PicoGreen dsDNA Reagent (Invitrogen, Eugene, OR, USA). Genomic DNA was amplified using the GenomiPhi DNA Amplification Kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Samples were diluted to a final solution of 50 ng/ml and stored at -20ºC.

The study was approved by the Ethics Committee of the Biomedical Research Institute - INCLIVA (Valencia, Spain). Written informed consent was obtained from all participants.

SNP Selection

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

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

binding energy changes in the interaction of miRNAs with the wild-type target sequence compared to the derived 3'UTR sequence. Only the duplexes with hybridization free energy ≤ −20 kcal/mol were chosen (35).

Identification of validated pathways targeted by *in silico* predicted microRNAs

In order to further investigate the miRNAs predicted to bind to the two 3'UTR SNPs highly associated with phenotypic traits (hsa-miR-149-5p, hsa-miR-892b, hsa-miR-185-3p and hsa-miR-762), we used DIANA-miRPath v2.0 (http://www.microrna.gr/miRPathv2) to identify the miRNA targeted pathways. The output provides intuitive heat maps and enriched KEGG pathway visualizations for easier inspection (36).

***In silico* quantitative analysis of tissue-specific expression**

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

Statistical Analysis

For each polymorphism studied, Fisher's exact test was used both to check for deviations from Hardy-Weinberg equilibrium (HWE) among controls and to compare differences in allele counts between cases and controls. In order to account for differences between populations, allele frequencies of our Spanish population were compared to those of both a North European population (CEU) and a Southern one from Tuscany (TSI) using Fisher's exact test.

Associations between the genotyped genes and various pigmentation characteristics were assessed via logistic regression. Association analyses were done for all samples pooled, with eye colour (blue/green *versus* brown/black), hair colour (brown/black *versus* blond/red), skin colour (fair *versus* brown), number of naevi (≥50 *versus* <50), presence of lentigines (yes *versus* no), and childhood sunburns (yes *versus* no) as the outcome variables. This was performed for four different patterns of inheritance: dominant (major homozygotes *versus* heterozygotes plus minor homozygotes), over-dominant (major homozygotes plus minor homozygotes *versus* heterozygotes), recessive (major homozygotes plus heterozygotes *versus* minor homozygotes), and additive (counting additively for each copy of minor allele). Genotype-related odds ratios (ORs), their corresponding 95% confidence intervals (CIs) and associated *P*-values were estimated. Association analyses with phenotypic traits were adjusted by sex, since sex-differentiated allelic effects for pigmentation traits, sensitivity to sunlight and melanoma have been previously shown (38–40).

In order to assess associations among genotypes and melanoma risk, genotype-related ORs, their corresponding 95% CIs and associated *P*-values were estimated via unconditional logistic regression. Multivariate logistic regression was also carried out combining sex and all significant risk factors revealed in Table S1. This was also done for all four patterns of inheritance.

Statistical analyses and plots were conducted using R statistical framework (http://www.R-project.org). All genetic analyses were performed estimating the effect of the minor allele in the Spanish population. Unknown and missing values were

excluded at each specific analysis. All *P*-values were two-sided, and those less than 0.05 were considered statistically significant.

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 (*DTNBP1*), 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

presented the most potential allelic effects for phenotypic traits in the Spanish population (*P*-value < 0.01). The rs2325813 SNP, located in the *MLPH* gene, was correlated with the presence of more than 50 naevi (P=8.97x10^{-4}). Two SNPs, *HOXC8* rs4142680 and *WNT3A* rs752107, correlated with the presence of lentigines (P=6.57x10^{-3} and P=4.53x10^{-4}, respectively); while *LYST* rs6696123 showed association with an absence of lentigines (P=2.56x10^{-3}). Two more SNPs, rs10270 in the *CLIP1* gene and rs4980113 in the *KCNMA1* gene, were associated with dark hair colour (P=1.44x10^{-3} and P=2.67x10^{-3}, respectively). Finally, *KIT* rs8022 was correlated with light eye colour (P=8.88x10^{-3}) (Table 1).

