Replication and Predictive Value of SNPs Associated with Melanoma and Pigmentation Traits in a Southern European Case-Control Study

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Abstract

Background: Genetic association studies have revealed numerous polymorphisms conferring susceptibility to melanoma. We aimed to replicate previously discovered melanoma-associated single-nucleotide polymorphisms (SNPs) in a Greek case-control population, and examine their predictive value.

Methods: Based on a field synopsis of genetic variants of melanoma (MelGene), we genotyped 284 patients and 284 controls at 34 melanoma-associated SNPs of which 19 derived from GWAS. We tested each one of the 33 SNPs passing quality control for association with melanoma both with and without accounting for the presence of well-established phenotypic risk factors. We compared the risk allele frequencies between the Greek population and the HapMap CEU sample. Finally, we evaluated the predictive ability of the replicated SNPs.

Results: Risk allele frequencies were significantly lower compared to the HapMap CEU for eight SNPs (rs16891982 – SLC45A2, rs12203592 – IRF4, rs258322 – CDK10, rs1805007 – MC1R, rs1805008 – MC1R, rs910873 – PIGU, rs17305573 – PIGU, and rs1885120 – MTAP) and higher for one SNP (rs6001027 – PLA2G6) indicating a different profile of genetic susceptibility in the studied population. Previously identified effect estimates modestly correlated with those found in our population (r = 0.72, P < 0.0001). The strongest associations were observed for rs401681-T in CDKN2A (odds ratio [OR] 1.60, 95% CI 1.22–2.10; P = 0.001), rs16891982-C in SLC45A2 (OR 0.51, 95% CI 0.34–0.76; P = 0.001), and rs1805007-T in MC1R (OR 4.38, 95% CI 2.03–9.43; P = 2 × 10^-5). Nominal statistically significant associations were seen also for another 5 variants (rs258322-T in CDK10, rs1805005-T in MC1R, rs1885120-C in MYH7B, rs2218220-T in MTAP and rs4911442-G in the ASIP region). The addition of all SNPs with nominal significance to a clinical non-genetic model did not substantially improve melanoma risk prediction (AUC for clinical model 83.3% versus 83.9%, p = 0.66).

Conclusion: Overall, our study has validated genetic variants that are likely to contribute to melanoma susceptibility in the Greek population.

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Introduction

Plentitude of studies has shown that ultra-violet (UV) light exposure and certain phenotypic traits, i.e. red or blonde hair, light-colored eyes, fair skin complexion, and prominent mole pattern are major risk factors for the development of cutaneous melanoma (CM) [1–6]. A strong genetic background has been supported by twin studies showing a 55% contribution of genetic effects in melanoma variation liability [7].

High-penetrance germline mutations in CDKN2A and CDK4 genes are rare (0.2–1.2%) in sporadic CM, but they are encountered in approximately 5% of families with only two members with CM, and in 30–40% of families with 3 or more affected members [8–10]. The advent of high-throughput genotyping technologies and their utilization in population-based
Validation of Melanoma-Associated SNPs in Greece

SNP selection and Genotyping
All variants included in this study were selected from the last update of the MelGene field synopsis (October 2011), a large online database that was created with the purpose of comprehensively collecting and meta-analyzing all published genetic associations of melanoma (http://www.melgene.org) [23]. More specifically, the 34 selected variants from MelGene were distinguished in two groups: 1) all variants associated with melanoma at a level of p<0.05 following meta-analysis of relevant data from at least 3 independent case-control datasets (28 variants) and 2) additional biologically plausible variants representing potential causal pathways and selected from GWAS (3 variants) and candidate gene studies (3 variants) with genome-wide (p<10^{-7}) or nominally significant (p<0.05) associations. These variants were also included in MelGene but not necessarily meta-analyzed due to insufficient number of available datasets. In all, of the 34 variants, 19 had reached genome-wide significance in a previous GWAS or in MelGene, and the other 15 had not.

DNA isolation, Genotyping and Quality control
Genomic DNA was isolated from peripheral blood using the QiAamp DNA blood mini kit (Qiagen). DNA concentration was quantified in samples prior to genotyping by using Quant-iT dsDNA HS Assay kit (Invitrogen). The concentration of the DNA was adjusted to 5 ng/μl.

A total of 50 ng from each DNA sample was used to genotype the selected 34 SNPs using the Sequenom iPLEX assay (Sequenom, Hamburg, Germany). Allele detection in this assay was performed using matrix-assisted laser desorption/ionization – time-of-flight mass spectrometry [30].

Our quality control criteria included the inclusion of SNPs with a genotype call rate of 95% or higher, as well as SNPs showing no deviation from Hardy-Weinberg equilibrium (HWE) in the controls using a chi-squared test (P>0.05).

Statistical Analysis
We examined the association of each SNP with CM by performing conditional logistic regression analyses assuming a multiplicative model of inheritance considering the minor allele as the reference allele. To control for the effect of the other covariates/risk factors on CM in the Greek population, each SNP was subsequently incorporated into multivariable logistic regression models using a stepwise variable selection approach. The covariates considered were eye color (light: blue, green/gray and light brown or dark: dark brown and black), hair color (light: blond/red and light brown or dark: dark brown and black), skin color (light: fair/pale and light brown or dark: dark brown), phenotype (type I, II, III or IV, according to the Fitzpatrick scale), tanning ability (burn, minimal tan, burn then tan or deep tan), and sunburn (presence or absence). We estimated odds ratios and 95% confidence intervals (95% CI) for all models. Missing values for any of the non-genetic risk factors were imputed using multiple imputation methods. Variables where all the required information was available were used for the construction of the models for the estimation of the imputed missing values.

