A Three–Single-Nucleotide Polymorphism Haplotype in Intron 1 of *OCA2* Explains Most Human Eye-Color Variation

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We have previously shown that a quantitative-trait locus linked to the *OCA2* region of 15q accounts for 74% of variation in human eye color. We conducted additional genotyping to clarify the role of the *OCA2* locus in the inheritance of eye color and other pigmentary traits associated with skin-cancer risk in white populations. Fifty-eight synonymous and nonsynonymous exonic single-nucleotide polymorphisms (SNPs) and tagging SNPs were typed in a collection of 3,839 adolescent twins, their siblings, and their parents. The highest association for blue/nonblue eye color was found with three *OCA2* SNPs: *rs7495174* T/C, *rs6497268* G/T, and *rs11855019* T/C (P values of 1.02×10^{-61}, 1.57×10^{-96}, and 4.45×10^{-54}, respectively) in intron 1. These three SNPs are in one major haplotype block, with TGT representing 78.4% of alleles. The TGT/TGT diplotype found in 62.2% of samples was the major genotype seen to modify eye color, with a frequency of 0.905 in blue or green compared with only 0.095 in brown eye color. This genotype was also at highest frequency in subjects with light brown hair and was more frequent in fair and medium skin types, consistent with the TGT haplotype acting as a recessive modifier of lighter pigmentary phenotypes. Homozygotes for *rs11855019* C/C were predominantly without freckles and had lower mole counts. The minor population impact of the nonsynonymous coding-region polymorphisms Arg305Trp and Arg419Gln associated with nonblue eyes and the tight linkage of the major TGT haplotype within the intron 1 of *OCA2* with blue eye color and lighter hair and skin tones suggest that differences within the 5′ proximal regulatory control region of the *OCA2* gene alter expression or messenger RNA–transcript levels and may be responsible for these associations.

The pigmentary traits of skin, hair, and eye color combined with high levels of environmental UV exposure are potential modulators of individual risk for developing both melanoma and nonmelanoma skin cancer (NMSC).[1] The incidence rate of both NMSC and melanoma is greatest in fair-skinned, sun-sensitive individuals, which indicates the importance of the innate ability to respond to UV light through an increased synthesis of melanin, known as the "tanning response." The quantity and quality of melanin pigmentation is central to the photoprotection of both melanocytes and keratinocytes. The human melanocortin-1 receptor (MC1R [MIM 155555]) protein, expressed on the surface of melanocytes, is a key determinant of photosensitivity,[2,3] with variant alleles of this seven-transmembrane G-protein–coupled receptor, present at high frequency in white populations, linked to the high incidences of NMSC and melanoma.[4]

Other genes involved in melanin biogenesis modify the penetrance of MC1R variant alleles. The gene responsible for oculocutaneous albinism type II (*OCA2*), which encodes the P protein, is an integral melanosomal membrane protein with an 838-aa ORF that contains 12 transmembrane-spanning regions.[5] *OCA2* maps[6] to chromosome 15q11.2-q12 and is the human homologue of the mouse pink-eyed dilution gene (*p*) (Entrez Nucleotide accession number NM_000275).[7,8] Epistatic interactions between MC1R and the *OCA2* gene were first reported to contribute to skin pigmentation phenotypes in a Tibetan population.[9] *OCA2* transcription is induced following UV-B irradiation of skin,[10] and, together with MC1R, plays important roles in control of pigmentation.[11]

Polymorphisms in *OCA2* occur in different populations,[12] and this locus underlies the genetic linkage of blue/brown eye (BEY2/EYCL3 [MIM 227220]) and brown hair (HCL3 [MIM 601800]) to chromosome 15, as reported elsewhere.[13] Two *OCA2* coding-region variant alleles—Arg305Trp and Arg419Gln—were recently shown to be associated with brown and green/hazel eye colors, respectively,[14,15] and blue eye color has also been linked to this locus through use of microsatellite[16,17] and SNP[18] markers. Indeed, our genomewide linkage scan for eye color suggested that 74% of variation in eye color in Europeans could be attributed to a QTL linked to the *OCA2* region of chromosome 15q.[16]

To understand how alleles of the *OCA2* gene influence eye color and pigmentation associated with skin-cancer risk, we sequenced all *OCA2* exons encoding the P protein. To test for statistical association with pigmentary traits, we typed the exonic polymorphisms detected in our preliminary study combined with those reported in the literature and haplotype-tagging SNPs identified from the

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Table 1. Distribution of Pigmentation Phenotypes among Genotyped Study Participants

Phenotype	No. (%) of Subjects with Phenotype		
	Females	Males	All
Eye color:			
Blue/gray	617 (45.5)	613 (47.7)	1314 (46.1)
Green/hazel	405 (29.8)	333 (25.9)	789 (27.7)
Brown	335 (24.7)	340 (26.4)	749 (26.3)
Total	1,357 (100)	1,286 (100)	2,852 (100)
Hair color:			
Red/auburn	81 (6.0)	46 (3.6)	146 (5.1)
Fair/blond	210 (15.5)	158 (12.3)	377 (13.2)
Light brown	464 (34.2)	445 (34.6)	974 (34.2)
Dark brown	570 (42.0)	574 (44.7)	1234 (43.3)
Black	32 (2.4)	62 (4.8)	120 (4.2)
Total	1,357 (100)	1,285 (100)	2,851 (100)
Skin Color:			
Fair/pale	547 (41.8)	512 (40.8)	1,099 (40.4)
Medium	630 (48.1)	580 (46.3)	1,295 (47.6)
Olive/dark	132 (10.1)	162 (12.9)	324 (11.9)
Total	1,309 (100)	1,254 (100)	2,718 (100)

HapMap database[19] (International HapMap Project) in a large twin family sample of adolescents phenotyped for melanoma risk factors, including eye, skin, and hair color; mole (melanocytic nevus) count; and degree of freckling (ephelidae).