Likewise, we carried out an association analysis between genotypes and melanoma risk. Five SNPs showed a tendency to correlate with melanoma susceptibility in the Spanish population. Among them, three SNPs (*HOXB7* rs1589, *MARCKS* rs28558559 and *ADAM9* rs4733967) showed a melanoma protective effect (OR<1). On the other hand, *PTCH2* rs41269085 and *ADAMTS20* rs6582463 displayed a melanoma risk effect (OR>1) (Table S3).

For the association results to be adjusted by the confounding variables, we performed a multivariate analysis including phenotypic risk factors (hair colour, solar lentigines and the presence of childhood sunburn) and sex as covariates. Polymorphisms located in *HOXB7, MARCKS, ADAM9* and *PTCH2* remained significant after the adjustment, with no substantial changes in allelic effects, confirming the putative role of these variants in melanoma susceptibility. Additionally, *KCNMA1* rs4980113 and *IRF4* rs9391997 were marginally associated with melanoma protection (Table S3).

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=4.24x10^{-5}), "MAPK signalling pathway-hsa04010" (P=1.07x10^{-4}) and "Basal cell carcinoma-

hsa05217" (P=2.52x10^{-3}). Figure S2 represents in detail these three KEGG pathways, highlighting the specific target genes of the selected miRNAs.

We further evaluated the association between the genotype of both *MLPH* rs2325813 and *WNT3A* rs752107 and the gene expression levels in sun-exposed skin by using the GTEx portal. Individuals carrying rs752107*T allele, which was predicted to decrease miRNA-mRNA binding efficiency, seem to present increased expression of *WNT3A* in sun-exposed tissue (Figure S3). No changes in *MLPH* expression regarding genotype were observed.

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

member of the exophilin subfamily of Rab effector proteins known as melanophilin, which acts as a link between the small GTPase melanosome-bound RAB27A and the actin-associated motor protein MYO5A (44). This protein complex plays a crucial role in the melanosome motility in melanocytes, and aberrations in any of the complex components has been shown to result in perinuclear localization of melanosomes and therefore failure to transfer mature melanosomes to adjacent keratinocytes, eventually causing hypopigmentation (45). Human individuals homozygous for a pathogenic *MLPH* mutation (c.102C>T; p.R35W) display Griscelli syndrome type 3, a pigmentary disorder characterized by a hypopigmented phenotype (45–47). The naevus-associated SNP in this work, rs2325813, is predicted to disrupt a binding site of two miRNAs (hsa-miR-185-3p and hsa-miR-762). The presence of the minor allele in the target sequence enhances miRNA binding efficiency, repressing mRNA translation of *MLPH*, and ultimately limiting the formation of RAB27A/Melanophilin/Myosin-5a complex. Thus, reduction of *MLPH* gene expression may cause an abnormal accumulation of mature melanosomes around the nucleus of melanocytes, resulting in light pigmentation and poor tolerance to sunlight. Interestingly, our results are consistent with the well-known correlation between melanocytic naevus number, a main risk-prediction factor for melanoma incidence, and the propensity to burn, rather than tan, of light-skinned individuals (48). Therefore, genes implicated in functions related with melanosome trafficking, especially the RAB27A/Melanophilin/Myosin-5a membrane transport pathway, would be relevant candidates for additional investigation in further pigmentation and melanoma studies.

WNT/β-catenin signalling has a pivotal role in the formation of melanocytes, since this pathway has been implicated in promoting the development of neural crest-derived