Additionally, we estimated the correlation of risk allele frequencies between the HapMap CEU sample and the Greek population across all the evaluated SNPs. Moreover, we estimated the correlation of the effect sizes found in the Greek population with those found previously in the original publications or MelGene dependent on the source of SNP selection. We examined whether the direction of the effect estimates was in the same or in opposite directions.
| SNP            | Minor/Major alleles in the Greek sample | Gene Locus | Position/Function | MAF (95% CI) in the Greek sample | MAF in the reference source | OR in the Greek sample (95% CI) | OR (95% CI) in the reference source | GWAS significant | Reference source |
|----------------|----------------------------------------|------------|-------------------|----------------------------------|---------------------------|-------------------------------|-------------------------------|-----------------|-----------------|
| rs16891982     | C/G                                    | SLC45A2    | exon              | 0.14 (0.11–0.17)                 | 0.068                     | 0.51 (0.34–0.76)               | 0.40 (0.33–0.47)               | Yes              | Melgene         |
| rs401681       | T/C                                    | CLPTM1L    | intron            | 0.40 (0.36–0.44)                 | 0.450                     | 1.60 (1.22–2.10)               | 1.15 (1.08–1.22)               | Yes              | Melgene         |
| rs12203592     | T/C                                    | IRF4       | intron            | 0.04 (0.03–0.07)                 | 0.2259                    | 1.08 (0.62–1.89)               | 1.04 (0.99–1.08)               | No               | Candidate gene study [44] |
| rs7023329      | G/A                                    | MTAP       | intron            | 0.40 (0.36–0.44)                 | 0.497                     | 0.95 (0.73–1.25)               | 0.84 (0.80–0.89)               | Yes              | Melgene         |
| rs11515        | G/C                                    | CDKN2A     | 3' UTR            | 0.18 (0.15–0.21)                 | 0.2279                    | 1.07 (0.70–1.53)               | 1.15 (1.01–1.32)               | No               | Melgene         |
| rs308440       | A/G                                    | CDKN2A     | 3' UTR            | 0.07 (0.05–0.09)                 | 0.2279                    | 1.07 (0.70–1.53)               | 1.15 (1.01–1.32)               | No               | Melgene         |
| rs4636294      | G/A                                    | MTAP region | intergenic      | 0.41 (0.36–0.45)                 | 0.485                     | N/A                           | 0.82 (0.75–0.90)               | Yes              | Melgene         |
| rs12203592     | G/T                                    | MTAP region | intergenic      | 0.31 (0.27–0.35)                 | 0.417                     | 0.86 (0.64–1.15)               | 0.83 (0.78–0.89)               | No               | Melgene         |
| rs2218220      | T/C                                    | MTAP region | intergenic      | 0.41 (0.37–0.46)                 | 0.487                     | 0.74 (0.56–0.97)               | 0.84 (0.80–0.89)               | Yes              | Melgene         |
| rs10757257     | A/G                                    | MTAP       | intron            | 0.31 (0.27–0.35)                 | 0.415                     | 0.87 (0.65–1.16)               | 0.81 (0.76–0.86)               | Yes              | Melgene         |
| rs1011970      | T/G                                    | CDKN2B     | 5' UTR            | 0.16 (0.13–0.20)                 | NA                       | 1.27 (0.91–1.78)               | NA                           | No               | GWAS [49]       |
| rs1408799      | T/C                                    | TIRP-1region | intergenic      | 0.32 (0.28–0.36)                 | 0.258                     | 1.16 (0.87–1.54)               | 0.86 (0.80–0.93)               | No               | Melgene         |
| rs1042602      | A/C                                    | TYR        | exon              | 0.46 (0.42–0.51)                 | 0.316                     | 1.11 (0.85–1.46)               | 0.95 (0.90–0.99)               | No               | Melgene         |
| rs1126809      | A/G                                    | TYR        | exon              | 0.20 (0.16–0.23)                 | 0.316                     | 1.11 (0.85–1.46)               | 0.95 (0.90–0.99)               | No               | Melgene         |
| rs1393350      | A/G                                    | TYR        | intron            | 0.19 (0.16–0.23)                 | 0.316                     | 1.11 (0.85–1.46)               | 0.95 (0.90–0.99)               | No               | Melgene         |
| rs1544410      | A/G                                    | VDR        | intron            | 0.42 (0.38–0.46)                 | 0.400                     | 0.95 (0.72–1.24)               | 0.89 (0.82–0.97)               | No               | Melgene         |
| rs1800407      | A/G                                    | OCA2       | exon              | 0.06 (0.04–0.08)                 | 0.070                     | 1.32 (0.81–2.16)               | 1.4 (1.07–1.82)                | No               | Melgene         |
| rs258322       | T/C                                    | CDK10      | intron            | 0.05 (0.03–0.07)                 | 0.095                     | 2.26 (1.32–3.88)               | 1.66 (1.48–1.86)               | Yes              | Melgene         |
| rs1800055      | T/G                                    | MC1R       | exon              | 0.13 (0.11–0.16)                 | 0.114                     | 1.59 (1.09–2.32)               | 1.13 (1.02–1.26)               | No               | Melgene         |
| rs1800007      | T/C                                    | MC1R       | exon              | 0.02 (0.01–0.03)                 | 0.078                     | 4.38 (2.03–9.43)               | 1.83 (1.56–2.15)               | Yes              | Melgene         |
| rs1800008      | T/C                                    | MC1R       | exon              | 0.02 (0.01–0.04)                 | 0.098                     | 1.64 (0.85–3.19)               | 1.54 (1.33–1.79)               | Yes              | Melgene         |
| rs1800009      | A/G                                    | MC1R       | exon              | 0                                 | 0.015                     | N/A                           | 1.89 (1.51–2.38)               | Yes              | Melgene         |
| rs1800006      | A/C                                    | MC1R       | exon              | 0.005 (0.001–0.02)               | 0.009                     | 0.33 (0.03–0.32)               | 1.47 (1.18–1.83)               | No               | Melgene         |
| rs1154746      | T/G                                    | MC1R       | exon              | 0                                 | 0.010                     | N/A                           | 1.67 (1.26–2.21)               | No               | Melgene         |
| rs4785763      | A/C                                    | AFG3L1     | 3' UTR            | 0.28 (0.24–0.32)                 | 0.328                     | 1.29 (0.97–1.72)               | 1.36 (1.27–1.45)               | Yes              | Melgene         |
| rs6059017      | G/A                                    | ASIP       | 3' UTR            | 0.14 (0.12–0.18)                 | 0.089                     | 2.19 (1.08–4.54)               | 1.53 (1.25–1.87)               | No               | GWAS [49]       |
| rs4914114      | T/G                                    | ASIP region | intergenic      | 0.24 (0.21–0.28)                 | 0.31                     | 0.86 (0.64–1.16)               | 1.21 (0.96–1.51)               | No               | Candidate gene study [50] |
| rs1015362      | A/G                                    | ASIP region | intergenic      | 0.27 (0.24–0.31)                 | 0.27                     | 0.91 (0.68–1.21)               | 0.89 (0.69–1.13)               | No               | Candidate gene study [50] |
| rs910873       | A/G                                    | PIK3G      | intron            | 0.02 (0.01–0.03)                 | 0.076                     | 2.11 (0.96–4.67)               | 1.52 (1.36–1.70)               | Yes              | Melgene         |
| rs17305573     | C/T                                    | PIK3G      | intron            | 0.02 (0.01–0.03)                 | 0.09                     | 2.11 (0.96–4.67)               | 1.52 (1.36–1.70)               | Yes              | Melgene         |
| rs1885120      | C/G                                    | MYH7B      | intron            | 0.02 (0.01–0.03)                 | 0.073                     | 2.22 (1.01–4.88)               | 1.59 (1.41–1.79)               | Yes              | Melgene         |
Table 1. Cont.