Material and Methods

Structure of the Study Population and Pigmentation Characteristics

Adolescent twins and their siblings were recruited for an investigation of genetic and environmental factors contributing to the development of pigmented nevi[20,21] and were also phenotyped for pigment traits, including skin, hair, and eye color. The pigmentation characteristics of the twins were examined on up to three occasions, at ages 12, 14, and 16 years, as described elsewhere.[22] Subjects were overwhelmingly (>95%) of northern European origin (mainly Anglo-Celtic). One research nurse (A. Eldridge) rated hair color on a five-category scale (1 = fair/blond, 2 = light brown, 3 = red/auburn, 4 = dark brown, 5 = black), eye color (1 = blue/gray, 2 = green/hazel, 3 = brown), and skin color at the inner upper left arm (1 = fair/pale, 2 = medium, 3 = olive/dark). Density of ephelidae was recorded at three body sites (face, dorsum of right hand, and shoulders) on a four-point scale (0 = none, 1 = mild/infrequent/sparse, 2 = moderate/evenly distributed, 3 = severe). For most analyses, a composite freckling score was constructed by summing the scores for the three sites. Parents were not clinically examined, so their skin, hair (as at age 21), and eye colors were obtained by self-report.

Melanocytic nevi were counted on all subjects by a single observer (A. Eldridge) on all parts of the body except chest, abdomen, buttocks, and scalp. Counts were recorded separately for 34 regions, by diameter (0–2 mm, 2–5 mm, or >5 mm) and were further classified as flat, raised, or atypical (see the work of Zhu et al.[21] for more detail).

There were 5,075 family members in 1,100 pedigrees with some phenotypic data, and DNA was available for genotyping for 3,839 individuals within 1,037 of these pedigrees. Phenotypic data for the genotyped subset are collated in table 1. With the exclusion of one member of each genotyped MZ twin pair, there were 3,011 individuals with complete record of *OCA2* genotype, hair color, eye color, and sex. We analyzed the data collected when the twins were aged 12 years, since our data for this age group were most complete.

OCA2 *Exon Amplification, Transgenomic Wave Mutation–Detection System, and DNA Sequencing*

To search for human *OCA2* polymorphisms present in the southeast Queensland twin collection, we analyzed genomic DNA samples from 40 individuals: 9 with red/auburn hair, 10 each with fair/blond and light brown hair, and 11 with dark brown hair (representing 23 blue/gray, 9 green/hazel, and 8 brown eye colors). PCRs were conducted for each *OCA2* coding-region exon (amplimers and size products for each *OCA2* coding-region exon are available on request) that encompassed the six reported amino acids changing alleles Ala257Asp, Arg305Trp, Arg419Gln, Leu440Phe, His615Arg, and Ile722Thr.[6,12,14,23,24] PCR products were denatured and analyzed on a Transgenomic Wave System (model 2100D), for mutation detection, by use of the Navigator Software package. Samples with a mismatch in the analyzed PCR fragment were sequenced from new PCR products through use of ABI BigDye Terminator version 3.1 chemistry and were separated on a capillary-based genetic analyzer (Applied Biosystems). Sequence chromatograms were analyzed using the Sequencher program (Gene Codes).

OCA2 *SNP Genotyping*

A combination of exonic and intronic SNPs (table 2) and 40 SNPs selected from the HapMap database[19] (International HapMap Project) as suitable for haplotype coverage of the *OCA2* locus were used to test for association with a range of pigmentary traits (table 3). Assays were designed for 75 SNPs through use of the Sequenom MassARRAY Assay Design software (version 3.0) (data available on request). Four SNPs (*rs3751651, rs728405, rs7171246,* and *rs7496968*) failed during the design and testing stage and were excluded. SNPs were typed using iPLEX chemistry on a Compact MALDI-TOF Mass Spectrometer (Sequenom) through use of standard methods.[25]

Statistical Methods

We performed standard linear and logistic regression analyses of the data, using the R computer package (version 2.3.1 [R Development Core Team 2006]), as well as recursive partitioning tree-based analyses, using the R party package.[26] We used MENDEL[27] to perform individual SNP and haplotypic association analysis, which correctly allows for the relatedness within the sample. Plots of inter-SNP linkage disequilibrium were prepared using the Haploview program.[28]

Table 2. *OCA2* Polymorphism Screen in Twins Selected for Hair and Eye Color

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Table 3. *OCA2* SNP Association with Blue Eye Color

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Results

Pigmentation Characteristics

The frequencies of eye, hair, and skin color ratings (table 1) are consistent with those reported elsewhere for the southeast Queensland population,[16,22,29] although a somewhat higher percentage of dark brown hair (43.3% vs. 34%) and medium skin (47.6% vs. 40.8%) colors, with a corresponding decrease in other hair-color classifications, were observed in the present combined study. The eye-color grade distributions were similar to our earlier reports, with 46.1% blue/gray, 27.7% green/hazel, 26.3% brown in the total sample collection. The genotyped subjects were ~52% female and ~48% male, and there were no significant differences in eye-color distributions between females and males. There was a very wide range in the number of freckles (nil to severe) and of nevi (2–426); 76% of the sample had some freckling on at least one of the three examined body sites. On average, males had nine more moles than did females, and a similar trend was observed for freckling, which was particularly obvious on the shoulders. This male excess has been noted elsewhere; red-haired subjects of both sexes showed the greatest number of freckles and the least number of moles, consistent with a number of other past[22] and recent[30] reports.

Search for Polymorphism in OCA2 *Exonic Regions in Adolescent Twins Selected for a Range of Hair and Eye Colors*

The human *P*-gene transcript encoded by the *OCA2* locus is divided into 24 exons (fig. 1*C*), covering >345 kb[12]; 23 of these exons span the 836-aa coding region, with exon 1 representing exclusively a noncoding 5′ UTR. The translational initiation codon is located in exon 2, and exon 24 includes the termination codon plus the 3′ UTR. A possible alternative spliced region,[8] previously referred to as "exon 19" and containing an in-frame stop codon, was neither included in the analysis nor used in our exon or P-protein numbering system.[5] At least 42 apparently nonpathogenic variant alleles of the *OCA2* gene have been identified in the literature, 22 of which are exonic; of these, 6 result in amino acid changes (see the Albinism Database). Some of these polymorphisms have markedly different frequencies in different populations, which indicates the potential to explain differences in pigmentation phenotypes among ethnic groups.[12,24]

DNA analysis of 40 individuals in our study identified 10 coding-region SNPs and 10 flanking intronic-region SNPs (table 2) that confirmed the presence and allele frequencies of several of the polymorphisms that had already been described in the literature [12] for the white population. During this screening, two novel nonsynonymous changes—Val380Met (National Center for Biotechnology Information [NCBI] number ss66538500) and Val519Ala (NCBI ss66538502)—were discovered that were present at low frequencies—0.01 and 0.04, respectively—in the test subcollection of 80 alleles. Notably, the Val443Ile change reported as an albinism allele[31] was also found at the low frequency of 0.02; although it resulted in a deficient P-protein activity, this may represent a hypomorphic allele in the general population rather than a private mutation, as it has also been reported by others.[15] The Ala481Thr albinism-related polymorphism,[31] present at high levels within the Japanese population,[32] was seen neither in this discovery search nor during the screening of the complete collection. Whereas no new synonymous changes were seen in the exonic regions of the PCR products, two changes that have not been reported elsewhere were seen within intronic flanks IVS5-53 (NCBI ss66538498) C/G and IVS18+45 (NCBI ss66538504) G/C at frequencies of 0.02 and 0.04, respectively.

