Melanocortin 1 Receptor Variants in an Irish Population

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The identification of an association between variants in the human melanocortin 1 receptor (*MC1R*) gene and red hair and fair skin, as well as the relation between variants of this gene and coat color in animals, suggests that the *MC1R* is an integral control point in the normal pigmentation phenotype. In order to further define the contribution of *MC1R* variants to pigmentation in a normal population, we have looked for alterations in this gene in series of individuals from a general Irish population, in whom there is a preponderance of individuals with fair skin type. Seventy-five per cent contained a variant in the *MC1R* gene, with 30% containing two variants. The Arg151Cys, Arg160Trp, and Asp294His variants were significantly associated with red hair ($p = 0.0015$, $p < 0.001$, and $p < 0.005$, respectively). Importantly, no individuals harboring two of these three variants did not have red hair, although some red-haired individuals only showed one alteration. The same three variants were also over-represented in individuals with light skin type as assessed using a modified Fitzpatrick scale. Despite these associations many subjects with dark hair/darker skin type harbored *MC1R* variants, but there was no evidence of any particular association of variants with the darker phenotype. The Asp294His variant was similarly associated with red hair in a Dutch population, but was infrequent in red-headed subjects from Sweden. The Asp294His variant was also significantly associated with nonmelanoma skin cancer in a U.K. population. The results show that the Arg151Cys, Arg160Trp, and Asp294His variants are of key significance in determining the pigmentary phenotype and response to ultraviolet radiation, and suggest that in many cases the red-haired component and in some cases fair skin type are inherited as a Mendelian recessive. ***Key words: genetics/hair color/pigmentation/skin type. J Invest Dermatol 111:119–122, 1998***

Pigmentation in animals and humans is in part determined by the relative amounts of pheomelanin (red/yellow) and eumelanin (brown/black) in the hair/skin, as well as by the presence or absence of melanocytes within the hair follicles or interfollicular epidermis in different skin regions (Thody *et al*, 1991; Halaban and Moellmann, 1993; Prota *et al*, 1995). Alterations in genes encoding for factors concerned with melanocyte survival (e.g., c-kit and c-kit ligand) and for enzymes involved in pigment production (including tyrosinase and P polypeptide) are responsible for certain well-recognized pigmentatory disorders (reviewed in Halaban and Moellmann, 1993; Spritz, 1994). In animals, expression of the agouti gene results in the preferential production of pheomelanin, with ectopic expression of this gene causing obesity, diabetes, and a predisposition to tumor formation, in addition to changes in coat color (Bultman *et al*, 1992). Conflicting views originally existed on whether the agouti product acts through the melanocortin 1 receptor (MC1R) or through its own specific receptor in directing the production of pheomelanin, but the evidence now suggests that agouti's effect on pigmentation is through its action as an inverse agonist at the MC1R (Conklin and Bourne, 1993; Jackson, 1993; Siegrist *et al*, 1997). Despite the variation in the cutaneous expression of agouti between and within animals of different coat colors, the level of expression of agouti seems similar in human skin from different ethnic backgrounds (Bultman *et al*, 1992; Vrieling *et al*, 1994; Wilson *et al*, 1995). Alpha-melanocyte stimulating hormone and other pro-opiomelanocortin peptides, which are agonists for MC1R, also alter the pheomelanin/eumelanin ratio, resulting in the preferential production of eumelanin (Burchill *et al*, 1986; Chhajlani and Wikberg, 1992; Mountjoy *et al*, 1992). Although an exogenous supply of alpha-melanocyte stimulating hormone can affect the degree of pigmentation of humans and animals, levels of this hormone in the plasma and the skin are similar in individuals with different skin type (Lerner and McGuire, 1961; Spiro *et al*, 1987).