| SNP            | Position/Function | OR in the Greek sample (95% CI) | OR in the Greek sample gene Locus Position/Function | OR in the Greek sample | MAE in the reference source (95% CI) | MAE in the reference source | Reference source | GWAS significant |
|----------------|-------------------|--------------------------------|---------------------------------------------------|-----------------------|--------------------------------------|---------------------------|------------------|-----------------|
| rs6001027      | intron            | 0.36 (0.27–0.48)               | 0.36 (0.27–0.48)                                   | 0.86 (0.77–0.96)      | Yes                                  | Yes                       | Melgene          | Yes              |
| rs4636294      | intron            | 0.30 (0.21–0.40)               | 0.30 (0.21–0.40)                                   | 0.86 (0.77–0.96)      | Yes                                  | Yes                       | Melgene          | Yes              |

Abbreviations: MAE, minor allele frequency; CI, confidence interval; OR, odds ratio.

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Results

Our sample included 284 patients with CM matched on age and sex to 284 controls; of those, 270 (48%) were men. Median age was 44 years (range 18–85) for patients and 42 years (range 18–81) for controls. Demographics and phenotypic traits are shown in Table S1. Missing values in phenotypic characteristics were due to the fact that blood samples and questionnaires in one participating center were collected in the early phase of this study, and the corresponding individuals could not be found in order to retrieve these data. A total of 34 variants were selected for genotype analysis (Table 1). All of them were successfully genotyped with call rates of 95% or above. Deviation from HWE in the control population was noticed for one single-nucleotide polymorphism (SNP) (rs4636294), which was subsequently excluded from further statistical analyses.

From the selected variants, four SNPs are found in the 3’-UTRs and one in the 5’-UTR of the respective gene loci; 13 are located in introns; and 10 are within exons. The remaining 6 variants are found in intergenic positions. We found evidence for strong pairwise LD ($r^2 > 0.85$) between rs2218220 and rs4636294 ($r^2 = 0.95$), which deviated from HWE; rs10757257 and rs1335510 ($r^2 = 0.96$); rs1939350 and rs1126809 ($r^2 = 0.94$); and rs1885120, rs910873 and rs1730575 ($r^2 = 0.90$). For the remaining, moderate LD was observed ($r^2 < 0.60$).

Association of variants with CM risk

Table 1 shows the 33 analyzed SNPs, their effect sizes, minor and major alleles and the corresponding frequencies in the Greek population. All alleles identified as minor in the Greek population were also minor alleles in the CEU HapMap sample with one exception (rs6001027 whose minor allele was T in the Greek population but C in HapMap CEU).