Assay of OCA2 *SNP Alleles and Haplotype Determination in an Adolescent Twin Collection*

No new common nonsynonymous variants in the *OCA2* gene were detected in the 23 coding-region exons assayed in the test subcollection. We typed all of the 30 reported synonymous and nonsynonymous coding-region polymorphisms and five flanking intronic SNPs showing high polymorphism, including the new IVS5-53 C/G change (table 2), together with 40 HapMap tagging SNPs (table 3). Of the 75 selected SNPs, four failed during the design and testing stage; of the remaining 71 SNPs, a further 13 were dropped from the analysis, either because of low minor-allele frequency or because of Hardy-Weinberg disequilibrium, suggestive of additional assay problems. A total of 3,839 subjects were genotyped, with the final 58 SNPs used for statistical analyses (table 3).

For coding-region SNPs, the synonymous changes Ala355Ala, Cys517Cys, Ala776Ala, and Ser788Ser were present at high frequency. The Gly780Gly variant was monomorphic for the T allele in our twin sample collection, in contrast to the report of Lee et al.,[12] who found the T allele at a frequency of 0.27 in whites. Only the nonsynonymous changes Arg305Trp at 0.05 and Arg419-Gln at 0.09 polymorphisms could be considered to be common in the southeast Queensland white population. The alanine amino acid allele was found exclusively at position 257, consistent with the report of Lee et al.[12] but in contrast to the RefSeq entry for the cDNA for this gene.

Figure 1*A* shows the linkage-disequilibrium plot for the 58 tagging SNPs covering the region from *OCA2* exon 1 to exon 24. Seven major haplotype blocks can be deduced, according to the criteria of Gabriel et al.,[33] with an eighth small block of three SNPs within intron 1 (shown in blue). The intron 1 haplotype block includes the three SNPs *rs7495174* T/C, *rs6497268* G/T and *rs11855019* T/C.

