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Melanocortin-1 Receptor Variant R151C Modifies Melanoma Risk in Dutch Families with Melanoma

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Germline mutations of the cell-cycle regulator p16 (also called "CDKN2A") in kindreds with melanoma implicate this gene in susceptibility to malignant melanoma. Most families with familial atypical multiple-mole melanoma (FAMMM) who are registered at the Leiden dermatology clinic share the same p16-inactivating deletion (p16-Leiden). Incomplete penetrance and variable clinical expression suggest risk modification by other genetic and/or environmental factors. Variants of the melanocortin-1 receptor (MC1R) gene have been shown to be associated with red hair, fair skin, and melanoma in humans. Carriers of the p16-Leiden deletion in Dutch families with FAMMM show an increased risk of melanoma when they also carry MC1R variant alleles. The R151C variant is overrepresented in patients with melanoma who are from families with the p16-Leiden mutation. Although some of the effect of the R151C variant on melanoma risk may be attributable to its effect on skin type, our analyses indicate that the R151C variant contributes an increased melanoma risk even after statistical correction for its effect on skin type. These findings suggest that the R151C variant may be involved in melanoma tumorigenesis in a dual manner, both as a determinant of fair skin and as a component in an independent additional pathway.

Introduction

Cancer is a complex multifactorial process in which gene-gene and gene-environment interactions are thought to play crucial roles. Identification of genes involved in melanoma skin cancer has been difficult, except in cases of very strong familial risk (Hussussian et al. 1994; Kamb et al. 1994*b;* Zuo et al. 1996). A candidate melanoma-susceptibility gene, p16 (also called "CDKN2A" [MIM 600160]), has been isolated from the 9p21 region (Kamb et al. 1994*a;* Nobori et al. 1994). Strong evidence for its candidacy as a melanoma-predisposition gene has been provided by germline mutations in ~40% of all kindreds with melanoma (Piepkorn 2000). p16 is a cell-cycle regulator, which fulfils its function by binding to cyclin-dependent kinases 4 (CDK4 [MIM 123829]) and 6 (CDK6 [MIM 603368]) (Serrano et al. 1993). In this way, p16 inhibits the kinase activity of CDK4/cyclinD and CDK6/cyclinD, which normally phosphorylate the retinoblastoma protein (pRB). Phosphorylation of pRB leads to progression of the cell into the S phase of the cell cycle (Serrano et al. 1993).

Fair skin is an additional known risk factor for melanoma (Kricker et al. 1991; Bliss et al. 1995). Variation in human skin pigmentation is due to varied amounts of eumelanin (brown/black) and pheomelanin (red) produced by melanocytes. The melanocortin-1 receptor (MC1R [MIM 155555]) is a regulator of eumelanin and pheomelanin production after binding of the melanotropic hormone, α-melanocyte–stimulating hormone (αMSH). In humans, variants of the MC1R gene are of key significance in determining the pigmentary phenotype and response to ultraviolet radiation (Valverde et al. 1995; Box et al. 1997; Smith et al. 1998; Healy et al. 2000; Palmer et al. 2000). The MC1R variants R151C, R160W, and D294H have been particularly associated with fair skin and with skin cancer (Valverde et al. 1995; Smith et al. 1998; Flanagan et al. 2000; Healy et al. 2000; Palmer et al. 2000). In these studies, one variant in the genotype turned out to be sufficient to cause fair skin and increased melanoma risk (Healy et al. 2000; Palmer et al. 2000). Several studies have shown that specific MC1R gene variants act as independent genetic risk factors for skin cancer in general and that these MC1R variants contribute to skin cancer risk at least as strongly as do fair skin and red hair (Palmer et al. 2000).

In six Dutch families with familial atypical multiple-mole melanoma (FAMMM), a 19-bp germline deletion (p16-Leiden) in exon 2 of the p16 gene has been found to be present (Gruis et al. 1995*b*). Incomplete penetrance and variable clinical expression suggest risk modification by other genetic and/or environmental factors

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in these kindreds (Gruis et al. 1995*a;* Hayward 1996). In the present study, we determined the role of MC1R variants as modifiers of melanoma risk in members of Dutch families with FAMMM who are known to carry p16-Leiden. In these families, variant alleles increase the risk of melanoma. In particular, the fair-skin MC1R variant R151C was found to be associated with melanoma risk in p16-Leiden carriers. Although it appears that some of the increased risk in R151C-positive p16-Leiden carriers can be attributed to the fair skin type that is associated with this variant, our stratified statistical analysis supports the notion that R151C also contributes an increased risk that is independent of its effect on skin type. This is consistent with the fact that MC1R is a member of the G-protein–coupled receptor superfamily, the members of which can act as oncogenes in other signal-transducing systems, including the p38 mitogen-activated protein (MAP) kinase pathway (Coughlin 1994; Simonds 1999; Smalley and Eisen 2000).

