

Melanocortin-1 Receptor (*MC1R*) Gene Variants and Dysplastic Nevi Modify Penetrance of *CDKN2A* Mutations in French Melanoma-Prone Pedigrees

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Abstract

Germline mutations in *CDKN2A* gene predispose to melanoma with high but incomplete penetrance. Penetrance of *CDKN2A* gene was found to be significantly influenced by host factors (nevus phenotypes and sunburn) on one hand and by variants of *MC1R* gene (RHC variants consistently associated with red hair and fair skin) on the other hand. Our goal was to examine the joint effects of *MC1R* variants and other potential risk factors [total nevi, dysplastic nevi, pigmentary traits (skin, hair and eye color), skin reactions to sunlight, and degree of sun exposure] on *CDKN2A* penetrance. Clinical, genetic, and covariate data were recorded in 20 French melanoma-prone families with cosegregating *CDKN2A* mutations. Analysis of the cotransmission of melanoma and *CDKN2A*

mutations was conducted by likelihood-based methods using the regressive logistic models, which can account for a variation of disease risk with age and can include the aforementioned risk factors as covariates. RHC variants, considered either alone or in the presence of pigmentation and nevus phenotypes, were found to increase significantly *CDKN2A* penetrance. Multivariate analysis, using a stepwise selection procedure, showed significant effects of two factors on melanoma risk in *CDKN2A* mutations carriers: RHC variants [odds ratio of hazard function (OR), 2.21; $P = 0.03$] and dysplastic nevi (OR, 2.93; $P < 0.01$). Such results may have important consequences to improve the prediction of melanoma risk in families. (Cancer Epidemiol Biomarkers Prev 2005;14(10):2384–90)

Introduction

Cutaneous malignant melanoma (CMM) is a complex disease resulting from genetic and other risk factors. Approximately 10% of malignant melanomas are observed in individuals with a family history. Epidemiologic studies have shown that exposure to sunlight is the major environmental risk factor associated with melanoma, whereas high numbers of melanocytic nevi, both clinically banal and atypical (dysplastic), hair color, eye color, skin color, extent of freckling, and skin reactions to sun exposure are the major host factors (see ref. 1, for a review).

Two melanoma high-risk genes have been identified in melanoma-prone families: the cyclin-dependent kinase inhibitor 2A (*CDKN2A*; refs. 2, 3) that encodes two distinct proteins translated in alternative reading frames, p16^{INK4A} and p14^{ARF}, and the cyclin-dependent kinase 4 (*CDK4*; ref. 4). The p16^{INK4A} and CDK4 proteins are implicated in the same cell cycle pathway, pRb-regulated G₁-S phase transition (5, 6), whereas the p14^{ARF} protein acts by the p53 pathway to induce cell cycle or apoptosis (7). Most germline *CDKN2A* mutations affect the p16^{INK4A} protein and such mutations have been observed in ~20% (range, ≥5% to <50%) of tested

melanoma-prone families from Australia, Europe, and North America (8, 9). *CDK4* acts as an oncogene and germline mutations of this gene have been found in only three melanoma-prone families worldwide (4, 8), whereas both *CDK4* and *CDKN2A* mutations have been found to segregate in one Australian kindred (10).

Besides these two high-penetrance genes, common genetic variants with a more modest effect on melanoma risk have been reported such as those encoding proteins involved in pigmentation, DNA repair, cell growth, and differentiation or detoxification of metabolites (see ref. 11, for a review). One of the most studied is the melanocortin-1 receptor gene (*MC1R*), which has been shown to play a crucial role in human pigmentation (12). Moreover, some variants of this gene were found to increase melanoma risk (13, 14). *MC1R* is a seven-pass transmembrane G-protein-coupled receptor for α -melanocyte-stimulating hormone (α -MSH) and is expressed on many cell types, including melanocytes (15). Binding of α -MSH to *MC1R* normally activates adenylylase and increases intracellular cyclic AMP production, which leads to a switch in melanin production from red/yellow pheomelanin pigments to brown/black eumelanins (16). Three variants, R151C, R160W, and D294H (also called RHC variants), were found to be consistently associated with fair skin and red hair (see ref. 16, for a review). RHC and other variants also increased risk for ephelides or solar lentigines (17). Case-control studies in different populations reported that RHC and other variants were associated with melanoma risk in individuals with dark skin (13) or independently of skin type (14) and, more recently, independently of UV exposure and clinical risk factors (skin, hair and eye color, solar lentigines, and nevus count; ref. 18). This suggests that *MC1R* gene may play a role in melanoma pathogenesis not only through its effect on melanin synthesis but also through a more direct effect on melanocytic cellular transformation.

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Although *CDKN2A* mutations confer a high risk of melanoma, penetrance of this gene is incomplete. Examination of the penetrance of *CDKN2A* mutations in 80 multiple-case families from Europe, Australia, and the United States, as part of the Melanoma Genetics Consortium, has indicated a variation of this penetrance according to geographic location (19). By age 80 years, *CDKN2A* mutation penetrance reached 58% in Europe, 76% in the United States, and 91% in Australia. A few host factors have been shown to enhance significantly *CDKN2A* penetrance in families with cosegregating *CDKN2A* mutations: total nevi and dysplastic nevi in 13 North American pedigrees (20) and dysplastic nevi and sunburn in 20 French pedigrees (21). Moreover, *MC1R* variants were found to increase penetrance of mutations in *CDKN2A* (22, 23). This increase was mainly due to RHC variants in 15 Australian pedigrees (22) and to R151C variant in six Dutch families (23). However, these two latter studies did not take into account the effects of other melanoma risk factors, such as nevus phenotypes, pigmentary traits, or sun-related covariates, except for skin color in the Dutch study (23).

