

Ocular melanoma is not associated with *CDKN2A* or *MC1R* variants – a population-based study

C. Vajdic^a, A. Kricger^b, D.L. Duffy^c, J.F. Aitken^d, M. Stark^c, J.A.C. ter Huurne^e, N.G. Martin^c, B.K. Armstrong^f and N.K. Hayward^c

Germline variants in the melanocortin 1 receptor gene (*MC1R*) and the p16 gene (*CDKN2A*) are associated with an increased risk of cutaneous melanoma. The frequency of these germline variants was examined in a population-based, incident series of 62 ocular melanoma cases and ethnicity-matched population controls. In both cases and controls, 59% of individuals carried at least one *MC1R* variant and there were no significant differences in the frequency of any of the five most common variants of *MC1R*. We also found no significant differences between cases and controls in the frequency of any of the four most common variants of *CDKN2A*, and no melanoma case carried a deleterious germline *CDKN2A* mutation. Our findings argue against an important predisposing effect of the *MC1R* and *CDKN2A* genes for ocular melanoma. *Melanoma Res* 13:409–413 © 2003 Lippincott Williams & Wilkins.

Introduction

Melanoma is the most common primary intra-ocular tumour in adults of mainly European origin; the incidence of microscopically verified ocular melanoma in these populations in 1988–1992 ranged up to 1.1 per 100 000 in men and 1.0 per 100 000 in women [1]. In hospital-based studies, few ocular melanoma cases (<1%) reported a family history of ocular melanoma [2]; about 4% reported a family history of cutaneous melanoma [3].

Germline mutations of the breast cancer susceptibility gene *BRCA2* have been reported in 2–3% of ocular melanoma cases [4–6], but no other ocular melanoma susceptibility genes have been identified. The search for candidate susceptibility genes has focused on those known to be associated with cutaneous melanoma, e.g. the cell cycle regulatory genes, *CDKN2A* [7] and *CDK4* [8], and the melanocortin 1 receptor gene (*MC1R*), a pigmentation-related gene [9,10], but no associations of germline mutations in these genes with ocular melanoma have been reported [8,11–15].

No population-based study of the relationship between ocular melanoma and *CDKN2A* and *MC1R* mutations has been reported. As ocular and cutaneous melanomas have been shown to share some phenotypic and environ-

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^aNational Centre for HIV Epidemiology and Clinical Research, Darlinghurst, NSW and The Cancer Council, NSW, Kings Cross, NSW, Australia, ^bNational Breast Cancer Centre, Camperdown, NSW, Australia, ^cCancer and Cell Biology Division, Queensland Institute of Medical Research, 300 Herston Rd, Herston, Qld 4029, Australia, ^dEpidemiology Unit, Queensland Cancer Fund, Spring Hill, Qld, Australia, ^eDepartments of Dermatology and Human Genetics, Leiden University Medical Center, Leiden, The Netherlands, and ^fSchool of Public Health, The University of Sydney, NSW and The Cancer Council NSW, Kings Cross, NSW, Australia.

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Correspondence and requests for reprints to N.K. Hayward, Cancer and Cell Biology Division, Queensland Institute of Medical Research, 300 Herston Rd, Herston, Qld 4029, Australia.
Tel: 61-7-33620306; fax: 61-7-38453508; e-mail: nickH@qimr.edu.au

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mental risk factors in population-based epidemiological studies [16,17], it could be argued that the association between germline *MC1R* and *CDKN2A* variants and ocular melanoma risk ought to be examined in a population-based study. We aimed to do this in a sample of cases from our population-based study of ocular melanoma [16] and controls genotyped in recent studies of cutaneous melanoma [7,9].