melanocytes (49,50). In humans, the WNT pathway is significantly up-regulated in solar lentigines, suggesting that overstimulation of melanocytes proliferation and differentiation play a crucial role in the pathogenic mechanism of solar lentigines (51). Interestingly, in this work we identify a polymorphism, rs7352107, located in the 3'UTR of the *WNT3A* gene that is strongly associated with the presence of solar lentigines. *WNT3A* (OMIM #606359) encodes a WNT ligand that acts through the WNT/β-catenin pathway promoting melanocyte differentiation, and may promote melanoma differentiation as well (49). Furthermore, the minor allele of rs7352107 is predicted to decrease the binding efficiency to the 3'UTR gene region of two microRNAs (hsa-miR-149-5p and hsa-miR-892b), leading to a weaker miRNA-mRNA interaction and therefore a higher level of secreted WNT3A ligand. This probably enhances the activation of the WNT/β-catenin signalling and subsequently the proliferation of melanocytes. These observations, together with the results from Yamada and cols. (2014) (51), suggest that abnormal regulation of melanogenesis via gene expression changes is expected to be involved in several pigmentary disorders and in melanoma risk phenotypes. Thus, studies focusing on the regulation of WNT/β-catenin signalling could potentially clarify the causal mechanisms of pathogenic hyperpigmentation and hypopigmentation conditions.

The miRNAs predicted to bind to *MLPH* rs2325813 (hsa-miR-185-3p and hsa-miR-762) and to *WNT3A* rs7352107 (hsa-miR-149-5p and hsa-miR-892b) seem to target genes involved in pigmentation mechanisms and skin cancer. Remarkably, out of all significant pathways, "Wnt signalling pathway" and "MAPK signalling pathway" were the only ones targeted by three of the four miRNAs. Furthermore, "Basal cell carcinoma" pathway was also targeted by hsa-miR-185-3p and hsa-miR-762. These

observations may corroborate the importance of these miRNAs in both human pigmentation and skin cancer pathways. Based on GTEx project data, genes encoding for these miRNAs, except for hsa-miR-892b, are expressed in sun-exposed skin (Figure S3), confirming the expression of these miRNAs in skin tissue, and suggesting a possible role of these miRNAs in skin regulation and function.

Additionally, five polymorphisms displayed a notable statistical association with phenotypic characteristics. Among these SNPs, we would like to highlight that the variant rs4142680, located in the 3'UTR of *HOXC8*, displays an interesting predisposition tendency towards sun-damaged phenotypes. The *HOXC8* gene has been shown to be massively up-regulated in melanoma cancerous cells as a consequence of diminished miR-196a levels, leading to an aggressive melanoma phenotype via the overexpression of several tumorigenic target genes (52). Curiously, the web-based miRNA binding prediction analysis in this work showed an intermediate free energy (-16.60 kcal/mol) for binding hsa-miR-4509 to the 3'UTR sequence containing the rs4142680*T allele, and predicted that presence of the C allele may break the putative binding site. Thus, the association between rs4142680*C and the presence of solar lentigines may be the result of increased *HOXC8* expression that could be possibly promoting melanocyte proliferation.

In summary, we analysed the potential implications of 3'UTR polymorphisms in pigmentation, sensitivity to sunlight and skin cancer. A plausible cause of the action of these 3'UTR SNPs in the appearance of different sun-related benign pigmented skin lesions might be the differential gene expression attained by disrupting putative

miRNA-binding sites. Specifically, we detected two potential associations with well-recognised skin cancer risk traits that modify miRNA-mRNA interactions: rs2325814 in the 3'UTR of the *MLPH* gene and rs752107 in the 3'UTR of the *WNT3A* gene. Future functional studies will be needed to determine the exact implications of these polymorphisms. In addition, we detected five genes that might contribute to pigmentation variation in our population. The fact that *MLPH, LYST* and *CLIP1* functions have been related to intracellular membrane trafficking and pigment disorders reinforces the need to explore more deeply the role of melanosome transport pathways in pigmentation and tanning ability. Similarity, the study of genes that are at least partially involved in melanocyte proliferation and differentiation, such as *WNT3A, KCNMA1, KIT* and *HOXC8*, may allow for the detection of novel low-penetrance genes involved in human pigmentation and in susceptibility to skin cancer.

ACKNOWLEDGEMENTS

We especially thank all the participants for giving their consent to take part in this study, and the medical staff from the different hospitals for collecting the samples. We would like to thank Maria Torres for her expert technical support with genotyping, carried out at the Spanish National Genotyping Centre in Santiago de Compostela (CEGEN-PRB2-ISCIII), and supported by grant PT13/0001, ISCIII-SGEFI/FEDER.