The MC1R is a seven pass transmembrane G-protein coupled-receptor of 317 amino acids, which is expressed in several cell types, including melanocytes and keratinocytes (Chhajlani and Wikberg, 1992; Mountjoy *et al*, 1992; Healy *et al*, 1998). Variants of this receptor, some of which are known to differ in their ability to activate adenylyl cyclase, are associated with various coat colors in mice, cattle, horses, foxes, and chickens, whereas the *MC1R* gene is deleted in the red guinea pig (Cone *et al*, 1996; Joerg *et al*, 1996; Marklund *et al*, 1996; Takeuchi *et al*, 1996; Vage *et al*, 1997). We have previously shown that variants of the human *MC1R* (especially the Asp294His variant, where aspartate is replaced by histidine at codon 294) are associated with red hair and fair skin in individuals from a (predominantly) British population, and this association of red hair with variants has recently been confirmed in a study of Australian monozygotic and dizygotic twins (Valverde *et al*, 1995; Box *et al*, 1997). The relation of *MC1R*

Manuscript received November 11, 1997; revised February 25, 1998; accepted for publication March 13, 1998.

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Abbreviations: MC1R, melanocortin 1 receptor; NMSC, nonmelanoma skin cancer.

variants to these phenotypic characteristics is likely to be due to the presence of (at least some of) these variants affecting the alpha-melanocyte stimulating hormone signaling pathway in follicular melanocytes so that pheomelanin is preferentially synthesized; this may also be the case in human skin, but the exact relationship between skin type and pheomelanin/eumelanin production in the interfollicular epidermis in humans does not seem straightforward (Thody *et al*, 1991; Hunt *et al*, 1995). *MC1R* variants (especially the Asp84Glu variant, where aspartate is replaced by glutamate at codon 84) are also associated with susceptibility to sporadic melanoma, a relationship that seems largely independent of skin type (Valverde *et al*, 1996).

The studies to date on *MC1R* variants in humans have identified three areas within the gene where variants cluster, including the first transmembrane domain/first intracellular loop/second transmembrane domain, the second intracellular loop, and the seventh transmembrane domain (Valverde *et al*, 1995, 1996; Box *et al*, 1997; Koppula *et al*, 1997). The identification of an association between red hair/fair skin and *MC1R* variants has provided a potential mechanism by which the normal variation in human skin and hair color can be investigated; however, in our original studies, individuals were selected from the extremes of hair color or skin type, and therefore they did not provide an adequate basis for the calculation of relative risks, for study of the mode of inheritance of the pigmentory phenotype, or for formal testing of which variants are significant in their effect on phenotype. We have therefore looked for *MC1R* variants in a consecutive series of volunteers from an Irish population, a population noted for sensitivity to ultraviolet radiation (Beirn *et al*, 1970). In addition, because the relative risk of developing red hair with the Asp294 variant was high, and because this change was the most frequent alteration detected in our original study (Valverde *et al*, 1995) and is easily detected using restriction fragment length polymorphism (RFLP) analysis, the frequency of this variant and its association with red hair was investigated in individuals from The Netherlands and Sweden. Furthermore, as an additional test of any association between genotype and phenotype, we have examined the frequency of the Asp294His variant in a group of subjects with nonmelanoma skin cancer(NMSC) and controls.

MATERIALS AND METHODS

Subjects from an Irish population One hundred and two consenting consecutive Caucasian individuals (patients with psoriasis and staff in the City of Dublin Skin & Cancer Hospital, Ireland) were recruited as volunteers for the study. None of the volunteers were known to be genetically related to any other volunteer, and individuals with skin cancer (melanoma and nonmelanoma) were excluded; patients with psoriasis were chosen because there is no known association between psoriasis and skin type/hair color, and such a group is unlikely to be biased with respect to referral for skin cancer or atypical moles or sun sensitivity. Skin type was assessed by detailed personal interview using our previously modified Fitzpatrick (1988) classification: I, always burns, never tans; between I and II, always burns, does not tan after one exposure, but tans lightly after several exposures; II, always burns, tans lightly after one exposure and after several exposures; between II and III, always burns but tans well; III, seldom burns, tans well; between III and IV, burns after longer exposure but tans very deeply *or* never burns but tans lightly; and IV, nevers burns, tans deeply (Valverde *et al*, 1996). Subjects were also graded on a four point scale (no tan, light golden, medium brown, and dark brown) according to how well they tanned following repeated exposures to natural sunlight. Hair color throughout life was documented using a chart of hair color standards (courtesy of Professor Hans Schaeffer, L'Oréal, Centre de Recherche, Clichy, France), and then classified into agreed hair color classes ("red," "fair," and "dark"); red hair color included strawberry blonde and auburn as well as red, whereas fair included blonde, fair, and light brown. Dark hair color consisted of medium brown, dark brown, and black. Scalp hair color at 20 y was employed in the analysis with *MC1R* variants. Other variables that were recorded included eye color at the time of interview (including the background iris color, the central iris color, and the presence of flecks of pigment scattered throughout the iris), the ability to freckle, and the individual's ancestry. A venous blood sample was collected from each subject, and genomic DNA was isolated as described previously (Valverde *et al*, 1995).

