Human Molecular Genetics, 2000, Vol. 9, No. 17 2531–2537

Pleiotropic effects of the melanocortin 1 receptor (*MC1R*) gene on human pigmentation

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Received 14 June 2000; Revised and Accepted 23 August 2000

Variants of the melanocortin 1 receptor (*MC1R*) gene are common in individuals with red hair and fair skin, but the relative contribution to these pigmentary traits in heterozygotes, homozygotes and compound heterozygotes for variants at this locus from the multiple alleles present in Caucasian populations is unclear. We have investigated 174 individuals from 11 large kindreds with a preponderance of red hair and an additional 99 unrelated redheads, for *MC1R* variants and have confirmed that red hair is usually inherited as a recessive characteristic with the R151C, R160W, D294H, R142H, 86insA and 537insC alleles at this locus. The V60L variant, which is common in the population may act as a partially penetrant recessive allele. These individuals plus 167 randomly ascertained Caucasians demonstrate that heterozygotes for two alleles, R151C and 537insC, have a significantly elevated risk of red hair. The shade of red hair frequently differs in heterozygotes from that in homozygotes/compound heterozygotes and there is also evidence for a heterozygote effect on beard hair colour, skin type and freckling. The data provide evidence for a dosage effect of *MC1R* variants on hair as well as skin colour.

INTRODUCTION

Pigmentation of the hair and skin is one of the most striking polymorphic human traits and is the major co-variant of ultraviolet sensitivity and skin cancer. The availability of many mouse and zebrafish pigmentary mutants suggests that pigmentation may provide a tractable system in which to study the genetics of complex traits in man. In addition, variation in human pigmentation is of great interest for studies of human evolution and migration (1,2). In the mouse, a large number of loci (>50) are important in the control of melanocyte development and melanogenesis (3). However, polymorphism at only one human locus, the melanocortin 1 receptor (*MC1R*), has been reported to date to be associated with physiological variation in hair and skin colour in otherwise normal humans (2,4–6).

MC1R is a seven-pass G protein coupled receptor, the natural ligand for which is believed to be αMSH, a tridecapeptide cleavage product of pro-opiomelanocortin (POMC) (7). Although ACTH is also active at this receptor in man, whether there are other physiological ligands is not clear. Though knowledge of the downstream signalling from the MC1R is incomplete, activation of the MC1R elevates intracellular cAMP which in turn influences a range of melanogenic enzymes that modulate the amounts of eumelanin (black/brown pigment) and phaeomelanin (red/yellow pigment) (8).

MC1R gene mutations are associated with changes in coat colour in various animals, including mouse, cow, horse, chicken, dog, fox, pig and sheep (8–16). Dominant mutations, which darken the coat through enhancement of eumelanin production, have been detected in several of these species and transfection studies have confirmed the ability of some of these alterations to constitutively activate the receptor (8,9,13–16). Conversely, recessive inactivating mutations of *MC1R*, which cause phaeomelanin synthesis and red or yellow fur, have been identified in some animals (8–11,13,14). Despite this, dominant activating mutations have been found in foxes with significant red coat coloration, suggesting a non-epistatic interaction between dominant *MC1R* mutations and agouti (an antagonist or inverse agonist at MC1R) (14).

Previous human studies, however, have left a number of issues unresolved (4–6). For instance, the extent of any heterozygote versus homozygous/compound heterozygous *MC1R* variant effects on several aspects of pigmentation, including hair colour (scalp, beard), freckling, eye colour, as well as on skin colour, has not been adequately investigated. Secondly, the mode of inheritance of red hair is unclear, with some redheads apparently harbouring only one variant allele, whereas others have two. The more recent description of red hair in subjects compound heterozygous for POMC mutations and comparison of the *MC1R* gene in dizygotic twins discordant for red hair also indicates that *MC1R* variants are