The goal of the present study was to examine the joint effects of *MC1R* variants, nevus phenotypes, pigmentary traits, history of sun exposure, and skin reactions to sunlight on *CDKN2A* penetrance using a different analytic approach from the ones previously used (22, 23). Analysis of the cotransmission of CMM and *CDKN2A* mutations in 20 French melanoma-prone families was conducted by a likelihood-based method using regressive logistic models. These models can include the effect of a major gene (known here as being *CDKN2A*) and the aforementioned potential risk factors as covariates to explain the distribution of melanoma and nonmelanoma cases in the whole family structures and can account for a variation of melanoma with age by incorporating survival analysis techniques (24-26).

Patients and Methods

Ascertainment of Families and Data Collection. The 20 melanoma-prone families available for the present study were identified as part of the French Familial Melanoma Project (as described in details in refs. 21, 27). In brief, a sample of 100 families with at least two melanoma cases has been recruited since 1986 from the Department of Dermatology at the Institut Gustave Roussy and other French hospitals forming the French Familial Melanoma Study Group. Eligible probands were defined as White subjects living in France for >10 years, who had a newly diagnosed and histologically confirmed melanoma. From the total sample of 100 families, 53 families were eligible for *CDKN2A* mutation testing, because they included at least two histologically confirmed melanoma cases with DNA available among first- or second-degree relatives. Genotyping for *CDKN2A* germline mutations has led to identification of 20 melanoma pedigrees who contained a total of 14 different *CDKN2A* mutations: L16P, 19ins6, G23N, R24P, M53I, S56I, V59G, L62P, del 67-71, A68L, N71K, G101W, FS102-119X, and V126D (21).

Family data were collected by interviewing the probands on their first-degree, second-degree, and third-degree relatives and included demographic characteristics (gender, date of birth, and if deceased, age at death and cause of death) and the occurrence of melanoma and any other cancer, along with the age at diagnosis. Confirmation of melanoma diagnosis was sought through medical records, review of pathologic material, and/or pathology reports. Only histologically confirmed melanomas were considered as affected in the analyses. Physical examination was conducted by a trained dermatologist in all probands and 70% of probands' relatives. A questionnaire, recording data on various risk factors, was completed by the probands and relatives seen in the hospital and was distributed by probands to all other relatives. The

data recorded on the questionnaire included skin color (pale or dark), eye color (pale or dark), hair color (red, blond, light brown, dark brown, or dark), number of nevi (<10, 10-50, >50 nevi), presence of atypical/dysplastic nevi (moles ≥ 5 mm in diameter with irregular margins and variegated color, only for those examined by a dermatologist), degree of exposure to sunlight (low, medium, or high) during holidays, leisure time and work time, artificial UV exposure (yes or no), long stay (>1 year) in a sunny country, skin reactions to sunlight evaluated by the ability to tan (low, medium, or high), and propensity to sunburn (low, medium, or high).

Written informed consent was obtained before participation under an Institutional Review Board-approved protocol.

Genotyping of *MC1R* Variants. Genomic DNA was extracted from peripheral blood lymphocytes using the QiaAmp DNA blood midi kit (Qiagen, Chatsworth, CA). A specific amplification of *MC1R*-coding sequence was done with the following primers: forward, NT-F 5'-GCAGCACCAT-GAACTAAGCA-3' and reverse, NT-R 5'-CAGGGTCACA-CAGGAACCA-3' (MWG, Ebersterg, Germany) as described by Kanetsky et al. (28). This primer set is located in the noncoding region enclosing the *MC1R* gene and resulting in a 1,125-pb PCR fragment. The PCR reactions were done in a final volume of 50 μ L with 50 ng of genomic DNA, 200 μ mol/L of each deoxynucleotide triphosphate (Pharmacia LKB Biotechnology, Orsay, France), 0.5 μ mol/L of each primer, 1.25 IU Taq DNA polymerase Hot Start (Qiagen), and 1 \times PCR hot start buffer (Qiagen). The reaction of PCR was initially denatured for 10 minutes at 94°C followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C and a final extension step at 72°C for 10 minutes. Five microliters of PCR products were purified with ExoSAP-IT method (U.S. Biochemical, Cleveland, OH).

To identify *MC1R* variants, PCR products were sequenced with the same primers used for PCR and two additional internal primer sets. The sequence of the internal primers were TM-F, 5'-AACCTGCACTCACCCATGTA-3'; TM-R, 5'-TTTAAGGCCAAAGCCCTGGT-3'; CT-F, 5'-TCGTCTTCAG-CACGCTCTTC-3'; and 5'-CAGGGTCACACAGGAACCA-3'. The sequencing of *MC1R* was done with 2.5 μ L of DNA template of PCR, 4 μ L of Big Dye (Perkin-Elmer, Applied Biosystem, Foster City, CA), and 1.5 pmol of each primer. PCR products were purified by solid-phase extraction through sephacryl G50 and subsequently analyzed using an ABI 377 sequencer (Perkin-Elmer, Applied Biosystem).

Genotyping of *MC1R* variants was conducted in all members of 20 melanoma families with DNA available and in a sample of 172 controls. Controls, free of melanoma, belonged to breast and ovarian cancer families followed at Institut Gustave Roussy.

Statistical Analysis. Comparison of *MC1R* allele frequencies in controls and members of the melanoma-prone families was assessed by either a Fisher's exact test or a Pearson's χ^2 test, depending on the sample size.

Effects of *MC1R* variants and other potentially risk factors (e.g., nevus phenotypes, pigmentary traits, history of sun exposure, and skin reactions to sunlight) on *CDKN2A* penetrance were estimated using combined segregation-linkage analysis. This analysis of the cotransmission of melanoma and *CDKN2A* mutations in the 20 melanoma-prone families was based on the regressive logistic models (24) extended to take into account a variable age at diagnosis of disease (26) and linked marker loci (25). The probability of observing a family with a particular configuration of affected and unaffected individuals (termed the "likelihood") is written as the product of the probability of the vector of genotypes at the disease-causing locus (*CDKN2A*) multiplied by the penetrance function summed over individuals with an unknown *CDKN2A* genotype. The probability of unobserved

genotypes is expressed in terms of the frequency of *CDKN2A* mutations in the general population for those individuals who have no ancestors in the pedigrees (founders of the pedigrees and spouses) and in terms of Mendelian probabilities for those with ancestors in the pedigrees. The frequency of *CDKN2A* mutations in the population was assumed to be 0.0001, as in previous analyses (19, 21).