BH is funded by the Jaume I University of Castellon under a Predoctoral Research contract (No. 15721). MI-V is funded by the Institute Carlos III of the Ministry of Health under a Sara Borrell contract (No. CD15/00153). GR is funded by the Institute Carlos III of the Ministry of Health under a Miquel Servet II contract (No. CPII14-00013). CIBERONC is an initiative of the Institute Carlos III of the Ministry of Health.

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 l., 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 Gilchrest B A, Eller M S. DNA photodamage stimulates melanogenesis and other photoprotective responses. J Investig 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.

16 Musunuru K, Darnell R B. Paraneoplastic neurologic disease antigens: RNA-binding proteins and signaling proteins in neuronal degeneration. Annu Rev Neurosci 2001: **24**: 239–262.

17 Vislovukh A, Vargas T R, Polesskaya A *et al.* Role of 3'-untranslated region translational control in cancer development, diagnostics and treatment. World J Biol Chem 2014: **5**: 40–57.

18 López de Silanes I, Quesada M P, Esteller M. Aberrant regulation of messenger RNA 3'-untranslated region in human cancer. Cell Oncol 2007: **29**: 1–17.

19 Filipowicz W, Bhattacharyya S N, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008: **9**: 102–114.

20 Przybyła A, Lamperska K, Mackiewicz A. Analysis of sequence variants in the 3'UTR of CDKN2A gene in melanoma patients. Contemp Oncol 2015: **19**: 276–279.

21 Chin L J, Ratner E, Leng S *et al.* A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. Cancer Res 2008: **68**: 8535–8540.

22 Bhaumik P, Gopalakrishnan C, Kamaraj B *et al.* Single Nucleotide Polymorphisms in MicroRNA Binding Sites: Implications in Colorectal Cancer, Single Nucleotide Polymorphisms in MicroRNA Binding Sites: Implications in Colorectal Cancer. Sci World J 2014: : e547154.

23 Ratner E, Lu L, Boeke M *et al.* A KRAS-variant in ovarian cancer acts as a genetic marker of cancer risk. Cancer Res 2010: **70**: 6509–6515.

24 Aitken J, Welch J, Duffy D *et al.* CDKN2A Variants in a Population-Based Sample of Queensland Families With Melanoma. J Natl Cancer Inst 1999: **91**: 446–452.

25 Sauroja I, Smeds J, Vlaykova T *et al.* Analysis of G1/S checkpoint regulators in metastatic melanoma. Genes Chromosomes Cancer 2000: **28**: 404–414.

26 Laayouni H, Calafell F, Bertranpetit J. A genome-wide survey does not show the genetic distinctiveness of Basques. Hum Genet 2010: **127**: 455–458.

27 Segura M F, Greenwald H S, Hanniford D *et al.* MicroRNA and cutaneous melanoma: from discovery to prognosis and therapy. Carcinogenesis 2012: **33**: 1823–1832.

28 Syed D N, Lall R K, Mukhtar H. MicroRNAs and photocarcinogenesis. Photochem Photobiol 2015: **91**: 173–187.

29 Barrett J C, Fry B, Maller J *et al.* Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005: **21**: 263–265.

30 Liu C, Zhang F, Li T *et al.* MirSNP, a database of polymorphisms altering miRNA target sites, identifies miRNA-related SNPs in GWAS SNPs and eQTLs. BMC Genomics 2012: **13**: 661.

31 Gong J, Liu C, Liu W *et al.* An update of miRNASNP database for better SNP selection by GWAS data, miRNA expression and online tools [Internet]. Database 2015: **2015**[cited 2016 Apr 21] DOI: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4397995/

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, Vaníček 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-Cardeñosa 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.

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.