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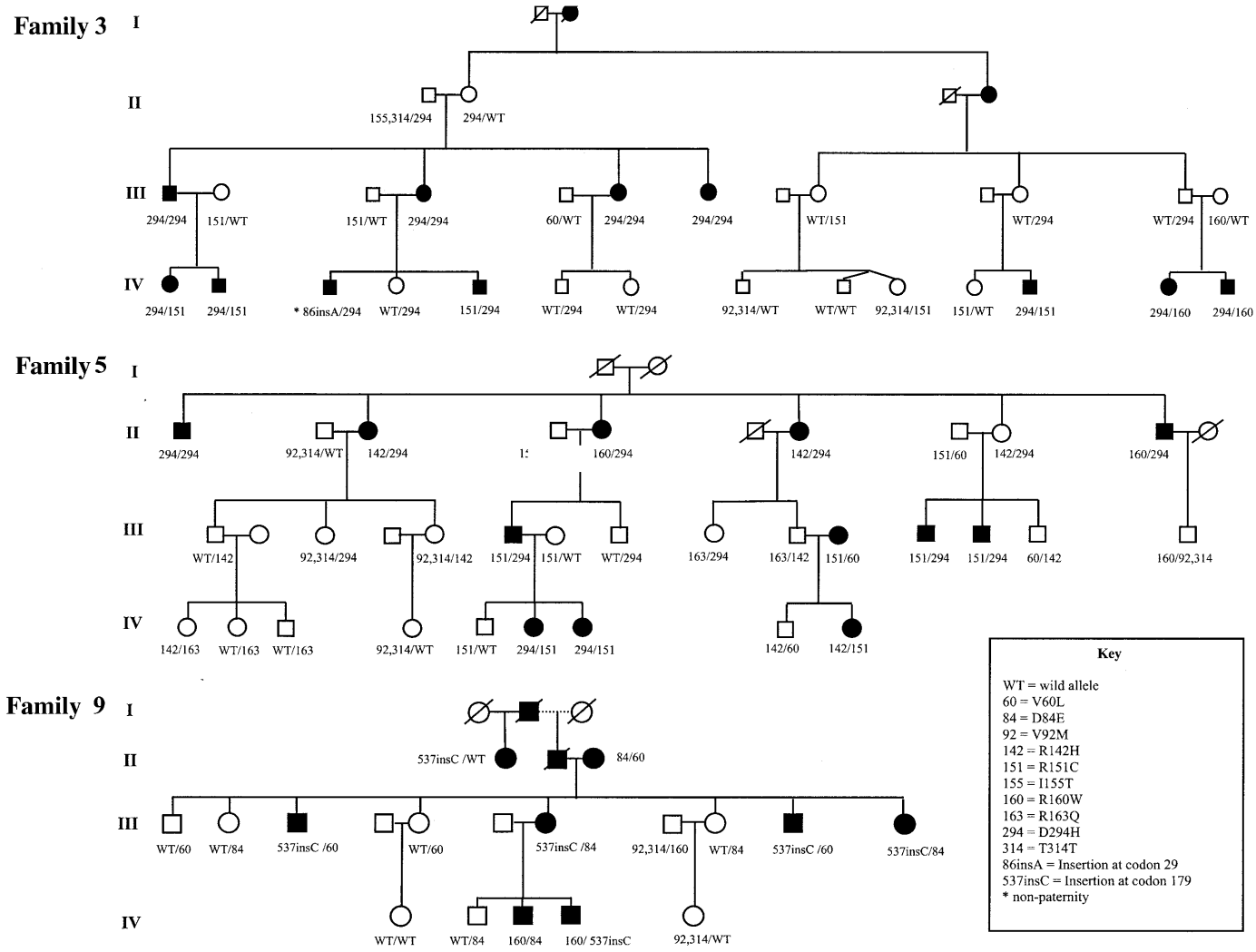

Figure 1. Family trees of kindreds F3, F5 and F9, containing several redheaded members. Most redheaded subjects in families F1, F2, F4, F6, F7, F8, F10 and F11 contained two variant *MC1R* alleles (R142H/R151C/R160W/D294H/86insA/537insC) similar to the pattern of inheritance seen in family F3.

not solely responsible for the red hair phenotype in man (5,17). Thirdly, not all studies have supported an association between *MC1R* and pigmentary phenotype (18); interpretation of many studies is difficult because ~30 variant sites have been identified in the human *MC1R* and the functional status of many of these is unknown.

