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Characterization of melanocyte stimulating hormone receptor variant alleles in twins with red hair

Neil F. Box[1], Jason R. Wyeth[1], Louise E. O'Gorman[2], Nicholas G. Martin[2] and Richard A. Sturm[1,*]

[1]Centre for Molecular and Cellular Biology, University of Queensland, Brisbane, Qld 4072 Australia and
[2]Queensland Institute of Medical Research, Brisbane, Qld 4029 Australia

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The association between *MSHR* coding region variation and hair colour in humans has been examined by genotyping 25 red haired and 62 non-red Caucasians, all of whom were 12 years of age and members of a twin pair study. Twelve amino acid substitutions were seen at 11 different sites, nine of these being newly described MSHR variants. The previously reported Val92Met allele shows no association with hair colour, but the three alleles Arg151Cys, Arg160Trp and Asp294His were associated with red hair and one Val60Leu variant was most frequent in fair/blonde and light brown hair colours. Variant *MSHR* genotypes are associated with lighter skin types and red hair ($P < 0.001$). However, comparison of the *MSHR* genotypes in dizygotic twin pairs discordant for red hair colour indicates that the *MSHR* gene cannot be solely responsible for the red hair phenotype, since five of 13 pairs tested had both haplotypes identical by state (with three of the five having both identical by descent). Rather, it is likely that additional modifier genes exist, making variance in the *MSHR* gene necessary but not always sufficient, for red hair production.

INTRODUCTION

Our understanding of the genetics of red hair in humans is incomplete, with the several reported attempts at gene linkage indecisive (1). However, the phenotype is generally considered to be recessive to dark hair and possibly dominant to fair/blonde. It has also been suggested that heterozygotes for this trait might have sandy-red hair, with the homozygous genotype associated with fair skin and freckling (2). Biochemical characterization and ultrastructural examination of the melanins synthesized and deposited within the hair follicle have shown that these compounds are the major determinant of the visible colour (3). Two broad classes of mammalian pigments may be synthesized by the follicular melanocytes and it is incorporation of eumelanin pigments, which are black or brown, that results in dark hair, while yellow or red hair results from incorporation of eu- and pheomelanins (4). It has also been shown that relatively minor differences in melanin quality or content can have dramatic effects on visible hair colour. Ultrastructurally, eumelanin synthesis is restricted to discrete organelles termed eumelanosomes, while the pheomelanins are synthesized in pheomelanosomes. In humans most hair types are the result of mixed melanogenesis, where different levels of both types of pigment are produced. It has been noted that the highest levels of pheomelanins in humans are present in Caucasian fiery red heads (3) and that this may be explained primarily by synthesis of pheomelanosomes within the follicular melanocytes. However, hair follicles from some types of human red hair have been found to contain not only pheomelanosomes but also eumelanosomes and an uncommon 'mosaic' form of melanosome which exhibits features of both eumelanosomes and pheomelanosomes (3).

The physiological signals that control the production of the melanosome and melanin type are extrinsic to the melanocyte. In mice these include the α-melanocyte stimulating hormone (MSH) and the Agouti protein. However, intrinsic to the melanocyte is the ability to respond to these proteins through their interaction with the melanocyte stimulating hormone receptor (MSHR). Genetic studies on mouse coat colours have provided insight into the involvement of MSHR and Agouti in pigmentation (5), with mutations at either locus affecting melanogenesis and producing complementary phenotypes. The human *MC1R* gene has been cloned (6–8), localized to chromosome 16q24.3 (9,10) and shown to encode the MSHR protein. MSHR is a seven transmembrane domain G protein-coupled receptor belonging to the melanocortin receptor subfamily homologous to the mouse extension locus (11).

Although it is presently unclear how MSHR directs the production of the different forms of melanosomes and, therefore, pigmentation phenotype in humans, there is little doubt that it has an important role. Recent research presented by Valverde *et al.* (12) has found polymorphisms within the *MSHR* coding region that are associated with red hair, reporting 82% of British or Irish individuals displaying different shades of red hair being either homozygous, heterozygous or compound heterozygous for nine different amino acid substitutions at the *MC1R* locus, compared with 22% of auburns, 33% of fair/blondes and <20% of brown or black haired individuals having one of these alleles. The relative likelihood for the red hair phenotype has been estimated to be 15-fold among individuals carrying one of the reported variant

