Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma

David L. Duffy[1], **Z. Z. Zhao**[1], **Richard A. Sturm**[2,1], **Nicholas K. Hayward**[1], **Nicholas G. Martin**[1], and **Grant W. Montgomery**[1]

[1]Queensland Institute of Medical Research, Brisbane, Australia

[2]The University of Queensland, Melanogenix Group, Institute of Molecular Bioscience, Brisbane, Australia

Abstract

We have previously described the role of red hair (Melanocortin 1 Receptor, *MC1R*) and blue eye (Oculocutaneous Albinism Type 2, *OCA2*) gene polymorphisms in modulating risk of cutaneous malignant melanoma (CMM) in a highly sun-exposed population of European descent. A number of recent studies, including genome-wide association studies (GWAS), have identified numerous polymorphisms controlling human hair, eye and skin colour. In this paper, we test a selected set of polymorphisms in pigmentation loci (*ASIP, TYR, TYRP1, MC1R, OCA2, IRF4, SLC24A4, SLC45A2*) for association with CMM risk in a large Australian population-based case control study. Variants in *IRF4* and *SLC24A4*, despite being strongly associated with pigmentation in our sample, did not modify CMM risk, but the other six did. Three SNPs (rs28777, rs35391, rs16891982) in the MATP gene (*SLC45A2*) exhibited the strongest crude association with risk, but this was attenuated to approximately the same effect size as that of a *MC1R* red hair color allele by controlling for ancestry of cases and controls. We also detected significant epistatic interactions between *SLC45A2* and *OCA2* alleles, and *MC1R* and *ASIP* alleles. Overall, these measured variants account for 12% of the familial risk of CMM in our population.

INTRODUCTION

Cutaneous malignant melanoma (CMM) is a common cancer in the fair skinned population of tropical and subtropical Queensland, Australia, with an estimated lifetime risk of 5% (Queensland Cancer Registry, 2008). Within fairer skinned populations, risk varies with degree of paleness, as well as with other pigmentation phenotypes such as hair and eye color, all of which are strongly genetically determined.

We have previously described association between variants in the pigmentation genes encoding the melanocortin-1 receptor (*MC1R*) (Palmer *et al.*, 2000; Box *et al.*, 2001) and the human melanocyte-specific P-protein (*OCA2*) with CMM risk (Sturm *et al.*, 2008). A number of recent genome-wide association studies (GWAS) have characterized variants in both known pigmentation pathway (*TYR, TYRP1, SLC45A2*) (Sulem *et al.*, 2007; Sulem *et al.*, 2008) and novel (*IRF4, SLC24A4*) (Sulem *et al.*, 2007; Han *et al.*, 2008) genes that influence pigmentation, and one study has reported on associations with CMM (Gudbjartsson *et al.*, 2008). Association to both pigmentation and CMM risk has also been

reported with variants in a region of chromosome 20q fairly close to *ASIP* (Brown *et al.*, 2008; Sulem *et al.*, 2008; Gudbjartsson *et al.*, 2008).

Variants in many of these genes exhibit significant differences in frequency, and underlie the pigmentation differences, between different ethnic groups. It is important therefore, to differentiate between disease associations due to a direct genotype-phenotype relationship, and those to confounding by ancestry.

Here, we replicate and extend association results with CMM to *MC1R*, *OCA2*, *TYR* (*OCA1*), *TYRP1* (*OCA3*), *IRF4*, *SLC24A4*, *SLC45A2 (MATP, OCA4)*, and *ASIP* and examine the nature of interaction between these loci on disease risk in a large sample from a population at high environmental and genetic risk of melanoma.

RESULTS

There were 1738 CMM cases and 4517 controls. Ancestry and coloring for the sample are shown in Table 1. Differences between cases and controls are in the expected direction, as reported previously for this sample (Palmer *et al.*, 2000), with cases tending to have lighter coloured eyes and hair. Participants were genotyped at up to 161 SNPs across the gene regions of interest (Supplementary Table 1). Table 2 shows case and control allele frequencies and Hardy-Weinberg equilibrium tests for selected SNPs. A number of SNPs exhibited extreme Hardy-Weinberg disequilibrium, but since the chosen SNPs are in pigmentation loci known to be highly informative for colouring and ancestry, it could be shown that the disequilibrium was due to ethnic stratification of the sample rather than to genotyping error (see Supplementary Table 2).

One or more of the SNPs selected in each gene region could be shown to be associated with skin, hair or eye colour (Table 3), and in the subsequent analyses, we only present results for association with CMM for the polymorphisms most penetrant for pigmentary characteristics from each gene. In keeping with the selection criteria, a number of these SNPs were also strongly associated with reported ancestry (see Supplementary Table 2). Among the CMM cases with ancestry information, only 2.7% reported other than 100% Northern European ancestry, as compared to 13.5% of the control sample. To control these effects on CMM association analyses below, we report analyses on the subset of 1438 cases and 3098 controls reporting four grandparents of Northern European ancestry. We found that further inclusion of an ancestry indicator for English, Scottish/Welsh/Irish, and continental Europe as a covariate did not detect finer levels of ethnic confounding on CMM association. Interestingly, rs12203592 (in *IRF4*) did exhibit significant allele frequency differences between controls from continental Europe, England, and Scotland, Ireland and Wales, but these could be safely ignored given that this locus does not exhibit significant association with CMM (see below).

