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Interactive effects of MC1R and OCA2 on melanoma risk phenotypes

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The relationships between MC1R gene variants and red hair, skin reflectance, degree of freckling and nevus count were investigated in 2331 adolescent twins, their sibs and parents in 645 twin families. Penetrance of each MC1R variant allele was consistent with an allelic model where effects were multiplicative for red hair but additive for skin reflectance. Of nine MC1R variant alleles assayed, four common alleles were strongly associated with red hair and fair skin (Asp84Glu, Arg151Cys, Arg160Trp and Asp294His), with a further three alleles having low penetrance (Val60Leu, Val92Met and Arg163Gln). These variants were separately combined for the purposes of this analysis and designated as strong '*R*' (OR = 63.3; 95% CI 31.9–139.6) and weak '*r*' (OR = 5.1; 95% CI 2.5–11.3) red hair alleles. Red-haired individuals are predominantly seen in the *R*/*R* and *R*/*r* groups with 67.1 and 10.8%, respectively. To assess the interaction of the brown eye color gene OCA2 on the phenotypic effects of variant MC1R alleles we included eye color as a covariate, and also genotyped two OCA2 SNPs (Arg305Trp and Arg419Gln), which were confirmed as modifying eye color. MC1R genotype effects on constitutive skin color, freckling and mole count were modified by eye color, but not genotype for these two OCA2 SNPs. This is probably due to the association of these OCA2 SNPs with brown/green not blue eye color. Amongst individuals with a *R*/*R* genotype (but not *R*/*r*), those who also had brown eyes had a mole count twice that of those with blue eyes. This suggests that other OCA2 polymorphisms influence mole count and remain to be described.

INTRODUCTION

Skin, hair and eye color are some of the most obvious forms of human diversity (1), yet the complexities of the genetic mechanisms underlying differences in these traits are only now beginning to be understood. It is likely that a relatively small set of genes contribute most of the variation in pigmentation phenotypes seen in human populations, and that they do this by regulating the level of synthesis, chemical composition, packaging and distribution of melanin (2,3). The melanocortin-1 receptor (MC1R) is the first human gene in which coding region polymorphisms have been shown to be a major determinant of variation in pigmentation phenotypes. However, the contribution of individual MC1R alleles to hair and skin color and other traits such as freckling (ephelidae), moliness (nevus density) and tanning ability (difference in reflectance of exposed and unexposed skin) has not yet been fully defined.

MC1R is a seven-transmembrane G-protein coupled receptor that activates adenylate cyclase to elevate cAMP levels upon hormone stimulation (4). The interaction of MC1R with the natural POMC (pro-opiomelanocortin) derived α-MSH (melanocyte-stimulating hormone) and ACTH (adrenocorticotropic hormone) peptide ligands influences the activity of enzymes and the structural proteins involved in the melanogenic pathway, leading to a switch from the synthesis of the red/yellow pheomelanin product to black/brown eumelanin (5). MC1R variant alleles with amino acid substitutions within the coding region result in an altered receptor activity (6,7), and are associated with the red hair and fair skin (8–10) a phenotype (denoted RHC; red hair color) that is caused by the synthesis of a high level of pheomelanin (11,12). Gene expression studies during eumelanogenesis have shown increased expression of tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and tyrosinase-related protein 2 (DCT) and the P-protein genes are

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directed through MC1R stimulation (13,14). However, in the absence of a strong MC1R signal lower levels of TYR and P-protein together with the absence of TYRP1 and DCT may be enough to switch synthesis to pheomelanin.

Hormonal stimulation of MC1R is also central to the tanning response of human melanocytes following UV irradiation (15,16). Although the hormonal signaling pathways are yet to be fully defined, a synergistic action of α-MSH together with other mitogens on protein phosphorylation has been reported (17). These pathways converge inducing changes in gene expression largely through the transcription factor MITF (microphthalmia transcription factor), which appears to be critical in activation of the eumelanogenic pathway (18,19). Combined treatment of melanocytes with UV and α-MSH also potentiates cell dendricity (20) and transfer of pigment to keratinocytes (21).

The MC1R transcript expressed in melanocytes has an intronless coding region and the gene is located at the telomeric end of chromosome band 16q24.3 and the complete transcription unit of this region has recently been defined (22,23). Population surveys have shown that the MC1R gene is highly polymorphic. Over 30 variant alleles have been reported in Caucasian populations from the British Isles, Australia, Holland and the USA (tabulated in 3,24). There are also alleles of high frequency in the Asian population (25,26), but there are no variants in African populations that result in amino acid changes, indicating an evolutionary history of strong selective pressure favoring the consensus sequence.

Several genetic studies of the relationship between MC1R variant alleles and the RHC phenotype also suggested their association with number of freckles (10,27,28) and sun-induced lentigines (29). Carriers of two MC1R variants are now thought to have as much as an 11-fold increased risk of freckling and 2-fold higher risk of solar lentigines (30), with MC1R contributing up to 60% of the attributable risk for ephelides. The influence of MC1R genetic status on skin sun-sensitivity has also been investigated in recent studies that have shown an association between the degree of tanning after repeated sun exposure and MC1R genotype (31). Although no difference was found between individuals with a single minimal erythemal dose of UV-B, MC1R did have an effect on the erythema dose-response gradient when treatments above this level were given (32). However, these reports have not presented quantitative data on the relationship of MC1R genotype to basal (unexposed) and facultative (sun exposed) skin reflectance measurements. Other genes are likely to modify MC1R genetic effects in determination of pigmentation phenotype and it is notable that an epistatic interaction with the OCA2 gene, which is mutated in Type II albinism and encodes the P-protein, has recently been suggested (33). Polymorphism of the OCA2 gene almost certainly underlies the previous assignment of the brown/blue eye (BEY2/EYCL3, MIM227220) and brown hair (HCL3, MIM601800) loci to chromosome 15q (34), with two OCA2 coding region variants Arg305Trp and Arg419Gln recently shown to be associated with non-blue eye colors (35).

With this in mind, we present data on the specific effects of MC1R variant alleles on hair, eye, spectrophotometric measurements of skin color, nevus and ephelide count, and how these allelic effects interact with each other (dominance) and with inferred allelotypes at the BEY2 locus (apparently the OCA2 gene) and the two OCA2 polymorphisms reported to influence eye color (epistasis).

RESULTS

Pigmentation characteristics

Nurse ratings of hair color were highly consistent with more objective gradings using standard hair color swatches. We therefore used the former as the categories coincided with those used by the parents to rate their own hair color. Use of the skin reflectance measurements, however, did refine information on skin color beyond the nurse ratings, so both are used. Frequencies of hair, skin and eye color ratings (Tables 1–4) are consistent with those found in our earlier reports for the South-East Queensland (27,29) and general Australian populations (36,37). The genotyped subjects were 52% female and 48% male. There were no significant differences in pigmentation phenotypes between the sexes, except that females were rated as having fairer skin ($P < 0.01$). However, skin reflectance measurements suggest this is due to differential tanning (51.7% reflectance for females on back of hand versus 50.4% for males) rather than a difference in constitutive pigmentation (61.4 versus 61.2% for inner upper arm).

There was a very wide range in the number freckles (nil to severe) and also of nevi (3–422), 76% of the sample having some freckling present on at least one of the three body sites examined. On average, males had 10 more moles than females and a similar trend was observed for freckling, which was particularly obvious on the shoulders. This male excess has been noted in several epidemiological studies (38).

Red-haired subjects had the greatest number of freckles and least number of moles and this has also been reported in other studies of juvenile subjects (39–47). There was a non-linear increase in freckling score when grouped by hair color (Fig. 1A). No individual designated as black-haired displayed freckling, scores were then lowest for dark brown, with slightly higher and equivalent levels in light brown and fair individuals. Freckles were present at multiple sites in red-haired individuals amongst whom were numbered the most extreme levels of freckling—80% of red heads were in the severe category compared with only 17% of other hair colors. A non-linear correlation was also apparent between total mole count and composite freckle score in the adolescent twin collection group plotted by hair color (Fig. 2A); those with red hair had the lowest mole numbers compared with the other colors up until the highest levels of freckling were reached, whereupon the fair, light brown and dark brown curves then overlapped. In the total group analysis presented as a box plot, those having greater numbers of freckles or more freckling sites also scored an increased number of nevi until severe freckling became apparent, whereupon mole number decreased.