Identification of variants Polymerase chain reaction (PCR) for the *MC1R* gene was carried out and variants were identified using a combination of sequencing and RFLP analysis. Automated and/or manual cycle sequencing of codons 1–200, which includes the first transmembrane domain/first intracellular loop/second transmembrane domain, and the second intracellular loop, was carried out for 71 samples, including all red haired individuals and a representative selection of all phenotypes present in the group of 102 subjects. PCR products were also analyzed by RFLP analysis using the enzymes *AvaII* (for codon 84 in the second transmembrane domain) and *TaqI* (for codon 294 in the seventh transmembrane domain); RFLP analysis for variants at codons 84 and 294 was carried out on all 102 samples. The oligonucleotide primers used for the initial PCR were 5′CTGGAGGTG-TCCATCTCTGAC3′ and 5′ATGAAGAGCG-TGCTGAAGACGA3′ for the second transmembrane region, 5′GGT-CCACCAGGGCTTTGGCCTT3′ and 5′TGCCCAGCACACTTAAAGC-GCGTGCA3′ for the seventh transmembrane region, and 5′AGCAC-CATGAACTAAGCAGGACACCT3′ and 5′TGATCACGTCAATGACAT-TGT3′ for codons 1–200. Examination of these areas and codons of the *MC1R* should detect over 95% of sequence changes so far described. Manual sequencing was performed using the Sequitherm Cycle Sequencing kit (Epicentre Technologies) according to the protocol using the above primers, and products were separated on a 6% polyacrylamide gel and visualized by autoradiography. Automated sequencing was carried out with an M13 Rev primer (on PCR products that had been originally amplified using an M13 Rev-linked primer) using dye primer chemistry (Perkin Elmer, Warrington, Cheshire, U.K.) according to the manufacturer's instructions, and products were run on an Applied Biosystems 377 (Warrington, Cheshire, U.K.) automated sequencer. All putative variants were confirmed by repeat PCR and sequencing or RFLP analysis from genomic DNA.

Table I. List of variants detected in the Irish population

Region of human MC1R	Variant	Number of individuals (%) in which variant observed
First transmembrane region	Val60Leu	14 of 71 (19.7%)[a,b,c,d,e,f]
Second transmembrane region	Asp84Glu	4 of 102 (3.9%)[b,g]
	Val92Met	10 of 71 (14.1%)[c,g]
Second intracellular region	Arg151Cys	17 of 71 (23.9%)[d,h,i]
	Ile155Thr	1 of 71 (1.4%)
	Arg160Trp	11 of 71 (15.5%)[h,j,k]
	Arg163Gln	4 of 71 (5.6%)[e]
Seventh transmembrane region	Asp294His	7 of 102 (6.9%)[f,i,k]

[a]Four individuals homozygous for Val60Leu; [b]two individuals with Val60Leu and Asp84Glu; [c]two individuals with Val60Leu and Val92Met; [d]one individual with Val60Leu and Arg151Cys; [e]one individual with Val60Leu and Arg163Gln; [f]one individual with Val60Leu and Asp294His; [g]two individuals with Asp84Glu and Val92Met; [h]three individuals with Arg151Cys and Arg160Trp; [i]one individual with Arg151Cys and Asp294His; [j]one individual homozygous for Arg160Trp; [k]two individuals with Arg160Trp and Asp294His. In addition, a silent change at codon 158 (Leu158Leu) was detected in nine individuals.