Figure 1 shows the correlation between the ORs identified for the 33 eligible SNPs in the Greek population and in the original source where these were selected. We noticed overall modestly high correlation of the respective effect estimates ($r = 0.72, P<0.0001$). No differences in ORs between the Greek population and the original source were beyond chance (i.e. 95% CI between the two populations showed overlap for each SNP). Overall, no nominally significant difference in ORs was noticed across all SNPs in the two populations ($P = 0.411$ for Mann-Whitney U).
When limited to SNPs that had previously reached genome-wide significance in either Melgene or a previous GWAS, the correlation of effect sizes was $r = 0.83$ ($P < 0.0001$) and the correlation of risk allele frequencies was $r = 0.98$ ($P < 0.0001$). Conversely, for the 14 SNPs that had not previously reached genome-wide significance, the respective correlation coefficients were $r = 0.24$ ($P = 0.43$) and $r = 0.72$ ($P = 0.003$).

### Figure 1. Correlation of effect sizes

Correlation of the effect sizes found in the Greek population and those described in the original publication or MelGene. Not shown are: rs4636294 (excluded from analyses because of HWE deviation); rs1011970 because OR was not available in the original publication and/or MelGene; rs1805009 and rs11547464 because all subjects were homozygous for the major alleles.

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### Table 2. Results of the univariable and multivariable analyses adjusting for hair color, skin color, eye color, phototype, sunburn and tanning and comparison with data from MelGene [23].

| SNP- Minor Allele | MAF | Gene Locus | Univariable analysis OR (95% CI) | P-value | Multivariable analysis OR (95% CI) | P-value | MelGene OR (95% CI) | p-value | Known associations | Nevi/Pigmentation |
|-------------------|-----|------------|----------------------------------|---------|-----------------------------------|---------|-------------------|--------|-------------------|------------------|
| rs258322-T        | 0.05| CDK10      | 2.26 (1.32–3.88)                  | 0.003   | 1.77 (0.68–4.62)                  | 0.241   | 1.66 (1.48–1.86)   | 4 × 10^{-18} | No/Yes            |                  |
| rs401681-T        | 0.40| CLPTM1L    | 1.60 (1.22–2.10)                  | 0.001   | 1.99 (1.21–3.36)                  | 0.006   | 1.15 (1.08–1.22)   | 9.6 × 10^{-6} | Weak/No           |                  |
| rs1805005-T       | 0.13| MC1R       | 1.59 (1.09–2.32)                  | 0.016   | 1.61 (0.81–3.20)                  | 0.179   | 1.13 (1.02–1.26)   | 0.024   | No/Yes            |                  |
| rs1805007-T       | 0.02| MC1R       | 4.38 (2.03–9.43)                  | 0.00002 | 5.50 (1.37–22.15)                 | 0.016   | 1.83 (1.56–2.15)   | 2.7 × 10^{-15} | No/Yes            |                  |
| rs1885120-C       | 0.02| MYH7B      | 2.22 (1.01–4.88)                  | 0.047   | 3.10 (0.89–10.82)                 | 0.0176  | 1.59 (1.41–1.79)   | 7.4 × 10^{-15} | No/Yes            |                  |
| rs2218220-T       | 0.41| MTAP       | 0.74 (0.56–0.97)                  | 0.032   | 0.54 (0.33–0.90)                  | 0.05    | 0.84 (0.80–0.89)   | 5.5 × 10^{-11} | Yes/No            |                  |
| rs4911442-C       | 0.05| (NCOA6) ASIP region | 1.79 (1.02–3.14) | 0.042 | 3.29 (1.21–8.93) | 0.02   | 1.2 (0.99–1.46) | 1.03 × 10^{-8} | No/Yes            |                  |
| rs16891982-C      | 0.14| SCL45A2    | 0.51 (0.34–0.76)                  | 0.001   | 0.39 (0.17–0.89)                  | 0.042   | 0.40 (0.33–0.47)   | 4 × 10^{-27} | No/Yes            |                  |

1Association analysis on negative strand. Abbreviations: NS, not significant.
2MelGene status = Data from MelGene, an online database of reported genetic associations of melanoma including a systematic meta-analysis of melanoma-associated variants from published datasets and grading of these associations for strength of epidemiological evidence [23]. OR (95% CI) and p value correspond to nominal association with melanoma after meta-analysis of data for each variant.
3For this variant no meta-analysis was performed in MelGene due to the lack of sufficient datasets. The data represent those derived from the initial GWAS reporting an association of this variant with melanoma [50].

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Univariable analysis using a multiplicative model revealed 8 SNPs that were nominally statistically significantly associated with melanoma at a P = 0.05 level (Table 2). All with the exception of rs1805005 had previously reached genome-wide significance in Melgene or GWAS. The strongest associations were observed for rs401681-T in locus CLPTM1L (OR 1.60, 95% CI 1.22–2.10; P = 0.001), rs16891982-C in locus SCL45A2 (OR 0.51, 95% CI 0.34–0.76; P = 0.001), and rs1805007-T in locus MCIR (OR 4.38, 95% CI 2.03–9.43; P = 2 × 10−5). These 3 variants would also withstand multiple-testing Bonferroni correction for n = 33. The remaining five significantly associated variants were rs258322-T in CDK10 (OR 2.26, 95% CI 1.32–3.88; P = 0.003), rs1805005-T in MC1R (OR 1.59, 95% CI 1.09–2.32; P = 0.016), rs1885120-C in MYH7B (OR 2.22, 95% CI 1.01–4.88; P = 0.047), rs2218220-T in MTAP (OR 0.74, 95% CI 0.56–0.97; P = 0.032) and rs4911442-G in ASIP region (OR 1.79, 95% CI 1.02–3.14; P = 0.042).