*To whom correspondence should be addressed. Tel: +61 7 3365 1831; Fax: +61 7 3365 4388; Email: r.sturm@mailbox.uq.edu.au

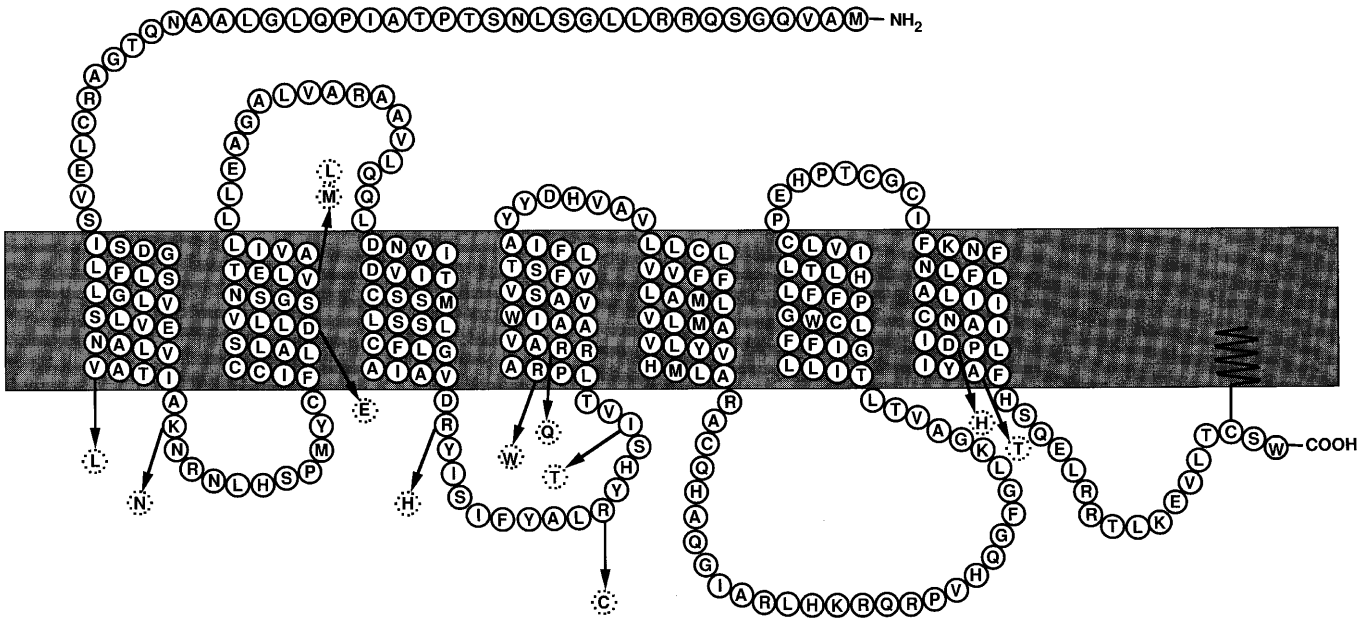

Figure 1. Position of amino acid missense mutations on the proposed schematic structure of the consensus human MSHR protein sequence described in this report. The replacement amino acid is highlighted in dashed circles. The cysteine residue at position 315 is thought to be covalently attached to palmitic acid (17).

alleles and 170-fold among individuals who carry two variant alleles (5), although this may depend upon which allele is being considered. Additional evidence of MSHR involvement in pheomelanogenesis comes with the association of null or missense alleles of the *MSHR* gene in red Norwegian (13) and Holstein (14) cattle, chestnut coat colour in horses (15) and determination of the colour of chicken feathers (15).

To further substantiate the reported association between the status of the human *MC1R* locus and the Caucasian red hair genotype, we have analysed the *MSHR* coding sequence alleles in a sample of young twins of red and non-red hair colour. We also describe variants that segregate with the red phenotype observed in four families.

RESULTS

MSHR protein sequences in Caucasians and Chinese

Of the three reported *MC1R* sequences which are of unknown racial origin, two are identical (7,8), with the third (6) differing by three MSHR amino acid changes: Thr90Ser, Pro162Arg and Ala164Arg. In our initial PCR experiments on Caucasian DNA samples we found a composite of amino acids at each of these positions, with consistent observation of Ser90, Arg162 and Ala164 (Fig. 1). Uncertain of which of these published sequences to define as a non-variant consensus, we decided to determine the MSHR protein sequence from a more heavily pigmented population that does not manifest any red hair phenotypes. The *MC1R* coding region was amplified using nested PCR from DNA isolated from 10 Chinese individuals from Jiangsu Province and subjected to direct automated sequence analysis. The derived sequences were in each case homozygous and consistent for Ser90, Arg162 and Ala164, suggesting that these amino acids probably do not contribute to red pigmentation in Caucasians.