The penetrance function (probability of disease phenotype, Y ; given the vector of genotypes, g and covariates, X) over n individuals in a family is decomposed in a product of penetrance functions for each individual i :

$$P(Y|g, X) = \prod_{i=1}^n P(Y_i|g_i, X_i)$$

where g_i is the i th person's genotype at *CDKN2A* locus and X_i is the vector of covariates for i . Survival analysis concepts were introduced to take into account a censored age at diagnosis of melanoma (26). Because there was no subject affected with melanoma under 15 years of age in the melanoma-prone families, the period of follow-up was taken from 15 years of age to age at diagnosis for affected individuals, age at examination for unaffected subjects (or affected subjects with unknown age at diagnosis), or age at death for deceased subjects. The penetrance function is then derived from the hazard function $\lambda(k)$, which is the probability of being affected in the k th interval given unaffected before. The hazard function $\lambda_i(k)$, for the i th individual in the k th interval, is a logistic function: $\lambda_i(k) = \exp\{\theta_i(k)\}/(1 + \exp\{\theta_i(k)\})$, where $\theta_i(k)$, the logit of the hazard function, is:

$$\theta_i(k) = \alpha_{g_i} + \beta_{g_i}X_i + u_{g_i}(k)$$

where α_g is the genotype-specific baseline parameter, β_g is the row vector of genotype-specific regression coefficients for covariates X , and $u_g(k)$ is a function of k that represents the variation of the logit of the hazard function with time and can be genotype dependent. The hazard function can be assumed to be constant over time [$u_g(k) = 0$] or varying with time using different parametric functions of k . We found that the function $u_g(k) = \delta_g \times \ln(k)$ fit better the data than a polynomial function of k and was then used in all analyses.

All covariates were dichotomized with the baseline and at-risk categories being defined as follows: hair color (dark or dark brown versus light brown, blond, or red), skin color (dark versus pale), propensity to sunburn (low or medium versus high), ability to tan (high or medium versus low), sun exposure (low or medium versus high), total nevi (≤ 50 versus > 50 nevi), and dysplastic nevi (absent versus present). For each *MC1R* variant, the baseline category included *MC1R* consensus homozygotes, whereas the at-risk category included subjects with one or two variant alleles. Only dominant effects were considered, because the frequency of homozygote subjects for each variant was $< 2\%$. The present analysis considered individually each *MC1R* variant with frequency $\geq 5\%$ in the family sample as well as the pool of RHC variants (R151C, R160W, and D294H). To deal with missing covariates, we used the complete case method, which implies coding subjects with missing covariates as unknown for disease status (29).

Likelihood computation of a family sample usually involves correction for ascertainment of the families when they are not drawn at random from the population. Each of the melanoma-prone families was selected, because it included at least two melanoma cases with DNA available for *CDKN2A* genotyping and at least one of them was carrier of *CDKN2A* mutations. This mode of ascertainment was too complex to be corrected analytically. In such situation, one strategy is to calculate the likelihood of the observed phenotypes (melanoma status, age, and covariates) and genotypes (*CDKN2A* mutation status),

conditional on the observed phenotypes in all family members. This conditional likelihood represents an assumption-free method of ascertainment and leads to unbiased parameter estimates when the ascertainment is based on phenotype (30). However, this approach may cause a substantial loss of the information and may hamper the estimation of the parameters of the regressive model, especially when covariates are included in the model. An alternative strategy is to calculate the joint likelihood of the observed phenotypes (melanoma status, age, and covariates) and genotypes (*CDKN2A* mutation status) without correcting for ascertainment but assuming that *CDKN2A* is the disease-causing gene. Although use of joint likelihood method may overestimate the parameters of the disease gene, tests of the effects of covariates are likely to be valid when the disease-causing gene is known and most subjects have been genotyped. Indeed, analysis of simulated data under different sampling schemes has shown that the joint likelihood approach was robust to noncorrection for ascertainment and detected the generated model in terms of genetic and risk factor effects as well as gene \times risk factors interactions when the disease-causing gene was known (31). Provided that conditional likelihood led to numerical problems (unstable parameters estimates), we used the joint likelihood strategy, restricting our analyses to (a) *CDKN2A* mutation carriers and (b) all individuals genotyped for *CDKN2A* (all nongenotyped subjects being coded as unknown for melanoma status and thus bringing no contribution to the likelihood). Indeed, restricting the analyses to genotyped subjects without inferring *CDKN2A* mutation status in untyped subjects can minimize possible bias on parameter estimates.

Parameter estimation and tests of models were carried out using maximum likelihood methods, as implemented in the computer program REGRESS (32). Nested models were compared by use of likelihood ratio tests. Effects of the covariates (*MC1R* variants and other potential risk factors) on the penetrance function were tested by comparing a model without the effect of the tested covariate (regression coefficient β for this covariate set to zero) with a model including this covariate (regression coefficient β for this covariate estimated), whereas taking into account the effect of *CDKN2A* (and eventually other covariates already included in the regressive model). The odds ratio (OR) of hazard function associated with each covariate [$OR = \exp(\beta)$] and 95% confidence interval (95% CI) were computed from the corresponding estimate of the β parameter. Effect of each *MC1R* variant (or pooled RHC variants) on *CDKN2A* penetrance was first tested in univariate analysis. For each variant showing a significant effect, we tested alternatively whether that effect remained in the presence of each host factor which may be a potential confounder. Finally, effects of *MC1R* variants, host-related phenotypes, and degree of sun exposure were considered jointly in multivariate analysis and their significance was tested using a stepwise procedure.

