The Asp84Glu variant of the melanocortin 1 receptor (*MC1R*) is associated with melanoma

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Melanocyte stimulating hormone (MSH) plays an important role in determining the cutaneous response to ultraviolet radiation and may also influence melanoma progression. We have previously shown that variants of the melanocortin receptor present on melanocytes, *MC1R*, are associated with sun sensitivity and red hair in a UK population and therefore now consider the gene as a candidate for melanoma susceptibility. We have compared the frequency of known *MC1R* variants in the second and seventh transmembrane domains in 43 melanoma cases and 44 controls. *MC1R* variants were more common in cases than controls ($\chi^2 = 6.75$, 1 d.f.; $P = 0.0094$) with a relative risk to carriers of variant alleles compared with normal homozygotes of 3.91 (95% c.i.: 1.48–10.35), and a population risk attributable to carriers of 34.6% (95% c.i. 10.7–52.1%). The Asp84Glu variant was only present in melanoma cases and appears to be of particular significance. The contribution of variant *MC1R* alleles was largely independent of skin type. Variants of the *MC1R* gene are likely to be causally associated with the development of melanoma.

INTRODUCTION

The incidence of melanoma is increasing in most Caucasian populations (1–3). The high case fatality, coupled with the ubiquity of the main environmental cause, ultraviolet radiation, has led to interest in identifying high risk groups and research into the underlying biological basis of susceptibility. There appears to be a significant genetic contribution to melanoma susceptibility (4), however, the attributable risk of certain genetic syndromes such as xeroderma pigmentosum and kindreds harbouring germline mutations of p16 may be small (5,6). On the other hand, pigmentation and the cutaneous response to ultraviolet irradiation, which also appear to be largely under genetic control, are major risk factors for melanoma (1,3). Melanoma rates are an order of magnitude higher in whites (Caucasians) than in blacks (Negroes), and even within many Caucasian populations a collection of pigmentary characteristics including propensity to freckling, pale skin, light or red hair, a tendency to burn and an inability to tan are associated with increased risk of melanoma (1,3,7).

The genetics of human pigmentation appears complex, but analogies can be drawn from other mammalian systems. In the mouse the product of the extension locus [the melanocyte stimulating hormone receptor, the melanocortin 1 receptor (*Mc1r*)] controls the switch from phaeomelanin (yellow) to eumelanin (black) (8,9). Mice homozygous for a loss-of-function mutation in the receptor produce only yellow pigment in the hair; (these mice do not spontaneously develop skin tumours, and to date no ultraviolet irradiation studies on mice with known *Mc1r* variants have been reported). Mutations in the same gene in cattle and horses results in red and chestnut animals respectively (10–12). We have recently shown that, in man, variants of the melanocyte stimulating hormone are associated with red hair and the propensity to burn rather than tan following sun exposure (13). Melanocyte stimulating hormone (αMSH) is a tridecapeptide cleaved from its precursor proopiomelanocortin which also gives rise to other peptide hormones with pigmentary activity including ACTH (14). αMSH is secreted by the anterior pituitary but is also produced peripherally in skin (15) and some, if not all, of its effects on melanocytes are mediated through binding to the *MC1R*, a member of the family of G protein coupled transmembrane receptors, and subsequent activation of adenyl cyclase (8,16,17).

There are at least two reasons for anticipating that allelic variants of the *MC1R* gene may be causally associated with melanoma susceptibility. First, given the associations between skin type and melanoma, and between *MC1R* variants and skin type, one might expect an over-representation of variant alleles in melanoma subjects. Importantly, although the association between skin type and melanoma is marked in some populations such as Australia (1,3), in UK studies and in others from Northern Europe the relative risk is more modest (18–20); this has been attributed to the low ambient ultraviolet irradiation and the high proportion of sensitive skin types in the general population (19), but may also reflect the methodological difficulties of classifying subjects by skin type (21). It is possible, therefore, that (genotypic) examination for the presence of variant alleles may be more revealing than examination of phenotype. A second unrelated, but no less cogent, reason for suspecting that *MC1R* may be a candidate susceptibility gene for melanoma, is that as

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well as playing an important physiological role in controlling the switch from phaeomelanin to eumelanin, αMSH with or without other growth factors including basic fibroblast growth factor may play an important role in stimulating melanoma cell growth and tumour progression (22,23). Therefore, we have examined a group of patients with melanoma and a control population for *MC1R* allelic variants.

RESULTS

We studied the *MC1R* gene of 43 unrelated patients with cutaneous melanoma and of 44 unrelated control patients. The skin type of each individual was assessed using a modified Fitzpatrick scale (24), and a history of freckling and hair colour taken. We found, as expected, a weak association between melanoma and skin type: if skin type was treated as a quantitative variable (25), the means of skin type for melanoma patients and controls were 2.03 and 2.33 respectively. This difference was just statistically significant at the 5% level for a one-tailed test in the predicted direction, i.e., lower skin type in the melanoma group ($t = 1.70$, 81 d.f.; $P = 0.046$).