Material and Methods

Family Material and Control Subjects

All of the pedigree data, the method of ascertainment, and the histopathological findings on the six 9p-linked Dutch families with FAMMM syndrome have been published elsewhere (Bergman et al. 1992). From these families, 101 carriers of p16-Leiden were included in the single-strand conformation polymorphism (SSCP; Orita et al. 1989) analysis of MC1R.

Skin type was assessed by the skin-type classification of Fitzpatrick and Breathnach (1963). In this classification, "Caucasian" (white) skin is divided into three skin phenotypes (Fitzpatrick and Breathnach 1963): skin type I represents fair skin that always burns and never tans but frequently freckles in the sun; skin type I is accompanied by red hair. Skin type II is lightly pigmented, easily burned, and yields a light sun tan; most individuals with skin type II have fair hair and blue eyes. Skin type III is still a white skin type, characterized by easy tanning and infrequent burning in the sun. These individuals tend to have brown hair and brown eyes. Skin type IV never burns, always tans with ease, and is not regarded as a white skin type.

Detection of MC1R Variants

Genomic DNA from members of the Dutch families with FAMMM was isolated from peripheral blood leukocytes by routine methods (Miller et al. 1988). A specific PCR product of MC1R coding sequence (GenBank accession number X65634) was digested by 2 U of either *Rsa*I or *Msp*I and was screened for mutations by SSCP analysis (Orita et al. 1989) on a 6% polyacrylamide gel with 10% glycerol. The gels were run at room temperature for 6 h at 26 W or for 16 h at 20 W, for *Msp*I and *Rsa*I digests, respectively. PCR reaction mixtures contained 60 mM Tris HCl, pH 10.0; 2.0 mM $MgCl_2$; 15 mM $(NH_4)_2SO_4$; 100 μM dGTP, dTTP, dATP, and dCTP; 1 μl α-[^{32}P]dCTP (3,000 Ci mmol^{-1}), 500 ng of each PCR primer, 2 U Ampli*Taq* (Perkin Elmer Cetus); and 10% dimethyl sulfoxide, in a total volume of 100 μl. Fifty nanograms of genomic template DNA was added to 10 μl of reaction mixture. Samples were covered with mineral oil, were denatured for 4 min at 92°C, and were passed through 33 cycles of amplification, which consisted of 50 s of denaturation at 92°C, 50 s of primer annealing at 60°C, and 2 min of elongation at 72°C. The amplifications were performed in 0.5 ml tubes (Perkin Elmer). The DNA sequences of the primers were as follows: forward, 5′-CAACGACT-CCTTCC-TGCTTC-3′ and reverse, 5′-TGCCCAGCACACTTAA-AGC-3′, resulting in a 1,018-bp PCR fragment.

Sequence Analysis

For sequence analysis, internal primers were used to amplify MC1R in two fragments, which were purified by the EasyPrep PCR Product Prep Kit (Pharmacia Biotech). Products were directly sequenced by the AmpliCycle Sequencing Kit (Perkin Elmer) with the amplification primers. The sequences of the internal primers were as follows: forward, 5′-ACCTGCAGCTCCAT-GCTGTC-3′ and reverse, 5′-GTCACGATGCTGTGGTAGC-3′.

Statistical Analysis

For comparison of the allele frequencies in different groups, either Pearson's χ^2 test or Fisher's exact test was applied, depending upon which test was more appropriate. The effect that the number of MC1R variants in the genotype had on melanoma risk was evaluated using logistic regression. The Mantel-Haenszel χ^2 test was used to test the effect of the MC1R variant R151C on melanoma risk, conditional on skin type.

Results

We studied the role of MC1R variants in six Dutch families with FAMMM, in whom p16-Leiden is segregating (Gruis et al. 1995*a*). Mutation analysis of the entire MC1R coding sequence was performed in 101 p16-Leiden carriers from these families, of whom 38 had developed a melanoma. Allele frequencies for six MC1R variants detected in p16-Leiden carriers are presented in table 1, together with the frequency data from a large Dutch control population. In general, frequencies of variants in the p16-Leiden carriers paralleled those in the control population; exceptions included the R151C variant, which was present in ~14% of the family members, in contrast with a general population frequency of 5%

Table 1

Allele Frequencies of MC1R Variants in 101 Typed p16-Leiden Carriers

Sample	Frequency (No.) of MC1R Variant					
	V60L	V92M	R151C	I155T	R160W	R163Q
All p16-Leiden carriers ($n = 101$)	.074 (15)	.089 (18)	.139 (28)	.035 (7)	.149 (30)	.069 (14)
p16-Leiden carriers without melanoma ($n = 63$)	.071 (9)	.095 (12)	.095 (12)	.016 (2)	.127 (16)	.087 (11)
p16-Leiden carriers with melanoma ($n = 38$)	.079 (6)	.079 (6)	.211 (16)	.066 (5)	.182 (14)	.039 (3)
General population ($n = 385$)[a]	.083 (64)	.069 (53)	.048 (37)	.003 (3)	.105 (81)	.049 (38)

[a] Allele frequencies estimated in 385 control individuals from The Netherlands (Bastiaens et al. 2001).