In univariate analyses, *MC1R, ASIP, TYRP1,* (*IRF4*) and *SLC45A2* SNPs were the markers most strongly associated with CMM risk in terms of crude risk (Table 4). Given the star-shaped genealogy of *MC1R* red-hair associated haplotypes (ie all these coding variants are in complete negative LD; in our population we have observed only three haplotypes with more than one coding variant present), we have recoded *MC1R* haplotypes as "*R*" (high red hair penetrance: rs1805006 (*D84E*), rs11547464 (*R142H*), rs1805007 (*R151C*), rs1805008 (*R160W*), rs1805009 (D294H) and "*r*" (low red hair penetrance: rs1805005, rs2228479, rs885479) (Sturm *et al.*, 2003) for this and subsequent analyses. On restricting the case-control analysis only to individuals reporting all four grandparents to be of Northern European ancestry, the significant predictors of CMM risk were four "R" SNPs in *MC1R* (rs1805007, rs1805008, rs1805006 and rs11547464); the three *SLC45A2* SNPs, rs1126809

in *TYR,* and rs4911442 in the region of *ASIP.* In the case of *SLC45A2,* the three SNPs were in very strong disequilibrium, and the noncoding SNPs rs35391 and rs28777 were not significant melanoma risk predictors in multivariate logistic regression models after adjusting for rs16891982.

When we further adjusted for hair, eye and skin colour, several SNPs exhibited a persistent association, suggesting they act as mediating variables (Table 4) between ancestry and melanoma risk. The *TYR* coding SNP rs1126809 remained important, but rs12913832 in *OCA2* (a regulatory SNP actually located in an intron of the adjacent *HERC2* gene, but the main determinant of blue eye colour, Sturm *et al.*, 2008) and the *TYRP1* SNPs were less impressive as CMM risk factors. The effect of *OCA2**R419Q was relatively small in its own right. When we combine the strongest associated SNPs from *TYR, SLC45A2, OCA2, MC1R* and *ASIP* in a multivariate logistic regression (Table 5), this gives a Nagelkerke R^2 index=0.059. For comparison, a regression including eye, skin and hair color obtained R^2=0.048, and supplementing this with the genotype data increased the R^2 to 0.08. Because logistic regression R^2 are difficult to interpret (Nagelkerke,1991), we also present locus-specific sibling recurrence risk ratios (λ_S). Under the usual multiplicative model assumption, these can be multiplied to give an total contribution of 1.14 to the sibling recurrence risk. We can calculate the equivalent contribution of measured pigmentation phenotype (skin, hair and eye colour) at 1.37, so that the current SNPs explain approximately 43% of the effects of pigmentation on familial risk (log(1.15)/log(1.37)), and about 15% of the total observed sibling risk λ_S=2.49 estimated previously in our population (Do et al, 2004).

In analyses including pairwise interactions, we observed an interaction only between OCA2*R419Q and rs1126809 (P=0.01, Supplementary Table 3), though there was a suggestive pattern of interaction between MC1R and ASIP genotypes and CMM which mirrored a highly significant interaction between these loci in their effects of red hair colour (Table 6).

As we noted elsewhere (Brown *et al.*, 2008), a number of SNPs spread over the region around ASIP exhibit association to CMM, and the analyses above have concentrated on rs4911442 only because it is genotyped in more controls than the other peak SNPs. The pattern of linkage disequilibrium around *ASIP* is quite complex, with "hotspots" just distal to the gene and 70 kbp proximally (see Figure 1), and the pattern of individual SNP association to CMM and to red hair color is bimodal, dipping rather than rising over *ASIP.*

Examining haplotypes comprised of 31 SNPs spanning from rs17305657 to rs1885120 (a distance of 1.77 Mbp) revealed that the commonest haplotype (present in 4% of controls) is significantly increased in CMM cases (7.3%, OR=1.89; see Supplementary Table 4). This haplotype is tagged by the SNPs that are most strongly individually associated with CMM (including rs4911442). When analysis is restricted just to six tagging SNPs, there are eight haplotypes present at greater than 1% study frequency, one (78% of control samples) being significantly increased in controls, and three (3.8%, 3.9%, 0.6%) increased in cases (global X^2=47.2, df=14, P=2×10^{-5}). These latter three share only the distal three markers in common, so the most likely location for the susceptibility locus lies downstream to *ASIP.*

Using a publicly available US population GWAS dataset (Simon-Sanchez *et al.*, 2007), we were able to show that the peak CMM SNP set described by Gudbjartsson *et al.* (2008) was in strong linkage disequilibrium with rs4911442, despite being 600 kbp distant (P=4×10^{-13}). Specifically, an individual carrying the G-A risk haplotype defined by Gudbjartsson *et al.* (2008) had a 50% probability of carrying the rs4911442*C on that haplotype, while the other 3 haplotypes were associated with rs4911442*T in 99% of cases.