We amplified the *MC1R* gene by PCR and examined for the presence of previously reported variants by sequencing or restriction enzyme digest. Twenty out of 43 melanoma patients, but only eight out of 44 controls were carriers of variant alleles (Table 1). The association of any variant allele with melanoma was statistically significant ($\chi^2 = 6.75$, 1 d.f.; $P = 0.0094$). The estimated risk of melanoma for those with one or two variant alleles relative to normal homozygotes was 3.91 (95% c.i.: 1.48–10.35). The population risk attributable to carriers was 34.6% (95% c.i.: 10.7–52.1); (this is an estimate of the amount by which the incidence of the disease would be reduced if the risk in carriers were reduced to the same level as that in non-carriers; it is a somewhat artificial concept but provides some measure of the population impact of the carrier status on disease incidence) (26). Of particular note was that the association between variant alleles and melanoma was largely accounted for by the Asp84Glu

variant (aspartate replaced by glutamate at codon 84) which was identified in 10 melanoma cases, with two of these individuals being homozygous for this change, whereas no control case showed this alteration. Clinically, the two subjects homozygous for the Asp84Glu variant did not differ from the other melanoma patients, in that these individuals developed their melanoma at 42 and 81 years old respectively, whereas the overall group developed melanomas at ages ranging from 28 to 86 years, neither patient developed more than one melanoma, and hair colour/skin type/freckling characteristics were red/skin type I–II/freckles and dark brown/skin type II/freckles respectively for the two homozygotes. Interestingly, 24 of the 43 melanoma subjects had been previously investigated for p16^{INK4} mutations (27); only one case contained a p16^{INK4} mutation which had occurred as a somatic event in an individual heterozygous for the Asp84Glu variant.

Three melanoma cases showed the Asp294His variant (aspartate replaced by histidine at codon 294), and one additional case showed an Asn91Asp (asparagine replaced by aspartate at codon 91) alteration; neither of these changes were seen in the control population. By contrast the Val92Met variant (valine to methionine at codon 92) was found at similar frequency in both melanoma and control groups. No case in which the melanoma and normal skin was sequenced demonstrated a *MC1R* variant in the melanoma alone, consistent with the variants being present in the germline rather than resulting from a somatic event; [in addition, a previous allelotype study on cutaneous melanoma (including 36 of the 43 melanomas in the present study) detected no allelic imbalance on chromosome arm 16q where the human *MC1R* gene is located] (32). The association between melanoma and carriers of variant *MC1R* alleles appeared largely independent of skin type when examined using the Mantel–Haenszel test (25) ($\chi^2 = 4.59$, 1 d.f.; $P = 0.032$; the four cases where skin type was not available were excluded). Within skin type categories the relative risk for carriers of variant alleles versus normal homozygotes was 3.57 (95% c.i.:1.25–10.14).

Table 1. Comparison of MC1R variants in 39[a] melanoma patients and 44 control patients with respect to skin type, hair colour at 20 years old, and ability to freckle

Melanoma		Asp84Glu	Asn91Asp	Val92Met	Asp294His	Total	Controls		Val92Met	Total
Skin type	I	2	0	0	0	4	Skin type	I	1	4
	I–II	4[bc]	1[c]	4[b]	0	12		I–II	1	9
	II	1	0	1	2	12		II	3	12
	II–III	0	0	0	1	3		II–III	0	3
	III	1[b]	0	2[b]	0	6		III	1	8
	III–IV	0	0	0	0	1		III–IV	1	5
	IV	0	0	1	0	1		IV	1	3
Hair colour	Red	3	0	1	1	6	Hair colour	Red	1	5
	Light	2[b]	0	4[b]	1	16		Light	3	14
	Brown/black	3[bc]	1[c]	3[b]	1	17		Brown/black	4	25
Freckles		6[bc]	1[c]	4[b]	1	25	Freckles		4	20

[a]This information was known for 39 of 43 melanoma cases in whom the *MC1R* gene was investigated; data was not available for two cases who contained an Asp84Glu alteration, and for one case with a Val92Met variant.
[b]Two individuals had Asp84Glu and Val92Met.
[c]One individual had Asp84Glu and Asn91Asp.

DISCUSSION

We show that certain variants of the *MC1R* are more common in individuals with melanoma than in control subjects and that this association is greater than the association between melanoma and skin type. Interestingly, one particular allele, the Asp84Glu variant which was present in 23% of the melanoma subjects but none of the controls (in the present study) accounts for most of this difference. Consideration of whether the association between variant alleles and melanoma is acting independently of skin type is of biological and clinical relevance. First, in a previous study relating *MC1R* variants to skin type and pigmentary characteristics in a selected British population (13), we found the Asp84 Glu change in only two out of 135 subjects (including 47 subjects with red/auburn hair and 77 who were skin type I or II). This variant is therefore not common even in those thought to be at increased risk of melanoma on the basis of pigmentary characteristics or history of sun sensitivity. The finding of two individuals homozygous for Asp84Glu in the melanoma group, a genotype not previously reported, emphasises the potential importance of this change. On the other hand, although the statistical analysis suggests that the effect of genotype appears largely independent of skin type, there are a number of legitimate criticisms of the way skin type was recorded in the present study. The Fitzpatrick classification, whilst widely used and superior to other measures such as measurement of erythema following experimental exposure to ultraviolet radiation, is known to be a crude measure of the cutaneous response to ultraviolet radiation (24), and telephone enquiry may be less sensitive than direct interview. (Self-reported hair colour, except for the extremes of hair colour is likely to be prone to considerable error which was why hair colour was not formally analysed in the present study.) Nevertheless if the association of *MC1R* alleles and melanoma were acting through skin type, on the basis of previous work (13), one would have expected an association to be seen with all the variant alleles. This was not the case, and in particular the Val92Met variant was seen at similar frequency in both controls and melanoma cases: an alternative explanation is that the Val92Met is a neutral polymorphism rather than being associated with skin type.