The upper inner arm and back of hand mean reflectances were 61.3 and 51.0% respectively, representing an average 20% increase over basal levels when considering facultative skin color on exposed skin. Those with red hair had slightly higher skin reflectance measurements with a mean on the dorsal hand of 55.7%, representing only a 10% increase in facultative skin

Table 1. Phenotypic characteristics of MZ and DZ twins, sibs and parents

	Female	(%)	Male	(%)
Hair color				
Red/auburn	54	(3.1)	35	(2.1)
Fair/blonde	166	(9.6)	130	(7.6)
Light brown	352	(20.4)	323	(18.8)
Dark brown	299	(17.3)	287	(16.7)
Black	23	(1.3)	52	(3.0)
Total	894	(52.0)	827	(48.0)
Eye color				
Blue/gray	384	(22.3)	387	(22.4)
Green/hazel	297	(17.2)	218	(12.7)
Brown	214	(12.5)	223	(12.9)
Total	895	(52.0)	828	(48.0)
Skin color				
Fair/pale	461	(26.8)	392	(22.7)
Medium	358	(20.8)	345	(20.0)
Olive/dark	76	(4.4)	91	(5.3)
Total	895	(52.0)	828	(48.0)

color. Comparison of pigmentation phenotypes at ages 12 and 14 found a trend towards darkening in hair and skin color, but no change in the number of red-haired individuals was seen.

Multiplicative penetrance of variant MC1R alleles in red hair

The genotype and allele frequencies at nine polymorphic amino acid sites within the MC1R coding region are summarized as a matrix of genotypes associated with red : non-red hair color in Table 5 (lower triangle). There was no Hardy–Weinberg disequilibrium for any of the variants, and analysis of a subsample using flanking polymorphisms showed most haplotypes carried no more than one variant from the consensus allele (9,23,24,27). However, double and triple variants have previously been noted (9,27) and two individuals in our collection carried genotypes with three variants Asp84Glu, Val92Met, Arg160Trp and Ile155Thr/Ile155Thr, Arg160Trp. RHC-variant alleles Arg151Cys, Arg160Trp, Asp294His are common in the Queensland population and are responsible for most of the red hair color in this community consistent with our earlier reports (9,27,29,48). Inspection of the genotype matrix shows that at least one of these three alleles is found in 93% of those with red hair. With the exception of the Arg142His and Ile155Thr variants, which occur at relatively low frequencies, all variants could be found in at least one red-haired individual, most commonly paired with one of the other RHC alleles as a compound heterozygote. The consensus allele in combination with a variant allele was found in only 11% of red heads.

Penetrance of each variant allele was modeled in a logistic regression analysis using red and fair/blonde hair phenotypes separately. This indicated the three RHC alleles Arg151Cys, Arg160Trp and Asp294His were highly associated with red hair and fair skin, showing odds ratios (OR) of 118, 50 and 94 respectively (Table 6) compared with low-strength alleles Val60Leu and Val92Met with OR 6 and 5 relative to the consensus allele for red hair. The Asp84Glu allele was relatively strongly associated with RHC, recording an OR of 62; the weakest allele was Arg163Gln and this gave only a 2-fold increase in association with red hair. The regression model assumes that these alleles act multiplicatively on expression of red hair. To test this we compared the predicted penetrance for the various compound heterozygote and homozygote genotypes (Table 5, upper triangle) to that observed in our sample (lower triangle). For each genotype there is a good fit to the observed data, supporting the assumption of multiplicative penetrance.

Table 2. Phenotypic characteristics of MZ and DZ twins, sibs and parents

Skin reflectance	All		Red hair	
Percentage reflectance[a]	Female	Male	Female	Male
Inner arm	61.4	61.2	63.3	63.5
Outer arm	51.7	50.4	55.9	55.6
Total	710	716	21	29

[a]Group mean percentage reflectance as measured by EEL reflectometer at 650 nM.

A similar model was investigated for fair/blonde hair (Table 6). Now it was the Asp84Glu and Ile155Thr variants that had the highest OR of 3.1 and 2.9, respectively. The RHC alleles were also moderately associated with fair/blonde color, although Val92Met and Arg163Gln showed no significant association. The population attributable risk of fair/blonde hair due to carrying an MC1R variant is 21%, and 6% for light brown hair color (data not shown). Also shown in Table 6 are the ORs for fair/pale skin, as assessed by the three-point rating scale (Materials and Methods). All MC1R variant alleles are significantly associated except the rare Arg142His, the largest ORs being with Asp84Glu (12.5) and Asp294His (7.5).

From these analyses it can be concluded that the Asp84Glu, Arg151Cys, Arg160Trp and Asp294His variants can be considered strong RHC alleles, which in further analyses below we will designate '*R*'. The Val60Leu,Val92Met, Arg163Gln variants are relatively weak RHC alleles and are designated '*r*'. The penetrances of the six genotypes formed upon combining these alleles grouped as weak and strong, together with the consensus '+' allele in each hair color are shown in Table 7. This shows that significant numbers of red-haired individuals are only seen in *R*/*R* and *R*/*r* genotypes with 67.1 and 10.8%, respectively. The frequency of red hair in those with a heterozygous *R*/+ genotype is only 1.5%, and less than 1% in those with an *r/r* or *r*/+ genotypes, while no redhead was observed with a +/+ consensus genotype. The concordance between the observed and expected frequencies of red hair for each grouped genotype again supports a multiplicative penetrance model.

Additive effects of variant MC1R alleles on skin reflectance

Self-reported skin color on the three point scale was found to be more consistent with the measurement of the constitutive than the tanned skin color, reflectance measurements from the inner arm being twice as important as those from the back of hand in predicting self-reported skin color (data not shown). Subjects with a consensus genotype showed the darkest induced skin color with mean skin reflectances of 60.5% for the inner arm and 49.8% for the back of hand. To address the quantitative relationship of variant MC1R alleles with skin reflectance, the

Table 3. Phenotypic characteristics of MZ and DZ twins, sibs and parents

Freckles	Face		Back right hand		Shoulders			
	Male and female	(%)	Male and female	(%)	Females	(%)	Males	(%)
0 = nil	290	(28.9)	785	(78.3)	252	(25.1)	207	(20.6)
1 = mild	304	(30.3)	126	(12.6)	133	(13.3)	133	(13.3)
2 = moderate	201	(20.0)	54	(5.4)	66	(6.6)	88	(8.8)
3 = severe	208	(20.8)	38	(3.8)	49	(4.9)	75	(7.5)
Total	1003		1003		500		503	

Table 4. Phenotypic characteristics of MZ and DZ twins, sibs and parents

Nevi	Female	Male	
Mean	109.6	120.5	$P = 0.009$[a]
Range	8–366	3–422	
25–75%	72–139	76–153	
Total	500	504	

[a]Mann–Whitney test excluding one MZ twin per family.

increase in the mean skin reflectance measurement per variant allele was calculated relative to the consensus genotype (Table 8). In general, alleles acted in an additive manner to increase skin reflectance, *R* alleles showing a greater effect (+1.9%) than *r* alleles (+0.9%) on inner arm reflectance, which persisted after adjustment for sex, hair and eye color. This difference was even more pronounced for exposed skin (+1.5% for *R* compared with +0.3% for *r* on back of hand), but was significantly reduced after adjustment for sex, hair and eye color.