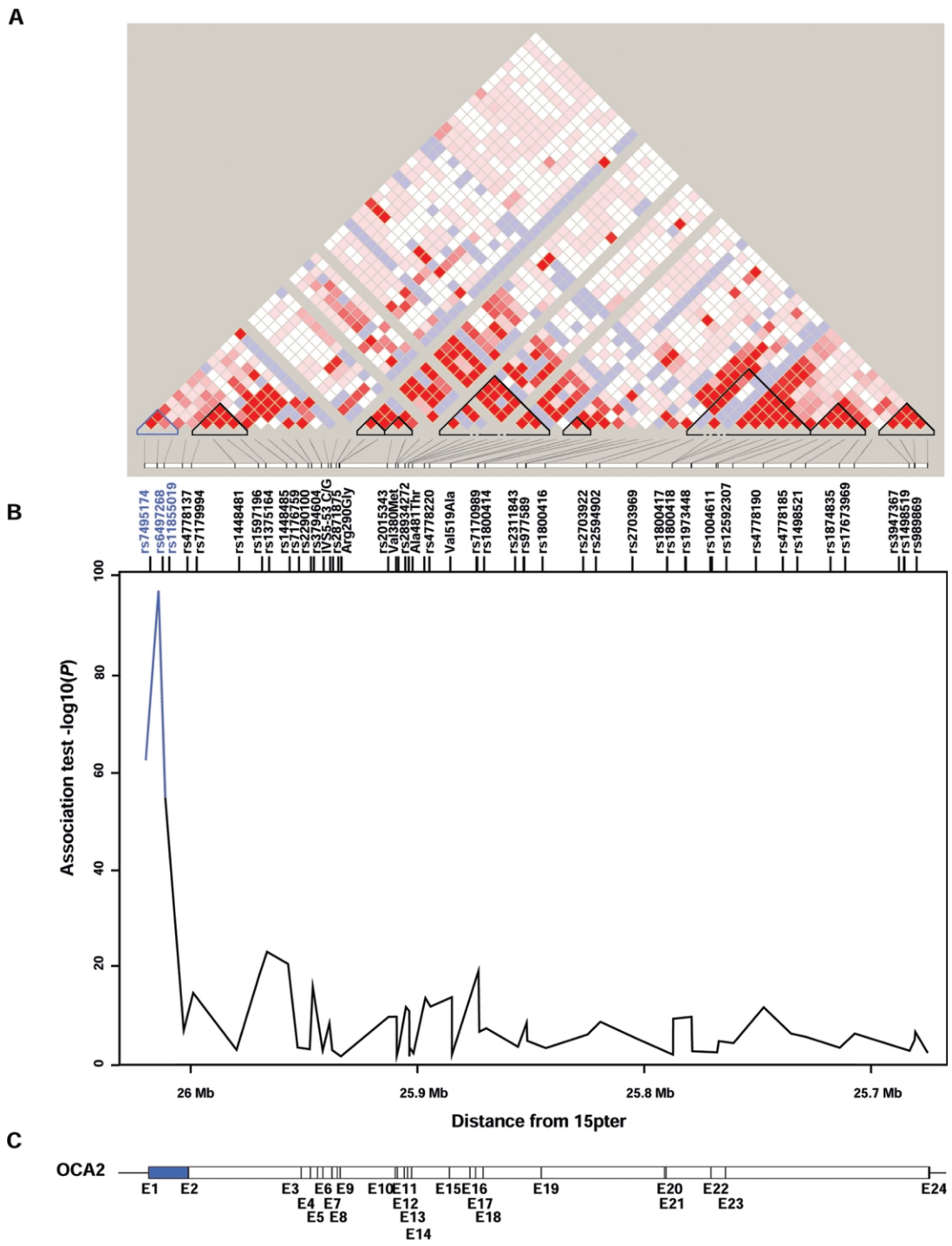

Figure 1. *A,* Positions of the *OCA2* haplotyping SNPs, shown on the bar in alignment with those indicated in panel B. These are connected by lines to the linkage disequilibrium (r^2) heat plot for the 58 tagging SNPs covering the region from *OCA2* exon 1 to exon 24. Haplotype blocks showing mean values of the correlation coefficient r^2 >0.8 are highlighted by triangles, with the intron 1 block in blue. *B,* Likelihood test statistic (*Y*-axis), plotted against the physical map distance from chromosome 15pter (*X*-axis) aligned with the exon positions as shown directly below (*panel C*). The score of the −log10 *P* values for each SNP association with blue/nonblue eye color (table 3) is shown here as a continuous line plot, with the intron 1 SNPs shown in blue. The positions of a selected subset of *OCA2* SNPs used for haplotype analysis are shown above the plot. *C,* Schematic representation of the physical structure of *OCA2* genomic locus, with the transcription unit blocked. Exons are indicated by black vertical lines and are numbered below, from exon 1 (E1) to exon 24 (E24), with intron 1 highlighted in blue.

Association of OCA2 *SNP Alleles with Blue Eye Color*

A plot of the maximum likelihood-ratio test statistic (LRTS) for association with blue/nonblue eye color is shown in fig. 1*B*. Allele frequencies and the allele linked with blue or nonblue colors is tabulated in table 3. The strongest associations were found for three SNPs (*rs7495174* T, *rs6497268* G, and *rs11855019* T) in the intron 1 haplotype block, with *P* values of 1.02×10^{-61}, 1.57×10^{-96}, and 4.45×10^{-54}, respectively. The LRTS curve also demonstrated another broad peak contained within intron 2, which includes SNP *rs1375164* ($P = 2.98 \times 10^{-22}$), and the third highest peak close to the intron 16 splice-acceptor junction, which includes IVS16-47 *rs7170989* ($P = 2.51 \times 10^{-18}$). Of the two common *OCA2* coding-region changing SNPs identified elsewhere as modifying the association of green/hazel or brown eye color,[14,22] the Arg305Trp *rs1800401* change did not show significant association in this expanded study ($P = .84$), although the Arg419Gln *rs1800407* polymorphism was strongly associated with nonblue eye colors ($P = 4.96 \times 10^{-10}$).

In a separate analysis combining all tested SNPs, a recursive partitioning approach was applied to predict eye color on the basis of the 58 informative SNPs.[26] A subset of 18 SNPs—beginning with the highest associated SNP *rs6497268,* then *rs7495174* and *rs11855019*—were selected by this algorithm as significant predictors of blue or brown eye color. Use of these SNPs correctly predicted blue or brown eye color with a specificity of up to 80% in several nodes, with >50% of a major node of 1,113 samples having blue eyes (fig. 2).

Association of OCA2 *SNP Haplotypes with Eye, Hair, and Skin Color*

Given the facts that the three *OCA2* intron 1 SNPs were most strongly associated with blue/nonblue eye color and were grouped together into a single haplotype block, the frequencies of the eight possible haplotype combinations present in the twin population were deduced using the program MENDEL (table 4). One major haplotype TGT (haplotype 1 in table 4) was predicted, representing 78.4% of alleles, with four minor haplotypes as TTT (haplotype 2 in table 4) at 7.9%, CTC (haplotype 8 in table 4) at 6.4%, with TGC (haplotype 3 in table 4) and TTC (haplotype 4 in table 4) each at 3.4%. The three other haplotypes—CGT (haplotype 5 in table 4), CTT (haplotype 6 in table 4), and CGC (haplotype 7 in table 4)—were considered rare in the twin collection, all falling well below 1%. The coding-region polymorphisms were predicted to occur as 305Arg-419Arg (haplotype 9 in table 4) at 87.2% of alleles, 305Trp-419Arg (haplotype 10 in table 4) at 5%, and 305Arg-419Gln (11) at 7.7%; the 305Trp-419Gln haplotype was not found in our sample. Logistic-regression analysis demonstrated that the remaining 55 SNPs made little additional contribution to the expression of blue eye color beyond that already deduced from the intron 1 haplotypes. Therefore, only these haplotypes and the highest frequency nonsynonymous coding-region 305 and 419 alleles were considered for association analysis with other pigmentation characteristics.

The frequencies of the predicted diplotypes for the three *OCA2* intron 1 SNPs sorted from those of highest to lowest in blue eye color are shown in table 5. The 1/1 diplotype was found in 62.2% of samples; of those, 62.5% had blue eyes, 28% had green eyes (combined nonbrown 90.5%), and only 9.5% had brown eyes. The 1/4 diplotype in 4.8% of samples also had a higher frequency of blue eyes (47.1%) than brown eyes (32.6%). All other diplotypes had higher frequencies of brown eye color. The 1/2 diplotype was the second-most-common genotype, at 12.8%, and had the highest frequency (38.5%) of green eyes. The third-most-common (8.9%) diplotype, 1/8, had eye-color frequencies reciprocal to those of the 1/1 diplotype, showing only 7.9% blue and 68.8% brown eyes (combined nonblue 92.1%). These data are consistent with the TGT haplotype 1 acting as a highly penetrant recessive blue-eye-color allele. The TTC haplotype 4 is also associated with blue eyes, and the remaining haplotypes were dominant green- or brown-eye-color alleles.

For hair and skin color, the 1/1 diplotype had the highest frequency (40.2%) in subjects with light brown hair and was also enriched (92.6%) for fair/pale and medium skin types (table 5). Each of the diplotypes 1/2 to 1/5 had higher frequencies in subjects with dark brown hair and medium skin types, again consistent with TGT haplotype 1 acting as a recessive modifier associated with lighter pigmentary phenotypes.