The molecular mechanism behind the reported association is not known. In comparison with other mammals, the genetics of the *MC1R* in man has several enigmatic features including the nature of the mode of inheritance (28), and at present the functional significance of the variants in terms of signal transduction is unclear. Although the conservative change from aspartate to glutamate appears minor, aspartate at codon 84 is highly conserved throughout not only the melanocortin receptor family but also other G protein coupled receptors (16,17,29–31). Whilst it is possible that the change we have observed may not be the direct cause, but rather may be in linkage disequilibrium with some other change outside the coding region of the *MC1R* gene, the high degree of conservation makes a direct causal role for this particular variant plausible. To resolve this, studies of the functional significance of the various alleles, and case control studies of melanoma in different populations are required. Furthermore, there seem at least two possible pathways in which the *MC1R* may be influencing melanoma development: through controlling the switch from phaeomelanin to eumelanin and hence determining the ability to protect against ultraviolet radiation; or alternatively, via the effects of MSH on melanocyte growth. Studies on non-melanoma skin cancer might prove informative in this regard. If no association of the Asp84Glu variant is seen with squamous malignancy then it is likely that the association is melanoma specific, a feature favouring an influence of MSH on melanocyte growth rather than on the development of pigmentary protection.

MATERIALS AND METHODS

Patients and controls

Genomic DNA was isolated as previously described (13,32) from either paraffin-embedded melanoma, normal skin, or from venous blood of 43 unrelated Caucasian patients with a cutaneous melanoma originally excised between 1987 and 1995, and from venous blood of 44 unrelated control Caucasian patients with psoriasis. Patients with psoriasis recruited from a population survey were used as controls because of sample availability, and because there is no evidence of an altered risk of melanoma in this population group, nor is there any known association between psoriasis and skin type, the use of other (non-melanoma) dermatology outpatients as controls was felt inappropriate because of potential bias towards patients with non-melanoma skin cancer or patients referred because of concern about skin cancer. The melanoma individuals were ascertained through the Departments of Dermatology and Pathology at the Royal Victoria Infirmary, Newcastle and Dryburn Hospital, Durham. Control subjects were originally ascertained through the response to a request for patients with psoriasis in a local televised program on research into psoriasis. All melanoma and control individuals (except one melanoma patient who was living in Southern Scotland) resided in the North East of England. Forty two of the 43 melanoma patients did not have a family history of melanoma (the one case with a family history of melanoma had an affected mother only, but did not fulfil the criteria for the diagnosis of atypical mole syndrome). Only one patient developed a second cutaneous melanoma, and two patients had clinical features consistent with atypical mole syndrome. The age range of the melanoma and control groups was 28–86 (median 57.5) and 12–72 (median 49) years respectively. Skin type was assessed using a modified Fitzpatrick scale (24) [types (a) I, (b) between I and II, (c) II, (d) between II and III, (e) III, (f) between III and IV and (g) IV], ability to freckle, and hair colour at age 20 years (except in one case who was younger than 20 years at the time of study) in 27 melanoma cases and 44 controls by a detailed telephone questionnaire or personal interview. In an additional 11 melanoma cases (10 deceased, 1 demented), this data was acquired from a first degree relative; in one deceased case a carer provided the information; in four remaining cases (deceased/change of address) no information was available.

Mutation analysis

The *MC1R* gene was amplified by nested PCR (13) except that 10% DMSO was employed in the PCR for paraffin-embedded material. Sequencing of regions previously reported to contain variants in Caucasians (13), including the second transmembrane domain (codons 64–106; sequencing primer 5′-TGATCACGT-CAATGACATTGT-3′) and the seventh transmembrane domain (codons 278–300; sequencing primer 5′-TGCCCAGCACACT-TAAAGCGCGTGCA-3′) was carried out for melanoma cases. Controls were similarly sequenced through the second transmembrane domain, and screened for codon 294 variants by restriction

fragment length polymorphism (RFLP) analysis or sequencing. Putative mutations were confirmed by repeat PCR from genomic DNA, and sequencing or RFLP analysis (codon 84, *Ava*II; codon 294, *Taq*I). Overall, optimal sequence was obtained in the melanoma samples in 13 cases, in the melanoma and in normal skin in 14 cases, in blood in seven cases and in the normal skin in nine cases.

ACKNOWLEDGEMENTS

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