Results

The 20 French melanoma-prone families (comprising a total of 326 subjects), in which 14 different *CDKN2A* mutations were segregating (21), included 61 melanoma cases (mean age at diagnosis of 39.70 ± 13.02 years), 214 unaffected subjects (mean age at examination of 46.0 ± 20.1 years), and 51 subjects with unknown affection status. *CDKN2A* mutation status was determined in 160 subjects (51% being mutation carriers). *MC1R* genotype was available for 159 family members (31% having one variant and 52% having more than one variant).

The distribution of a total of 153 subjects genotyped for both *CDKN2A* and *MC1R* according to melanoma status is shown in Table 1. Among the 44 melanoma cases, only two subjects were noncarriers of *CDKN2A* mutations; whereas among the

Table 1. Genotyped family members by melanoma affection status

CDKN2A and MC1R genotypes	No. individuals	Affected subjects		Unaffected subjects		Unknown melanoma status*, no. subjects
		No. cases	Mean age at onset \pm SD	No. unaffected	Mean age at exam \pm SD	
153 subjects genotyped for both CDKN2A and MC1R						
CDKN2A, MC1R						
WT, consensus	15	1	40.00	13	40.77 \pm 15.05	1
WT, one variant	21	0	—	20	40.50 \pm 22.07	1
WT, two variants	32	1	28.00	31	40.97 \pm 19.21	0
WT, three variants	5	0	—	5	46.60 \pm 24.91	0
Mut, consensus	10	5	52.60 \pm 12.10	5	48.80 \pm 19.56	0
Mut, one variant	29	8	48.13 \pm 15.75	20	34.10 \pm 20.07	1
Mut, two variants	28	17	46.71 \pm 13.33	11	35.09 \pm 18.12	0
Mut, three variants	13	12	50.08 \pm 13.39	1	23.0	0
13 subjects genotyped for either CDKN2A or MC1R						
CDKN2A, MC1R						
WT, unknown	5	0	—	5	48.20 \pm 15.35	0
Mut, unknown	2	2	46.00 \pm 11.31	0	—	0
Unk, consensus	2	0	—	2	29.00 \pm 4.24	0
Unk, two variants	3	1	44.00	2	64.50 \pm 16.26	0
Unk, three variants	1	0	—	1	59.00	0

Abbreviations: WT, wild type for CDKN2A; Mut, mutation for CDKN2A; unk, unknown genotype for CDKN2A.

*Age at exam is unknown for individuals with unknown melanoma status.

106 unaffected subjects, 37 subjects were carriers of CDKN2A mutations (three subjects had an unknown melanoma status). The frequency of subjects carrying none, one, two, or three MC1R variants was significantly different in affected and unaffected carriers of CDKN2A mutations ($P = 0.001$). Interestingly, in these CDKN2A mutation carriers, there was a higher number of unaffected than affected subjects with one MC1R variant, whereas the inverse trend was observed in subjects with at least two MC1R variants. Moreover, the mean age at diagnosis of melanoma in affected individuals and the mean age at examination in unaffected subjects did not differ according to the number of MC1R variants in both carriers and noncarriers of CDKN2A mutations ($P > 0.40$).

Allele frequencies of the 14 MC1R variants, detected in the present sample, are shown in Table 2. All MC1R variants were in Hardy-Weinberg equilibrium ($P > 0.15$). The allele frequencies were not significantly different between affected and unaffected members of the 20 melanoma-prone families when we considered all genotyped subjects or CDKN2A mutation carriers only ($P > 0.06$). When comparing 159 members of melanoma-prone families (pool of affected and unaffected) and 172 controls, we found a significantly higher frequency of

R151C, D294H, and T314T variants in family members than in controls ($P < 0.05$). Preliminary analyses (results not shown) examining the frequency distribution of each MC1R variant according to skin, hair and eye color, skin reactions to sunlight, and nevus phenotypes showed a significantly higher frequency of RHC variants in subjects with red-blond than dark-brown hair color ($P < 0.04$), whereas the inverse trend was observed for T314T and R163Q variants ($P < 0.03$). The D294H variant alone was significantly more frequent in subjects with pale than dark skin color ($P = 0.02$) and the R163Q variant was less frequent in individuals with high than low or medium propensity to sunburn ($P = 0.05$). These analyses were also carried out in unaffected subjects, because host-related factors and some MC1R variants have been reported to be risk factors for melanoma by case-control studies. The R151C variant or pooled RHC variants were found to be significantly associated with hair color ($P < 0.01$), skin color ($P < 0.05$), and tanning ability ($P < 0.03$).

Combined segregation-linkage analyses of cutaneous malignant melanoma and CDKN2A mutations status were first conducted in the 20 French melanoma-prone families by considering MC1R variants alone (Table 3). Two of the seven

Table 2. Frequencies of MC1R variants in members of 20 melanoma-prone pedigrees and controls

Variants	All family members ($n = 159$)	Controls ($n = 172$)	Carriers and noncarriers of CDKN2A mutations		Carriers of CDKN2A mutations	
			Affected family members* ($n = 45$)	Unaffected family members* ($n = 111$)	Affected family members* ($n = 42$)	Unaffected family members* ($n = 37$)
V60L	16.0	14.2	20.0	14.4	20.2	14.9
S83P	0.6	0.6	1.1	0.5	0.0	0.0
S83L	0.6	0.0	1.1	0.5	0.0	1.4
D84E	0.9	0.6	2.2	0.5	2.4	0.0
V92M	11.3	8.4	14.4	10.4	15.5	8.1
A111V	0.9	0.0	0.0	1.4	0.0	0.0
R151C [†]	11.0	4.9	11.1	11.3	11.9	8.1
I155T	1.3	0.3	2.2	0.9	2.4	1.4
R160W	6.6	3.5	7.8	6.3	7.1	9.5
R163Q	4.4	4.4	6.7	3.6	7.1	2.7
F196L	0.6	0.3	0.0	0.9	0.0	1.4
I264I	0.6	0.6	0.0	0.9	0.0	0.0
D294H [†]	5.0	1.2	8.9	3.6	9.5	2.7
T314T [†]	16.7	9.9	16.7	16.7	17.9	14.9

*There was no significant difference for each MC1R variant frequency between affected and unaffected members of the 20 melanoma-prone families regardless of CDKN2A genotype.