In the present study we have studied the inheritance of red hair and *MC1R* sequence variation in a number of pedigrees and in a case–control series. We also describe the prevalence of *MC1R* mutations in: (i) subjects with red hair and pale skin; (ii) subjects with similar (pale) skin types but without red hair; and (iii) in a non-red control group. Finally, we have examined the effects of *MC1R* on other pigmentary phenotypes including beard, eye colour and freckling. We show that the majority of red-haired individuals are homozygote or compound heterozygote *MC1R* mutants and, furthermore, that those redheaded individuals who are heterozygous at this locus in general have a different shade of red hair from homozygotes/compound heterozygotes. In addition, non-redheaded individuals with pale skin (low skin type) are more likely to be heterozygote than those without red hair and non-pale skin (high skin type). Differences in *MC1R* variant gene dosage therefore appear to cause a range of pleiotropic effects.

RESULTS

Family and case–control studies

Samples were obtained from 219 individuals from 11 families based on 11 index cases of red hair. The MC1R sequence of 74 individuals with red hair and 100 without was determined (Table 1 and Fig. 1). The family studies are limited in that certain combination of alleles may not be segregating within the pedigrees. We therefore extended our earlier population studies (confining the ascertainment to north European populations where red hair is relatively common) by phenotypically

Table 1. Family red hair genotypes

	60	84	92	142	151	155	160	163	294	86 InsA	537 InsC	+
60	0/0											
84	1/1	0/0	0/0									
92	0/0	0/0	0/2	0/1								
142	0/2	0/0	0/1	0/0								
151	3/8	0/0	0/4	2/2	16/16	0/0						
155	0/0	0/0	0/0	0/0	0/0	0/0	0/0					
160	0/1	1/1	0/4	0/0	8/9	0/0	0/0					
163	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0				
294	0/4	0/0	0/1	2/3	23/24	0/1	5/5	0/0	2/2			
86InsA	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0		
537InsC	2/2	2/2	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/0	
+	0/6	0/3	0/6	0/1	1/29	0/0	0/4	0/0	0/3	0/0	1/1	0/5

Proportion of red hair in genotyped individuals from family studies. The rows and columns are the *MC1R* genotypes on each chromosome. The fraction in each cell is the proportion of red-haired individuals with each genotype. Variants are abbreviated to the codon number but are: V60L, D84E, R142H, R151C, R160W, R163Q, D294H.

Table 2. Population red hair genotypes

	60	84	92	95	142	151	155	160	163	294	86 InsA	537 InsC	+
60	0/6												
84	0/2	0/0											
92	0/5	0/2	0/0										
95	0/0	1/1	0/0										
142	0/0	0/0	0/0	0/0	0/0								
151	1/5	0/0	1/3	0/0	0/0	1/1							
155	0/0	0/0	0/1	0/0	0/0	0/0	0/0						
160	2/5	0/0	0/0	0/0	1/1	4/5	0/0	0/2					
163	0/3	0/0	0/3	0/0	0/0	0/1	0/0	0/1	0/1				
294	1/3	0/0	0/0	0/0	0/0	1/1	0/0	2/2	0/0	0/0			
86InsA	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0		
537InsC	0/0	0/0	0/0	0/0	0/0	1/1	0/0	0/0	0/0	0/0	0/0	0/0	
+	0/15	0/0	0/14	0/0	0/1	1/14	0/2	0/11	0/7	0/5	0/0	0/0	0/42

Proportion of red hair in genotyped individuals from case-control studies. The rows and columns are the *MC1R* genotypes on each chromosome. The fraction in each cell is the proportion of red-haired individuals with each genotype. Variants are abbreviated to the codon number as in Table 1 with additional variant, T95M.

assessing and sequencing the *MC1R* gene of 167 individuals (18 redheads, 149 non-reds) not selected for any pigmentary characteristic (71 Irish, 70 British, 26 Swedish, including 96 from an earlier study), as well as an additional 85 unrelated redheads. The genotype data for this group of 85 with red hair were combined with data of 14 redheads from the group of 167 randomly recruited individuals giving a total of 99 unrelated redheads and their results are shown in Table 2. The 167 random individuals can be used to estimate the allele frequency of the variants in the population (Table 3).