Genotypic mean reflectances were largely consistent with an additive model, except for the *r*/*r* homozygote genotype, which was 0.3% darker than the consensus homozygote on the inner arm. This may indicate a darker constitutive skin color in these individuals but as the back of hand reflectance was considerably lighter (by +1.8%), they are still not as responsive as the consensus genotype to sun exposure. The skin lightening detected on the basis of *R*/*R* genotype, with +3.4% on the inner arm and +5.0% on the back of hand, was consistent with the differences seen with the red hair phenotype (Tables 1–4), possibly reflecting their reduced tanning capacity on sun exposed sites. A heterozygote effect is seen on both unexposed and exposed skin, with reflectance increases for both *r*/+ (+0.9/+0.6%) and *R*/+ (+1.5/+1.2%) genotypes intermediate between the consensus (taken as 0/0%) and *R*/*R* (+3.4/+5.0%) homozygotes. The heterozygote effect becomes less apparent after adjustment for hair and eye color as may be expected for coinheritance of these traits.

The inner and outer arm reflectance is similarly affected by the presence of an *R* allele but this is not the case for *r*. In a GEE regression the *r* allele effect was significantly smaller for the inner arm than that on the back of hand ($P = 0.02$).

Association between MC1R genotype, freckling and mole count

Since red-haired subjects had the greatest number of freckles and lowest number of moles (Tables 1–4), we also tested for association with MC1R genotype. When the degree of freckling was plotted against composite MC1R genotypes (Fig. 1B), there was remarkable similarity to that seen plotted by hair color phenotype (Fig. 1A). A dosage effect of the RHC alleles commensurate with their penetrance for red hair was apparent. The +/+ and *r*/+ genotypes had the lowest levels, followed by *R*/+ and *r*/*r* with almost equivalent levels, with high scores in *R*/*r* and extreme freckling in *R*/*R* carriers.

No consensus homozygote had a severe freckling score, although there was a positive correlation between total mole count and freckling score seen in the curves plotting these characteristics by +/+, *R*/+ and *R*/*R* genotypes (Fig. 2B). There was little effect of the *r* allele upon this correlation, but the *R*/R genotype had the lowest number of moles and greatest number of freckles, consistent with the phenotypic association found in red heads (Fig. 2A). In the heterozygous state, the *R*/+ genotype displays an initial positive association with moliness until severe freckling is reached, after which there is a significant decrease in the number of moles. This effect on mole count was evident across the different levels of freckling (including the mildest levels), arguing against it being purely an artifact of counting difficulty. However, the non-linearity of the freckle score–mole count regression at high freckling scores may well reflect difficulty in this small subgroup. The overall positive correlation between freckling and nevus count, over which the MC1R effect is laid, may reflect sun exposure history.

Notably, of the 25 individuals with the most extreme freckling score there were only 13 with red hair and 12 of these were of *R*/*R* genotype (including five Arg151Cys and one Asp294His homozygotes) with one *r*/+ (see below). The remaining 12 individuals had non-red hair, including one fair/blonde, five light brown and six dark brown. The fair/blonde individual was *R*/*R* and the remaining individuals included 6 *R*/*r*, one *r*/*r*, two *r*/+ and two *R*/+ genotypes. Of the five heavily freckled individuals who were scored as consensus heterozygotes at the nine SNPs screened, three new MC1R variants were found upon complete sequencing, Val38Met (GTG to ATG), Leu80Pro (CTG to CCG in the 13th *r*/+ individual with red hair), and Arg213Trp (CGG to TGG); none of these missense changes have previously been reported (24). That 23/25 of our heavily frecked subjects were either homozygotes or compound heterozygotes for variant MC1R alleles confirms the very strong association of the MC1R gene with severe freckling, even in the absence of red hair. The attributable fraction due to carrying at least one variant MC1R allele increased linearly with the degree of freckling from 1 to 9 ranging from 23.4% for mild to 100% for severely freckled subjects (tabulated in Fig. 2B).

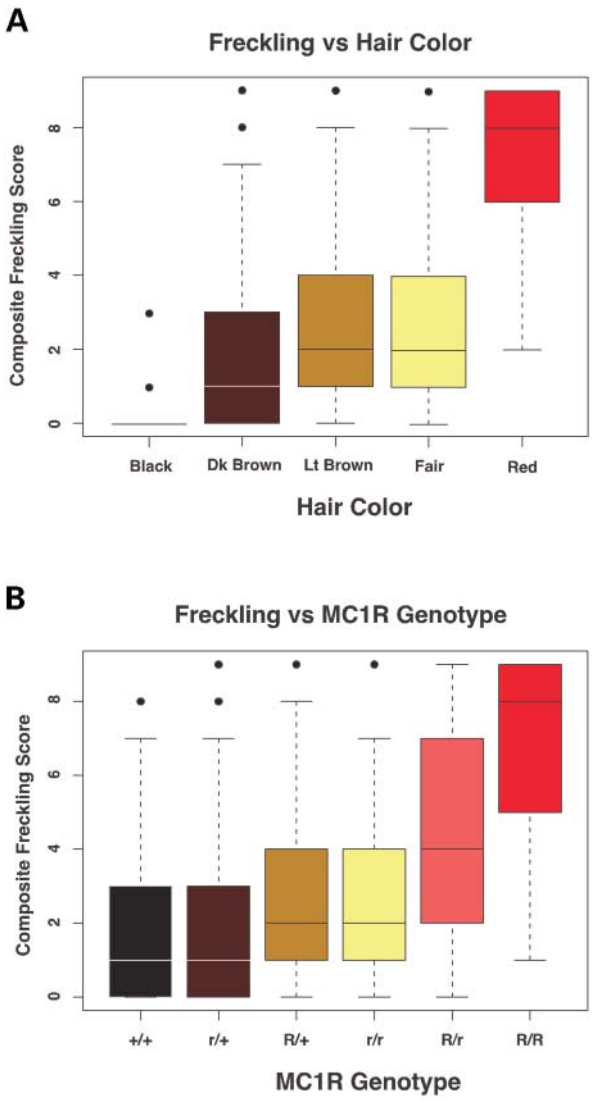

Figure 1. (**A**) Association between freckling and hair color. The composite freckling score was the summation of the degree of freckling, using the 0–3 scale of each individual on the face, shoulders and back of the right hand (Table 3) giving a range from a baseline of 0 to a total maximum of 9 (*y*-axis). This value is plotted for each hair color defined as red/auburn (red), fair/blonde (yellow), light brown (tan), dark brown (brown) and black (black) (*x*-axis). The Tukey box plot is used to represent the median, 75 and 25% limits by the vertical boxes, the 97.5% upper and 2.5% lower range of the samples indicated by the hatched bars, and the outliers as solid circles respectively. (**B**) Association between freckling and MC1R genotype. The composite freckling score (*y*-axis) is plotted against each MC1R genotype combination of +, *r* and *R* alleles as defined in the tables (*x*-axis).