[†]Frequencies of R151C, D294H, and T314T were significantly different in 172 controls and in all members of the 20 families ($P < 0.044$). No difference was found for all other comparisons.

Table 3. Combined segregation-linkage analyses of melanoma and CDKN2A mutation status incorporating MC1R variants as covariates

Model	All genotyped subjects*					CDKN2A mutation carriers					
	α_{aa}^\dagger	α_{aA}^\dagger	δ^\ddagger	OR [‡] _{MC1R variant} (95% CI)	Test statistic (χ^2) [§]	P	α_{aa}^\dagger	δ^\ddagger	OR [‡] _{MC1R variant} (95% CI)	Test statistic (χ^2) [§]	P
R160W	-10.27	-6.45	0.93	2.06 (0.85-4.96)	2.20	0.14	-6.57	0.96	2.13 (0.82-5.49)	2.03	0.15
D294H	-10.53	-6.58	0.95	3.23 (1.36-7.67)	5.69	0.02	-6.79	1.01	3.58 (1.49-8.60)	6.49	0.01
R163Q	-10.44	-6.46	0.92	2.59 (1.00-6.45)	3.45	0.06	-6.67	0.98	2.93 (1.16-7.35)	4.21	0.04
R151C	-10.05	-6.21	0.86	1.27 (0.58-2.76)	0.31	0.57	-6.36	0.91	1.34 (0.61-2.91)	0.48	0.49
V60L	-10.00	-6.20	0.86	1.14 (0.63-2.03)	0.18	0.68	-6.32	0.89	1.09 (0.59-1.99)	0.07	0.79
V92M	-10.24	-6.42	0.90	1.59 (0.78-3.20)	1.58	0.21	-6.61	0.95	1.71 (0.83-3.50)	2.03	0.15
T314T	-9.92	-6.10	0.85	0.89 (0.48-1.63)	0.15	0.70	-6.25	0.89	0.94 (0.50-1.73)	0.05	0.82
RHC variants	-10.70	-6.72	0.96	2.15 (1.07-4.28)	4.71	0.03	-6.90	1.00	2.30 (1.15-4.59)	5.26	0.02

*Genotyped subjects include carriers and noncarriers of CDKN2A mutations.

[†] α_{aa} and α_{aA} are genotype-specific baseline risks in noncarriers and carriers of CDKN2A mutations, respectively; δ is the regression coefficient specifying the variation of the hazard function with time. In the subsample of CDKN2A mutations carriers, only α_{aA} is estimated.

[‡]The OR of hazard function associated with each covariate [OR = exp(β)] and 95% CI were computed from the corresponding estimate of the regression parameter of that covariate (β).

[§]Tests are based on likelihood ratios and are two sided. Likelihood ratio test compares a model where the effect of the tested factor (MC1R variant) is absent versus a model where it is present and follows a χ^2 with 1 degree of freedom.

common MC1R variants (frequency $\geq 5\%$ in the family sample) significantly increased CMM risk in carriers of CDKN2A mutations: D294H (OR of hazard function, 3.58; 95% CI, 1.49-8.60; $P = 0.01$) and R163Q (OR, 2.93; 95% CI, 1.16-7.35; $P = 0.04$). Pooled RHC variants increased also significantly CDKN2A penetrance (OR, 2.30; 95% CI, 1.15-4.59; $P = 0.02$). When repeating analyses by considering all genotyped subjects (carriers and noncarriers of CDKN2A mutations), similar results were obtained, except for the R163Q variant, which was only borderline significant ($P = 0.06$).

We then tested for the effect of RHC variants in presence of each host factor significantly associated with melanoma risk (as found in preliminary analyses) in CDKN2A mutations carriers. As seen in Table 4, having at least one RHC variant remained significant in presence of either hair color ($P = 0.05$), sunburns ($P = 0.02$), high number of nevi ($P = 0.03$), or dysplastic nevi ($P = 0.03$). Moreover, each host factor showed an additional significant effect ($P < 0.04$), except for hair color ($P = 0.08$). Similar results were observed in the whole sample of all genotyped subjects (data not shown). We did not consider in these analyses D294H or R163Q, because the number of subjects carrying these variants and having known covariates was too small.

Finally, multivariate analysis (Table 5), examining together RHC variants, host factors, and sun exposure, showed that only RHC variants and dysplastic nevi increased significantly CDKN2A penetrance ($P = 0.03$ for RHC variants and $P < 0.01$ for dysplastic nevi). The next factor to enter into the model was high number of nevi, which was borderline significant ($P = 0.053$). Analyses of the sample of carriers and non carriers of CDKN2A mutations led to similar effects of RHC variants (OR, 1.99; 95% CI, 1.01-3.91; $P = 0.05$) and dysplastic nevi (OR, 3.14; 95% CI, 1.59-6.20; $P < 0.01$) on melanoma risk.

Discussion

The MC1R gene was reported to be a low-penetrance melanoma predisposing gene as well as a modifier of CDKN2A penetrance especially with respect to RHC variants (22, 23). The present study extends these previous results by showing the significant joint effects of RHC variants and dysplastic nevi on the penetrance of CDKN2A mutations in 20 French melanoma-prone families.