Materials and methods

Cases

Patients with ocular melanoma were eligible for this study if they were diagnosed at 50 years of age or younger or had bilateral disease diagnosed at any age. These restrictions were applied to maximize the likelihood of finding germline mutations. A total of 107 eligible cases was ascertained for a population-based case–control study covering all cases of ocular melanoma diagnosed between 1 January 1996 and 31 July 1998 in Australia [16]. Ninety-nine (93%) of these patients were interviewed for the study. Eighty (81%) gave a blood sample, and sufficient DNA for analysis was extracted from 71. The homogeneity of ethnicity in cases and controls (see below) was considered to be important, and analyses for the present study were restricted to 62 cases with reported British or other northern European ethnicity (defined as having three or

more grandparents born in England, Ireland, Scotland, Wales, Isle of Man, UK undefined, Austria, Belgium, Denmark, Finland, France, Germany, Netherlands, Norway, Sweden or Switzerland).

All cases had melanomas originating in the choroid, ciliary body, iris or conjunctiva and were included whether or not the diagnosis had been confirmed histopathologically. The distributions of primary tumour site, morphology, size and stage at diagnosis in these cases were similar to those of other cases in the larger population-based study, except for a relatively smaller proportion of conjunctival melanomas (Table 1). Most (84%) originated in the choroid or ciliary body and 54% were histopathologically confirmed. One case had a first-degree relative with confirmed cutaneous melanoma and three others reported a family history of cutaneous melanoma that was not confirmed. None had a first-degree relative with confirmed ocular melanoma.

Controls

Controls were subjects sampled from the Australian National Health and Medical Research Council Twin Register and members of their families who had been used as controls in studies of cutaneous melanoma and *MC1R* [9] and *CDKN2A* [7] mutations or polymorphisms.

Table 1 Distributions of tumour characteristics for study subjects with genetic analyses and the incident series from which they were drawn

Tumour characteristic	Genetic study subjects		Incident cases ^a	
	<i>n</i>	%	<i>n</i>	%
<i>Ocular site of origin</i>				
Choroid or ciliary body	52	83.9	102	79.1
Iris	8	12.9	15	11.6
Conjunctiva	2	3.2	12	9.3
<i>Histopathologically verified</i>				
33	53.2	76	58.9	
<i>Tumour morphology^b</i>				
Spindle	18	54.5	34	44.7
Mixed	10	30.3	25	32.9
Epithelioid	3	9.1	7	9.2
Unspecified melanoma	2	6.1	10	13.2
<i>Tumour size^{c,d}</i>				
Small	17	32.7	27	26.5
Medium	18	34.6	36	35.3
Large	17	32.7	39	38.2
<i>Tumour stage^e</i>				
Localized to site of origin	44	84.6	80	78.4
Spread to another ocular site ^e	8	15.4	20	19.6
Unknown	0	0.0	2	2.0

^aCases diagnosed between 1 January 1996 and 31 July 1998, and aged less than or equal to 50 years at diagnosis or with bilateral ocular melanoma diagnosed at any age.

^bHistopathologically verified cases only.

^cChoroid and ciliary body melanomas only.

^dSmall, thickness ≤ 3.0 mm and diameter < 15.0 mm; medium, 3.1 mm \leq thickness ≤ 8.0 mm and diameter < 15.0 mm; large, thickness > 8.0 mm, any diameter or diameter ≥ 15.0 mm, any thickness.

^eNot including sclera and optic nerve.

isms. They included a sample of 200 unrelated individuals drawn from 3300 Register participants [7] and 738 individuals who were parents of twins in a study of naevus counts, including 218 who had been controls in a study of *MC1R* polymorphisms and melanoma risk [9]. The total pool of 938 controls was restricted for these analyses to 738 individuals with self-reported British or other northern European ancestry as defined for the cases above; no age restrictions were applied. All 738 selected controls had been genotyped for five common *MC1R* variants, and four common *CDKN2A* polymorphisms had been sought in 131.

Data collection

Cases reported their ethnicity in a telephone interview [16], whereas controls supplied their ancestry in a mailed, self-administered questionnaire [7,9].

DNA extraction

DNA was extracted using a modified 'salting out' procedure as described previously [7].