Five SNPs were significantly associated with CM in the multivariable analyses after controlling also for hair color, skin color, eye color, phototype, sunburn and tanning (Table 2).

Power Considerations

The power of our study to detect ORs similar to those previously found, given the allele frequencies observed in the Greek population, ranges from 5.2% for rs12203592 to 100% for rs16891982 at α = 0.05. By summing the power estimates for all SNPs to detect the respective ORs seen previously, we estimated that if ORs were identical in the Greek population and the frequencies in both the Greek sample and HapMap CEU, our study would be expected to have found 8 nominally statistically significant associations among the 33 tested. Among the 18 variants that had been previously identified with genome-wide significance and did not show deviation from HWE, our study would be expected to have found 6 nominally statistically significant associations and 7 were indeed nominally significant.

Comparison of risk allele frequencies between Greek sample and HapMap CEU

For 20 SNPs, the respective minor alleles were the risk alleles for melanoma. Table 3 shows risk alleles in the Greek sample and their frequency in both the Greek sample and HapMap CEU. The risk alleles in the Greek population had a median frequency of 20% (IQR, 4–60%), while their median frequency in HapMap CEU was 32% (IQR, 12–62%) (P = 0.243 for Mann-Whitney U). The correlation between the two populations was very high (r = 0.95, P < 0.0001) (Fig. 2).

The risk allele frequencies of nine SNPs (rs6001027-C, rs16891982-G, rs12203592-T, rs258322-T, rs1805007-T, rs1885120-C, rs1805008-T, rs910873-A, rs17305573-C, and rs1805120-C) were different beyond chance between the Greek sample and HapMap CEU (i.e. 95% CI of risk allele frequencies in the Greek population and the HapMap sample did not overlap). All these variants (except for rs6001027, a nevi-related SNP in PLA2G6) had significantly lower frequencies of risk alleles in the Greek population compared to HapMap CEU, while six of those are variants of genes with well-established role in the genetic control of pigmentation (rs16891982 in SCL45A2, rs12203592 in IRF4, rs1805007 in MC1R, rs1885120 in MYH7B, rs1805007 and rs1805008 both in MC1R).

Predictive value of predisposing SNPs in melanoma-associated risk factor models

Figure 3 shows the areas under the curve (AUC) for 3 models considering different levels of genetic information. Compared to the phenotypic traits alone, models including the CM-associated SNPs only slightly improved the AUC. The AUC for the model that included only the nominally significant phenotypic traits (i.e. eye color, skin color, sunburn, phototype and tanning) (model 1) was 83.3%, whereas for the model that included these traits along with the 3 SNPs that remained significant after Bonferroni correction in the univariable analysis (model 2) was 83.7%, and
The AUC for the model including the traits and all 8 SNPs with nominal significance (model 3) was 83.9%. Compared to the baseline non-genetic model, the genetic models did not confer a nominally significant improvement to the prediction of CM (P = 0.42 for model 1 vs. model 2, and P = 0.66 for model 1 vs. model 3).

**Discussion**

We have replicated SNP-melanoma associations, with MAFs ranging from 2% to 41%. Eight associations were nominally statistically significant in the Greek population, the majority of which (87%) had previously reached genome wide significance. The replication of variants deriving from GWAS-discovered loci in our cohort, such as 20q11.2 (ASIP region), 9p21 (MTAP region), 16q24 (MC1R region) and 5p13 (CLPTM1L region), underscores the important contribution of the agnostic approach of GWAS in revealing genuine associations of genetic factors in complex diseases. For 8 SNPs the risk alleles had significantly lower frequencies in the Greek population compared to the HapMap CEU sample, while for 1 SNP the risk allele in the Greek population was higher than HapMap. The genetic models containing the SNPs that confer risk for melanoma improved the AUC compared with the model including only the phenotypic risk factors, but the improvement was of small magnitude.

The aim of our study was to validate a selected panel of SNPs in a case-control cohort of Greek descent, given our recent findings of a higher than expected genetic contribution of CDKN2A/CDK4.

**Table 3.** List of genotyped SNPs, risk alleles in the Greek sample, and risk allele frequency in the Greek sample and HapMap CEU.