DISCUSSION

This paper attempts to address two topics. The first is to confirm and quantify the overall contribution of currently recognized pigmentation loci to variation in risk of cutaneous melanoma. Secondly, we expand the evidence of the recently recognized association between SNPs near *ASIP* and CMM, and attempt to reconcile results from different studies (Gudbjartsson *et al.* 2008; Brown *et al.*, 2008).

We have replicated association of SNPs at our chosen loci *MC1R*, *OCA2*, *ASIP*, *SLC45A2*, *SLC24A4*, *TYR*, *TYRP1*, and *IRF4* with skin, hair and eye color, and shown that most of these SNPs are also significant predictors of CMM risk in our population. Because some SNPs are not genotyped in all subjects, the dataset suitable for multivariate analysis is reduced, so the confidence intervals around simultaneously adjusted effect sizes are broader than for (most of) the univariate analyses. The pattern is broadly consistent with multiplicative epistasis., with only weak evidence for non-multiplicative interactions The combination of SNPs in the five associated loci (*MC1R*, *SLC45A2*, *OCA2*, *TYR*, *ASIP*) explains roughly one-third to one-half of the variation in risk due to observed pigmentation phenotype. Pigmentation phenotype itself explains approximately one-third of the familial aggregation of melanoma. By contrast, the high penetrance CDKN2A mutations segregating in families with multiple cases of melanoma explain only 0.1% of recurrence risk in the total population. This is because they are extremely rare, involved in 0.2% of all Queensland melanoma cases (Aitken et al, 1999), with an allele frequency $\sim 5 \times 10^{-6}$. Attributable risk is another measure of the importance of a risk locus, but is not completely satisfactory in that genotypes that are rare in our population may offer the lowest absolute reduction in risk (for example, the rare protective allele in *SLC45A2*).

This is only the third study reporting an association between *SLC45A2* and CMM. Fernandez and coworkers (Fernandez *et al.*, 2008) genotyped the coding polymorphism rs16891982 (F374L) in a collection of Spanish CMM cases and controls, while Guedj *et al.* (2008) report results from a French case-control study. In keeping with our findings, the minor (*L374*) allele was protective (7% frequency in Spanish cases and 16% frequency in Spanish controls; 4% and 10% in the French sample). This protective allele is associated with olive and dark skin, and so is least common in northern European populations (1.7% in the Utah-derived CEU HapMap sample), more common in southern Europeans, but is fixed in African and most Asian populations (Yuasa *et al.*, 2006). In our Australian sample, examination of the 3 SNP haplotypes, as well as logistic regression analysis showed that rs16891982 is the SNP most strongly associated with melanoma risk in our population.

The variant in the tyrosinase gene most strongly associated with CMM risk by Gudbjartsson and coworkers (2008) was rs1126809 (*R402Q*). We found significant association with rs1126809, and any association to the intronic SNP rs10765198 disappeared in multivariate models where rs1126809 was included as a covariate. The *Q402* variant form of tyrosinase has 25% of the catalytic activity of the *R402* form at 37°C (Tripathi *et al.*, 1991), and causes autosomal recessive ocular albinism when co-occurring with more penetrant OCA mutations in compound heterozygote form (Fukai *et al.*, 1995; Hutton *et al.*, 2008). It is therefore a strong candidate for the causative risk variant in this gene. Like Gudbjartsson and coworkers (2008), we did not detect any association between rs1042602 (*TYR**S192Y) with CMM in our Australian sample, even though those authors did detect an association with freckling in their Scandinavian sample.

In the case of *OCA2*, we found that the SNP (rs12913832) most strongly associated with blue eye colour in our sample (Sturm *et al.*, 2008) was not associated with CMM, especially after adjusting for ancestry. Rather, the less common coding mutation *R419Q* (rs1800407)

was a significant risk factor, notably in the multivariate analysis adjusting for other pigmentation loci. This association has also been seen in a Spanish dataset (Fernandez *et al.*, 2009), with similar effect sizes. In the French case-control study of Jannot *et al.* (2005), *R419Q* was not a significant risk factor for melanoma, but the nearby *R305W* and neighbouring intronic SNPs were. The *R305W* and *R419Q* variants are in complete linkage disequilibrium in our population. The *OCA2* variants screened by Gudbjartsson *et al.* (2008) were not associated with CMM.

The *TYRP1* (*OCA3*) variants we genotyped have been previously shown to be associated with eye and hair colour (Sulem *et al.* 2008), and with CMM risk (Gudbjartsson *et al.*, 2008). We confirmed these associations and effect size estimates. These variants differ significantly in frequency between the main HapMap ethnic samples, and we could demonstrate a gradient with proportion Northern European ancestry paralleling hair and eye colour. We did not detect specific interactions between *TYR* and *TYRP1* in their effects on melanoma risk or pigmentation phenotypes.