A very common Arg163Gln variant was also found in 16 of the 20 sequenced Chinese haplotypes. This polymorphism, and an Arg67Val variant observed in one individual, may be distinctive to the Chinese population, as they have either not yet been encountered (Arg67Val) or have only been rarely seen in Caucasians (Arg163Gln, Table 1), suggesting that it is unlikely that they are associated with red hair colour. Sequence data from the 10 Chinese individuals also revealed four Val92Met variant heterozygotes. This variant is also common in British and Irish red heads (12), but its high frequency in Chinese suggests that this *MSHR* allele is unlikely to contribute to the red hair phenotype in Caucasians. Of course, this conclusion assumes the absence of any epistatic interaction that may mask the functional consequences of *MSHR* variant alleles in Chinese.

MSHR sequences in red and non-red haired twins

Twins collected for a study designed to investigate the hereditary and environmental factors contributing to the development of pigmented naevi are also being phenotyped for skin, hair and eye colour at 12, 14 and 16 years of age. Among the 365 pairs collected to date there were 44 pairs in which one or both of the twins were recorded as having red/auburn hair, representing 6% of all twins. Genomic DNA was isolated from blood or a buccal swab from each twin pair, with eight red monozygotic and 17 dizygotic sets with one or both having red hair available for analysis. Templates for sequencing of the *MC1R* locus from one of each pair of red haired monozygotic twins and from both members of dizygotic pairs (with one exception) concordant or discordant for red hair were amplified by nested PCR and subjected to direct automated sequence analysis. This sequencing screen was used to search for *MSHR* alleles already reported (12) and also to detect novel variants. Where heterozygosity was detected the individual haplotypes were determined by genotyping of the parents or separated by molecular cloning of the PCR product followed by manual sequencing of the isolated template expressed in bacteria.

Table 1. Association of variant *MSHR* alleles with hair and skin type

Variant allele	Genotype	Hair colour					Skin type			
		Fair/blonde	Light brown	Dark brown	Red	χ_3^2	Fair/pale	Medium	Olive/dark	χ_2^2
V60L	0	13	15	20	24	11.67**	40	28	4	1.20
	1	7	6[a]	1	1		10[a]	5	0	
K65N	0	20	21	21	24		49	33	4	
	1	0	0	0	1		1	0	0	
D84E	0	19	21	20	23		47	32	4	
	1	1	0	1	2		3	1	0	
V92M	0	17	19	15	21	2.86	41	27	4[b]	0.87
	1	3	2	6[b]	4		9	6	0	
V92L	0	20	21	20	24		49	32	4	
	1	0	0	1	1[a]		1[a]	1	0	
R142H	0	20	21	21	24		50	32	4	
	1	0	0	0	1		0	1	0	
R151C	0	15	16	17	7	18.89***	27	24	4	5.44
	1	5[a]	5	4	18[c]		23[c]	9[a]	0	
I155T	0	20	21	21	24		50	32	4	
	1	0	0	0	1		0	1	0	
R160W	0	15	16	21	16	8.97*	38	26	4	1.26
	1	5	5	0	9[b]		12[a]	7[a]	0	
R163Q	0	19	21	21	24		48	33	4	
	1	1	0	0	1		2	0	0	
D294H	0	20	21	19	20	8.48*	44	32	4	2.53
	1	0	0	2	5		6	1	0	
A299T	0	20	21	21	24		49	33	4	
	1	0	0	0	1		1	0	0	

Contingency χ^2 is given where numbers of variant genotypes are sufficient.
For individual alleles genotype 0 = homozygous wild-type or 1 = variant heterozygote (except where homozygous indicated).
[a]Includes one homozygote.
[b]Includes two homozygotes.
[c]Includes five homozygotes.
*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

In total, 12 different amino acid substitutions within the MSHR sequence were found in this sample at 11 different sites (Fig. 2). Based on the proposed structure for the MSHR protein (17,18), the Val60Leu polymorphism lies in the first transmembrane region, Lys65Asn in the first intracellular loop, Asp84Glu and Val92Met/Val92Leu in the second transmembrane segment, Arg142His, Arg151Cys and Ile155Thr in the second intracellular loop, Arg160Trp and Arg163Gln in the fourth transmembrane segment and Asp294His and Ala299Thr in the seventh transmembrane segment (Fig. 1). One silent nucleotide change Thr314Thr (ACA→ACG) was also observed during the analysis of *MSHR* alleles.