($P < 10^{-6}$), and the I155T variant ($P \approx .001$). The excess of the R151C allele in the p16-Leiden carriers is mostly due to a high frequency in the melanoma patients (21%; table 1). Carriers without melanoma also present a higher frequency of the R151C allele than does the general population (10% vs. 5%); this indicates that these melanoma pedigrees are enriched for this allele. A plausible reason for this enrichment can be found in the ascertainment procedure: only families with many melanoma patients were collected in the very early stages of our study. If the R151C allele indeed increases melanoma risk, as our results indicate, the frequency of this allele should be increased in these families, as a consequence of the ascertainment procedure. Therefore, we have determined the transmission distortion of the R151C allele, and not merely the allele frequency, in the families included in our study. Within the families, the R151C allele was transmitted from heterozygous parents carrying p16-Leiden to affected offspring more often than expected by chance. Of 12 p16-Leiden–positive offspring with melanoma, 8 had received the R151C allele from their heterozygous parents; however, of 16 unaffected offspring (all carrying p16-Leiden), only 6 had inherited the R151C allele ($P \approx .07$, one sided).

When all MC1R variant alleles were considered jointly as a potential melanoma risk factor in p16-Leiden carriers, a similar pattern was observed. Overall, 38% of the p16-Leiden carriers developed melanoma. Table 2 shows a melanoma frequency of 55% in p16-Leiden carriers with two MC1R variants, compared to a melanoma frequency of 18% in MC1R wild-type p16-Leiden carriers. Logistic regression showed that the melanoma risk increases with the number of MC1R variants in the genotype ($P \approx .01$), with an odds ratio (OR) of 2.4 for each additional MC1R variant in the genotype. When this analysis was performed with subjects who had R151C alleles excluded, the OR per MC1R variant was still increased (OR 1.8), but it did not differ significantly from 1.0 ($P \approx .16$). The age at onset for melanoma was very similar in all genotype groups, varying between 34 years in the group with one MC1R variant and 42 years in the group with two MC1R variants. No significant differences were found between the genotype groups for the current age distribution in unaffected individuals. The distribution of skin types in p16-Leiden carriers was determined and found not to differ significantly from the control population (table 3); however, within the group of p16-Leiden carriers, a significant difference in the skin type distribution existed between subjects with and without melanoma ($P \approx .02$), with a preponderance of fair skin types among melanoma patients. Subsequently, the effect of the MC1R variant R151C on melanoma risk was evaluated with a correction for the existing relationship between R151C and fair skin and for the increased melanoma risk in people with fair skin. A Mantel-Haenszel test conditional on skin type yielded a χ^2 of 2.78 ($P \approx .05$, one sided), which supports, albeit with marginal significance, that the R151C variant independently contributes to melanoma risk.

Discussion

The incidence of cutaneous melanoma in white individuals has doubled roughly every 10 years since the early part of last century (Henderson et al. 1991). Much of this increase has been attributed to excessive sun exposure. Despite this major environmental risk factor, it is clear that individuals are variably susceptible to melanoma development. For example, melanoma is essentially a tumor of white-skinned people (Armstrong and Kricker 1996), and, within this population, individuals with skin that burns easily in the sun (skin types I and II), seem to be at significantly increased risk (Osterlind et al. 1988). Melanin pigment protects the skin from the damaging effects of ultraviolet radiation. Whereas >50

Table 2

Association between MC1R Variants and Melanoma Risk in 101 p16-Leiden Carriers

MC1R Genotype	No. of Carriers	No. of Melanoma Cases (Frequency)	Age at Onset (years)
Wt/Wt	17	3 (.18)	40
Wt/Var	55	19 (.35)	34
Var/Var	29	16 (.55)	42
Wt/R151C	10	5 (.50)	42
Var/R151C	17	11 (.65)	45

Note.—Wt = wild type; Var = variant.