Table 5 also lists the 305 and 419 coding-region polymorphism diplotypes in eye, hair, and skin types, sorted by frequency of blue eye color. The 10/11 diplotype found in only 0.9% of samples had the highest frequency of blue eye color (50%), despite the fact that the 419Gln change on haplotype 11 was strongly associated with green eye color.[22] In contrast, the 10/10 diplotype (0.3% of samples) had equal frequencies of blue and brown eye color, at 42.9% each. The 11/11 diplotype (305Arg-419Gln/305Arg-419Gln [50% of samples]) had the highest frequency (66.7%) of green eye color and no blue eyes. The 9/11 diplotype showed the second highest frequency (39.3%) of green eye color. These results highlight the association of the 419Gln change on haplotype 11 with green eye color. The most common diplotype (9/9 [75.6% of samples]) had the second highest frequency (49%) of blue eyes, greater than either the blue or brown frequencies in those of the 9/11 diplotype and again consistent with the association of the 419Gln allele with green eye color.

Association of Eye Color and OCA2 *SNP Alleles with Freckling and Mole Count*

We examined the density of facial freckling in the sample collection, sorted by eye color, on the basis of a four-point scale, as shown in fig. 3*A*. Blue eye color was associated with the highest percentage of individuals rated as "se-

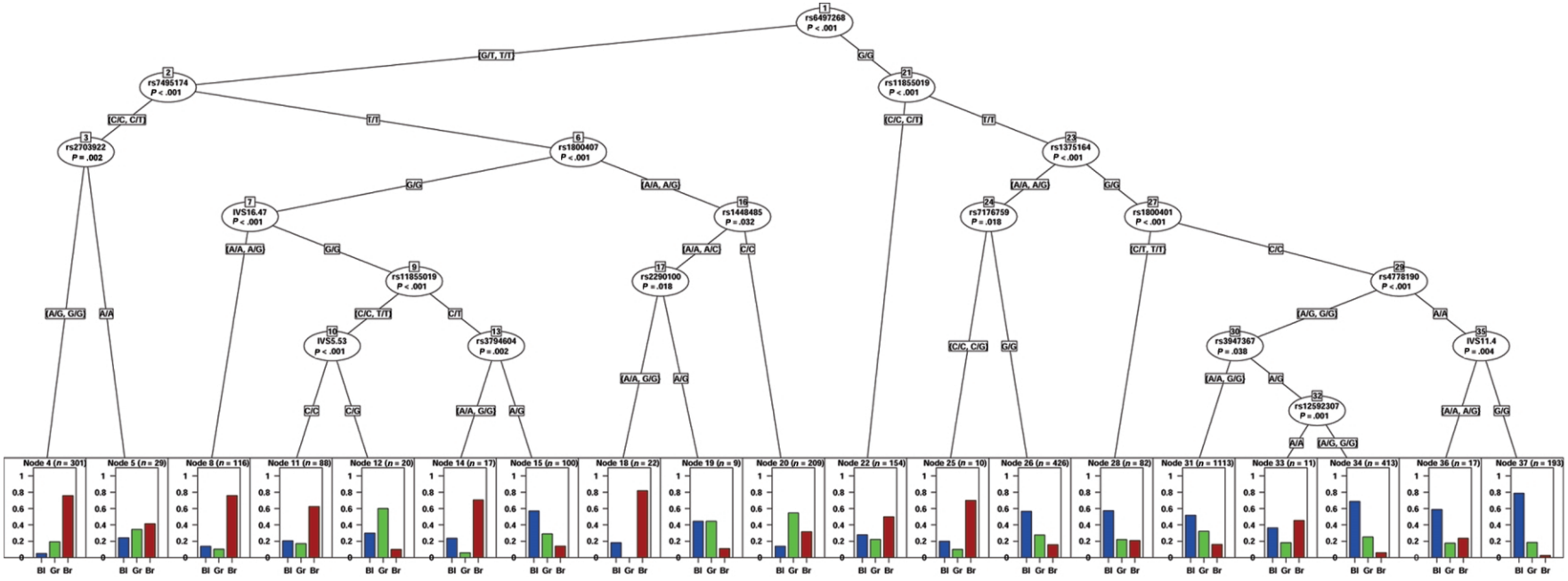

Figure 2. *OCA2* SNP prediction of eye color. A regression tree of 37 nodes based on 18 *OCA2* tagging SNPs is shown with nodal *P* values and genotypes. A histogram of percentage eye color as blue (Bl), green (Gr), and brown (Br) for the 19 branching haplotype combinations is plotted, with the number of twin samples (n) indicated above each panel. Nodes shown branching to the left are higher for brown, and those to the right are higher for blue. Most striking are the nodes with high sample numbers, which approach 80% of individuals who have brown (node 4, $n = 301$; node 8, $n = 116$) or blue (node 34, $n = 413$; node 37, $n = 193$) eyes, with the major node 31 ($n = 1{,}113$) comprising 80% of individuals with blue or green eyes. Minor nodes are predominantly higher for brown or green eyes.

Table 4. *OCA2* Intron 1 and Coding-Region Haplotype Frequencies

Haplotype	Nucleotides[a]	Frequency
1	TGT	.7844
2	TTT	.0788
3	TGC	.0340
4	TTC	.0340
5	CGT	.0033
6	CTT	.0010
7	CGC	.0001
8	CTC	.0644
9	RR[b]	.8723
10	WR	.0504
11	RQ	.0772

[a] Nucleotides at *rs7495174/rs6497268/rs11855019*.

[b] Amino acids 305 and 419 correspond to SNP changes *rs1800401* = Arg305Trp and *rs1800407* = Arg419Gln, respectively.

vere" frecklers and the lowest proportion of nonfrecklers. Reciprocally, those with brown eyes had the lowest proportion of severe frecklers and the highest proportion of nonfrecklers. Subjects with green eyes had degrees of freckling intermediate between those of subjects with eye color between blue and brown. The combined frequencies of mild-to-moderate freckling were similar for subjects with all three eye colors.

Assessment of freckling on three body sites, on a nine-point scale, gave median freckle scores for blue-eyed subjects at 2.0 (95% CI 1.8–2.2), green-eyed subjects at 1.0 (95% CI 0.8–1.2), and brown-eyed subjects at 1.0 (95% CI 0.9–1.1), with means of 2.25, 2.06, and 1.29, respectively. A similar trend was found for mole density (fig. 3*B*), with a slightly greater number and larger range of mole counts in individuals with blue eyes (88.4%) and green eyes (89.0%) compared with those with brown eyes (76.0%).