Inspection of Tables 1 and 2 gives an indication that the relatively common alleles, R151C, R160W and D294H, almost always result in red hair when homozygous. Using the random (non-family) cases to exclude ascertainment bias for non-MC1R effects, we can calculate the penetrance of the alleles, when homozygous or heterozygous with each other as 0.787 (95% CI 0.553–0.936). The genotype tables suggest that other variants, D84E, R142H and I155T, which are rare in the population, act as recessive red hair alleles in combination with the three commoner variants, as do the single base insertion/

Table 3. Allele frequency of MC1R variants

Allele	Number in 149 non-reds	Number in 18 reds	Number in 167 total	Frequency
V60L	46	4	50	0.15
D84E	4	1	5	0.015
V92M	28	1	29	0.087
T95M	0	1	1	0.003
R142H	1	1	2	0.006
R151C	21	12	33	0.099
I155T	3	0	3	0.009
R160W	20	9	29	0.087
R163Q	16	0	16	0.048
D294H	7	5	12	0.036
537InsC	0	1	1	0.003
Wild-type	152	1	153	0.458

Allele frequency of coding variants in MC1R in a random Caucasian population of 167 individuals.

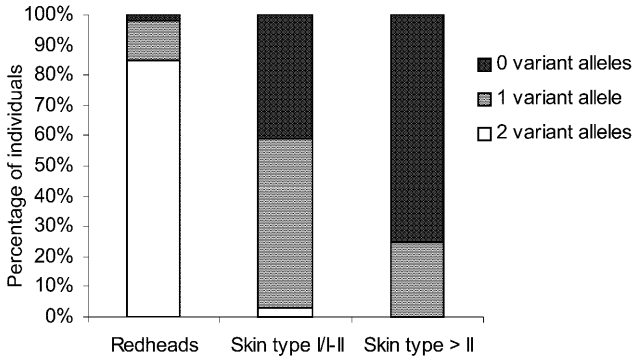

Figure 2. Percentage of unrelated redheaded subjects (Redheads, $n = 99$), non-redheaded individuals with fair skin (Skin type I/II = skin types I, I–II, or II, $n = 61$), and non-redheaded dark-skinned individuals (Skin type > II, $n = 60$) with 1 or 2 *MC1R* alleles of variants D84E, R142H, R151C, I155T, R160W, D294H, 86insA and 537insC.

frameshift mutations 537InsC and 86InsA. The risk ratio for the R142H allele acting as a recessive is significant (risk ratio 2.2, 95% CI 1.3–2.9), whereas for the D84E allele ratio encompasses zero (risk ratio 2.2, 95% CI 0.9–2.8). The numbers of the other alleles are too small to allow meaningful analysis. Conversely, the variants V92M and R163Q do not appear to produce red hair when in combination with the highly penetrant alleles and are probably silent variants.

The V60L variant is particularly interesting as it is present at high frequency in the population (15%) and may act as a low penetrant recessive red hair allele. Analysis of the frequency of red hair in individuals homozygous for V60L, or compound heterozygous with R151C, R160W and D294H, indicates that V60L has a recessive penetrance of 0.103 (95% CI 0.044–0.212). The risk ratio for red hair with the V60L acting as a recessive was 2.4 but the 95% CI encompasses zero (0.8–6.7).

The pedigree data provided by the families (Fig. 1) give further support to the inheritance of red hair as a recessive trait. One family may provide an exception. Family F9 is unusual in that two red-haired parents produced eight children, four of whom have red hair and four do not. The sequence of the *MC1R* gene from individuals in this family reveals that the father (deceased) can be inferred to be heterozygous for 537InsC with wild-type. His half-sister has the same *MC1R* genotype and, although elderly and grey when examined, her niece supported her description of red hair when younger. Each of the four red-haired children has inherited 537InsC, in two cases as compound heterozygote with V60L and in two cases with D84E. The four non-red children who inherited the wild-type *MC1R* have it opposite either V60L or D84E; this indicates that this allele is probably truly wild-type and does not harbour undetected deleterious changes. It thus appears that in this family 537InsC is acting as a dominant allele. An alternative hypothesis would be that there is another (non-*MC1R*) dominant red hair gene segregating in this family; however, pedigree analysis of the family using MLINK indicates that dominant red hair is linked to 537InsC with a LOD score of 2.6, just below statistical significance.