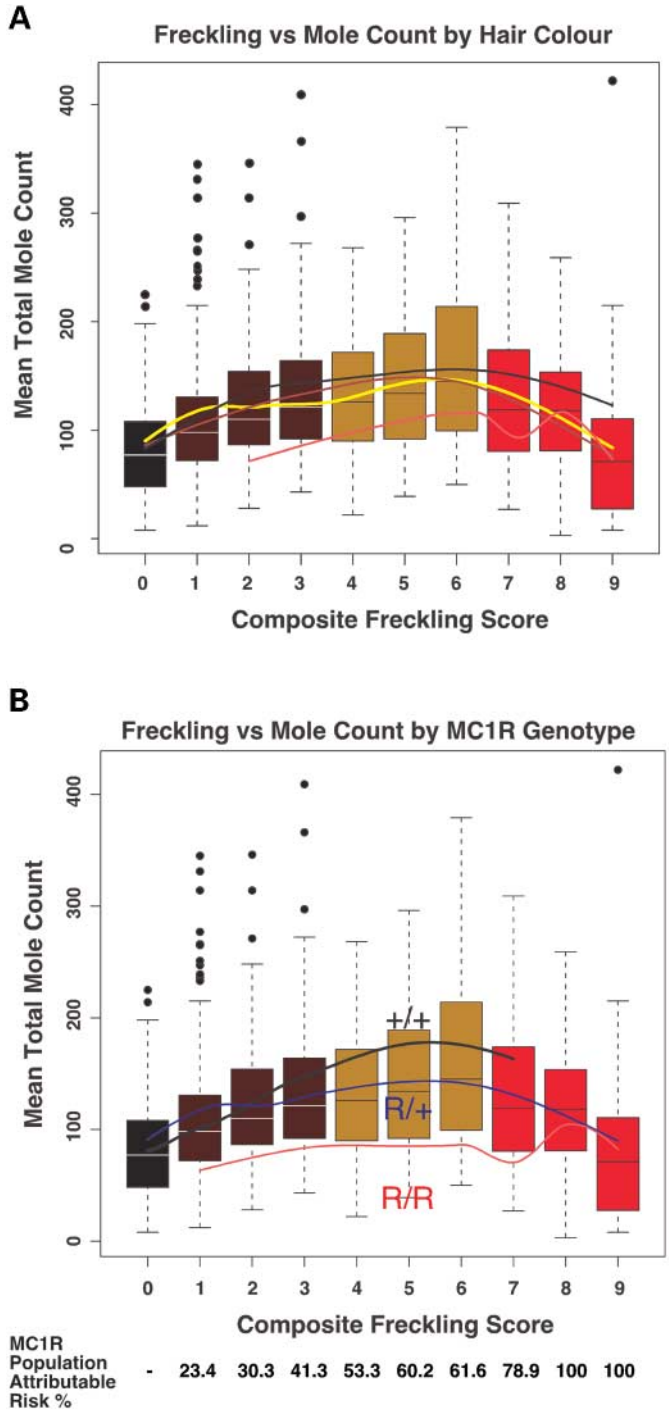

Figure 2. (**A**) Association between freckling, moliness and hair color. The composite freckling score (*x*-axis) is plotted against the mean of mole count (*y*-axis) for individuals with that score as described in Figure 1. Mole count versus composite freckling score for each hair color as represented as red/auburn (red), fair/blonde (yellow), light brown (brown) and dark brown (black) are indicated by the curves fitted by locally weighted Poisson Regression (92). (**B**) Relationship between freckling and moliness allowing for MC1R genotype. The composite freckling score (*x*-axis) was plotted against the mean of mole count (*y*-axis) for individuals with that score. The LOESS (92) fit of the mean value of mole count per composite freckling score for each MC1R genotype is indicated by the curves, *R/R* (red), *R*/+ (blue) and consensus +/+ (black). The population attributable risk of the MC1R gene relative to each freckling score is shown below the *x*-axis.

Interaction between MC1R and eye color on freckling and mole count

There are strong correlations between skin, hair and eye color, but no effect of MC1R genotype on eye color has been reported (10,27,28,49). Furthermore, eye color is a risk factor for melanoma independent of hair color and this is likely to be due to a non-MC1R mediated relationship with a poor skin response to UV-light (50).

Table 5. MC1R genotypes and penetrances of red hair phenotype

Allele	Consensus	V60L	D84E	V92M	R142H	R151C	I155T	R160W	R163Q	D294H
	0[a]	*0.2*	*1.6*	*0.2*	*0*	*3.3*	*0*	*1.6*	*0.1*	*2.7*
Consensus	**0**									
	0 : 440[b]									
		1.3	*8.7*	*1.2*	*0*	*16.4*	*0*	*8.8*	*0.6*	*13.9*
V60L	**0.5**	**3.2**								
	1 : 197	1 : 30								
			41.6	*8.1*	*0.1*	*59.5*	*0*	*41.9*	*4.0*	*54.7*
D84E	**6.2**	**0**	—							
	1 : 15	0 : 5	0 : 0							
				1.1	*0*	*15.4*	*0*	*8.2*	*0.5*	*13.1*
V92M	**1.7**	**0**	**0**	**0**						
	3 : 171	0 : 32	0 : 5	0 : 9						
					0	*0.1*	*0*	*0.1*	*0*	*0.1*
R142H	**0**	**0**	—	**0**	—					
	0 : 10	0 : 2	0 : 0	0 : 1	0 : 0					
						75.2	*0.1*	*59.8*	*8.0*	*71.4*
R151C	**1.0**	**17.6**	**50**	**20.5**	—	**76.2**				
	2 : 193	9 : 42	1 : 1	8 : 31	0 : 0	16 : 5				
							0	*0*	*0*	*0.1*
I155T	**0**	**0**	**0**	**0**	—	**0**	**0**			
	0 : 19	0 : 3	0 : 1	0 : 3	0 : 0	0 : 3	0 : 1			
								42.3	*4.1*	*55.1*
R160W	**2.4**	**5.9**	**50**	**0**	**0**	**64**	—	**50**		
	3 : 121	2 : 32	4 : 4	0 : 28	0 : 1	16 : 9	0 : 0	3 : 3		
									0.2	*6.7*
R163Q	**0**	**0**	**0**	**0**	—	**15.4**	**0**	**10**	**0**	
	0 : 73	0 : 20	0 : 1	0 : 21	0 : 0	2 : 11	0 : 3	1 : 9	0 : 5	
										67.3
D294H	**0**	**7.7**	**100**	**13.3**	—	**85.7**	**0**	**50**	**0**	**100**
	0 : 38	1 : 12	4 : 0	2 : 13	0 : 0	6 : 1	0 : 1	3 : 3	0 : 5	1 : 0

[a]Upper triangle matrix (italics) is the expected frequency (%) of red-haired individuals using a linear multiplicative inheritance model for each genotype (allele 1 OR × allele 2 OR, from Table 6).
[b]Lower triangle matrix gives the penetrance (in bold) and below that the numbers of red : non-red haired individuals (RHC : NRHC) of each MC1R genotype [penetrance = 100 × RHC/(RHC + NRHC)];—indicates not observed in this sample; the two triple variant genotypes are considered independently for this analysis.

Before assessing the interaction of the eye color with variant MC1R alleles, we performed a combined segregation-linkage analysis of eye (and hair) color with genotype at the D15S165 marker which is about 2 Mb centromeric of the OCA2 gene on chromosome 15q11.2–15q12 (51). This confirmed recessive inheritance of blue eye color. We estimated the recombination distance to D15S165 as zero and a frequency of 21% for the dominant brown eye *B* allele, consistent with the estimate of 26% for a US Caucasian population (52). The corresponding genotype frequencies expected for *B/B*, *B/b*, *b/b* are 4.4, 33.2 and 62.4%. From the segregation analysis, the predicted penetrance of a *b/b* genotype is 6% for brown, 22% green/hazel and 73% blue/grey, leading to an expected frequency of blue eye color of 45.6%, in close agreement with the 44.7% that is found in the study population (Table 1). Under this model, blue-eyed individuals in this sample are 97% likely to be assigned as *b*/*b*. This analysis confirms the presence of a major locus for eye color at or near the OCA2 locus that has been previously designated BEY2, and that eye color can be used as a proxy for genotype at this locus.

As expected, brown eye color was associated with dark brown hair color and with medium and olive/dark skin color; blue eye color was associated with fair/blonde hair and fair/pale skin (but with only marginal effect on light brown hair color). We tested for the genetic interaction between eye color and strong MC1R variant *R*-alleles associated with fair/pale skin in the determination of skin color, degree of freckling and mole count (Table 9; Fig. 3). The percentage of fair-skinned individuals of *R*/*non-R* heterozygote genotype (*R*/−) is between that of the *non-R*/*non-R* (−/−) and *R*/*R* homozygotes for both the corresponding brown/green and blue eye color genotypes. This is expected, given the previously reported heterozygote effect of MC1R RHC variants on skin color (27) and effect on skin reflectance (Table 8). All blue-eyed R/R individuals were in the fair/pale skin category but this decreased to 85.4% with fair/pale skin for brown/green-eyed R/R individuals, the remainder having medium skin color. This proportionate lightening in all genotypic groups when carrying both recessive blue-eyed *b* and red-hair *R* alleles indicates additive action of MC1R and BEY2/OCA2 loci on constitutive skin color.