Four double variant haplotypes were detected, all in individuals who are red haired: Val92Met, Asp294His; Arg160Trp, Ala299Thr; Val92Met, Arg151Cys; Val92Leu, Arg151Cys. This latter haplotype occurred in combination with a triple variant haplotype, Lys65Asn, Val92Leu, Arg151Cys.

MSHR variant frequency in red and non-red hair and association with skin type

As nine of the 12 *MSHR* polymorphisms detected in the red haired twin samples had not previously been reported, in addition to the 25 red heads we also determined the frequency of each of the variants in one member of twin pairs with fair/blonde (19), light brown (20) or dark brown (20) hair colour, to secure independence of the haplotypes. The genotypes of all 87 individuals are tabulated according to hair colour and skin type (Table 1).

The sample size was insufficient to statistically test the association of all of the variants with hair or skin type. The single Lys65Asn, Arg142His, Ile155Thr and Ala299Thr polymorphisms were only detected in the red haired samples; the Asp84Glu, Val92Leu and Arg163Gln variants were detected once each in red and non-red hair colours. The Val92Met polymorphism was found to be present in all hair colour divisions and shows no

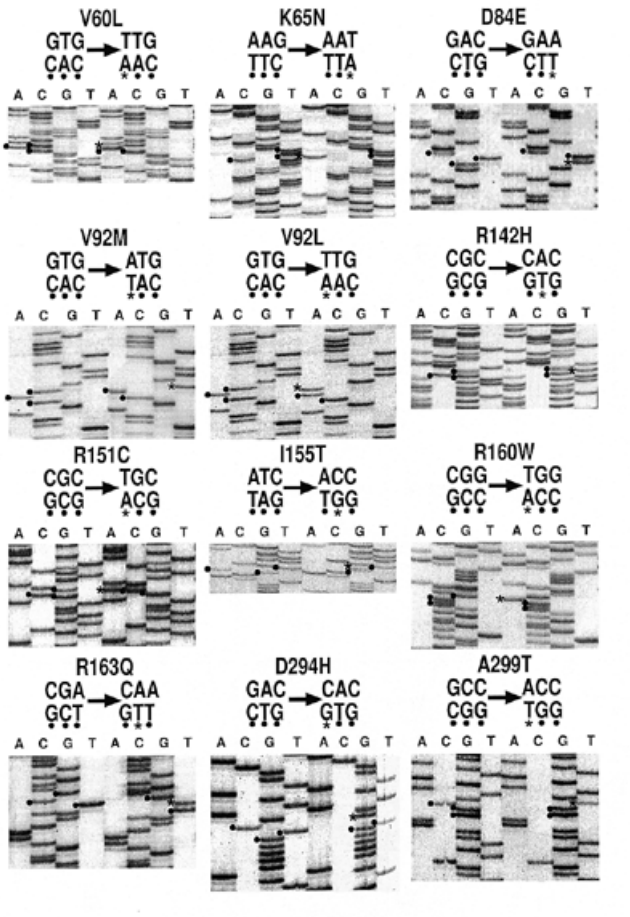

Figure 2. Sequencing autoradiograms showing nucleotide changes in *MSHR* alleles. The consensus and variant sequences reading in the non-coding direction are shown to the left and right in each panel respectively. Listed above is the affected codon with the nucleotide substitution highlighted by an asterisk on the gel to the left of the respective bands. The R160W sequence was resolved on an 8 M urea–30% formamide sequencing gel.

association with any hair or skin classification, the two individuals homozygous for the Val92Met variant having dark brown hair colour and medium skin type.

Four of the variant alleles were found to have statistically significant associations with hair colour, but no variant by itself was associated with any skin type. The Val60Leu variant was the most frequent variant haplotype in both fair/blonde and light brown hair, but was found only once in the red and dark brown groups. The Arg151Cys, Arg160Trp and Asp294His variants are all associated with red hair, with homozygosity of Arg151Cys and Arg160Trp found in five and two cases respectively.