The two recent reports of association of SNPs in the region of *ASIP* to CMM risk (Gudbjartsson *et al.*, 2008; Brown *et al.*, 2008) implicated widely separated SNPs. We observed that the association of CMM to the *ASIP* region closely paralleled that with red hair color, and seems to be due largely to a single long (approximately 1.8 Mb) haplotype that straddles a number of haplotype block boundaries. This is likely to represent the effects of selection along with a relatively recent origin, as has been observed around other pigmentation loci (Barreiro *et al.*, 2008), given that this particular haplotype is also associated with lighter hair and skin color.

Given the role of Agouti signalling protein (*ASIP*) as an antagonist of the melanocortin pathway, it seems biologically plausible that there would be an interaction between *MC1R* genotype and *ASIP* genotype. For red hair color, this was markedly so, with the rs4911442*C allele being roughly equivalent to an "r" MC1R haplotype. This is seen most easily seen comparing the penetrances of the joint "R/w; C/T" genotype to the "R/r; T/T" genotype, and in the linear dose-response relationship between number of rs4911442*C alleles on a "R/r" background (Table 8). In the same fashion, MC1R "R" haplotypes were dominant to rs4911442*C. Therefore *ASIP* is a red hair color locus, in the same way as *MC1R*. In keeping with this, rs4911442 genotype was a significant predictor of facial freckling (P=0.0001), as noted by others (Gudbjartsson *et al.*, 2008). A similar pattern was observed for CMM, but did not reach formal statistical significance. The association signal for red hair color and CMM across chromosome 20 did track quite strongly, so we expect that the epistatic pattern seen for these two loci in respect to hair color would also be demonstrable for CMM given a larger sample size.

An interesting negative finding in this study is the absence of an association between *IRF4* genotype and melanoma risk, despite the very strong evidence for association with this locus and skin coloring in this and other studies (Han *et al.*, 2008; Sulem *et al.*, 2008). This was also noted by Gudbjartsson *et al.* (2008), and is in some ways the obverse of our previous observation (Palmer *et al.*, 2000) that some pigmentation loci such as MC1R seem to affect CMM risk by pathways other than that mediated by measurable changes in skin coloring or tanning ability. Here, we observe an effect on coloring which one would expect to significantly influence response of the organism to exposure to ultraviolet light, but appears neutral with respect to CMM risk. In the case of *OCA2*, we and others have previously described how a single SNP rs12913832 strongly predicts eye color (Sturm *et al.*, 2008; Kayser *et al.*, 2008; Eiberg *et al.*, 2008), but has little or no effect on CMM risk. We do see an effect of another *OCA2* polymorphism rs1800407 on CMM risk, but this is less impressive in terms of statistical significance.

A peculiarity of these analyses is that most of these pigmentation loci markedly differ in allele frequencies between different ethnic groups, in a way that parallels the differences in CMM rates between these groups. The simplest hypothesis, given that we have shown that all the selected variants are associated with pigmentation, is that these variants underlie ethnic variation in CMM risk. To definitively exclude ethnic confounding as a cause of the observed association between ancestry informative variants and disease risk, we would need either a cohort design, where we could show that the rate of CMM in carriers of all low risk variants is comparable to that seen in ethnic groups of known low "intrinsic" risk, or a family based design where we could also demonstrate linkage of these variants. Unfortunately, the age of onset of CMM in our sample and sampling design means that we cannot pursue the latter strategy, although we can demonstrate significant transmission-disequilibrium test results for pigmentation phenotypes such as skin colouring.

In conclusion, the variants in pigmentation genes we describe explain approximately 40% of the variation in familial risk of melanoma ascribable to phenotypes such as skin, hair and eye color. In the cases of *TYR*, *SLC45A2*, *OCA2* and *MC1R*, it is likely that the major causative risk variants have been identified, but this is still open for *ASIP*, and other minor risk variants at all loci await characterization.

MATERIALS AND METHODS

Study participants

CMM cases were a stratified sample of all cases of CMM diagnosed in the state of Queensland in the period 1982–1990, as described in detail elsewhere (Baxter *et al.*, 2008). These individuals were originally studied 1991–1994, but were recontacted and interviewed in 2002–2004.

The controls for the present analysis come from the Brisbane Twin Nevus Study. As described in detail elsewhere (Sturm *et al.*, 2008), adolescent twins, their siblings and parents have been recruited over sixteen years into an ongoing study of genetic and environmental factors contributing to the development of pigmented nevi and other risk factors for skin cancer. The proband twins are recruited at age twelve years via schools around Brisbane, Australia, and followed up at age fourteen. All controls are screened to be unaffected by CMM. The sample is overwhelmingly (>95%) of northern European origin (mainly Anglo-Celtic). All cases and controls gave informed consent to participation in this study, and the study protocol was approved by appropriate institutional review boards.