Subjects from other populations and individuals with NMSC Genomic DNA samples were also extracted from venous blood from 95 individuals from The Netherlands and 91 individuals from Sweden, who had been phenotyped with regard to hair color and skin type, and RFLP with *TaqI* (for codon 294) was carried out on these samples. Genomic DNA was also extracted from 57 subjects with NMSC (clinical and histologically confirmed), including 46 with basal cell carcinoma, eight with squamous cell carcinoma, 15 with actinic keratoses, and five with Bowen's disease (some patients had more than one type of NMSC lesion) from a U.K. population. The hair color and skin type of these subjects were recorded, and RFLP with *TaqI* (for codon 294) was carried out on their DNA. The control group from the U.K. population included 25 individuals who were friends or spouses of the NMSC patients, and a previously reported group of 44 subjects with psoriasis (Valverde *et al*, 1996); subjects with skin cancer were excluded from the control group.

RESULTS

The wild-type *MC1R* sequence was that reported by Chhajlani and Wikberg (1992), except for Ala164Arg (alanine rather than arginine at codon 164) as this difference was observed in all 71 samples sequenced. Fifty-three of the 71 (75%) Irish individuals who were sequenced contained a variant allele, with 21 of these 53 (30% of the 71 individuals) having two variants; this latter group included subjects with homozygous variants. None of the other 31 Irish individuals in whom RFLP for codons 84 and 294 had been performed contained variants at these codons. The list of variants detected in the study are outlined in **Table I**. The presence of any *MC1R* variant was associated with red hair in the Irish population (**Table II**), and three variants in particular were over-represented in this red-headed group; Arg151Cys

Table II. Relation of *MC1R* variants to phenotypic characteristics in the Irish population

Phenotypic characteristic	MC1R variant	Number (%) with phenotypic characteristic having variant	Number (%) without phenotypic characteristic having variant	Odds ratio	p value[a]
Red hair	Any	13 of 13 (100)	40 of 58 (69)	–	0.029
	Any (excluding Val92Met)	13 of 13 (100)	34 of 58 (58.6)	–	0.003
	Two variants	8 of 13 (61.5)[b]	12 of 58 (20.7)	6.1	0.006
	Two variants (excluding Val92Met)	8 of 13 (61.5)[b]	8 of 58 (13.8)	10.0	<0.001
	Arg151Cys	8 of 13 (61.5)	9 of 58 (15.5)	8.7	0.0015
	Arg160Trp	7 of 13 (53.8)[d]	4 of 58 (6.9)	15.8	<0.001
	Asp294His	5 of 13 (30.8)	3 of 89 (3.4)	12.7	0.005
Fair skin	Any	37 of 45 (82.2)	16 of 26 (61.5)	–	NS
	Any[c]	36 of 44 (81.8)	14 of 24 (58.3)	3.2	0.047
	Two variants	17 of 45 (37.8)	3 of 26 (11.5)	4.7	0.027
	Arg160Trp	10 of 45 (22)	1 of 26 (4)	7.1	0.046
	Arg151Cys[c]	14 of 44 (31.8)	2 of 24 (8.3)	5.1	0.037
	Val60Leu	9 of 45 (20)	7 of 26 (26.9)	–	ND
Freckles	Any	43 of 53 (81.1)	10 of 18 (55.6)	–	NS
	Arg151Cys	7 of 75 (9.3)	0 of 27 (0)	–	NS
	Arg160Trp	11 of 53 (20.8)	0 of 18 (0)	–	NS
	Asp294His	15 of 53 (28.3)	2 of 18 (11.1)	–	NS

[a]NS, not significant; ND not determined.
[b]Includes seven people heterozygous for each variant and one person homozygous for one variant.
[c]Fair skin graded as outlined in text according to degree of tanning alone (no tan or light golden).
[d]Includes one individual homozygous for this variant.

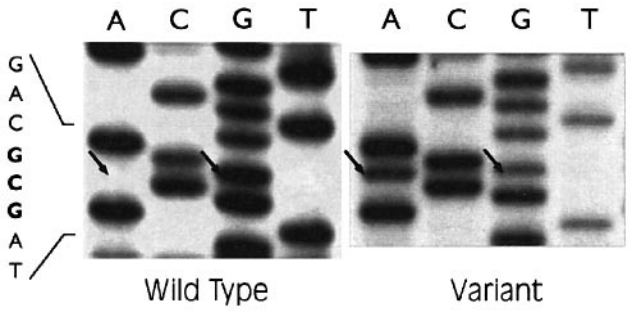

Figure 1. Sequence of the anti-sense strand of the *MC1R* gene in two Irish individuals. The sequence on the left is from a subject with a wild-type sequence, whereas the sequence on the right is from a subject heterozygous for the Arg151Cys variant (GCG altered to ACG, i.e., CGC altered to TGC on sense strand).