| SNP          | Risk allele in the Greek sample | Risk allele frequency in the Greek sample (95% CI) | Risk allele frequency in HapMap CEU (95% CI) |
|--------------|--------------------------------|-----------------------------------------------|---------------------------------------------|
| rs16891982 T | C                              | 0.86(0.83–0.89)                               | 0.98(0.94–0.99)                             |
| rs401681 T   | T                              | 0.40(0.36–0.44)                               | 0.43(0.36–0.50)                             |
| rs12203592 T | T                              | 0.04(0.03–0.07)                               | 0.16(0.11–0.21)                             |
| rs7023329 A  | A                              | 0.60(0.56–0.64)                               | 0.51(0.44–0.57)                             |
| rs11515 C    | C                              | 0.82(0.79–0.85)                               | 0.88(0.80–0.92)                             |
| rs3098440 A  | A                              | 0.07 (0.05–0.09)                              | 0.110(0.07–0.15)                            |
| rs4636294 T  | N/A                            | N/A                                           | 0.50(0.43–0.57)                             |
| rs1335510 T  | T                              | 0.69 (0.65–0.73)                              | 0.62(0.54–0.67)                             |
| rs2218220 C  | C                              | 0.59 (0.54–0.63)                              | 0.50(0.43–0.57)                             |
| rs10757257 G | G                              | 0.69(0.65–0.73)                               | 0.62(0.55–0.68)                             |
| rs1011970 T  | T                              | 0.16 (0.13–0.2)                               | 0.17(0.12–0.23)                             |
| rs1408799 T  | T                              | 0.32 (0.28–0.36)                              | 0.30(0.24–0.36)                             |
| rs1042602 A  | A                              | 0.46 (0.42–0.51)                              | 0.43(0.36–0.49)                             |
| rs1126809 A  | A                              | 0.20 (0.16–0.23)                              | 0.22(0.14–0.29)                             |
| rs1393350 A  | A                              | 0.19 (0.16–0.23)                              | 0.23(0.17–0.28)                             |
| rs1544410 G  | G                              | 0.58(0.54–0.62)                               | 0.56(0.49–0.62)                             |
| rs1800407 A  | A                              | 0.06(0.04–0.08)                               | 0.08(0.04–0.11)                             |
| rs258322 T   | T                              | 0.05(0.03–0.07)                               | 0.14(0.09–0.18)                             |
| rs1805005 T  | T                              | 0.13(0.11–0.16)                               | 0.08(0.04–0.12)                             |
| rs1805007 T  | T                              | 0.02(0.01–0.03)                               | 0.12(0.08–0.17)                             |
| rs1805008 T  | T                              | 0.02(0.01–0.04)                               | 0.13(0.08–0.17)                             |
| rs1805009 G  | G                              | N/A                                           | 1 (NA)                                      |
| rs1805006 C  | C                              | 0.995(0.98–0.999)                             | 1 (NA)                                      |
| rs11547464 G | G                              | N/A                                           | 1 (NA)                                      |
| rs4785763 A  | A                              | 0.28(0.24–0.32)                               | 0.38(0.31–0.44)                             |
| rs6058017 G  | G                              | 0.14(0.12–0.18)                               | 0 (NA)                                      |
| rs4911414 G  | G                              | 0.76(0.72–0.79)                               | 0.69(0.62–0.74)                             |
| rs1015362 G  | G                              | 0.73(0.69–0.76)                               | 0.74(0.67–0.79)                             |
| rs910873 A   | A                              | 0.02(0.01–0.03)                               | 0.08(0.05–0.12)                             |
| rs17305573 C | C                              | 0.02(0.01–0.03)                               | 0.08(0.05–0.12)                             |
| rs1885120 C  | C                              | 0.02(0.01–0.03)                               | 0.07(0.04–0.11)                             |
| rs4911442 G  | G                              | 0.04(0.03–0.07)                               | 0.06(0.02–0.12)                             |
| rs2284063 G  | G                              | 0.36(0.32–0.40)                               | 0.32(0.25–0.38)                             |
| rs6001027 C  | C                              | 0.70(0.66–0.73)                               | 0.32(0.25–0.38)                             |

N/A: not applicable because all subjects were found homozygous for the major alleles.

1Deviation from HWE, excluded from analyses.

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mutations in a sizable cohort of sporadic and familial cases of our population [28]. Recent GWAS employing a higher density SNP tagging in large patient datasets has revealed a number of variants in genes involved in cell cycle regulation, telomere maintenance and DNA damage response, such as MTF, ATM, PARP-1, TERT, CASP8, CCND1, as well as polymorphisms in MX2, SETDB1 and ARNT/LASS2/AXX19 region [31–34]. Although this study was based on earlier GWAS findings and certain candidate gene studies, our findings underscore the role of genes controlling pigmentation traits and DNA damage response in melanoma susceptibility in our population. This may reflect the importance of these pathways in melanoma development in a darker-skin population residing at an area of high year-round UV-influx. Most of the SNPs with significantly lower risk allele frequencies compared to HapMap CEU are found in loci implicated in pigmentation (SLC45A2, IRF4, CDK10, MIH7B, MC1R) and all but 2 (rs16891982, rs258322, rs1805007, and rs1885120) were replicated in the Greek population according to univariable analysis. These findings imply that there might be some differences in the genetic background underlying the phenotypical differences between the Greek and other European populations, and could partially explain the lower melanoma incidence in a population of darker skin complexion residing in a country with intense year-round UV exposure. In addition, our results may underscore the role of natural selection which tends to eliminate the prevalence of predisposing alleles in a population with high sun exposure and increase the frequency of protective alleles which also act through the protective pathways of pigmentation, However, Greeks harboring certain pigmentation-related risk alleles are at risk of developing melanoma.

In the case of melanocytic nevi, the comparison of allele frequencies between nevi-related variants in our cohort and the HapMap were less conclusive, with one variant (rs6001027) showing a higher allele frequency in our population. Only one (rs2218220 in the MTAP region, chrom. 9p21) of the previously nevi-associated SNPs was found to be positively associated with melanoma in our analysis. Given that nevi have been shown to be a strong risk factor of melanoma in the Greek population [35], it is likely that our study was not powered enough to detect smaller effect sizes conferred by these variants. In addition, other nevus-associated variants, yet uncovered, may play a role in melanoma risk.

Among the three top variants of our analysis, the most prominent locus was located within the cleft lip and palate transmembrane 1-like (CLPTM1L) gene and the telomerase reverse transcriptase (TERT) gene. The major C allele of rs401681 has been repeatedly reported to confer risk for BCC and protection against melanoma [36–39], and was recently replicated at a GWAS of 2,981 melanoma patients and 1,982 controls [31]. In addition, a meta-analysis including data from an Australian case-control study showed that TERT-CLPTM1L variants do influence melanoma risk, albeit with a relatively small effect size [32]. The “red hair” variant rs1805007 of the MC1R gene has been consistently linked to melanoma risk in relevant studies. In meta-analyses, rs1805007 showed the highest attributable risk for melanoma among MC1R variants [13,40] with effect estimates similar to those found in this study and a previous Greek case-control study [16]. rs16891982 of the SLC45A2, influences skin pigmentation and exhibits substantially different frequencies among populations, thus determined as an ancestry informative marker. The ancestral Leu allele (rs16891982-C) has been associated with dark skin, eye, and hair color in whites [41], while exhibiting a protective effect against melanoma [39,42–44].