MC1R genotyping

Cases

To obtain sufficient DNA for sequencing of the entire *MC1R* gene, a nested primer polymerase chain reaction (PCR) strategy was used, as described previously [18], for the amplification of extracted genomic DNA from each subject. The first primer set, hMC1R N-outer and C-outer [18], was used in a 25 μ l PCR containing 25 ng of genomic DNA, 1 \times PCR buffer containing 20 mM Tris-HCl and 50 mM KCl (Promega, Madison, WI, USA), 10% dimethyl sulphoxide (DMSO), 1.5 mM MgCl₂, 25 pmol of each primer, 200 μ M of each deoxynucleotide triphosphates (dNTP) and 1.25 U of *Taq* DNA polymerase (Promega). Cycle conditions were: initial denaturation for 3 min at 94°C, followed by 34 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, concluding with a 7 min extension at 72°C; 5 μ l of the first-round reaction was used as a template to seed a second 25 μ l reaction that used 25 pmol of the N-inner and C-inner primer pairs [18]. The amplification conditions were identical to the first round and the products were used for direct sequencing of the *MC1R* coding region [9,18]. Sequencing was performed by the addition of 100–200 ng of DNA template to 8 μ l of Applied Biosystems Incorporated (ABI) Prism dye terminator premix (utilizing *AmpliTag* DNA Polymerase FS [Perkin Elmer]) and 3.2 pmol primer. Reactions were made up to 20 μ l with MilliQ water and covered with paraffin oil. Three sequencing primers were utilized per template — the N-inner and C-inner primers, as well as an internal (Seq3) primer [18]. Cycling was carried out using: 30 s at 96°C, 15 s at 50°C and 4 min at 60°C for 25 cycles. PCR reaction products were ethanol precipitated, dried for 1 min at 95°C and submitted to an ABI 373 automated sequencer. Se-

quence data were analysed utilizing the program Sequencher (Genecodes).

Controls

Adult twins ($n = 200$) were genotyped at V60L, D84E, R151C, R160W and D294H, whilst the parents of adolescent twins ($n = 738$) were genotyped at V60L, D84E, V92M, R151C, I155T, R160W, R163Q, D294H and R142H, as described previously [9,18]. Only variants common to the two sources of controls were included in the analyses.

CDKN2A genotyping

CDKN2A was amplified as described previously [19,20]. Briefly, the promoter region was amplified using primers 96F and 968R, the 5' untranslated region (UTR) and exon 1 were amplified using primers 781F and 1424R, exon 2 was amplified with primers 42F and 551R, and exon 3 was amplified with primers X3P2F and X3P2R [20]. All PCRs involved a 'touchdown' thermal cycling routine of two cycles at each annealing temperature, decreasing by 2°C, followed by 25 cycles at the lower temperature. Each cycle consisted of 45 s at 94°C, 90 s at the annealing temperature and 90 s at 72°C. A 12 min initial denaturing step at 94°C and a 3 min final extension step at 72°C were also employed. PCR reactions consisted of 100–200 ng DNA, 10–30 pmol of each primer and 200 nM dNTPs (Promega) and were performed with 1.25 U of Amplitaq Gold (Hoffman-La Roche, Basel, Switzerland) in 100 mM Tris-HCl, pH 8.3, 500 mM KCl and 1.5 mM MgCl₂. Due to the high GC content of the *CDKN2A* gene, reactions were performed in a final concentration of 500 mM betaine (Sigma Chemical Company, St Louis, MO, USA). All products were electrophoresed on a 1.5% TAE agarose gel, excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

The fragments were sequenced with the same primers as used for the PCR step, and the 5'UTR was additionally sequenced with primer 427F [20] using ABI dye terminator sequencing kits according to the manufacturer's specifications. Sequencing products were run on an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). The whole of the *CDKN2A* coding region was screened for mutations in cases, whilst four common *CDKN2A* polymorphisms were assessed in control subjects.

Statistical analysis

We compared gene variant frequencies in cases and controls using the chi-squared test; $P < 0.05$ was considered to be significant. Standard multivariate logistic regression analyses were performed using the R statistics package [21]. These analyses included models containing age and sex, as well as a full model that included age, sex and genotype for the five 'red hair colour' *MC1R* and the four *CDKN2A* polymorphisms. Linkage disequilibrium (association) between the different variants was automatically adjusted for in the multiple regression framework. When multiple variants are included as predictors in the logistic framework, this appears in the correlation matrix for the predictor variables and so is conditioned on.