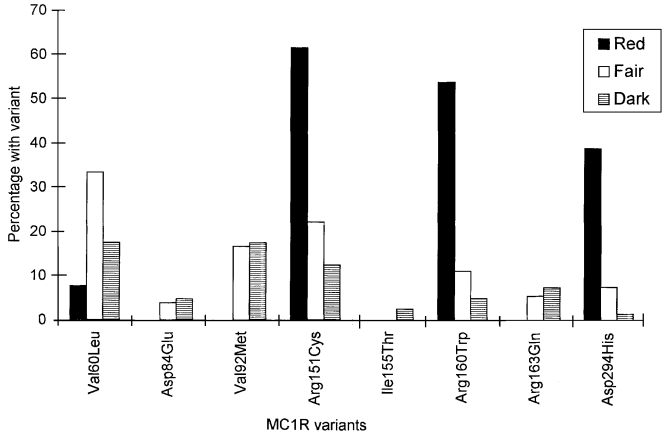

Figure 2. Association of *MC1R* variants with scalp hair color at age 20 in individuals from an Irish population.

(arginine altered to cysteine at codon 151), Arg160Trp (arginine to tryptophan at codon 160), and Asp294His (**Figs 1**, **2**). All 13 individuals with red hair in comparison with 16 of 58 subjects without red hair contained one of these three variants ($p < 0.001$), with eight of the 13 red heads and none of the 58 non-red heads either heterozygous for any two of these variants or homozygous for one of these variants ($p < 0.001$). Although three of five males with the Arg151Cys, Arg160Trp, and Asp294His variants who did not have red scalp hair had red facial (i.e., beard) hair, this was not significantly different to the frequency of red facial hair in males without red scalp hair and without any of these three variants (seven of 21 males whose DNA was sequenced). No individual without red scalp hair at age 20 y had red scalp hair at any other age, or red axillary or red pubic hair. The Val60Leu variant (valine to leucine at codon 60) was also observed frequently in the 71 individuals who were sequenced, and was over-represented in people with fair scalp hair, although this association was not statistically significant.

MC1R variants were more frequent in Irish subjects with fairer skin type (I, I–II, and II) than in those with darker skin type (II–III, III, III–IV, and IV), with 37 of 45 subjects (82.2%) with fair skin and 16 of 26 subjects (61.5%) with darker skin having a variant, and the presence of two variants was particularly associated with fair skin (**Table II**). All of the variants that were related to red hair color were more frequent in the fair skinned individuals, although only the Arg160Trp was statistically significant ($p = 0.046$); none the less the Arg151Cys and Asp294His were more common in those with light skin type [14 of 45 (31%) *vs* three of 26 (12%), and seven of 63 (11%) *vs* one of 39 (3%)], respectively. When the skin pigmentation was analyzed according to the degree of tanning following repeated exposure to natural sunlight, the presence of any variant and the Arg151Cys variant was significantly more common in individuals who did not tan or tanned poorly (**Table II**). The Val60Leu variant was not related to skin type. *MC1R* variants (including the Arg151Cys, Arg160Trp, and Asp294His variants) were more frequent in individuals who had freckles (**Table II**); however, the Arg151Cys and Asp294His variants were associated with a greater degree of freckling as assessed by the number of skin sites where freckling occurred (four or more of the following sites; face, neck, shoulders, upper limbs, back, chest, and lower limbs) ($p = 0.03$ and $p = 0.009$, respectively). There was no association between the presence of *MC1R* variants (or any particular variant) and eye color. Despite the associations outlined above, *MC1R* variants were found in some individuals from all hair color and all skin type categories.