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	*MLPH*	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	*WNT3A*	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	*LYST*	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	*HOXC8*	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	*CLIP1*	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	*KCNMA1*	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	*KIT*	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]
DTNBP1	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
E2F1	rs3213180	C==>G	hsa-miR-1182	break	-31.30	0.00	-31.30
FOXO3	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
KIT	rs8022	G==>T	hsa-miR-548as-3p	create	0.00	-20.80	20.80
MLPH	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
MYO5A	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
SOX9	rs1042667	A==>C	hsa-miR-1181	create	0.00	-23.60	23.60
WNT3A	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 (-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 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

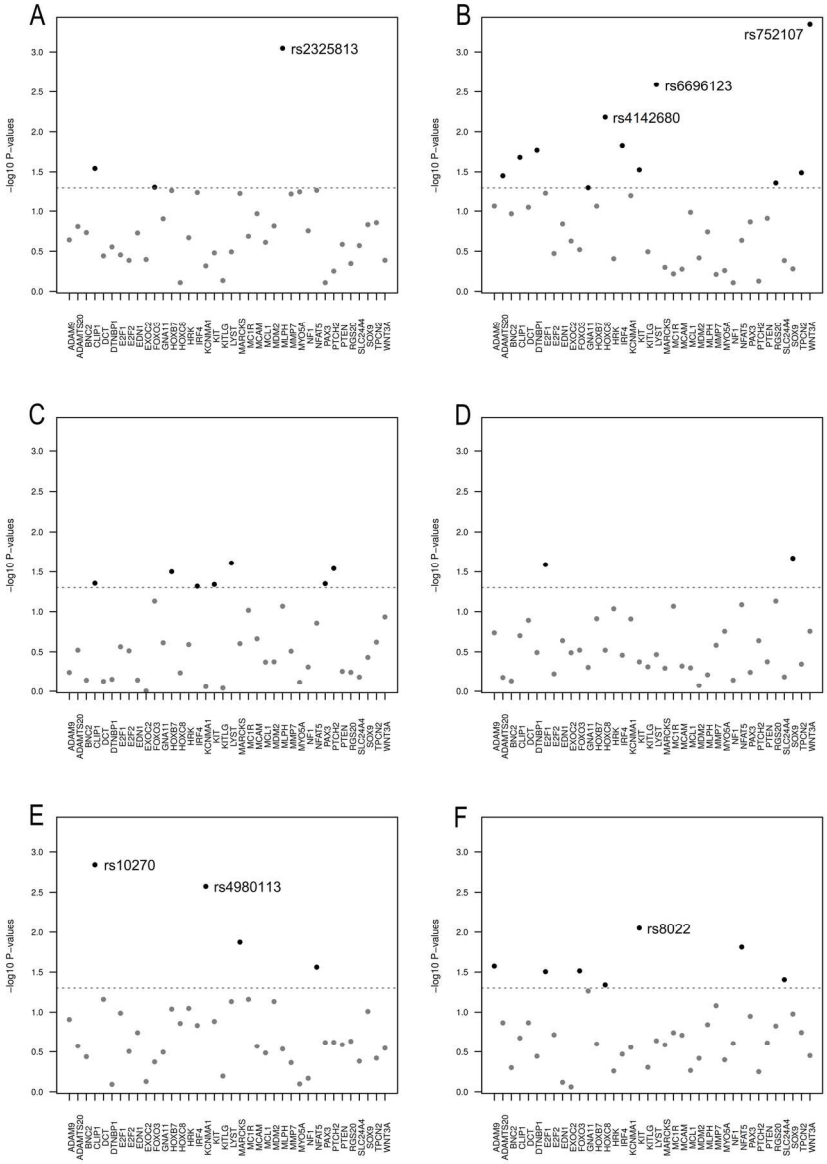

Figure 1. Manhattan plots display the significance of associated allelic effects (-log10 P-values) for each phenotypic trait. (a) naevus count, (b) solar lentigines, (c) childhood sunburns, (d) skin colour, (e) hair colour, and (f) eye colour. Each dot represents one of the 38 3'UTR SNPs genotyped. Black 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)