The variants selected for this study were based on the results of a large field synopsis and on-line database that scrutinized all published data on the genetic association of melanoma and subjected them to systematic meta-analyses. All but one (rs1805005) nominally significant associations in our selected set of SNPs came from a subgroup of variants which had p values of 10^{-7} and are likely to represent genuine associations [45]. We were also able to assess the predictive value of genetic factors in models incorporating various phenotypical and genetic risk factors. In the examined models, the predictive value of AUC did not substantially improve by the addition of genetic variants, compared with the model that involved only the clinical risk factors. Although these genetic models do not seem to contribute substantially to melanoma risk prediction, they are nevertheless suggestive of the contribution of low-penetration gene variants to melanoma risk. Failure of models relying on common gene variants to improve substantially the predictive discrimination of traditional risk factors is a common problem encountered in complex diseases. Much larger effect sizes and a very large number of genetic variants are needed to improve perceptively the predictive value of genetic models [46]. Moreover, our findings show that statistical significance of a risk model does not guarantee clinical utility highlighting the distinction between the statistical and clinical perspectives of genetic risk models [47].

The current study has some limitations. First, the sample size is modest resulting probably in limited power to detect small or even moderate effects for additional SNPs. Second, no data were recorded on the number of nevi, a well-known melanoma risk factor for melanoma. Nevertheless, only one (rs2218220) in MTAP of the eight SNPs associated with melanoma has been reported to be also associated with nevus count [48]. It is possible that rs2218220 would lose its significance as a melanoma-associated variant if the number of nevi were included in the multivariate analyses. Third, failure to replicate candidate loci in pigmentation-associated genes other than MC1R, SLC45A2, CDK10, MIH7B and
**Validation of Melanoma-Associated SNPs in Greece**

Further validation of newly described variants and a better understanding of the gene-environment interaction may provide valuable insight in the variation of melanoma risk among white populations of different ancestry.

**Supporting Information**

Table S1 Demographic characteristics and pigmented phenotype of melanoma cases and control subjects. (DOCX)

**Author Contributions**

Critically commented on and approved the final version of the manuscript: IS OAP EE AJS. Performed the experiments: IS OAP EE. Designed the experiments: IS OAP EE.

**References**

1. Tucker MA, Goldstein AM (2003) Melanoma etiology: where are we? Oncogene 22(20): 3042–3052.

2. Gandini S, Sera F, Cattaruzza MS, Pasquali P, Zanetti R, et al. (2005a) Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors. Eur J Cancer 41: 2040–2049.

3. Gandini S, Sera F, Cattaruzza MS, Pasquali P, Abeni D, et al. (2005b) Meta-analysis of risk factors for cutaneous melanoma: I. Common and atypical naevi. Eur J Cancer 41: 28–44.

4. Han J, Colditz GA, Hunter DJ (2006) Risk factors for skin cancers: a nested case-control study within the Nurses’ Health Study. Int J Epidemiology 35: 1314–1321.

5. Tsitoura-Ioannou E, Perry AE, Spencer SK, Gibson JJ, Cole BF, et al. (2005) Pigmentary characteristics and moles in relation to melanoma risk. Int J Cancer 116: 144–149.

6. Battalio V (2003) Genetic epidemiology of melanoma. Eur J Cancer 39: 1341–1347.

7. Shekar SN, Duffy DL, Youl P, Baxter AJ, Kvaksmo M, et al. (2009) A population-based study of Australian twins with melanoma suggests a strong genetic contribution to liability. J Invest Dermatol 129:2211–9.

8. Berwick M, Orlow I, Hummer AJ, Armstrong BK, Kricker A, et al. (2006) Pleiotropic effects of the melanocortin 1 receptor (MC1R) gene on human pigmentation. Hum Mol Genet 9: 2531–7.

9. Macgregor S, Montgomery GW, Liu JZ, Zhao ZZ, Henders AK, et al. (2011) Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. Hum Mol Genet 13: 47–51.

10. Flanagan N, Healy E, Ray A, Philips S, Todd C, et al. (2006) Pleiotropic effects of the melanocortin 1 receptor (MC1R) gene on human pigmentation. Hum Mol Genet 9: 2531–7.

11. Fargioli MC, Aragonzio G, Zalaudek I, Peris K (2006) High- and low-penetration cutaneous melanoma susceptibility genes. Expert Rev Anticancer Ther 6: 657–670.

12. Charalambous F, Lim CM, Kyriou K, Stefanaki I, Nicolaou V, et al. (2011) Comprehensive Field Synopsis and Systematic Meta-analyses of Genetic Association Studies in Cutaneous Melanoma. J Natl Cancer Inst 103: 1227–35.

13. Yokoyama S, Woolf SL, Boyle GM, Aoudie LG, MacGregor S, et al. (2011) A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. Nature 480: 99–103.

14. Bertolotto C, Lesueur F, Giuliano S, Strub T, de Lichy M, et al. (2011) A SUMOylation-defective MITF germline mutation predisposes to melanoma and renal carcinoma. Nature 480:94–9.