To test for any association of freckling or mole count with specific *OCA2* SNPs, multiple-regression analysis was performed. Results indicated that the *rs11855019* SNP contained within the *OCA2* haplotype 1 for blue eyes had the strongest association with both a high freckling score and a high mole count. Testing of this SNP in combination with all others, including *rs7495174* and *rs6497268,* that define the *OCA2* haplotype 1, did not lead to any statistically significant improvement of these associations. In comparison of the histograms in figure 3*A,* the same trend of association of freckling score with eye color is apparent in the association of *rs11855019* genotypes. Assessment of freckling on three body sites, on a nine-point scale, gave mean freckle scores for T/T at 1.89 (95% CI 1.78–2.00), C/T at 1.61 (95% CI 1.40–1.81), and C/C at 0.91 (95% CI 0.25–1.57). As seen in figure 3*B,* mole counts in this case showed a significant median difference between T/T and C/T, compared with C/C genotypes at 78 (95% CI 75.4–80.6), 76 (95% CI 71.3–80.7), and 41 (95% CI 33.7–48.3), with means of 87.2, 85.7, and 53.4, respectively, which echoes the relation between blue/green and brown eye color, which is also shown.

Discussion

Understanding the genetic basis of variation in human skin, hair, and eye color has been a major goal for elucidation of the correlation of pigmentation phenotype with skin-cancer risk. Genes mutated in cases of oculocutaneous albinism are likely to have polymorphic alleles responsible for pigmentary variation within the general population[34,35] and for differences among major population groups.[36] *OCA2* is one such gene, initially identified as the human ortholog of the *p* locus of mice, and, when mutated, as underlying oculocutaneous albinism type II.[7,8] Deletion of the region encompassing the *OCA2* gene on chromosome 15 has been associated with the hypopigmentation of the skin, hair, and eyes found in Prader-Willi and Angelman syndromes[7,37,38] and with extra copies of this chromosomal region, which results in generalized hyperpigmentation of the skin.[39,40] The distribution of *OCA2* alleles has also been shown to affect normal pigmentation variation in admixed African American and African Caribbean populations.[41] Moreover, this region has recently been reported to encompass the third-longest haplotype span of diminished heterozygosity in the genome of modern Europeans,[42] which implies intense selection at this locus in ancestral European populations.

An assessment of the level of polymorphism of the *OCA2* coding region in several populations was first conducted by Lee et al.,[12] who reported 28 nonpathogenic sequence variants, some with disparate frequencies among white, African American and African, Asian, and Indo-Pakistani populations. Later studies[6,23,24,32] that included SNPs reported in GenBank identified 11 nonsynonymous amino acid substitutions within *OCA2,* with a further two (Val380Met and Val519Ala) newly discovered in our southeast Queensland twin collection, bringing the total to 13 (table 2). Only two of these, Arg305Trp at 0.05 and Arg419Gln at 0.09, were found to be present at any significant frequency in the 3,839 samples genotyped in this study, so *OCA2* coding-region alleles account for little variation in pigmentation phenotype, at least within white populations. Moreover, similar frequencies of these nonsynonymous SNPs in other ethnic groups[12] (table 6) indicate that they are not population specific. This is in contrast to the significant number of nonsynonymous variant alleles of *MC1R* that have been associated with red hair and fair skin in European populations.[22,34,43,44]

The distribution of melanin with the melanocytes of the uveal tract of the eye is the physical basis of eye color,[45–48] and brown irides have up to 70% higher concentrations than do those of other colors.[49,50] Age-related changes in eye color do occur, but eye color becomes stable by age 6 years.[51] The genetics of human eye color has been studied for over a century,[52] and, for most of that time, it was considered a simple Mendelian recessive trait, with

Table 5. Frequencies (%) of *OCA2* Intron 1 Diplotypes, Arg305Trp, and Arg419Gln in Eye, Hair, and Skin Color Phenotypes

Genotype	Diplotype Number	*N* (%)[a]	Percentage of Subjects: Eye Color: Blue/Gray	Eye Color: Green/Hazel	Eye Color: Brown	Hair Color: Red/Auburn	Hair Color: Fair/Blond	Hair Color: Light Brown	Hair Color: Dark Brown	Hair Color: Black	Skin Color: Fair/Pale	Skin Color: Medium	Skin Color: Olive/Dark
1:													
TGT/TGT	1/1	1,772 (62.22)	62.5	28.0	9.5	5.1	16.2	40.2	37.0	1.5	46.5	4.61	7.4
TGT/**TTC**	1/4	138 (4.85)	47.1	20.3	32.6	3.6	15.2	29.7	44.2	7.2	35.6	47.4	17.0
TGT/**CGT**	1/5	7 (.25)	**28.6**	**14.3**	**57.1**	.0	.0	14.3	57.1	28.6	14.3	57.1	28.6
TGT/**TGC**	1/3	154 (5.41)	**27.9**	**22.1**	**50.0**	7.8	9.7	26.0	49.4	7.1	29.3	56.7	14.0
TGC/TTC	**3/4**	12 (.42)	**25.0**	**08.3**	**66.7**	8.3	8.3	8.3	16.7	58.3	27.3	18.2	54.5
TTT/TGC	**2/3**	29 (1.02)	**20.7**	**31.0**	**48.3**	.0	3.4	37.9	51.7	6.9	40.0	36.0	24.0
TTT/CGC	**2/7**	5 (.18)	**20.0**	**20.0**	**60.0**	20.0	20.0	40.0	20.0	.0	60.0	20.0	20.0
TGT/**TTT**	1/2	364 (12.78)	**17.6**	**38.5**	**44.0**	6.3	9.1	25.8	53.0	5.8	35.1	53.0	11.9
TGT/**CTC**	1/8	253 (8.88)	**7.9**	**23.3**	**68.8**	3.6	4.3	21.3	62.5	8.3	25.1	50.6	24.3
TTT/TTC	**2/4**	18 (.63)	**5.6**	**11.1**	**83.3**	5.6	16.7	16.7	61.1	.0	16.7	44.4	38.9
CTC/CTC	**8/8**	22 (.77)	**4.5**	**00.0**	**95.5**	.0	4.5	4.5	45.5	45.5	10.5	42.1	47.4
TTT/TTT	**2/2**	17 (.60)	**.0**	**35.3**	**64.7**	5.9	.0	17.6	70.6	5.9	26.7	60.0	13.3
TTC/CGT	**4/5**	3 (.11)	**.0**	**33.3**	**66.7**	.0	.0	33.3	66.7	.0	66.7	33.3	.0
TTC/CTC	**4/8**	13 (.46)	**.0**	**23.1**	**76.9**	.0	7.7	23.1	53.8	15.4	9.1	63.6	27.3
TTT/CTC	**2/8**	14 (.49)	**.0**	**21.4**	**78.6**	14.3	7.1	14.3	50.0	14.3	35.7	50.0	14.3
TGC/CTC	**3/8**	18 (.63)	**.0**	**5.6**	**94.4**	.0	.0	11.1	72.2	16.7	23.5	11.8	64.7
TTC/TTC	**4/4**	6 (.21)	**.0**	**.0**	**100.0**	.0	.0	.0	33.3	66.7	.0	.0	100.0
TGT/**CTT**	1/6	1 (.03)	**.0**	**.0**	**100.0**	.0	.0	.0	100.0	.0	.0	100.0	.0
TTT/CGT	**2/5**	1 (.03)	**.0**	**.0**	**100.0**	.0	100.0	.0	.0	.0	…	…	…
TGC/TGC	**3/3**	1 (.03)	**.0**	**.0**	**100.0**	.0	.0	100.0	.0	.0	.0	100.0	.0
2:													
WR/RQ[b]	10/11	24 (.86)	50.0	29.2	20.8	.0	12.5	29.2	54.2	4.2	23.8	71.4	4.8
RR/RR	9/9	2,114 (75.55)	49.0	26.5	24.5	4.9	13.2	36.0	41.6	4.2	40.6	47.3	12.1
RR/WR	9/10	251 (8.97)	46.6	19.1	34.3	6.4	15.5	25.9	46.2	6.0	36.4	46.9	16.7
WR/WR	10/10	7 (.25)	42.9	14.3	42.9	14.3	.0	.0	57.1	28.6	.0	1.00	.0
RR/RQ	***9/11***	387 (13.83)	***31.8***	***39.3***	***28.9***	5.7	11.9	29.5	50.6	2.3	43.5	48.0	8.5
RQ/RQ	***11/11***	15 (.54)	***.0***	***66.7***	***33.3***	.0	6.7	40.0	46.7	6.7	33.3	66.7	.0