Skin colour (phototype) versus hair colour and an *MC1R* heterozygote effect

Most individuals with red hair have pale skin, tend to burn on exposure to ultraviolet radiation and tan poorly. Conversely, however, there are individuals with pale skin who do not have red hair. In order to understand whether these phenotypic differences could be explained in terms of the *MC1R* we compared individuals with red hair (99 unrelated redheads) with persons with similar pale skin/phototype but without red hair (skin types I, I–II and II, $n = 61$). For further comparison a group with non-pale skin and non-red hair was included (skin type > II, $n = 60$). There was clear evidence for a dosage effect of *MC1R* status with most redheads being homozygous/compound heterozygous for *MC1R* variants D84E, R142H, R151C, I155T, R160W, D294H and the two insertions and non-redhead pale skin types being more likely to be *MC1R* heterozygotes than either the red-haired group or the individuals without red hair who tanned well ($P < 0.001$) (Fig. 2).

There was also evidence of a heterozygote effect on the shade of hair colour in the 99 unrelated redheads. Fifty-eight of the 84 (69%) individuals with two *MC1R* variant alleles (D84E, R142H, R151C, I155T, R160W, D294H and the two insertions) had 'pure' red hair colour and only 26 of 84 (31%) had strawberry blonde or auburn hair. In contrast, 11 of 13 (85%) redheads with a single *MC1R* variant allele had strawberry blonde or auburn hair and only 2 of 13 (15%) had 'pure' red hair ($P < 0.001$) (Fig. 3). Further evidence of a heterozygote effect comes from an analysis of the number of freckling sites in relation to *MC1R* status in the random population of 141 (71 Irish, 70 British) individuals who were phenotyped for freckling, where again there is a clear dosage effect (genotype categories as above) ($P < 0.001$) (Fig. 4). There was a similar trend in terms of beard colour although the numbers were too small for formal analysis. No relation was found between the number of variants and eye colour.

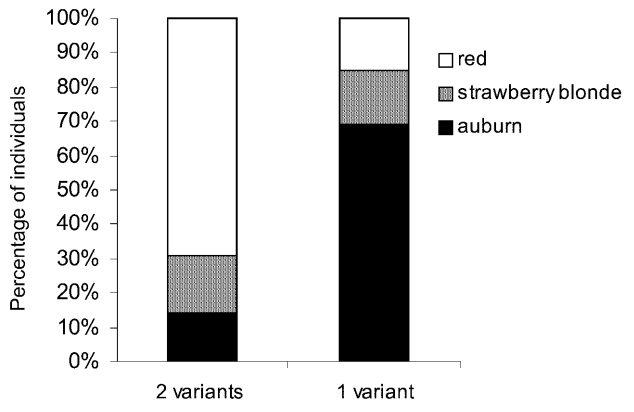

Figure 3. Shade of red hair in unrelated redheaded individuals with two (n = 84) or a single (n = 13) *MC1R* variant alleles (D84E, R142H, R151C, I155T, R160W, D294H, 86insA and 537insC) or no variants (n = 2).

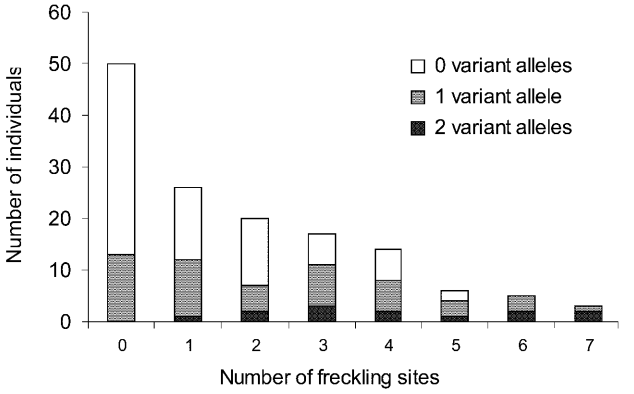

Figure 4. Relation between number of freckling sites and number of *MC1R* variants (D84E, R142H, R151C, I155T, R160W, D294H, 86insA and 537insC) in 141 controls.