Table 3

Skin-Type Distribution of the Dutch General Population and of p16-Leiden Carriers

	No. of Individuals with (Frequency of) Skin Type			
Sample	I	II	III	IV
All p16-Leiden carriers (n = 90)[a]	10 (.11)	38 (.42)	42 (.47)	…
p16-Leiden carriers without melanoma (n = 58)	3 (.05)	23 (.40)	32 (.55)	…
p16-Leiden carriers with melanoma (n = 32)	7 (.22)	15 (.47)	10 (.31)	…
General population (n = 385)[b]	25 (.07)	155 (.40)	181 (.47)	24 (.06)

[a] Skin-type data were avaliable for 90 of the 101 p16-Leiden carriers.
[b] Skin-type assessment is from the study by Bastiaens et al. (2001).

loci determine coat color in mice (Jackson et al. 1994; Jackson 1997), variation in hair and skin color in humans has so far been associated with polymorphisms at only 1 locus, MC1R (Valverde et al. 1995), although others are clearly involved, as is shown by sib pairs identical by descent for both parental MC1R alleles having quite different pigmentation (Box et al. 1997). In several studies, mutation screening in the general population has shown that MC1R variants are common among all skin types, although some are significantly associated with fair skin—for example, R151C, and, to a lesser extent, R160W (Smith et al. 1998; Flanagan et al. 2000; Healy et al. 2000; Palmer et al. 2000). Initial studies of the relationship between MC1R gene variants and melanoma risk were not conclusive, possibly because of small sample sizes (Valverde et al. 1996; Healy et al. 1999); however, several more-recent studies (Healy et al. 2000; Palmer et al. 2000; Box et al. 2001 [in this issue]; Kennedy et al., in press) have shown a relationship between the MC1R genotype and both familial and sporadic melanoma risk. In an Australian population-based sample consisting of 460 individuals with familial and sporadic melanoma and 399 control individuals, a strong relationship was observed between MC1R variants and skin type (Healy et al. 2000; Palmer et al. 2000). Furthermore, MC1R variants were present in 72% of melanoma cases and in 56% of the control individuals. The variant alleles R151C, R160W, and D294H resulted in doubled melanoma risk for each additional allele carried; this was also observed in the study by Box et al. (2001 [in this issue]) on familial melanoma. In the study by Palmer et al. (Healy et al. 2000; Palmer et al. 2000) the association between melanoma and MC1R variants persisted among individuals reporting a medium or olive/dark complexion. Similar results were obtained in a study of 123 patients with sporadic melanoma and 385 controls in The Netherlands (Kennedy et al., in press). Compound heterozygote and homozygote carriers of the V60L, V92M, R142H, R151C, R160W, R163Q, and H260P alleles had ORs of ~4 to develop melanoma, whereas heterozygotes for these variants had half the risk. Again, the predisposition turned out to be largely independent of skin type. The effect of MC1R variant alleles on both sporadic melanoma and familial melanoma therefore seems to be partly mediated via determination of pigmentation phenotype, and the MC1R alleles involved seemingly negate the protection normally afforded by a darker skin color. This is in agreement with the findings presented here. Although the sample size in our study is modest, the effects of various tentative melanoma risk factors can be evaluated more easily, since they are measured in a sample of individuals who all carry the same p16 mutation. We found an increased frequency of MC1R variants—in particular, of variant R151C—in patients with melanoma. Furthermore, we were able to show that within families the R151C allele was transmitted from heterozygous parents to >50% of melanoma-positive offspring and to <50% of melanoma-free offspring. This transmission distortion indicates that the increased frequency of R151C in melanoma patients is not simply a consequence of the relatively close family relationships that exist between some of the individuals in our sample. Although it appears that some of the increased risk in R151C-positive p16-Leiden carriers can be attributed to the fair skin type that is associated with this variant, our stratified statistical analysis supports the notion that R151C also contributes an increased risk that is independent of its effect on skin type.

The best-described signal transduction pathway for the G-protein–coupled receptor MC1R results in elevated levels of intracellular cAMP, which lead to an increased expression of tyrosinase and a switch from the synthesis of pheomelanin to that of eumelanin (Robbins et al. 1993). MC1R regulates the cAMP levels in the cell, via adenylate cyclase. The R151C amino acid change is present in the second cytoplasmatic loop of the receptor. Limited functional studies have suggested that R151C creates a change in MC1R function, affecting cAMP signaling (Frandberg et al. 1998; Rees et al. 1999; Schioth et al. 1999). When expressed in heterologous cells, the receptor bearing the R151C amino acid change showed binding affinity to α-MSH comparable to that of wild-type MC1R, although the variant was unable to stimulate cAMP production as strongly as the wild-type receptor. For us to better understand the relevance of MC1R mutations in mela-

noma risk, future studies will have to elucidate the functional properties of the variants in the target cell itself, the melanocyte. Although molecular details of MC1R signaling are not fully understood, the preliminary new insights in MC1R signal transduction (Coughlin 1994; Simonds 1999; Smalley and Eisen 2000) assume that the receptor is involved not only in pigment production but also in additional signaling pathways which evoke different biological responses.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for MC1R [accession number X65634])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CDKN2A [MIM 600160], CDK4 [MIM 123829], CDK6 [MIM 603368], and MC1R [MIM 155555])

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