Fourteen MC1R variants were detected in our melanoma-prone families. Seven of these variants (V60L, V92M, R151C, R160W, D294H, R163Q, and T314T) have frequencies higher than 5%. When comparing MC1R allele frequencies observed in Dutch, Australian, and French melanoma-prone families,

V60L, V92M, and D294H frequencies were higher in our sample, whereas R151C and R160W frequencies were higher in Australian and Dutch kindreds, respectively (22, 23). The T314T variant has only been detected in French families. Similar results were observed when comparing MC1R allele frequencies between control samples of these three populations. Note that in all three studies, frequency of MC1R variants was higher in the melanoma-prone families than in controls.

Only two (D294H and R163Q variants) of the seven common MC1R variants, considered individually, increased significantly CDKN2A penetrance in our sample. D294H is a RHC variant, being found associated with red-blond hair and fair skin in our sample, and reported to increase melanoma risk by case-controls studies (13, 14, 18). R163Q, weakly associated with fair skin (14, 33) and inversely associated with red-blond hair and sunburn in our sample, was reported by a case-control study to weakly increase melanoma risk (14). Note that the R151C variant, associated with melanoma in case-control studies (13, 14, 18) and in Dutch families segregating CDKN2A mutations (23), did not modify CDKN2A penetrance in our

Table 4. Effect of RHC variants in presence of host-related phenotypes using combined segregation-linkage analyses in CDKN2A mutation carriers

Model	OR* _{covariate} (95% CI)	Test statistic (χ^2) [†]	P
RHC + hair color			
1. Effect of RHC	2.08 (1.03-4.22)	3.94	0.05
2. Effect of red/blond hair	1.96 (0.96-3.97)	3.15	0.08
RHC + sunburns			
1. Effect of RHC	2.33 (1.16-4.67)	5.28	0.02
2. Effect of high propensity to sunburn	3.08 (0.91-10.46)	4.17	0.04
RHC + dysplastic nevi			
1. Effect of RHC	2.21 (1.11-4.44)	4.73	0.03
2. Effect of dysplastic nevi	2.93 (1.46-5.88)	8.53	<0.01
RHC + number of nevi			
1. Effect of RHC	2.26 (1.13-4.52)	5.02	0.03
2. Effect of high number of nevi	2.47 (1.25-4.90)	6.57	0.01

*The OR of hazard function associated with each covariate [OR = exp(β)] and 95% CI were computed from the corresponding estimate of the regression parameter of that covariate (β).

[†]Tests are based on likelihood ratios and are two sided. Test of RHC variants was conducted by comparing a model with a given host factor (and no effect of RHC) to a model including both RHC and the host factor. Test of a given host factor was conducted by comparing a model with RHC variants (and no effect of the host factor) to a model including both RHC and the host factor. These likelihood ratio tests follow a χ^2 with 1 degree of freedom.

Table 5. Outcomes of combined segregation-linkage analyses selecting the effects of RHC variants, host factors and sun exposure by a stepwise procedure in *CDKN2A* mutation carriers

Model	α_{AA}^*	δ^*	OR [†] _{dysplastic nevi} (95% CI)	OR [†] _{RHC variants} (CI)	OR [†] _{high number of nevi} (CI)	Test statistic (χ^2) [‡]	P
1. Dysplastic nevi	-7.17	1.07	3.02 (1.51-6.05)	—	—	9.06	<0.01
2. RHC variants added to model 1	-7.79	1.19	2.93 (1.46-5.88)	2.21 (1.11-4.44)	—	4.73	0.03
3. High number of nevi added to model 2	-8.26	1.27	2.46 (1.19-5.05)	2.19 (1.10-4.39)	2.03 (0.99-4.12)	3.74	0.053

* α_{AA} is the genotype-specific baseline risk in carriers of *CDKN2A* mutations; δ is the regression coefficient specifying the variation of the hazard function with time.
[†]The OR of hazard function associated with each covariate [OR = exp(β)] and 95% CI were computed from the corresponding estimate of the regression parameter of that covariate (β).

[‡]Tests are based on likelihood ratios and are two sided (the number of degrees of freedom is 1). Likelihood ratio test comparing the absence of a given risk factor versus a model including that risk factor was done at each step of the stepwise procedure.

study. The significant effect of pooled RHC variants in *CDKN2A* mutation carriers of our sample was similar to that found in the Australian family-based study (22): ORs associated with RHC variants being 2.02 and 2.30 in Australian and French families, respectively.

The increase in melanoma risk according to the number of *MC1R* variants observed in Dutch p16-Leiden carriers (23) was also found in our sample (results not shown). Indeed, in *CDKN2A* mutation carriers, the ORs associated with the presence of at least two *MC1R* variants was 3.58 (95% CI, 1.20-10.67), whereas the OR associated with the presence of only one variant was 1.57 (95% CI, 0.45-5.46). Case-control studies also reported a higher melanoma risk in carriers of two variants rather than just one (13, 14, 18). However, carrying two *MC1R* variants did not add further risk than carrying one variant in the Australian *CDKN2A* penetrance analysis (22).

No effect of *MC1R* genotype on the age at onset for melanoma was observed in the present sample, in agreement with the Dutch study (23) but differing from the Australian study, where a significant decrease in mean age at onset was observed in carriers of both *CDKN2A* mutation and *MC1R* variant when compared with carriers of *CDKN2A* mutation alone (22). These different results may be partly due to a difference in age distribution between samples.

Although case-control studies have indicated that *MC1R* variants increased melanoma risk independently of pigmentary traits (13, 14, 18), this issue was scarcely investigated in Australian and Dutch families (22, 23). First, the effect of RHC variants on *CDKN2A* penetrance remained significant in presence of host-related phenotypes (mainly hair color, sunburns, dysplastic nevi, and high number of nevi). Moreover, each of these latter factors, except hair color, had a significant effect in presence of RHC variants. When examining jointly all potential risk factors, only RHC variants and dysplastic nevi increased significantly *CDKN2A* penetrance. ORs associated with these two factors were almost unchanged whether the model included either one or both of them, indicating independent effects of RHC variants and dysplastic nevi on *CDKN2A* penetrance. Analyses done in the sample of carriers and noncarriers of *CDKN2A* mutations led to similar conclusions. Interestingly, RHC variants had a slightly higher effect on melanoma risk in the subsample of *CDKN2A* mutation carriers than in the whole sample of genotyped subjects, whereas the inverse trend was observed for dysplastic nevi.