The modifying effect of OCA2 on MC1R is also seen for freckling score; blue eye color increases freckling, and *R*/− and *R*/*R* genotypes further increase the freckling score in an additive fashion (Table 9). The significance of these effects was confirmed by ordinal GEE regression analysis (not shown). When the freckling score is plotted by MC1R genotype in the three eye color groups there is a consistent

Table 6. MC1R variant allele frequencies in south-east Queensland and odds ratios for red hair, fair/blonde hair and fair/pale skin

Variant allele	Frequency[a] (%)	Red hair OR (95% CI)[b]	Fair/blonde hair OR (95% CI)[b]	Fair/pale skin OR (95% CI)[b]
Consensus (+)	50.4	1	1	1
V60L	12.2	6.4 (2.8–14.9)	1.4 (1.1–1.9)	1.7 (1.3–2.1)
D84E	1.2	62.8 (17.6–223.7)	3.1 (1.4–6.9)	12.5 (4.8–42.8)
V92M	9.7	5.3 (2.2–12.9)	1.0 (0.7–1.4)	2.3 (1.8–3.1)
R142H	0.4	0.0 (0.0–∞)[c]	1.9 (0.5–6.9)	1.8 (0.6–5.7)
R151C	11.0	118.3 (51.5–271.7)	2.5 (1.8–3.4)	4.4 (3.3–5.7)
I155T	0.9	0.0 (0.0–∞)[c]	2.9 (1.4–6.1)	2.1 (1.0–4.3)
R160W	7.0	50.5 (22.0–115.8)	1.6 (1.1–2.3)	3.2 (2.4–4.4)
R163Q	4.7	2.4 (0.5–11.3)	0.7 (0.4–1.1)	2.0 (1.4–2.9)
D294H	2.7	94.1 (33.7–263.1)	2.2 (1.3–3.9)	7.5 (4.4–13.7)
r	26.7	5.1 (2.5–11.3)	1.0 (0.8–1.3)	1.9 (1.6–2.3)
R	21.8	63.3 (31.9–139.6)	1.4 (1.2–1.8)	4.2 (3.4–5.2)

[a]The total number of individuals ranged from 1747 to 1787 scored at the different variants.
[b]Odds ratios relative to consensus genotype (95% confidence interval).
[c]Insufficient numbers to test for statistical association.
r = V60L, V92M and R163Q.
R = D84E, R151C, R160W and D294H.

Table 7. Observed and expected frequencies of MC1R genotypes in penetrance of red hair color

MC1R genotype	No.	No. red	Expected[a] frequency (%), red	Observed frequency (%), red	Observed frequency (%), non-red			
					Fair/blonde	Light brown	Dark brown	Black
+/+	425	0	*0*	0	14	37	42	8
r/+	412	4	*0.2*	1.0	15	40	39	5
R/+	344	5	*2.5*	1.5	21	42	33	3
r/*r*	111	1	*1.0*	0.9	15	43	34	6
R/*r*	203	22	*11.8*	10.8	21	44	23	1
R/*R*	73	49	*62.1*	67.1	22	8	3	0
Total	1568	81						

+ = Consensus.
r = V60L, V92M and R163Q.
R = D84E, R151C, R160W and D294H.
[a]Expected frequency for red hair shown in italics using a linear multiplicative inheritance model for each genotype (allele 1 OR × allele 2 OR, Table 6); model goodness-of-fit $\chi^2_3 = 9.1$, $P = 0.02$.

Table 8. Relative mean skin reflectance increase for each MC1R variant allele and genotype

Variant allele	Inner arm percentage reflectance (CI 95%)[a]	Inner arm percentage reflectance (CI 95%)[a] adjusted[b]	Back of hand percentage reflectance (CI 95%)[a]	Back of hand percentage reflectance (CI 95%)[a] adjusted[b]
Consensus (+/+)	60.5 (60.0, 60.9)	60.8 (60.1, 61.5)	49.8 (49.2−50.4)	51.1 (50.0, 52.2)
r	+0.9 (+0.3, +1.5)	+0.2 (−0.2, +0.5)	+0.3 (−0.1, +0.7)	+0.7 (+0.1, +1.3)
R	+1.9 (+1.2, +2.6)	+1.1 (+0.6, +1.6)	+1.5 (+1.0, +1.9)	+1.0 (+0.3, +1.7)
r/+	+0.9 (+0.2, +1.6)	+0.8 (+0.1, +1.4)	+0.6 (−0.5, +1.7)	+0.4 (−0.6, +1.4)
r/*r*	−0.3 (−1.4, +0.8)	−0.3 (−1.3, +0.8)	+1.8 (+0.1, +3.5)	+1.9 (+0.3, +3.4)
R/+	+1.5 (+0.8, +2.2)	+1.3 (+0.8, +1.9)	+1.2 (+1.0, +2.3)	+0.9 (−0.1, +2.0)
R/*r*	+2.1 (+1.2, +2.9)	+1.5 (+0.6, +2.3)	+2.5 (+1.2, +3.9)	+1.4 (+0.2, +2.7)
R/*R*	+3.4 (+2.1, +4.7)	+2.8 (+1.3, +4.4)	+5.0 (+2.9, +7.0)	+1.9 (−0.5, +4.3)

+ = Consensus.
r = V60L, V92M and R163Q.
R = D84E, R151C, R160W and D294H.
[a]Increase relative to consensus genotype (95% confidence interval) per allele, or genotype.
[b]Adjusted for sex, hair and eye color.