DISCUSSION

Recent transfection studies have demonstrated that several human *MC1R* variants are compromised in their ability to stimulate the production of intracellular cAMP (19,20). Although previous association studies have reported on red hair in individuals with either one or two *MC1R* variant alleles, the results from the present kindred and case–control studies suggest that red hair most frequently arises as a result of two loss-of-function alleles at this locus and indicates that red hair is usually inherited as an autosomal recessive trait. The data provide evidence for R151C, R160W and D294H as highly penetrant recessive red hair alleles, equivalent to loss-of-function mutations in other species and for V60L as a low penetrant allele. The statistical evidence for other variants is less well supported, but points to R142H, V84E, 86insA and 537insC as recessive, loss-of-function alleles *in vivo* and suggests that the V92M and 163 variants are not functionally significant alleles. The family studies have revealed individuals with red hair with an insertion allele opposite a wild-type allele, suggesting dominance (or haploinsufficiency) of the 537InsC. However, this cannot be the normal mode of action of this variant as the majority of random sampled redheaded individuals do who harbour 537InsC are also carrying a second MC1R variant, rather than 537InsC alone providing evidence against haploinsufficiency or dominance action of the allele.

Whereas most redheads are homozygous or compound heterozygous for two variant *MC1R* alleles, a significant proportion (13%) have a single variant allele and the relative risk for having red hair with a single variant *MC1R* allele is increased. The majority of the single variant redheads have auburn or strawberry blonde hair, rather than pure red, consistent with a heterozygote effect on the extent of red hair colour. This association is based on a visual inspection of hair colour using a hair colour chart, but more refined assays including the use of a colorimeter or quantification of phaeomelanin/eumelanin ratios may help define in more detail the extent of the heterozygote effect by *MC1R* variants on human hair colour. There are also some rare individuals with definite red hair who are wild-type at *MC1R* and whose red hair may be due to another locus, but the results overall suggest that the percentage of redheads due to changes in *MC1R* outside the *MC1R* coding region (e.g. promoter alterations) or from another genetic locus is low. Although Tan *et al.* (21) have reported on an additional 65 amino acids resulting in a hitherto unknown *MC1R* isoform through alternative mRNA splicing, we have not identified any alterations to date in this second exon in several human populations (data not shown).

We have recently reported a heterozygote effect on an individual's ability to tan by certain *MC1R* variants in a randomly ascertained population (22). In the present case–control study, we have investigated a larger group of subjects with fair skin type (who do not have red hair) and made comparisons with a group of individuals with darker skin types and have confirmed the effect of a single variant allele on fair skin type. Further evidence for a heterozygote effect comes from the association of a single variant allele with red beard hair colour and freckling. This is despite the lack of evidence for a phenotypic effect from a single loss-of-function *Mc1r* allele in the mouse and in other mammals (8–13,15). Whereas a red beard might have been predicted from animal work to result from another locus, for example a ventral specific promoter of the *agouti signalling protein*, the human homologue of the mouse *agouti* gene (23,24), the data on *MC1R* and beard hair colour suggest that most red beards can be explained in terms of *MC1R* variants. Other genetic loci are also likely to be relevant because several red-bearded individuals did not contain detectable *MC1R* variants.

Both the V60L and V92M alleles are present at a high frequency in Caucasians (1,2,5,25). Transfection studies have suggested that the V60L variant differs significantly from wild-type MC1R in its ability to stimulate intracellular cAMP and that the V92M variant has ~2-fold lower affinity for αMSH than wild-type MC1R (20,26). Despite these *in vitro* observations, the V92M allele has been observed frequently in dark-skinned Caucasians and Asian populations as well as in fair-skinned individuals (1,2,5) and our present population data

fail to provide supporting evidence for V92M being associated with human red hair.