When the frequency of the consensus *MSHR* haplotype alone is examined even stronger associations with hair colour and skin type are found ($P < 0.001$, Table 2). No red haired individual was found to have a normal *MSHR* genotype and all four individuals with olive/dark skin were found to have a wild-type genotype, suggesting that variation in the MSHR protein is a strong determinant of red hair colour and is associated with lighter skin types. As the Val92Met variant was apparently unrelated to hair colour or skin type (Table 1), we redefined the consensus haplotype frequencies without regard to Val92Met as consensus/Val92Met (Table 2). Using this definition, no individual with dark brown hair was found to have a double variant haplotype and the associations with hair colour and skin type were even stronger.

Comparison of *MSHR* alleles in red/non-red haired dizygotic twin pairs and inheritance from red/non-red haired parents

In addition to their inclusion in the analysis of the frequencies of variant *MSHR* alleles in relation to hair and skin colour, the genotypes of 16 of the 17 dizygotic twin partners were available to test segregation of *MSHR* variants, with three twin pairs concordant and 13 discordant for the red hair phenotype. If red hair is a monogenic recessive trait the genotype of the red twin would be anticipated to contain two variant *MSHR* alleles and the genotype of the non-red partner should contain either two normal *MSHR* alleles or possibly one normal and one variant. Thus a discordant pair should share either zero or one alleles identical by state (IBS0 or IBS1). Given the selection for a red haired co-twin it is not surprising that none of the 13 non-red haired twins were found to have two normal *MSHR* alleles, but neither was any non-red twin found to have a homozygous variant genotype.

Table 2. Association of the consensus *MSHR* haplotype with hair and skin type

Variant allele	Genotype	Hair colour					Skin type			
		Fair/blonde	Light brown	Dark brown	Red	χ_6^2	Fair/pale	Medium	Olive/dark	χ_4^2
Consensus	0	5	5	6	0	38.90***	5	7	4	22.23***
	1	7	13	13	3		20	16	0	
	2	8	3	2	22		25	10	0	
Consensus/	0	7	6	12	0	46.04***	8	13	4	16.23**
V92M	1	6	13	9	5		21	12	0	
	2	7	2	0	20		21	8	0	

The consensus haplotype is wild-type for all 12 possible variants; 0 = homozygous consensus, 1 = variant heterozygote, 2 = homozygous variant (all but five *in trans*; see text).
For the consensus/V92M haplotype the same applies but the V92M variant is ignored and considered equivalent to wild-type.
**$0.001 < P < 0.01$; ***$P < 0.001$.

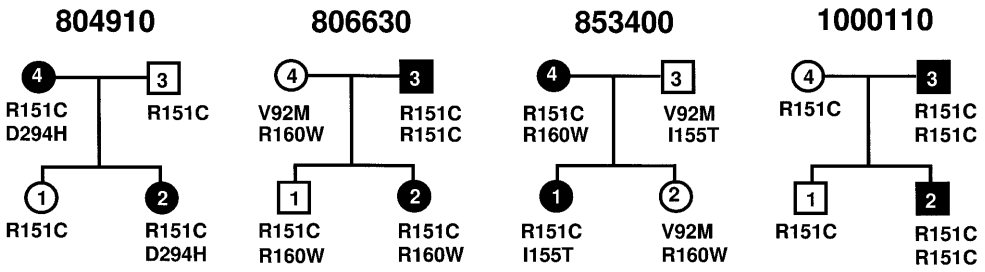

Figure 3. Inheritance of *MSHR* variants in red/non-red haired twins/sibs and parents. Segregation of *MSHR* variants within families including red/non-red dizygotic twins/sibs and red/non-red parents are shown with red hair indicated by the shaded figure. The number of the family is indicated above with the attached number for the twin/sib (1 or 2), mother (4) and father (3) enclosed in the symbol.

Table 3. Allele sharing by state in dizygotic twin pairs concordant and discordant for red hair

Alleles shared by state	Concordant red/red twins	Discordant red/non-red twins
0	0	2
1	1	6[a]
2	2	5[b]
Total	3	13

[a]Excludes Val92Met.
[b]Of these three are IBD2, one is probably IBD2 and one is either IBD1 or IBD2 ($P = 0.5$ in each case).