Ancestry was measured via questions to either the individual or a parent about the country of birth and ancestry of each of the grandparents of the individual. Since most subjects were of European ancestry, we have constructed three ancestry scores based on the proportion of grandparents of Northern European (British, Scandinavian, Danish, Dutch, German, French), Southern European (Spanish, Italian, Greek) and Eastern European ancestry. In addition, the Northern indicator was supplemented by "Celtic" (Ireland, Scotland and Wales), English and Continental indicators, to test whether there were further substructure effects. Ancestry data is available for all cases and controls included in these analyses.

Genotyping and statistical analysis

We selected SNPs for association testing based on association with pigmentation phenotypes in our own and other studies (Palmer *et al.*, 2000; Sturm *et al.*, 2008; Sulem *et al.*, 2007;, Han *et al.*, 2008). In the case of SNPs near *ASIP* (Brown *et al.*, 2008), we had observed maximal association to CMM at SNPs away from *ASIP* itself, so the two SNPs reported to be most strongly associated to CMM in the study of Gudbjartsson *et al.* (2008) were not genotyped. We did not genotype within *SLC24A5* ("Golden"), given that the variant

associated with human skin colour is almost monomorphic in whites. SNPs were typed using iPLEX™ Gold chemistry on a MALDI-TOF Mass Spectrometer (Sequenom Inc, San Diego). PCR reactions were carried out in 2.5 ul in standard 384-well plates. PCR was performed with 10ng genomic DNA, 0.5 unit of *Taq* polymerase (HotStarTaq, Qiagen, Valencia, CA), 500 umol of each dNTP, and 100 nmol of each PCR primer. PCR thermal cycling in an ABI-9700 instrument was 15 min at 94°C, followed by 45 cycles of 20 sec at 94°C, 30 sec at 56°C, 60 sec at 72°C. To the completed PCR reaction, 1 µl containing 0.15 units Shrimp Alkaline Phosphatase was added and the reaction incubated for 30 min at 37°C followed by inactivation for 5 min at 85°C. After adjusting the concentrations of extension primers to equilibrate signal-to-noise ratios, the post-PCR primer extension reaction of the iPLEX assay was performed in a final 5 ul volume extension reaction containing 0.1 ul of termination mix, 0.02 ul of DNA polymerase (Sequenom, San Diego, CA), and 600 nM to 1200 nM extension primers. A two step 200 short cycles program was used for the iPLEX reaction: initial denaturation was 30 sec at 94°C followed by 5 cycles of 5 sec at 52°C and 5 sec at 80°C. An additional 40 annealing and extension cycles were then looped back to 5 sec at 94°C, 5 sec at 52°C and 5 sec at 80°C. The final extension was carried out at 72°C for three minutes and the sample was cooled to 20°C. The iPLEX reaction products were desalted by diluting samples with 15 ul of water and adding 3 ul of resin, centrifuged to remove resin. The products spotted on a SpectroChip (Sequenom Inc, San Diego), processed and analysed in a Compact Mass Spectrometer by MassARRAY Workstation (version 3.3) software (Sequenom Inc, San Diego). Since genotyping has been carried out in various subsets of the data, the number of genotyped cases and controls varies from marker to marker. Specifically, some markers are genotyped in the entire collection, while others have been genotyped only in a nested sample of unrelated cases and controls.

Since some cases (105 individuals) are from 48 multiplex families and all our controls come from twin families, the association analyses were carried out using Sib-pair 1.0 (Duffy *et al.*, 2008). The association analysis in Sib-pair implements a logistic regression penetrance analysis of measured genotypes within pedigrees that may include monozygotic twins, and uses Monte Carlo (gene-dropping) simulation to obtain P-values adjusted for the relationships of cases and controls. Other analyses have been carried out in the R statistical analysis environment (R Core Development Team, 2008), using especially the *haplo.stats* package (Sinnwell *et al.*, 2007), and also using Haploview (Barrett *et al.*, 2005) to generate plots and long range haplotypes. We express the overall contribution of the measured loci to risk of melanoma in logistic regression analyses as the Nagelkerke R^2 (Nagelkerke 1991), and have also calculated the locus-specific sibling recurrence risk (James, 1971; Risch, 1990) predicted for that locus under an assumed lifetime risk of 5.2% (Queensland Cancer Registry, 2008). This further assumes a proportional hazards model is appropriate, but is quite robust to the assumed lifetime risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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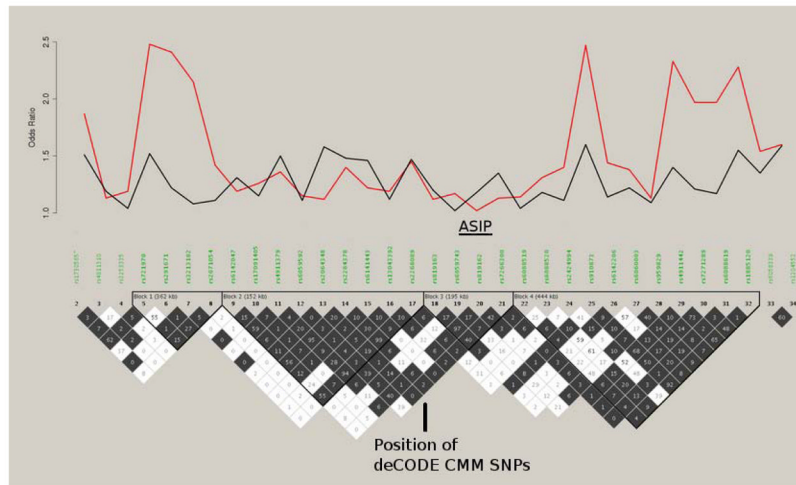
Figure 1.
Odds Ratios for red hair color (red line) and cutaneous malignant melanoma (black line) and pairwise linkage disequilibrium (r^2, with colors generated using the "4 gamete rule") versus SNPs around *ASIP.*