15. Lasithiotakis K, Kruger-Kragasakis S, Ioannidou D, Pediatidin I, Tosca A (2004) Epidemiological differences for cutaneous melanoma in a relatively dark-skinned Caucasian population with chronic sun exposure. Eur J Cancer 40: 2502–2507.

16. Lasithiotakis K, Kruger-Kragasakis S, Manousakis S, Ioannidou D, Panagiotidou I, et al. (2006) The incidence of cutaneous melanoma on Cete, Greece. Int J Dermatol 45: 397–401.

17. Nikolau V, Kang X, Stratigos A, Gogas H, Latorre MC, et al. (2011) Comprehensive mutational analysis of CDKN2A and CDK4 in Greek patients with cutaneous melanoma. Br J Dermatol 165: 2119–22.

18. Stratigos AJ, Yang G, Dimitianos R, Nicolaou V, Stefanaki I, et al. (2006) Germline CDKN2A mutations among Greek patients with early-onset and multiple primary cutaneous melanoma. J Invest Dermatol 126: 399–401.

19. Gabriel S, Zawazka L, Tabbaa D (2008) SNP Genotyping Using the Sequenom MassARRAY iPLEX Platform. In: Centro Protoc Hum Genet Chapter 2: Unit 2.12.

20. Barrett JH, Ben MM, Harland M, Taylor JG, Aitken JF, et al. (2011) Genomewide association study identifies three new melanoma susceptibility loci. Nat Genet 43: 1108–13.

21. Macgregor S, Montgomery GW, Liu JZ, Zhao ZZ, Henders AK, et al. (2011) Genome-wide association study identifies a new melanoma susceptibility locus at 1p11.3. Nat Genet 43: 111–13.

22. Amos CI, Wang LE, Lee JE, Gershenwald JE, Chen WV, et al. (2011) Genomewide association study identifies novel loci predisposing to cutaneous melanoma. Hum Mol Genet 20: 5012–23.

23. Law M, MacGregor S, Hayward NK (2012) Melanoma Genetics: Recent Findings Take Us Beyond Well-Traveled Pathways. J Invest Dermatol 132(7):1763–74.

24. Nikolau VA, Sypsa V, Stefanaki I, Gogas H, Papadopoulos O, et al. (2008) Risk associations of melanoma in a Southern European population: results of a case/control study. Cancer Causes Control 19: 671–9.

25. Dahlin T, Sulem P, Stefansson K, Gudmundsson J, et al. (2009) Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. Nat Genet 41: 221–227.
37. Han J, Qureshi AA, Prescott J, Guo Q, Ye L, et al. (2009) A prospective study of telomere length and the risk of skin cancer. J Invest Dermatol 129: 415–421.
38. Nan H, Qureshi AA, Prescott J, De Vivo I, Han J (2011) Genetic variants in telomere-maintaining genes and skin cancer risk. Hum Genet 129: 247–53.
39. Stacey SN, Sulem P, Mason G, Gudjonsson SA, Thorleifsson G, et al. (2009) New common variants affecting susceptibility to basal cell carcinoma. Nat Genet 41: 909–14.
40. Gerstenblith MR, Shi J, Landi MT (2010) Genome-wide association studies of pigmentation and skin cancer: a review and meta-analysis. Pigment Cell Melanoma Res 23: 587–606.
41. Graf J, Hodgson R, van Daal A (2005) Single nucleotide polymorphisms in the MATP gene are associated with normal human pigmentation variation. Hum Mutat 25: 278–284.
42. Fernandez LP, Milne RL, Pita G, Avilés JA, Lázaro P, et al. (2008) SLC45A2: a novel malignant melanoma-associated gene. Hum Mutat 29: 1161–7.
43. Guédj M, Bourillon A, Combadie`res C, Rodero M, Dieude` P, et al. (2008) Variants of the MATP/SLC45A2 gene are protective for melanoma in the French population. Hum Mutat 29: 1134–1160.
44. Duffy DL, Zhao ZZ, Sturm RA, Hayward NK, Martin NG, et al. (2010) Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma. J Invest Dermatol 130: 520–9.
45. Panagiotou OA, Ioannidis JP, Genome-Wide Significance Project (2012) What should the genome-wide significance threshold be? Empirical replication of borderline genetic associations. Int J Epidemiol 41:273–86.
46. Ioannidis JP (2012) Invited commentary-genetic prediction for common diseases. Arch Intern Med 72:744–6.
47. Wacholder S (2012) Clinical utility in evaluation of risk models. Am J Epidemiol 176:493–6.
48. Falchi M, Bataille V, Hayward NK, Duffy DL, Bishop JA, et al. (2009) Genome-wide association study identifies variants at 9p21 and 22q13 associated with development of cutaneous nevi. Nat Genet 41: 915–9.
49. Bishop DT, Demenais F, Iles MM, Harland M, Taylor JC, et al. (2009) Genome-wide association study identifies three loci associated with melanoma risk. Nat Genet 41: 920–5.
50. Nan H, Kraft P, Hunter DJ, Han J (2009) Genetic variants in pigmentation genes, pigmentary phenotypes, and risk of skin cancer in Caucasians. Int J Cancer 125:909–17.
51. Brown KM, Macgregor S, Montgomery GW, Craig DW, Zhao ZZ, et al. (2008) Common sequence variants on 20q11.22 confer melanoma susceptibility. Nat Genet 40: 838–40.