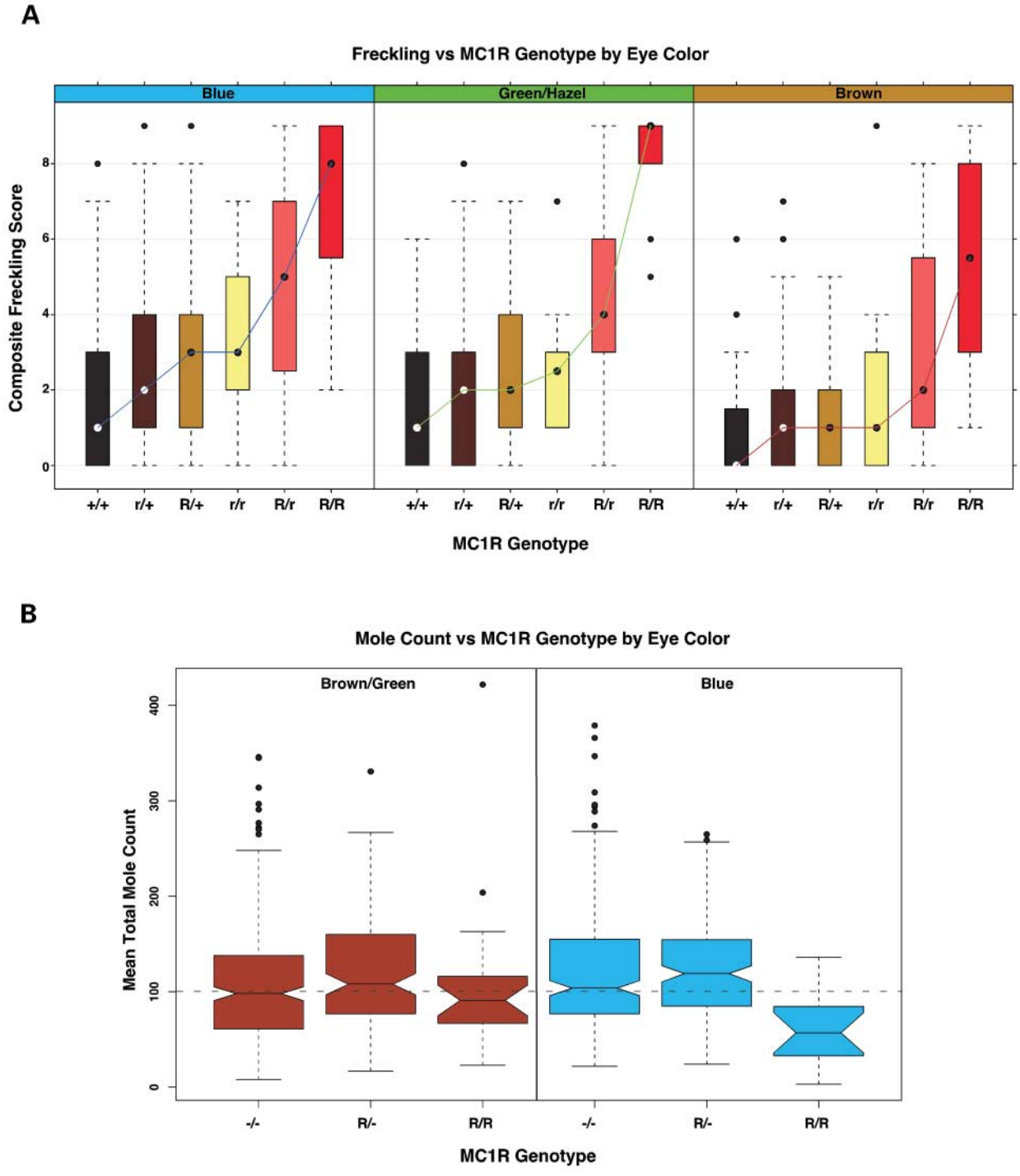

Figure 3. (**A**) Genetic interaction of MC1R and BEY2/OCA2 in freckling. The composite freckling score (*y*-axis) is plotted against each MC1R genotype combination of +, *r* and *R* alleles as defined in the tables (*x*-axis) for blue, green/hazel and brown eye color as described in Figure 1. (**B**) Genetic interaction of MC1R and BEY2/OCA2 in mole count. The mean total mole count (*y*-axis) is plotted against each MC1R genotype combination of non-*R* (−) and *R* alleles as defined in the tables (*x*-axis) for brown-green and blue eye color.

higher rate of freckling for each genotype in those with non-brown eye colors, those with blue eyes showing a slightly increased levels relative to green/hazel with the exception of *R*/*R* genotypes which were all extreme frecklers (Fig. 3A).

Blue eye color was found to slightly increase the mean nevus count for those of −/− and *R*/− MC1R genotype (Fig. 3B). In contrast, there was a significant decrease in the number of moles in blue-eyed *R*/*R* individuals from a geometric mean of 122.5 to 61.7 ($P = 0.0001$). The MC1R modifier effect is purely

Table 9. Genetic interaction of MC1R and BEY2 in skin color, freckling and mole number

		No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
BEY eye color		Brown/green	Brown/green	Brown/green	Blue	Blue	Blue
MC1R genotype		−/−[a]	*R*/−	*R*/*R*	−/−	*R*/−	*R*/*R*
Skin color							
Fair/pale	788	162 (29.8)	151 (53.2)	35 (85.4)	199 (49.0)	208 (79.1)	33 (100)
Medium	638	275 (50.6)	117 (41.2)	6 (14.6)	188 (46.3)	52 (19.8)	0 (0)
Olive/dark	144	106 (19.5)	16 (5.6)	0 (0)	19 (4.7)	3 (1.1)	0 (0)
Total		543	284	41	406	263	33
Grand total[b]	1570						
Mean freckling score[c]		1.5 (1.2, 1.7)	2.5 (2.1, 2.9)	6.7 (5.6, 7.8)	2.4 (2.1, 2.8)	3.9 (3.3, 4.2)	6.7 (5.5, 7.8)
Nevi count							
Geometric mean $P = 0.007$		109.5 (101.3, 118.5)	121.4 (111.0, 132.8)	104.3 (77.0, 141.3)	122.0 (112.5, 132.3)	123.1 (114.7, 132.2)	61.7 (43.8, 87.0)

[a] − is any non-*R* allele.
[b]The number of individuals here includes both DZ and MZ twins.
[c]Using 1–9 scale as shown in Figure 1 (95% CI).
R = D84E, R151C, R160W and D294H.
95% CIs calculated using GEE robust standard errors.

recessive as no decrease in mole count is seen in the *R*/− heterozygotes. *R*/*R* genotype explains 3.3% of the variance of log total mole count in the sample. Eye color directly explains 2.6% of the log total mole count variance but a further 2.1% is explained by the interaction of R/R and BEY2. Thus, although there is a significant interaction between BEY2 and MC1R in determination of mole number, they are not major genes for mole formation. Elsewhere we have reported that the broad heritability for flat mole count is 42% of which up to 80% is explained by a locus close to CDKN2A (53).

Interaction between MC1R and OCA2 on red hair, freckling and mole count

The two common polymorphisms within the OCA2 locus reported to be associated with non-blue eye color were also typed in the twin sample and these genotypes were analyzed in relation to the BEY2 eye color genotype for each individual predicted from combined segregation/linkage analysis (see above; Table 10). The OCA2 Arg305Trp and Arg419Gln polymorphisms occurred at frequencies of 0.05 and 0.08 respectively, in line with two earlier reports of 0.17 and 0.09 (54) and 0.07 and 0.08 (35) in the USA white population for each allele pair. The 305Trp change was strongly associated with green/hazel and brown eye color and 305Arg with blue eye color ($P = 0.003$); whereas the 419Gln change was associated with dark brown hair color and green/hazel eye color and 419Arg with blue eye color ($P = 10^{-8}$).

Interestingly the OCA2 305Trp allele did seem to act as a modifier of MC1R penetrance for red hair. In a logistic regression analysis including MC1R *R*, *r* and OCA2 Arg305Trp, Arg419Gln alleles the interaction term between MC1R and OCA2 305Trp was significant. Essentially the same results were obtained fitting the equivalent GEE model, except that the *r* by OCA2 305Trp interaction also reached significance via the Wald test (robust $Z = 2.4$; for *R* by OCA2 305Trp robust $Z = 2.1$). Specifically looking at *R*/*R* carriers, 25% of Arg305Trp heterozygotes were red-haired whereas 71% of Arg305Trp homozygotes were red-haired (Fisher's exact $P = 0.09$).

In contradistinction to the association of the blue-eyed (*b*/*b*) genotype with freckling and skin reflectance, neither OCA2 polymorphism was associated with these pigmentation phenotypes and no interaction between MC1R and these OCA2 alleles on freckling and skin reflectance was found. However, carriers of the 305Trp allele had on average 18 fewer nevi than 305Arg homozygote individuals (Kruskal–Wallis $P = 0.01$) while no effect was seen with carriers of the 419Gln allele ($P = 0.38$).

DISCUSSION

Previous studies examining variant MC1R alleles in relation to hair color (8–10,27–29,49,55) have all been consistent in finding the red-hair phenotype to be a recessive trait, although the reported influence of some alleles was variable between studies (reviewed in 24). Homozygote or compound heterozygote variant MC1R genotype carriers are generally red-haired, but as this is not always the case it is likely that other loci are involved in the expressivity of the trait (9). In addition, red hair occurs in a significant proportion of heterozygote variant MC1R allele carriers, some alleles displaying greater influence than others. It is unlikely that each of these variant alleles represents complete loss of receptor function or that they have a simple Mendelian recessive mode of inheritance.

It is known that some variant MC1R receptors fail to couple to cAMP activation when tested in heterologous expression systems (6,7). This loss of receptor-activated intracellular signaling is consistent with the results found in testing of primary human melanocyte strains of defined MC1R genotype (56). The Arg160Trp homozygote and Arg151Cys/Asp294His, Arg160Trp/Asp294His compound heterozygote melanocytes, demonstrating an impairment of tyrosinase activation in response to α-MSH stimulation and pronounced sensitivity to UV radiation treatment. Moreover, there are significant heterozygote effects on skin phototype (31), skin color and melanoma risk (27), which indicate that variant MC1R alleles do not

Table 10. Frequencies of OCA2 genotypes versus eye color

OCA2 genotype			Eye color no. (%)		
R305W	R419Q	No.	Blue	Green/hazel	Brown
R/R	R/R	1057	517 (48.9)	310 (29.3)	230 (21.8)
R/R	R/Q	204	54 (26.5)	92 (45.1)	58 (28.4)
R/R	Q/Q	7	0 (0)	4 (57.1)	3 (42.9)
R/W	R/R	126	60 (47.6)	22 (17.5)	44 (34.9)
R/W	R/Q	10	3 (30.0)	5 (50.0)	2 (20.0)
	Total		634	433	337
	Grand total[a]	1404			

[a]The number of individuals here excludes one MZ twin per family.
[b]Log–linear analysis of this table finds R305W ($\chi^2_2 = 12.2$, $P = 0.002$) to have an effect independent of R419Q on eye color ($\chi^2_2 = 48.3$, $P = 8 \times 10^{-10}$).

behave in a strictly recessive manner. Expression constructs for the three common RHC variant alleles Arg151Cys, Arg160Trp and Asp294His have been tested in MC1R-deficient transgenic mice to determine their effects on coat color. The resultant phenotypes suggest that these alleles do not result in complete loss of function, nor are they functionally equivalent in rescuing coat color pigmentation (57).