Mechanisms underlying the increased risk of CMM in subjects carrying *MC1R* variants are not well understood. Functional analyses of different *MC1R* variants have shown that V60L, R160W, D294H, and R151C variants are unable to stimulate cyclic AMP production as strongly as the wild-type allele, in response to α -MSH stimulation and alter melanin synthesis (34-36). The effect of V92M variant is not well established with discordant results (37, 38). Although no functional analysis was done for R163Q, it was found at high frequency in Asian population (39, 40), suggesting an

association with pheomelanin-rich skin. More recently, an evolutionary-based approach, conducted to predict *MC1R* variants with most likely functional significance, detected R160W and D294H among high-risk variants, whereas V60L, V92M, and R163Q were predicted to be low-risk variants (28).

Because *MC1R* is known to be involved in the UV sensitivity pathway, *MC1R* variants may have an effect on human melanocytes response to α -MSH and UV radiation. Indeed, melanocyte cells expressing *MC1R* variants produce increased levels of pheomelanin leading to limited photoprotection and exhibit increased sensitivity to cytotoxic effects of UV radiation (41).

The modifier effect of *MC1R* variants on *CDKN2A* penetrance might suggest a link between p16^{INK4A} and *MC1R* in response to UV radiation. It has been clearly shown that expression of p16^{INK4A} in human skin cells is increased after UV radiation, suggesting an implication in protective cell cycle response to UV (42); this expression being induced via interactions of α -MSH with *MC1R* (43). The presence of *MC1R* variants may alter the protective p16^{INK4}-mediated cell cycle response to UV and thus increase melanoma risk in carriers of both *CDKN2A* mutations and *MC1R* variants.

MC1R variants might also exert a non-UV-related effect on the propensity of melanocytic cellular transformation. It has been recently shown that human melanoma cells overexpressed *MC1R* compared with normal melanocytes (44), and melanoma cells synthesize and release α -MSH suggesting an autocrine effect of this protein on melanoma cells. It has been shown that α -MSH significantly reduces the proliferation and cellular invasion, through the decrease of fibronectin binding in human melanoma cells expressing the wild-type *MC1R*, whereas the RHC variants have no effect on growth and adhesion to extracellular matrix specifically with fibronectin (45, 46). Thus, *MC1R* variants could alter melanogenesis and induce melanoma development and progression via non-pigmentary mechanisms.

In conclusion, this study shows *CDKN2A* penetrance is modified by *MC1R* gene variants and presence of dysplastic nevi. It emphasizes the complexity of melanoma pathogenesis, which is likely to involve several pathways (cell cycle pathway, UV sensitivity pathway, nevus pathway, proliferation and invasion pathways, and DNA repair pathway). Investigation of other genes such as those involved in pigmentation, DNA repair, and carcinogen metabolism may help in disentangling these complex underlying mechanisms. Confirmation of these findings in other populations seems warranted. Moreover, such studies may have important consequences to improve the prediction of melanoma risk in families.