Of the discordant twins, two pairs shared neither haplotype, six pairs shared one haplotype in common and, contrary to the recessive hypothesis, shared haplotypes (IBS2) were found in five of the 13 twin discordant pairs (Table 3). Sequencing of parental *MSHR* alleles in these cases showed that three of the five share both parental alleles (IBD2) and the other two pairs probably do, excluding the possibility that the *MSHR* variants analysed in these individuals are the sole determinants of red hair.

If the variant *MSHR* alleles that we have described are associated with recessive expression of the red hair phenotype, it is of interest to determine how they segregate with hair colour in families. Of the 13 discordant dizygotic twin pairs, there were three families in which one parent was red and one non-red; an additional non-twin family (1000110) is also included (Fig. 3). In all four families both the red haired child and parent is a compound heterozygote or homozygous variant. However, there are four other individuals who are compound heterozygotes who are non-red, but three of these have Val92Met, which we have argued is phenotypically equivalent to wild-type. That more genes than *MC1R* determine red hair colour is seen from individual 80066301, who is red haired but has the same Arg151Cys/Arg160Trp genotype as his red haired sib. Since they are of opposite sex, it is possible that sex-related factors may be important.

DISCUSSION

Of the nine MSHR amino acid replacement variants that were described in the British and Irish populations (12) only three of these have been identified in our southeast Queensland twin collection. These are Asp84Glu, Val92Met and Asp294His. However, the Val92Met change is commonly seen in the Chinese population and is found in our four categories of hair colour without apparent association (Table 1), indicating that it represents a polymorphism of the MSHR receptor neutral with respect to red hair phenotype, contrary to its first description (12). The neutrality of this polymorphism was also suggested in a later study which investigated the frequency of the British/Irish *MSHR* mutations in melanoma patients (21). Functional testing of the Val92Met variant has suggested that it may display an altered affinity for the MSH ligand (19), but if this is the case any loss of binding potency is not seen when testing the ability of the receptor to activate adenylyl cyclase (20), predicting its functional equivalence to the wild-type state.

An additional nine variant *MSHR* alleles (Fig. 1) are described for the first time in this report, the most common being Arg151Cys and Arg160Trp (Table 1). Given the high frequency of these two alleles in our population it is perhaps surprising that they were not observed in the British and Irish population, which were the main contributors to the population of southeast Queensland. The latter also accounts for the incidence of red hair in our group, with the 6% frequency of red hair in our twin collection similar to the reported UK frequency of between 5.3 and 7.7% (22). Four of the variants were found to be associated with hair colour but none was by itself associated with skin type. The Val60Leu change may well be associated with fair/blonde and light brown hair colours whereas Arg151Cys, Arg160Trp and Asp294His variants are now shown to be associated with red hair colour. Considering all variants together, an association of the consensus wild-type haplotype with darker skin types is apparent and the association of total variant haplotypes with hair colour is highly significant ($P < 0.001$).

Although it is clear from these results and those of Valverde *et al.* (12) that human red hair colour is associated with variant *MSHR* alleles, our study has also shown that polymorphisms in the *MC1R* locus encoding MSHR are necessary but not sufficient for expression of the red hair phenotype. This conclusion is drawn from the observation that some dizygotic twins who share both *MSHR* haplotypes by state can be discordant for red hair colour (Table 3). Of the five red/non-red IBS2 twin pairs three are definitely identical by descent (IBD2) and one is probably IBD2, with the remaining pair sharing either one or both haplotypes by descent (IBD1 or IBD2), discounting the possibility that other polymorphisms not detectable in the *MSHR* coding region are responsible for the hair colour difference. It may be of interest that four of these five twin pairs are of opposite sex, with the female being red, suggesting that sex differences may play a role in

expression of the red hair phenotype. The lack of absolute correlation between *MSHR* genotype and red hair phenotype is perhaps not unexpected given the polygenic nature of the pigmentary trait in other animal species. The chemical characteristics of the melanin polymer are formed in a complex biochemical pathway dependent on a number of enzymes and intermediates (23), allowing genetic interaction and modification to determine the final phenotype (24).