Because the relative risk (odds ratio) of developing red hair with the Asp294His variant was high in the Irish population, and because this variant was the most frequent in red heads in our original study (Valverde *et al*, 1995), we subsequently examined for this variant in a group of 95 individuals (including 33 with red scalp hair) from The Netherlands, and in a group of 91 subjects (20 with red hair) from Sweden. This variant was significantly more common in red headed

people from The Netherlands (six of 33 *vs* none of 62, p = 0.001), but this was not the case in the subjects from Sweden (one of 20 *vs* none of 71). In the British NMSC patients, the Asp294His variant was also over-represented, with seven NMSC patients containing this variant in contrast to its complete absence in the control group (p = 0.003).

DISCUSSION

This population-based genetic study of the contribution of *MC1R* variants to the pigmentary phenotype, in contrast to our previous studies, concerns a population that was relatively unselected and the results provide an adequate basis for the study of relative risks and the mode of inheritance of the pigmentary phenotype. The Irish population was chosen because of the known high frequency of sun sensitive individuals. Our results suggest that the Arg151Cys, Arg160Trp, and Asp294His variants are of key relevance in determining the red-haired phenotype, and that the Asp294His variant is also an important determinant of red hair in Dutch people (as well as in the British population as reported in our original study on *MC1R* variants) (Valverde *et al*, 1995). All three variants showed some relation to fair skin, either using the modified Fitzpatrick classification, or by analysis with the degree of tanning.

The weaker association with skin type than with hair color may be due to the limitations imposed by the use of the Fitzpatrick classification (even in its modified form), because sensitivity to ultraviolet radiation-induced erythema is an important component of this classification, yet the relation of sensitivity to ultraviolet radiation-induced erythema to cutaneous pigmentation is not straightforward (Rampen *et al*, 1988). The use of the degree of tanning may circumvent this problem, but whether the degree of tanning is determined by the absolute amounts of eumelanin or phaeomelanin or the phaeomelanin/eumelanin ratio in the skin is also unclear (Thody *et al*, 1991; Hunt *et al*, 1995); however, the association of the Asp294His variant with NMSC provides independent evidence of the role of this locus in determining sun sensitivity, this analysis avoiding any subjectivity of assessment that might occur in the recording of pigmentation. Although we did not test for the codon 151 and 160 variants in this group, we would expect them also to be over-represented in NMSC patients.

The association of red hair and fair skin with either one or two (particular) *MC1R* variants is striking: in all cases with two variants where this included a combination of Arg151Cys, Arg160Trp, or Asp294His, the hair color was red and the skin type was fair; however, the presence of the exact same two variants (e.g., Arg151Cys and Arg160Trp) could result in either a definite red, a strawberry blonde, or an auburn hair color. Equally important, although the Arg151Cys, Arg160Trp, and Asp294His variants were detected in subjects without red hair, none of these cases were homozygous for any of these variants or had more than one of these three variants. These results shed important light on several aspects of pigmentation genetics. First, they imply a recessive mode of inheritance for red hair at this locus. In addition, because the presence of the same single variant in some people was associated with red hair but in other people was associated with another hair color, it raises the possibility that the gene function from the second wild-type allele may be reduced by alternative mechanisms in the red heads, or that these alleles harbor mutations outwith the area examined in this study. Second, other loci are clearly important in determining the exact color of the hair (i.e., auburn *versus* red in individuals with two identical variants). Evidence that the agouti product (at least in animals) can act as a competitive antagonist or an inverse agonist at the MC1R suggests other ways in which the relation between genotype and phenotype may be mediated, and may allow a fuller and tighter understanding of the role of the MC1R as a control point in human pigmentation. Third, our study only suggests a functional role for the Arg151Cys, Arg160Trp, and Asp294His variants. It is not possible to exclude a role for the other changes, due to the weak power of statistical testing of rarer alleles, but, for instance, in this study no evidence of a association between pigmentation and codon 92 variants was found. Finally, the high frequency of apparent polymorphisms of the *MC1R* is intriguing and suggests that the gene may be of interest in studying wider aspects of human evolutionary adaptation to the environment.

We are grateful to the staff of the City of Dublin Skin and Cancer Hospital for their assistance during this study. EH is a Medical Research Council Clinician Scientist Fellow.

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