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References

- Tucker MA, Goldstein AM. Melanoma etiology: where are we? *Oncogene* 2003;22:3042–52.
- Hussussian CJ, Struwing JP, Goldstein AM, et al. Germline p16 mutations in familial melanoma. *Nat Genet* 1994;8:15–21.
- Kamb A, Gruis NA, Weaver-Feldhaus J, et al. A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994;264:436–40.
- Zuo L, Weger J, Yang Q, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat Genet* 1996;12:97–9.
- Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993;366:704–7.
- Serrano M, Gomez-Lahoz E, DePinho RA, Beach D, Bar-Sagi D. Inhibition of ras-induced proliferation and cellular transformation by p16INK4. *Science* 1995;267:249–52.
- Pomerantz J, Schreiber-Agus N, Liegeois NJ, et al. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 1998;92:713–23.
- Soufir N, Avril MF, Chompret A, et al. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. *Hum Mol Genet* 1998;7:209–16.
- Goldstein AM, Struwing JP, Chidambaram A, Fraser MC, Tucker MA. Genotype-phenotype relationships in U.S. melanoma-prone families with CDKN2A and CDK4 mutations. *J Natl Cancer Inst* 2000;92:1006–10.
- Holland EA, Schmid H, Kefford RF, Mann GJ. CDKN2A (P16(INK4a)) and CDK4 mutation analysis in 131 Australian melanoma probands: effect of family history and multiple primary melanomas. *Genes Chromosomes Cancer* 1999;25:339–48.
- Hayward N. Genetics of melanoma predisposition. *Oncogene* 2003;22:3053–62.
- Valverde P, Healy E, Jackson I, Rees JL, Thody AJ. Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nat Genet* 1995;11:328–30.
- Palmer JS, Duffy DL, Box NF, et al. Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? *Am J Hum Genet* 2000;66:176–86.
- Kennedy C, ter Huurne J, Berkhout M, et al. Melanocortin 1 receptor (MC1R) gene variants are associated with an increased risk for cutaneous melanoma which is largely independent of skin type and hair color. *J Invest Dermatol* 2001;117:294–300.
- Chhajlani V, Wikberg JE. Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. *FEBS Lett* 1992;309:417–20.
- Sturm RA, Teasdale RD, Box NF. Human pigmentation genes: identification, structure and consequences of polymorphisms variations. *Gene* 2001;277:49–62.
- Bastiaens M, ter Huurne J, Gruis N, et al. The melanocortin-1-receptor gene is the major freckle gene. *Hum Mol Genet* 2001;10:1701–8.
- Matchard E, Verpillat P, Meziani R, et al. Melanocortin 1 receptor (MC1R) gene variants may increase the risk of melanoma in France independently of clinical risk factors and UV exposure. *J Med Genet* 2004;41:e13.
- Bishop DT, Demenais F, Goldstein AM, et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. *J Natl Cancer Inst* 2002;94:894–903.
- Goldstein AM, Martinez M, Tucker MA, Demenais F. Gene-covariate interaction between dysplastic nevi and the CDKN2A gene in American melanoma-prone families. *Cancer Epidemiol Biomarkers Prev* 2000;9:889–94.
- Chaudru V, Chompret A, Bressac-de Paillerets B, Spatz A, Avril MF, Demenais F. Influence of genes, nevi, and sun-sensitivity on melanoma risk in a family sample unselected by family history and in melanoma-prone families. *J Natl Cancer Inst* 2004;96:785–95.
- Box NF, Duffy DL, Chen W, et al. MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. *Am J Hum Genet* 2001;69:765–73.
- van der Velden PA, Sandkuijl LA, Bergman W, et al. Melanocortin-1 receptor variant Arg¹⁵¹Cys modifies melanoma risk in Dutch families with melanoma. *Am J Hum Genet* 2001;69:774–9.
- Bonney GE. Regressive logistic models for familial disease and other binary traits. *Biometrics* 1986;42:611–25.
- Bonney GE, Lathrop GM, Lalouel JM. Combined linkage and segregation analysis using regressive models. *Am J Hum Genet* 1988;43:29–37.
- Abel L, Bonney GE. A time-dependent logistic hazard function for modeling variable age of onset in analysis of familial diseases. *Genet Epidemiol* 1990;7:391–407.
- Grange F, Chompret A, Guillaud-Bataille M, et al. Comparison between familial and nonfamilial melanoma in France. *Arch Dermatol* 1995;131:1154–9.
- Kanetsky PA, Ge F, Najarian D, et al. Assessment of polymorphic variants in the melanocortin-1 receptor gene with cutaneous pigmentation using an evolutionary approach. *Cancer Epidemiol Biomarkers Prev* 2004;13:808–19.
- Greenland S, Finkle WD. A critical look at methods for handling missing covariates in epidemiologic regression analyses. *Am J Epidemiol* 1995;142:1255–64.
- Hodge SE, Elston RC. Lods, wrods, and mods: the interpretation of lod scores calculated under different models. *Genet Epidemiol* 1994;11:329–42.
- Martinez M, Goldstein AM, O'Connell JR. Comparison of likelihood approaches for combined segregation and linkage analysis of a complex disease and a candidate gene marker under different ascertainment schemes. *Genet Epidemiol* 2001;21 Suppl 1:S760–5.
- Demenais F, Lathrop M. REGRESS: a computer program including the regressive approach into the LINKAGE programs. *Genet Epidemiol* 1994;11:291.
- Sturm RA, Duffy DL, Box NF, et al. Genetic association and cellular function of MC1R variant alleles in human pigmentation. *Ann N Y Acad Sci* 2003;994:348–58.
- Frandsberg PA, Doufexis M, Kapas S, Chhajlani V. Human pigmentation phenotype: a point mutation generates nonfunctional MSH receptor. *Biochem Biophys Res Commun* 1998;245:490–2.
- Schioth HB, Phillips SR, Rudzish R, Birch-Machin MA, Wikberg JE, Rees JL. Loss of function mutations of the human melanocortin 1 receptor are common and are associated with red hair. *Biochem Biophys Res Commun* 1999;260:488–91.
- Healy E, Jordan SA, Budd PS, Suffolk R, Rees JL, Jackson JJ. Functional variation of MC1R alleles from red-haired individuals. *Hum Mol Genet* 2001;10:2397–402.
- Xu X, Thornwall M, Lundin LG, Chhajlani V. Val92Met variant of the melanocyte stimulating hormone receptor gene. *Nat Genet* 1996;14:384.
- Koppula SV, Robbins LS, Lu D, et al. Identification of common polymorphisms in the coding sequence of the human MSH receptor (MC1R) with possible biological effects. *Hum Mutat* 1997;9:30–6.
- Rana BK, Hewett-Emmett D, Jin L, et al. High polymorphism at the human melanocortin 1 receptor locus. *Genetics* 1999;151:1547–57.
- Peng S, Lu XM, Luo HR, Xiang-Yu JG, Zhang YP. Melanocortin-1 receptor gene variants in four Chinese ethnic populations. *Cell Res* 2001;11:81–4.
- Scott MC, Wakamatsu K, Ito S, et al. Human melanocortin 1 receptor variants, receptor function and melanocyte response to UV radiation. *J Cell Sci* 2002;115:2349–55.
- Pavey S, Conroy S, Russell T, Gabrielli B. Ultraviolet radiation induces p16CDKN2A expression in human skin. *Cancer Res* 1999;59:4185–9.
- Pavey S, Gabrielli B. α -melanocyte stimulating hormone potentiates p16/CDKN2A expression in human skin after ultraviolet irradiation. *Cancer Res* 2002;62:875–80.
- Mas JS, Gerritsen I, Hahmann C, Jimenez-Cervantes C, Garcia-Borrón JC. Rate limiting factors in melanocortin 1 receptor signalling through the cAMP pathway. *Pigment Cell Res* 2003;16:540–7.
- Robinson SJ, Healy E. Human melanocortin 1 receptor (MC1R) gene variants alter melanoma cell growth and adhesion to extracellular matrix. *Oncogene* 2002;21:8037–46.
- Eves P, Haycock J, Layton C, et al. Anti-inflammatory and anti-invasive effects of α -melanocyte-stimulating hormone in human melanoma cells. *Br J Cancer* 2003;89:2004–15.