Table 1

Descriptive statistics for genotyped CMM cases and controls (percentages are of total with data for that phenotype, except for ancestry).

	CMM Cases	**Controls**
All four grandparents of northern European ancestry	1483 (93.0%)	3098 (83.3%)
Blue/grey eyed	758 (43.7%)	1838 (44.7%)
Green/hazel eyed	692 (39.9%)	1212 (29.5%)
Brown/black eyed	283 (16.3%)	1059 (25.8%)
Blond hair	419 (24.1%)	569 (23.7%)
Light brown hair	653 (37.6%)	1391 (33.9%)
Red hair	193 (11.1%)	217 (5.3%)
Dark brown hair	402 (23.1%)	1705 (41.5%)
Black hair	71 (4.1%)	226 (5.5%)
Male	967 (47.8%)	2129 (47.2%)
Total genotyped individuals	1738	4517

Table 2

Allele frequencies and tests of Hardy-Weinberg Equilibrium for pigmention-associated polymorphisms in Australian CMM cases and controls.

Chr	Locus/Variant	rs ID	Alleles	Controls			CMM Cases		
				N	MAF[a]	HWE-P[b]	N	MAF[a]	HWE-P[b]
5	SLC45A2 i3 [c]	rs35391	A (G)	4107	0.0321	0.1527	1708	0.0088	1.0000
5	SLC45A2 i3	rs28777	G (T)	4111	0.0429	0.0001	1716	0.0118	1.0000
5	SLC45A2*F374L	rs16891982	G (C)	4032	0.0518	0.0000	1731	0.0133	0.0346
6	IRF4 i4	rs12203592	T (C)	4113	0.2259	0.8696	1727	0.2107	0.0264
9	TYRP1	rs1408799	T (C)	4110	0.3275	0.0766	1703	0.2877	0.0980
9	TYRP1 i6	rs2733832	C (T)	4098	0.4119	0.0492	1732	0.3753	0.1514
11	TYR i3	rs10765198	C (T)	4103	0.3138	0.1739	1721	0.3405	0.2930
11	TYR*S192Y	rs1042602	A (C)	4112	0.3486	0.7407	1728	0.3390	0.0150
11	TYR*R402Q	rs1126809	A (G)	4102	0.3061	0.7688	1731	0.35	0.08
14	SLC24A4	rs12896399	T (G)	4102	0.4493	0.2857	1705	0.4564	0.1512
15	OCA2*R419Q	rs1800407	A (G)	3996	0.0798	0.6061	1052	0.1042	1.0000
15	HERC2 i86	rs12913832	T (C)	4102	0.2315	0.0190	1747	0.2116	0.1133
16	MC1R*V60L	rs1805005	T (G)	2849	0.1212	0.6061	1945	0.1181	0.2041
16	MC1R*D84E	rs1805006	T (G)	2851	0.0111	0.4651	1947	0.0194	0.0008
16	MC1R*V92M	rs2228479	A (G)	1882	0.0961	0.0794	1286	0.0995	0.5023
16	MC1R*R142H	rs11547464	A (G)	1805	0.0044	0.4878	1114	0.0198	1.0000
16	MC1R*R151C	rs1805007	T (C)	1876	0.1096	0.9091	1126	0.1567	0.5223
16	MC1R*R160W	rs1805008	T (C)	2848	0.0697	0.6061	1947	0.1122	0.8365
16	MC1R*R163Q	rs885479	A (G)	1896	0.0466	0.7407	1291	0.0476	0.6452
16	MC1R*D294H	rs1805009	C (G)	2851	0.0266	0.9091	1947	0.0328	1.0000
20	ASIP region [d]	rs4911442	C (T)	4089	0.1306	0.3003	1745	0.1848	0.2003

[a] MAF = minor allele frequency

[b] HWE-P = Hardy-Weinberg Equilibrium test P-value

[c] The letter i denotes intron, so HERC2 i86 denotes the 86th intron of the HERC2 gene

[d] Actually in intron of *NCOA6*, 500 kbp distal to *ASIP*

Table 3

Results of association analyses of selected SNPs with skin, eye and hair color in individuals with all four grandparents of northern European ancestry.