Our study has attempted to quantify the penetrance of each of nine relatively common variant MC1R alleles for pigmentary traits including red hair, fair skin, freckling and nevus count in a representative sample of the white Australian population. There is a clear distinction in the strength of these alleles in relation to the penetrance for red hair, with RHC alleles Asp84Glu, Arg151Cys, Arg160Trp and Asp294His showing much higher OR compared with low-penetrance Val60Leu, Val92Met and Arg163Gln alleles (Table 6), although each of these latter alleles can occur with red hair (Table 5). The division in allele strength supported the classification of the MC1R alleles into strong and weak variants, designated *R* and *r* respectively, for some of our analyses. Modeling of the penetrance of each variant allele was found to be consistent with a multiplicative model for the effect of each allele on risk of red hair (Table 6). The expected frequencies of red hair based on genotypes derived from the defined consensus, *r*, and *R* alleles were in good agreement with those observed in our sample (Table 7). In contrast, the allelic effects were additive for untanned skin color as determined by reflectance measurements of the inner upper arm (Table 8).

A comparison of the penetrance of MC1R variant alleles for the red hair phenotype from several other studies where sufficient numbers for statistical analysis have been analyzed is possible (28,49,55). There is general agreement on the effects of each allele, although the frequency in each population varies. Understandably, there is an overrepresentation of red hair together with Arg151Cys and Asp294His in the families studied by Flanagan *et al.* (28), since these alleles are known to be at high frequency in the Scottish/Irish population (10,48). The only allele that was inconsistent and not seen to be highly associated with red hair in the present study was Arg142His, although neither this nor Ile155Thr were found in sufficient numbers in our sample to determine their effect on hair color. The low-penetrance Val92Met and Arg163Gln alleles were either found not to have an effect in the Flanagan study or to be at too low a frequency to permit a conclusion. Thus, studies of MC1R allelic effects in red hair families allow functional insights that complement conclusions we have reached in the general population.

While the MC1R variant alleles produce dramatic qualitative differences in hair color, their effects on skin color are more modest mean shifts on a continuous scale. This may indicate a fundamental difference in the pigmentary response of follicular melanocytes to those within the epidermis. Within the skin each melanocyte is surrounded by a consistent number of keratinocytes (~36) forming an epidermal melanin unit (EMU) which acquires, transports and metabolizes the melanin produced from one cell (58). Epidermal pigmentation manifests as a mosaic of individual melanocytes contributing millions of EMUs to the surface area of the skin. Melanocytes in a hair follicle are found at higher density placed as a cone surrounding the dermal papilla and adjoining cells of the differentiating hair shaft, with recent evidence suggesting that tyrosinase produced by the melanocyte remains active in the medullary cells of the hair shaft after transfer of the melanosome (59). Given these different cutaneous environments it is possible that the levels or accessibility of the α-MSH ligand to melanocytes within the hair follicle is much greater than when these cells are distributed in the basal layer of the epidermis, so explaining the greater effects of MC1R signaling in melanocytes of the follicles than of the skin. Perhaps for the same reason, MC1R has different influences on coat, skin and nose/pad color in dogs, nose and pad color being independent of MC1R genotype (60). Alternatively, interfollicular melanocytes may have some degree of endogenous eumelanogenesis, acting independent of MC1R, which hair melanocytes do not possess.

Apart from cellular environments, the physical differences of these tissues must also be considered in the interpretation of the different genetic models that have been put forward. The skin is observed as a two-dimensional self renewing sheet of keratinizing tissue served by single EMU melanocytes that are not actively cycling but contributing melanin-laden particles to dividing and differentiating keratinocytes that migrate to the surface of the skin. By comparison, the hair shaft is visible as a three-dimensional cylindrical elongated structure with melanocyte cellular activity cycling in synchrony with the keratinocytes within each hair generation, and being replenished with stem melanoblasts derived from the follicle bulb for each new hair growth cycle (61,62). Thus, there are fundamental differences in the milieu of follicular and epidermal melanocytes and it is possible that these explain penetrance

differences between tissues (hair and iris) in the pigmentary effects of MC1R variant alleles.

There have been conflicting reports on the association between nevi and freckling in many studies that have been conducted on risk factors for skin cancer. In adolescents, a positive relationship has been reported, with increased numbers of ephelides co-occurring with high nevus counts (44), but other studies have found no association in adults (38,45). This may be understandable given the lifetime changes that are known to occur in nevus and freckle numbers, nevus density peaking in late adolescence (47,63). There are also skin type and age prevalence differences in ephelides (64), problems in differentiating solar lentigines from ephelides (65), and measurable turnover of nevi even early in life (47). The latitudinal gradient relationship with nevus number may be explained by geographical increases in incident UV-exposure levels (41), although there is still far more variance within cities than between (66). There was an apparent correlation between mole and freckle number in our adolescent sample (Fig. 2A), those having more freckles also reporting an increased number of nevi, consistent with several other juvenile studies from Australia and New Zealand (39,41,42,67) and the Northern hemisphere (68,69). This has now been confirmed at the genotypic level where *R*/+ heterozygotes have a phenotype in between the freckling scores of +/+ consensus and *R*/*R* homozygotes (Fig. 2B).

The population-attributable risk of MC1R variant alleles for freckles in a collection of Dutch skin cancer patients has been estimated at 60%, albeit with half of those carrying MC1R variants not expressing ephelides (30). Measuring the degree of freckling on a nine-point scale, we have estimated the population-attributable risk due to carrying at least one MC1R variant allele at 100% for the most severe forms of freckling with a linear reduction to only 23.4% for those with mild freckling at only one site (Fig. 2B). Therefore, although the MC1R gene is the major freckling gene for those with severe freckling, our result indicates that other genes must also contribute to the phenotype in those with milder freckling. Intriguingly, we have found that the BEY2 locus contributes to the freckling phenotype, most notably in individuals with MC1R consensus and heterozygote genotypes (see below).

Within the pale-skinned highly freckled group, red hair was associated with a decreased mole count (Fig. 2A). This phenotypic association was further examined at the molecular level by plotting the strong *R* allele MC1R variant genotype against mole count and freckling score (Fig. 2B). In addition to confirming genotypically that *R*/*R* homozygotes had more freckles and fewer moles, an *R*/+ heterozygote effect was apparent, with lower mole counts seen as freckle score increased. This effect on mole count was evident across the different levels of freckling (including the mildest levels), arguing against it being purely an artifact of counting difficulty. The overall positive correlation between freckling and nevus count, over which the MC1R effect is laid, may reflect sun exposure history. The mechanism by which MC1R RHC variant alleles prevent mole formation is entirely unknown. It may be postulated that there is a mutually exclusive relationship between freckling and mole formation. The number and density of melanocytes within a mole is greatly increased (70), while in a freckle the number of melanocytes within the macule does not increase significantly but rather the pigmentation activity is enhanced (71). Some of the conflicting conclusions on the phenotypic relationship of mole and freckle number reported in the literature (38) may be due to the variable penetrance of MC1R genotypes for these traits, suggesting future genotypic classification may more accurately portray this relationship. Notably, it has been suggested that freckling and nevus formation represent independent forms of irregular or abnormal reactions of melanocytes to some environmental factor (sunlight exposure, sunburn etc.) (68). Moreover, freckling and nevus count represent different risk factors for melanoma (50), supporting the physiological distinction between these acquired pigmented lesions.