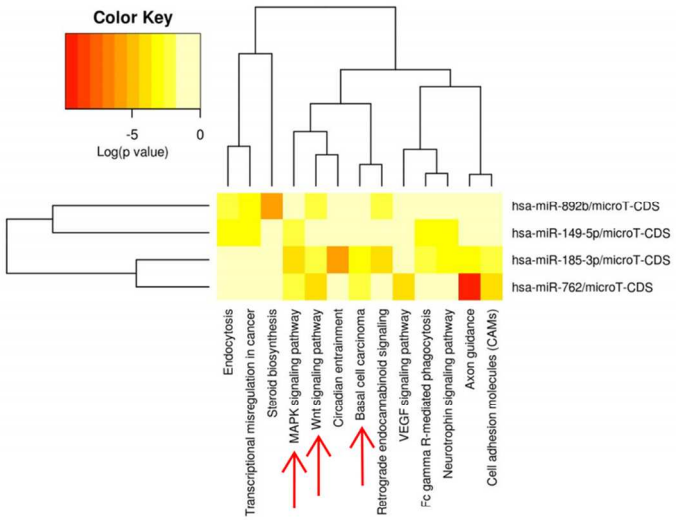

Figure 2. Heat map of selected miRNAs versus pathways. Darker colours represent higher significance. The attached dendrograms on both axes represent hierarchical clustering results for miRNAs (by exhibiting similar pathway targeting patterns) and pathways (by related miRNAs). Arrows indicate pathways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

Figure 2
209x148mm (150 x 150 DPI)

SUPPLEMENTARY MATERIAL

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

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

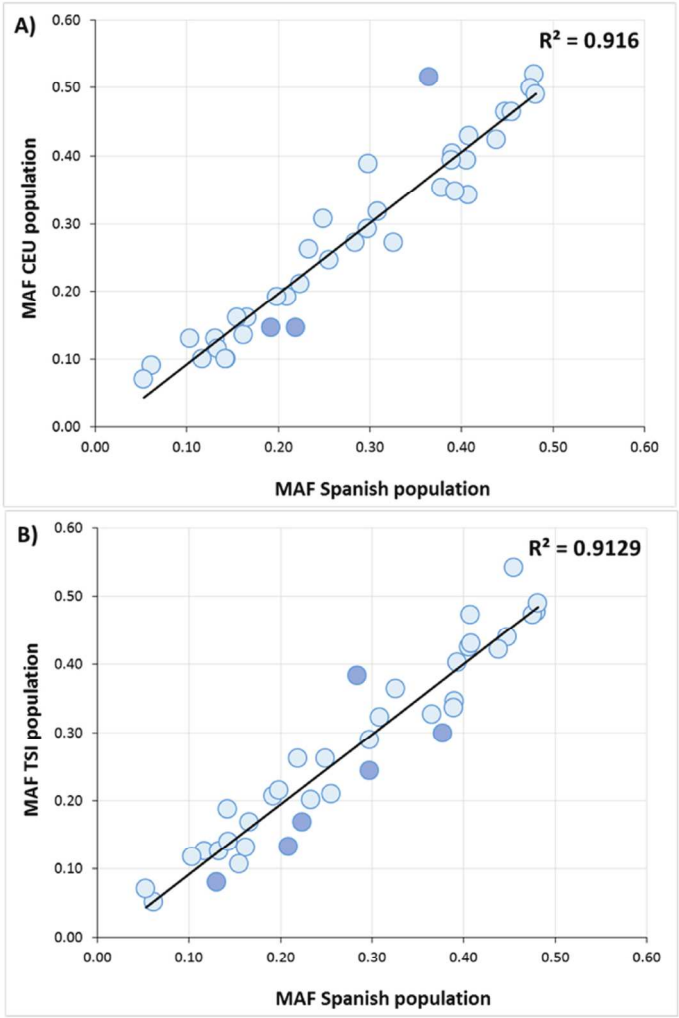

Figure S1. Comparison of minor allele frequencies between our Spanish sample and two different European populations. A) Northern Europeans from Utah (CEU), and B) Southern Europeans from Tuscany (TSI). Dark dots represent values that significantly differ from 1000 Genome Project data, when sample size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