MATERIALS AND METHODS

Family studies

Following a publicity campaign via radio and local newspapers, 11 index cases with red hair were selected for study on the basis of family size, number of redheaded family members, willingness to participate and geographical location. Two hundred and one individuals from these 11 families underwent a detailed personal interview by one observer (N.F.). Hair colour throughout life and at different body sites was documented using a chart of hair colour standards (courtesy of Professor Hans Schaeffer, L'Oreal). Hair colour was classified into agreed hair colour classes: red (including strawberry blonde and auburn), fair (blonde, mousy and light brown) and dark (medium brown, dark brown and black). For the purpose of this analysis, hair colour was operationally defined as scalp hair colour at the age of 21 years. Skin type was assessed using our previously described modified Fitzpatrick classification, where: (i) I, always burns, never tans; (ii) I–II, always burns, does not tan after one exposure, but tans lightly after several exposures; (iii) II, always burns, tans slightly after one exposure and after several exposures; (iv) II–III, always burns but tans well; (v) III, seldom burns, tans well; (vi) III–IV, burns after longer exposure but tans very deeply or never burns but tans lightly; and (vii) IV, never burns, tans deeply. The number of freckling sites was recorded by history up to a maximum of seven sites, i.e. face, shoulders, chest, back, abdomen, arms and legs.

A 10 ml sample of venous blood was collected from 170 subjects for DNA extraction. Seven to twelve scalp hairs were plucked from a further 13 children and from two adults who did not wish to undergo venesection. For geographical reasons, it was not feasible to visit a minority of additional family members in person. For these 28 individuals, hair colour was ascertained over the phone and a mouthwash sample, taken first thing in the morning, sent by post for DNA isolation.

In addition a further 85 unrelated UK redheads, 32 fair-skinned (type I or I–II) individuals (without red hair) and 70 random UK subjects (not selected on the basis of phenotype) were characterized as above with respect to pigmentary phenotype and blood was taken for DNA analysis. These results from the randomly recruited individuals were pooled with 71 previously characterized Irish individuals and 26 Swedish controls (total $n = 167$) for the purposes of examination for a heterozygote effect.

Sequencing of the *MC1R*

Following genomic DNA extraction, PCR for codons 1–317 of the *MC1R* gene was performed and variants were identified by dye primer automated DNA sequencing. The oligonucleotide primers for the initial PCR were: 5′-TGTAAAACGACGGCCAGTGAACTAAGCAGGACACCTGG-3′ (-21 M13 forward-linked primer) and 5′-CAGGAAACAGCTATGACCGGACCAGGGAGGTAAGGAACTGC-3′ (M13 reverse-linked primer). Where appropriate, a nested PCR was performed using the following primers: 5′-TGTAAAACGACGGCCAGTGACCTGGAGGCCTCCAACGAC-3′ (-21 M13 forward-linked primer) and 5′-CAGGAAACAGCTATGACCCCACCACCTCCCTCTGCCCAG-3′ (M13 reverse-linked primer).

Following PCR amplification (2 cycles of 95°C for 60 s, 57°C for 60 s, 72°C for 100 s followed by 32 cycles of 95°C for 60 s, 62°C for 60 s, 72°C for 100 s) the products were separated on a 1% agarose gel and purified by gel extraction (Qiagen, Crawley, UK). Sequencing was performed using the ABI PRISM dye primer cycle sequencing kit (Perkin Elmer, Warrington, UK) according to the manufacturer's instructions and products were run on an Applied Biosystems 373 automated sequencer. All sequencing results were read by two observers.

Risk ratios were calculated using Arcus Quickstat and proportions compared using Fisher's exact test or χ^2 (Arcus Quickstat). Pedigree analysis used the mlink utility of the FASTLINK programs (MRC HGMP Resource Centre; http://www.hgmp.mrc.ac.uk).

ACKNOWLEDGEMENTS

We thank Andrew Carrothers for help with statistics and Jo Dukes-McEwan and Caroline Hayward for pedigree analysis. The study was supported in part by the Leech Trust, Cancer Research Campaign and Medical Research Council. E.H. is a MRC Clinician Scientist Fellow. A.R. is a Leech Postdoctoral Fellow.

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