It is notable that two of the variant alleles found in the segregating families, Arg151Cys and Ile155Thr, occur close together in the second intracellular domain. This region contains two consensus sequences for cAMP-dependent protein kinase recognition between amino acids 142–145 and 151–154 (6,17). These changes in or immediately adjacent to the second kinase site may block phosphorylation and hence receptor signalling, with a similar situation likely to occur with the Arg142His change in the first kinase site. This would give a biochemical explanation for the apparent recessive nature of these variants. Statistical evidence for association of the remaining amino acid variants we describe with either hair colour or skin type and association with epistatically interacting genes will require a larger sample to be studied.

MATERIALS AND METHODS

Twins and DNA sample collection

The subjects studied were adolescent twins and their parents obtained through southeast Queensland schools with the aim of identifying major genes affecting mole frequency, pigmentation and other risk factors for melanoma. Twins were examined at age 12, with number of naevi counted and pigmentation characteristics, including hair colour, recorded. A 10 ml blood sample was collected to prepare genomic DNA (25) for zygosity diagnosis and DNA typing. A longitudinal study on mole formation in the twins is being performed with follow-up examination at ages 14 and 16. Parental blood samples were also collected and DNA prepared using the same technique. From the available collection of >300 twin pairs, 25 pairs where one or both had red/auburn hair were selected for study, as well as samples of approximately equal size in the three broad hair colour categories of fair/blonde, light brown and dark brown. Hair colour was assigned to one of these categories by the research nurse coordinating the study, who also classified untanned skin type as fair/pale, medium or olive/dark. Hair samples and colour photographs were also taken for validation.

PCR amplification of *MSHR* alleles

A nested primer strategy was used to obtain sufficient DNA from the *MSHR* locus for direct sequence analysis and to allow molecular cloning of alleles and identification of haplotypes. The primers for amplification of the *MSHR* coding region exon were based on the nucleotide sequence reported by Mountjoy *et al*. (7) (accession number X65634). The first primer set, hMSHR N-outer (5′-AGATGGAAGGAGGCAGGCAT-3′) and C-outer (5′-CCGCGCTTCAACACTTTCAGAGATCA-3′) (12), was used in a 25 µl Pfu DNA polymerase amplification reaction (according to the conditions recommended by Stratagene) containing 25 ng genomic DNA, 10% DMSO (26), initially denatured for 3 min at 94°C, followed by 30 cycles of 1 min 94°C, 1 min 55°C and 3 min 72°C, ending with a 7 min 72°C extension. Internal primers hMSHR N-inner (5′-CCCCTGGCAGCACCATGAACT-3′) and C-inner (5′-TGCCCAGGGTCACACAGGAAC-3′) were used in a second 25–50 µl reaction seeded using 5 µl of the first round reaction as template. Amplification conditions were identical to the first round; however, Pfu was used for cloning of amplification products while Taq DNA polymerase was utilized for preparing template for direct sequencing.

Molecular cloning and sequencing

DNA products from the Taq DNA polymerase amplification reactions were purified by agarose gel electrophoresis, isolated by Bandpure (Progen) and eluted in a 20 µl volume. Automated sequencing reactions were performed by addition of 4 µl template DNA to 8 µl ABI Prism dye terminator premix/AmpliTaq DNA polymerase FS (Perkin Elmer), 3.2 pmol primer and made up to 20 µl with water. Standard cycle conditions were used for a Thermal Cycler Model 480 (Perkin Elmer), with reaction products ethanol precipitated, dried and applied to an ABI 373 automated sequencer. Sequencing oligonucleotides spaced intermittently and in both directions throughout the coding region included forward hMSHR N-inner, Seq1 (5′-TCTGACGGGCTCTTCCTC-3′), Seq3 (5′-TCCAGCCTCTGCTTCCTG-3′) and Seq4 (5′-GCCCGGCTCCACAAGAGG-3′) and reverse hMSHR C-inner, Seq5 (5′-GCGCTGCCTCTTGTGGAG-3′) and Seq2 (5′-ATGGAGCTGCAGGTGATC-3′).

Fragments obtained by Pfu DNA polymerase amplification were purified by agarose gel electrophoresis, isolated by Bandpure (Progen) and eluted in a 20 µl volume. An aliquot of 10 µl product was 5′-phosphorylated using ATP/polynucleotide kinase with half of this insert then ligated to *Hin*cII-digested/dephosphorylated pBS vector. Single-stranded DNA prepared from recombinant clones was used as template for manual sequencing reactions (Pharmacia T7 kit) using the primers listed above, with results compiled from at least four clones or until both haplotypes were obtained and confirmed by restriction digestion (27).

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