Lastly, a modifier effect of eye color (and thus BEY2 genotype) on several of the human pigmentary traits associated with MC1R variant alleles was found (Table 9; Fig. 3). A previous report proposed an interaction of these two loci in the determination of skin color through the measurement of skin reflectance on limited data in a Tibetan population. The contribution of the OCA2 and MC1R genes to interindividual variation in skin pigmentation was assessed but failed to demonstrate an association between any gene polymorphisms in a single-locus analysis. However, when an epistatic model was applied to the data this suggested a gene–gene interaction between homozygosity at two OCA2 synonymous SNP markers and the Val92Met MC1R allele (33). Further support for a recessive interaction between the OCA2 and MC1R pigmentation genes is evident in the more severe phenotypes in the coat colors of mice that harbor mutations in each of these gene orthologs (72,73) and modifier effects seen in type II human albinism (74).

Our studies support and greatly extend the influence of interactions between the BEY2/HCL3 locus (now thought to be OCA2) and variant MC1R alleles on skin color, freckling and nevus count in the Caucasian population. The significant protective effect of the *b*/*b*, *R*/*R* genotype (blue-eyed red head) on nevus formation (Table 9) indicates that this is a true genetic recessive effect. The clinical distinction between flat nevi and freckles is sometimes difficult, but given the strict classification regime followed throughout this study it is unlikely that misclassification is the explanation for this effect. The effect of *R*/*R* genotype on melanocyte phenotype suggests that cAMP regulation is likely to be an important mechanism for limiting melanocyte cell growth or density within the epidermis responsible for mole formation. Nevi represent melanocyte cell clones that have undergone cellular expansion followed by growth arrest (75). A loss of the cAMP response in melanocytes may block the initial growth stimulus necessary to initiate mole formation, but still enhance melanocyte activity (rather than clonal cell expansion), giving rise to pigmented macules rather than moles. The finding that α-MSH synergizes with UV radiation to increase CDKN2A expression in both basal layer keratinocytes and melanocytes (76) points to other consequences of MC1R genotype. It is also known that the cAMP pathway initiated by α-MSH binding to melanocytes leads to an increase in CDKN2A as cells approach senescence (77,78). The negative influence of MC1R genotype on mole formation is a connection that must be examined in finer detail.

The contribution of the two OCA2 coding region polymorphisms assayed in this study to the BEY2 locus associations

with eye and hair color, freckling and skin reflectance is relatively minor. This was tested by including both eye color and SNP genotype in regression analyses, but also in a combined segregation-association analysis (not shown). In the latter, the estimated linkage disequilibrium coefficient between OCA2*Arg419Gln and the BEY2**Brown* eye allele was $r = 0.20$. A large number of OCA2 polymorphisms have been reported (54,79–81) and examination of a wider range of common alleles together with a comprehensive search for variation within the OCA2 gene is needed to fully understand the genetic relationship to the BEY2 locus.

In conclusion it must be emphasized that human pigmentation is a polygenic trait and that gene interactions will be an important determinant of pigmentation phenotype. Our results begin the task of dissecting the complexity of these interactions.

MATERIALS AND METHODS

Structure of the study population and pigmentation characteristics

Adolescent twins and their siblings previously studied in an investigation of genetic and environmental factors contributing to the development of pigmented nevi (53,82) were also phenotyped for pigmentary traits including skin, hair and eye color. Twins have been examined on up to three occasions: at 12, 14 and 16 years of age. Subjects were overwhelmingly (>95%) of northern European origin (mainly Anglo-Celtic).

Hair color was rated by one research nurse (AE) on a five-category scale (1, fair/blonde; 2, light brown; 3, red/auburn; 4, dark brown; 5, black); it was also matched by two nurses (AE and MG, blind to each other) to the closest of 30 standard hair color swatches of the Haarfarbentafel Fischer-Saller (83). If hair had been dyed then subjects were asked to match their natural color to the hair swatches.

Eye color was rated by AE as 1, blue/grey; 2, green/hazel; 3, brown. *Skin color* (inner upper left arm) was rated by AE as 1, fair/pale; 2, medium; 3, olive/dark. Skin color was also measured on two separate occasions using percentage skin reflectance at 650 nM on sun-exposed and non-exposed sites (back of left hand and inner upper left arm), using an EEL reflectometer, following a protocol described elsewhere (84). Reflectance at this frequency was chosen for this study as being most strongly correlated with inter-racial differences in skin color, as well as the most heritable (84). At age 12 there was no significant seasonal variation in skin reflectance.

Density of ephelidae (*freckling)* was recorded on a four point scale as 0, none; 1, mild/infrequent/sparse; 2, moderate/evenly distributed; and 3, severe at three body sites (face, dorsum of right hand, shoulders). For most analyses, a composite freckling score was constructed by summing the scores for the three sites.

Melanocytic nevi (*moles*) were counted on all subjects by a single observer (AE) on all parts of the body except chest, abdomen, buttocks and scalp. Counts were recorded separately for 34 regions, by diameter (0–2, 2–5 and >5 mm) and were further classified as flat, raised or atypical (see 53 for more detail). A reliability study showed that our nevus counts are highly reproducible between observers and over time (85).

Parents were not clinically examined, so only skin, hair (as at age 21) and eye color were obtained by self-report.

There were 2331 family members in 645 pedigrees for whom some phenotypic data were recorded, and DNA was available for genotyping for 1779 individuals within 460 of these pedigrees. MC1R genotypes were not obtained for 32 individuals due to PCR failure, inconsistencies or bad calls. Phenotypic data for the genotyped subset are collated in Tables 1–4. Excluding one member of each genotyped MZ twin pair, there were 1568 individuals with complete MC1R genotype, hair color, eye color and sex recorded, with 1463 also able to be assayed for OCA2 genotype. We chose to analyze the data collected when the twins were 12 years old, as these were most complete.

MC1R and OCA2 genotyping

PCR was performed in 96-well plates with the MC1R coding region amplified using 25–50 ng of genomic DNA in a 25 µl reaction volume which included 25 pmol of each primer and 1.25 units of *Taq* DNA polymerase (Promega). Amplification conditions and the sequence analysis strategy used for the MC1R coding region have been previously described (9,48). Allele-specific hybridization was performed as previously described (27,29,48) using 15-base ^{32}P-radiolabeled oligonucleotide probes with the mismatch centrally positioned to provide maximal binding stability to the complementary sequence and instability of a mismatch. MC1R genotypes were visualized using autoradiography.

In addition, we utilized genotypes at the marker D15S165, closely linked to the OCA2 gene on chromosome 15q11.2–15q12. D15S165 was typed as part of a genome scan of 274 of the twin families (642 individuals) in this sample at the Australian Genome Research Facility using ABI-Prism 377 automated sequencers (86). Allele calling was performed using GENOTYPER 2.0 (PE Applied Biosystems) and data used in joint segregation-linkage analysis to infer genotypes at the BEY2 locus (see below).

Genotyping of two OCA2 SNPs, Arg305Trp in exon 9 and Arg419Gln in exon 13 (54), was performed by primer extension followed by mass spectroscopy (Sequenom MassArray). PCR reactions for the two gene fragments were duplexed and run in the same primer extension reaction using appropriate nucleotide termination mix, spotted on a chip and subjected to mass spectrometry (87). The extension primers (GACGGTGTCCATCAGCATC for Arg305Trp; TGGCCCACACCCGTCCC for Arg419Gln) allowed spectral resolution and automatic genotyping by Sequenom software.

Statistical methods

We have performed standard linear and logistic regression analyses of the data using the R package (Version 1.5) (88). We used PAP 5.0 (89) to produce genotypic probability estimators for each individual in the pedigree (90). These are the posterior probabilities of each unobserved genotype (at the modelled 'BEY2' locus) for each individual. These probabilities were then used to simulate multiple replicates of the pedigrees with

imputed BEY2 genotypes. These replicates were subsequently bootstrap resampled for conventional linear, logistic and proportional odds regressions, giving robust standard errors and confidence intervals. This procedure avoids the problems of straightforward single imputation of genotypes. We have also fitted GEE models (91) using an exchangeable working correlation matrix blocking on family. This is one approach to dealing with residual familial correlations for the phenotypes in conventional regression analyses.

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