Figure S1
191x240mm (150 x 150 DPI)

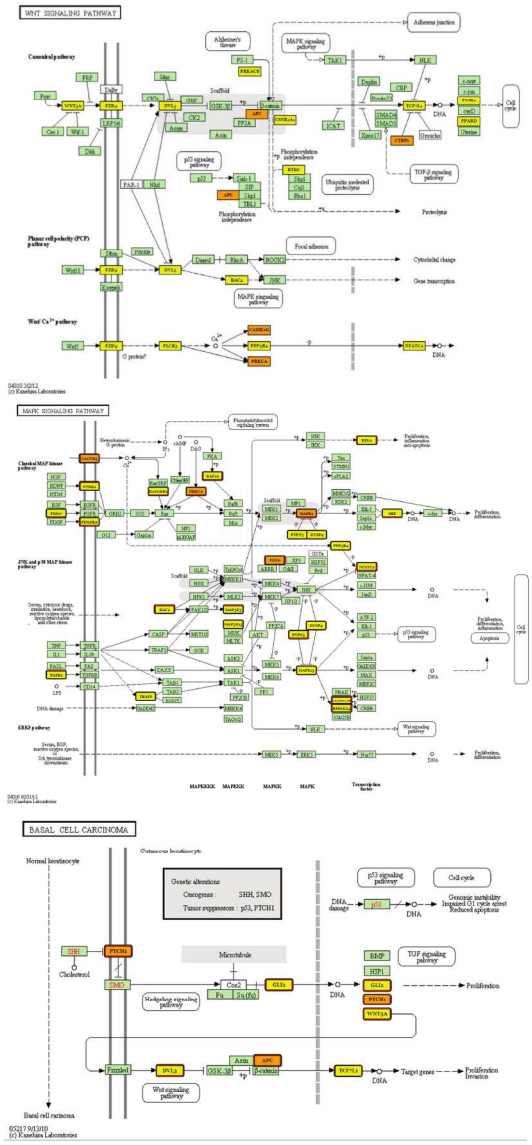

Figure S2. Enriched KEGG pathways involved in pigmentation and skin cancer that are targeted by miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs. Yellow denotes genes targeted by one miRNA of the list. Orange denotes genes targeted by more than one miRNA of the list.

Figure S2
265x470mm (150 x 150 DPI)