NOTE.—Genotypes and diplotype numbers are as listed in table 4 for *rs7495174/rs6497268/rs11855019*. Genotypes and diplotypes associated with blue/gray eyes are underlined, with haplotype 1 (TGT) always underlined. Genotypes and diplotypes associated with brown eyes are shown in bold. Genotypes and diplotypes associated with green/hazel eyes are shown in bold italics.

[a] For eye color, there were 2,848 subjects assessed in sample 1 and 2,798 subjects assessed in sample 2; for hair color, there were 2,847 subjects assessed in sample 1 and 2,797 assessed in sample 2; for skin color, there were 2,717 subjects assessed in sample 1 and 2,664 subjects assessed in sample 2.

[b] Amino acids corresponding to SNP changes *rs1800401* = Arg305Trp and *rs1800407* = Arg419Gln.

brown eye color dominant over blue. More recently, eye color has been accepted as being a polygenic trait, with multiple genes contributing to the expressivity of eye color.[18,35] However, estimates that one or more loci within the linkage region on chromosome 15 that contains the *OCA2* gene explain up to 74% of the QTL variance in eye color[16] confirm that there is a predominant genetic basis for eye-color variation. Here, we have shown that three SNPs within intron 1 of the gene have the highest statistical association with blue eye color (fig. 1). Moreover, these are found in a tight linkage-disequilibrium block, with the TGT haplotype 1 representing 78.4% of alleles in our sample. Given the fact that nonbrown eye colors are found at high frequency only in white populations, it is notable that the major *OCA2* haplotype 1 is found at 82.5% in Europeans and only at minor frequencies—7.4% in those of African and 12.1% of East Asian descent (HapMap database[19] [International HapMap Project] and table 6)—which suggests strong positive selection for TGT in Europeans[53,54]

The most striking statistical finding of this study is that the *OCA2* 1/1 homozygote diplotype is at a frequency of 65% in those with blue eyes and of only 9.5% in those with brown eyes (table 5). In our previous modeling studies of the twin sample with the *D15S165* marker close to the *OCA2* locus, we estimated the frequencies of a dominant brown eye B allele as 21% and of a recessive b allele as 79%,[22] which was close to the 26% B and 74% b allele modeled for the U.S. white population.[55] The similarity of the predicted b allele and the observed *OCA2* TGT haplotype 1 frequencies and the recessive action of this allele in combination with all other haplotypes (apart from TTC haplotype 4 [table 5], all other haplotypes acting like B) in the expressivity of blue eye color indicates that the intron 1 *OCA2* haplotypes are diagnostic for the dominant brown eye versus the recessive blue eye trait. The recessive action of the TGT haplotype is likely to explain effects of loss or gain of alleles at this locus on the pigmentation changes associated with Prader-Willi and Angelman syndromes[7,37,38] noted earlier. Nevertheless, the modeling of eye-color inheritance by use of a single locus determining eye color is insufficient to explain the range of eye color phenotypes, and the description of other loci influencing the appearance of green eye color (GEY/EYCL1 [MIM 227240]) has been noted.[56]

Elsewhere, we reported an interaction between eye color (by inferring *OCA2* genotypes as dominant brown B and recessive b alleles) and variant MC1R alleles responsible

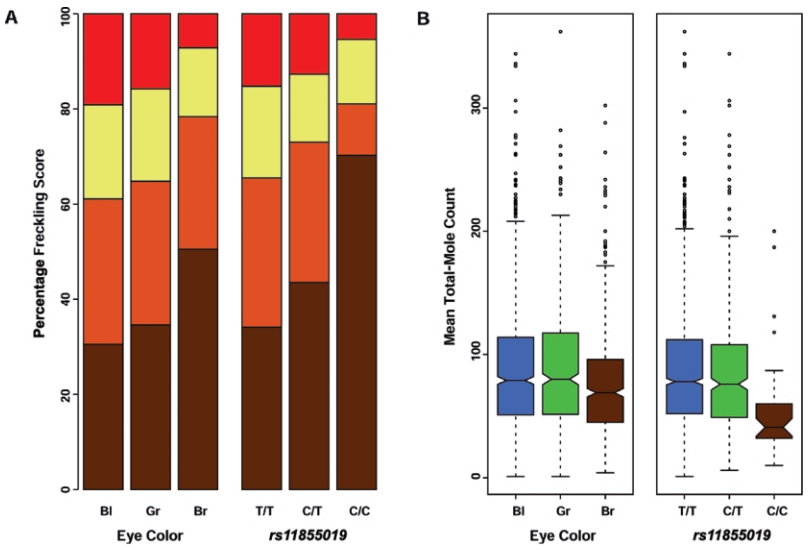

Figure 3. *A,* Histogram of percentage facial ephelidae score (*Y*-axis) in the twin collection divided on a four-point scale (none, mild, moderate, or severe), as indicated by the color shading. The left three bars are plotted by eye color as blue (Bl), green (Gr), and brown (Br), and the right three bars are plotted by genotype at *OCA2* SNP *rs11855019*. *B,* Mean total-nevus count (*Y*-axis), plotted against eye color in the left panel and by genotype at *OCA2* SNP *rs11855019* in the right panel.