Locus/Variant	SNP	Allelic Odds Ratio for pale skin (P-value)	Allelic Odds Ratios for blue eye color (P-value)	Allelic Odds Ratios for light hair (P-value)[a]
SLC45A2 i3	rs35391	3.42 (<1.0e-5)	2.60 (0.001)	5.34 (<1.0e-5)
SLC45A2 i3	rs28777	4.31 (<1.0e-5)	2.56 (0.017)	6.30 (<1.0e-5)
SLC45A2*F374L	rs16891982	3.80 (<1.0e-5)	2.50 (<1.0e-5)	6.72 (<1.0e-5)
IRF4 i4	rs12203592	1.63 (<1.0e-5)	1.28 (0.0017)	2.22 (<1.0e-5)
TYRP1	rs1408799	1.06 (0.2985)	1.22 (0.018)	1.22 (<1.0e-5)
TYRP1 i6	rs2733832	1.05 (0.3333)	1.22 (0.007)	1.22 (<1.0e-5)
TYR i3	rs10765198	1.25 (0.0015)	1.33 (<1.0e-5)	1.01 (0.77)
TYR*S192Y	rs1042602	1.06 (0.11)	1.04 (0.57)	1.13 (0.13)
TYR*R402Q	rs1126809	1.27 (<1.0e-5)	1.43 (<1.0e-5)	
SLC24A4	rs12896399	1.17 (0.061)	1.85 (<1.0e-5)	1.45 (<1.0e-5)
OCA2*R419Q	rs1800407	1.10 (0.59)	2.37 (<1.0e-5)	1.21 (0.14)
HERC2 i86	rs12913832	1.74 (<1.0e-5)	30.77 (<1.0e-5)	2.95 (<1.0e-5)
MC1R*V60L	rs1805005	1.01 (0.85)	1.00 (0.80)	1.13 (0.008)
MC1R*D84E	rs1805006	5.19 (<1.0e-5)	1.23 (0.61)	1.74 (0.041)
MC1R*V92M	rs2228479	1.38 (0.024)	1.06 (0.61)	1.17 (0.016)
MC1R*R142H	rs11547464	1.31 (0.54)	1.03 (0.71)	1.28 (0.74)
MC1R*R151C	rs1805007	2.57 (<1.0e-5)	1.09 (0.94)	2.13 (<1.0e-5)
MC1R*R160W	rs1805008	1.72 (<1.0e-5)	1.12 (0.17)	1.82 (<1.0e-5)
MC1R*R163Q	rs885479	1.10 (0.53)	1.21 (0.15)	1.12 (0.014)
MC1R*D294H	rs1805009	2.88 (<1.0e-5)	1.16 (0.74)	1.68 (<1.0e-5)
MC1R "R"[b]		3.70 (<1.0e-5)	1.09 (0.43)	2.30 (<1.0e-5)
MC1R "r"[c]		1.84	1.10	1.26
ASIP region	rs4911442	1.43 (<1.0e-5)	1.01 (0.87)	1.28 (<1.0e-5)

[a] Light hair = blond, light brown, red.

[b] High penetrance red hair color MC1R haplotype: variant at D84E, R142H, R151C, R160W, or D294H.

[c] Low penetrance red hair color MC1R haplotype: variant at V60L, V92M, or R163Q.

Table 4

Results of association analyses of individual pigmentation-associated SNPs and risk of CMM.

Locus/Variant	SNP	Allelic Odds Ratio for CMM (P-value)			
		All cases and controls	Subset: 100% Northern European ancestry	Same subset, adjusted for hair, eye and skin color[a]	Same subset, adjusted for MC1R genotype
SLC45A2 i3	rs35391	3.71 (<1.0e-4)[d]	2.49 (0.0014)	1.53 (0.11)	1.68 (0.16)
SLC45A2 i3	rs28777	3.75 (<1.0e-4)	2.37 (0.0012)	1.68 (0.045)	2.28 (0.0028)
SLC45A2*F374L	rs16891982	3.44 (<1.0e-4)	2.86 (<1.0e-5)	1.68 (0.0001)	1.68 (0.0004)
IRF4 i4	rs12203592	1.04 (0.32)	1.18 (0.0075)	1.05 (0.45)	1.03 (0.77)
TYRP1	rs1408799	1.20 (0.0007)	1.14 (0.0117)	1.166 (0.014)	1.06 (0.56)
TYRP1 i6	rs2733832	1.16 (0.0018)	1.12 (0.0243)	1.121 (0.025)	1.06 (0.33)
TYR*R402Q	rs1126809	1.20 (0.002)	1.13 (0.001)	1.19 (0.0059)	1.37 (0.0004)
TYR*S192Y	rs1042602	1.05 (0.30)	1.10 (0.10)	1.14 (0.040)	1.17 (0.041)
SLC24A4	rs12896399	1.11 (0.24)	1.12 (0.37)	1.07 (0.30)	1.09 (0.24)
OCA2*R419Q	rs1800407	1.31 (0.004)	1.18 (0.20)	1.23 (0.096)	1.35 (0.022)
HERC2 i86	rs12913832	1.15 (0.042)	1.06 (0.51)	1.20 (0.078)	1.21 (0.048)
MC1R*V60L	rs1805005	1.01 (0.80)	1.02 (0.87)	1.02 (0.83)	-
MC1R*D84E	rs1805006	1.61 (0.013)	2.15 (0.004)	1.58 (0.10)	-
MC1R*V92M	rs2228479	1.07 (0.53)	1.12 (0.42)	1.08 (0.62)	-
MC1R*R142H	rs11547464	2.25 (0.055)	3.33 (0.014)	3.85 (0.011)	-
MC1R*R151C	rs1805007	1.58 (7.0e-4)	1.39 (0.0042)	1.00 (0.99)	-
MC1R*R160W	rs1805008	1.68 (<1.0e-4)	1.53 (<1e-4)	1.29 (0.092)	-
MC1R*R163Q	rs885479	1.15 (0.54)	1.04 (0.99)	1.04 (0.83)	-
MC1R*D294H	rs1805009	1.42 (0.019)	1.27 (0.87)	1.01 (0.87)	-
MC1R "R"[b]		1.65 (<1e-5)	1.56 (<1e-5)	1.12 (0.1481)	
MC1R "r"[c]		1.10	1.08	0.95	
ASIP region	rs4911442	1.49 (<1e-5)	1.46 (<1e-5)	1.32 (8.0e-4)	1.39 (0.0037)