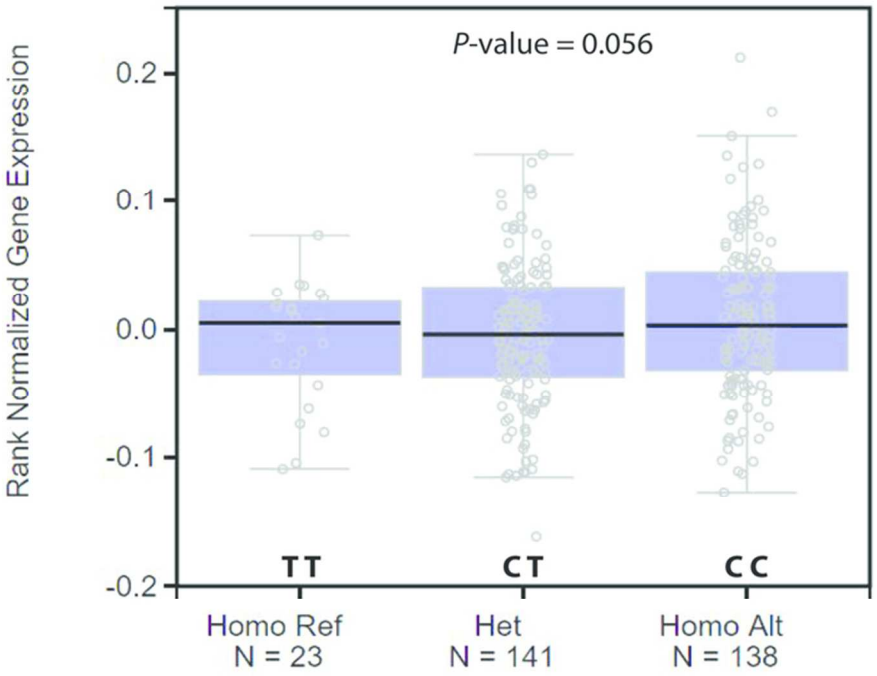

Figure S3. Box plot showing WNT3A expression according to SNP rs752107 genotype. T is the minor allele. Data taken from GTEx Portal.
Figure S3
135x96mm (150 x 150 DPI)

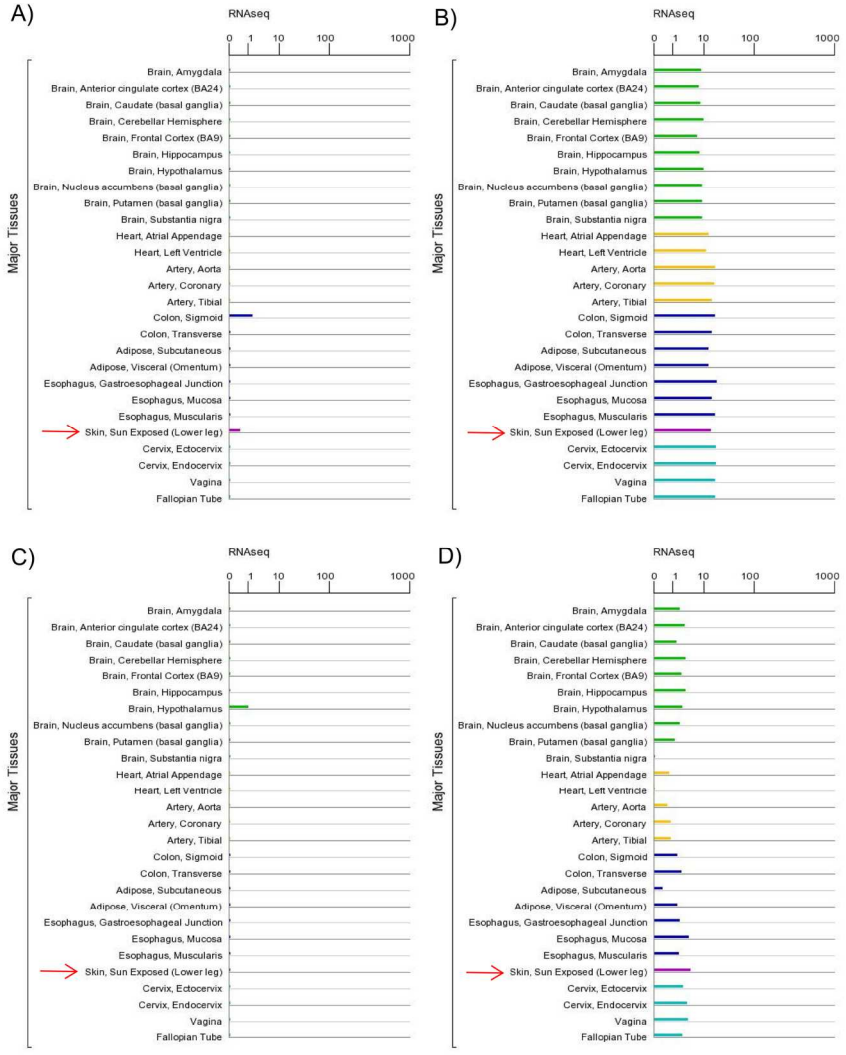

Figure S4. Expression in different tissues of the four miRNAs predicted to interact with highly-associated 3'UTR pigmentation SNPs. A) hsa-miR-185-3p; B) hsa-miR-762; C) hsa-miR-892b; D) hsa-miR-149-5p. Red arrow indicates sun-exposed skin tissue. Data taken from GTEx Portal, and images downloaded from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

Figure S4
297x420mm (150 x 150 DPI)