for red hair color (RHC) by examining statistical associations of a range of pigmentary traits in a collection of white adolescent twins and family members.[22,43] A modifying effect of *OCA2* genotype on highly penetrant RHC alleles (indicated as "R") was seen for constitutive skin color, freckling, and mole count. Most notable was that those carrying recessive blue eye color b/b and R/R genotypes had a significant reduction in nevus counts.

Numerous studies have shown light eye color to be associated with a higher risk of melanoma,[57] but this is confounded with other host pigmentary factors such as freckling[58] and is also modified by MC1R genotype.[22] The association of 10 *OCA2* intragenic SNPs with pigmentary traits and susceptibility to melanoma in a French population has recently been reported.[15] The combination of two SNPs—IVS13+112 and Ala776Ala (*rs1800419*)—formed a stronger association with melanoma risk than did any of the independent SNPs tested alone. Of these two SNPs, only the synonymous Ala776Ala change was tested in our twin sample, and it was found to be highly significantly associated with eye color ($P = 6.9 \times 10^{-4}$). In combination with SNP IVS13+112, the Ala776Ala major T allele associated with blue eye color and the minor non-blue C allele (table 3) were reported to be highest in melanoma cases and controls, respectively. However, this combination persisted after stratification for eye color, and the biological relevance of these associations is not immediately apparent. An epidemiological examination of the contribution of the *OCA2* haplotype 1 identified in this study to melanoma risk must now be considered a priority.

The position of the three major diagnostic SNPs for eye color located at the 5′ end of the *OCA2* gene near proximal regulatory regions immediately suggests that transcriptional regulation may be important in the action of the TGT haplotype 1. It is not apparent that the three SNPs in themselves play a direct role in expression of the *OCA2* transcript levels, since scanning of the intron 1 sequence with RepeatMasker shows that the *rs6497268* and *rs11855019* SNPs are within interspersed repeats, and, although the *rs7495174* SNP is within a unique sequence region, it does not appear to be in any transcription-factor binding site seen with the MatInspector program.[59] Thus, although unlikely to be responsible for regulation of the *OCA2* gene in themselves, they may be in tight linkage with regulatory elements that are affected by other changes.

Another human locus that has been tested for association with pigmentary traits is the agouti signaling protein gene (ASIP).[18] A g8818A/G SNP in the 3′ UTR of this gene has been reported to be associated with brown eye color[60] and dark hair[61] and is thought to destabilize the ASIP mRNA, which leads to premature degradation of the transcript. Quantification of the ASIP transcripts in melanocytes genotyped for this SNP did show decreases in levels of the ASIP mRNA,[61] which suggests that expression of human pigmentary genes at the level of transcription occurs in genes other than *OCA2*. Similar analysis of *OCA2* transcripts in primary melanocyte strains of defined *OCA2* haplotype 1 remain to be determined.

Polymorphism of gene-regulatory regions is likely to be one of the major contributors to phenotypic variation between and within human populations.[62] Our data demonstrate that variation in the *OCA2* gene 5′ region explains most human eye-color variation, but the molecular basis

Table 6. *OCA2* Intron 1 and Coding-Region Haplotype Frequencies in World Population Groups

A. Intron 1 Haplotype

Haplotype Number	Nucleotides[a]	Frequency			
		African	East Asian	European	Twin Collection[b]
1	TGT	.074	.121	.825	.784
2	TTT	.173	.102	.075	.079
3	TGC	.285	.018	.042	.034
4	TTC	.313	.051	…	.034
5	CGT	.020	.014	…	.003
6	CTT	…	…	…	.001
7	CGC	…	…	…	.000
8	CTC	.135	.690	.042	.064

B. Coding-Region Polymorphism

Haplotype Number	305/419[c]	Frequency				
		African African[d]	Pacific Rim[d]	Native American-Hispanic[d]	White[d]	Twin Collection[b]
9	RR	1.0	.96	.89	.84	.872
10	WR	…	.04	.07	.06	.050
11	RQ	…	…	.04	.10	.077

NOTE.—HapMap population descriptors are as follows: European subjects are Utah residents with ancestry from northern and western Europe (CEPH); East Asian includes Japanese (from Tokyo) and Han Chinese (from Beijing) (EAS); African subjects are Yoruba (from Ibadan, Nigeria) (YRI). For more information, see the International HapMap Project Web site.

[a] At *rs7495174/rs6497268/rs11855019*.

[b] Data from the southeast Queensland twin collection are shown in this table.

[c] Amino acids corresponding to SNP changes *rs1800401* = Arg305Trp and *rs1800407* = Arg419Gln.

[d] Population data from the SNP500Cancer Panel.

of the association with the blue/brown eye color phenotype remains to be elucidated.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Albinism Database, http://albinismdb.med.umn.edu/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP/
Entrez Nucleotide, http://www.ncbi.nlm.nih.gov/entrez/ (for human homologue of the mouse pink-eyed dilution gene [accession number NM_000275])
International HapMap Project, http://www.hapmap.org/
NCBI, http://www.ncbi.nlm.nih.gov/ (for Val380Met [ss66538500], Val519Ala [ss66538502], IVS5-53 [ss66538498], IVS18+45 [ss66538504], IVS5-39 [ss66538499], IVS13-15 [ss66538501], IVS15+78 [ss66538503], and IVS21+18 [ss66538505])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for MC1R, BEY2/EYCL3, HCL3, and GEY/EYCL1)
SNP500Cancer, http://snp500cancer.nci.nih.gov/

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