[a]Hair color encoded as 4 category categorical variable (fair, light brown, red, dark). Eye color as 3 category ordinal trait. Skin color as 3 category ordinal trait.

[b]High penetrance red hair color MC1R haplotype: variant at D84E, R142H, R151C, R160W, or D294H.

[c] Low penetrance red hair color MC1R haplotype: variant at V60L, V92M, or R163Q.

[d] Bolding denotes P value $< 10^{-3}$ (equivalent to P<0.04 after a Bonferroni correction for forty tests).

Table 5

Multivariate analysis of CMM versus 5 polymorphic pigmentation loci, and pigmentation phenotype in individuals of 100% Northern European ancestry (1062 cases, 1262 controls).

Variant	Allelic Odds Ratio (no covariates)	Allelic Odds Ratios (pigmentation as covariate)	Locus-specific Sibling Recurrence Risk Ratio[5] (Attributable Risk[6])
MC1R "r"	1.10 (0.94–1.27)	1.09 (0.94–1.27)	1.047 (19.4%)
MC1R "R"	1.74 (1.49–2.01)	1.46 (1.24–1.72)	
rs16891982*C SLC45A2	2.47 (1.51–4.07)	2.04 (1.27–3.40)	1.058 (90.6%)
rs1800407*A OCA2*R419Q	1.32 (1.09–1.61)	1.34 (1.10–1.64)	1.008 (5.1%)
rs1126809*A TYR*Q402R	1.24 (1.09–1.41)	1.18 (1.04–1.34)	1.010 (8.0%)
rs4911442*C ASIP	1.44 (1.23–1.69)	1.40 (1.19–1.65)	1.016 (10.8%)
Hair colour[1]	-	0.88, 1.50, 0.72, 0.59 (P=1.8e-5)[4]	1.374
Skin Colour[2]	-	0.98, 0.63 (P=0.007)[4]	
Eye Colour[3]	-	1.22, 1.05 (P=0.10)[4]	
Total Model R^2	5.9%	8.3%	

[1] Five categories (fair, light brown, red, dark brown, black); reference category, fair.

[2] Three categories (light, medium, dark); reference category, light.

[3] Three categories (blue/grey, green/hazel, brown/black); reference category, blue/grey.

[4] P-value from sequential likelihood ratio test.

[5] Assuming a lifetime melanoma risk of 5.2% in the Queensland population

[6] Proportional reduction in disease rate if entire population carried lowest risk genotype.

Table 6

Interaction between rs4911442 (near ASIP) and *MC1R* effects on red hair color, skin color and CMM risk.

Joint Genotype		Cutaneous Melanoma			Red Hair		
MC1R	rs4911442	Cases	N	Odds Ratio	Red	Total	Penetrance
R/R	C/C	1	2	5.00	1	2	0.500
	C/T	17	34	5.00	30	38	0.789
	T/T	45	94	4.59	73	108	0.676
R/r	C/C	3	11	1.88	5	12	0.417
	C/T	27	60	4.09	12	74	0.162
	T/T	73	226	2.55	13	250	0.052
R/w	C/C	2	5	3.33	0	6	0.000
	C/T	43	112	3.12	8	140	0.057
	T/T	99	343	2.03	13	412	0.032
r/r	C/C	3	5	7.50	0	5	0.000
	C/T	20	35	6.67	2	39	0.051
	T/T	19	83	1.48	0	100	0.000
r/w	C/C	2	16	0.71	0	19	0.000
	C/T	33	127	1.76	3	148	0.020
	T/T	74	323	1.49	2	417	0.005
w/w	C/C	2	2	24.81	0	3	0.000
	C/T	24	101	1.56	0	135	0.000
	T/T	52	312	1.00	0	412	0.000