Results

Frequency of *MC1R* variants

Identical proportions (59%) of cases and controls had at least one *MC1R* variant. There were no significant differences between cases and controls in the frequency of any of the five most common variants of *MC1R* (Table 2). There was also no significant association with ocular melanoma when all variant alleles were pooled (Table 3).

Table 2 Frequency of common variants in the *MC1R* and *CDKN2A* genes amongst ocular melanoma cases and controls of northern European descent

Variant	Cases				Controls ^e			
	C/C ^a	C/V ^b	V/V ^c	Frequency ^d	C/C ^a	C/V ^b	V/V ^c	Frequency ^d
<i>MC1R</i>								
V60L	43	17	2	0.17	567	159	12	0.12
D84E	61	1	0	0.01	717	21	0	0.01
R151C	51	9	2	0.10	585	143	9	0.11
R160W	49	13	0	0.10	623	113	2	0.08
D294H	59	3	0	0.02	694	44	0	0.03
<i>CDKN2A</i>								
nt-191	29	26	7	0.32	46	56	18	0.38
nt442	59	3	0	0.02	128	3	0	0.01
nt500	50	11	1	0.10	105	24	0	0.09
nt540	52	9	1	0.09	105	26	0	0.10

^aC/C denotes the homozygote consensus genotype.

^bC/V denotes the heterozygote genotype.

^cV/V denotes the homozygote variant genotype.

^dOf the variant allele.

^eSome control genotypes were missing for certain variants.

CDKN2A genotype

We found no significant differences between cases and controls in the frequency of any of the four most common variants of *CDKN2A* (Table 2), and none of these polymorphisms was associated with an increased risk of ocular melanoma (Table 4). No case of ocular melanoma carried a deleterious germline *CDKN2A* mutation.

Discussion

In our series of young, non-familial, ocular melanoma cases in a population-based case-control study, we found no deleterious mutations of *CDKN2A* and no evidence of associations between ocular melanoma and a number of polymorphisms in *CDKN2A* and *MC1R*.

Deleterious germline mutations of *CDKN2A* have also not been found in previous studies of familial [11,12, 14] and sporadic [8,13] ocular melanoma cases and ocular melanoma cases with a family history of cutaneous melanoma [11,14]. There is, however, some evidence that a loss of *CDKN2A* function may play a role in ocular melanoma. Although no evidence has been found of somatic mutation of *CDKN2A* in this disease [8,22], homozygous deletion of the gene or inactivation of it through hypermethylation of its pro-

motor has been found in some cases [22,23]. Thus, the possibility of a role for the loss of *CDKN2A* function in ocular melanoma remains open.

Our finding that *MC1R* polymorphisms that increase the risk of cutaneous melanoma were not associated with the risk of ocular melanoma was similar to that of the only other study to examine this relationship in ocular melanoma [15]. These findings are compatible with the observation that red hair is not consistently associated independently with ocular melanoma [16]. Red hair is a known risk factor for cutaneous melanoma and the 'red hair colour' variant alleles of *MC1R* (R151C, R160W and D294H) have been associated with an approximately two-fold increased risk [9,10].

A similarity between the genetic determinants of ocular and cutaneous melanoma would not necessarily be expected. Although the melanocytes of the uvea, conjunctiva and skin have a common embryologic origin [24], there are important differences between them. Unlike cutaneous melanocytes, there is no evidence that incident ultraviolet radiation initiates melanogenesis in ocular melanocytes [25]. It is also uncertain whether uveal melanocytes produce melanin *in vivo* during adulthood [26]; ocular melanosomes are relatively inactive and remain within the melanocyte, whereas epidermal melanocytes continuously synthesize melanosomes and transfer them to keratinocytes [27]. Finally, the occurrence of ocular albinism, characterized by depigmentation of the eye but not the skin, suggests different melanogenesis pathways in ocular and cutaneous melanocytes [26]. To the contrary, however, recent evidence indicates that ocular melanocytes express melanocortin 1 receptors (*MC1R*) on their surface and are responsive to α -melanocyte-stimulating hormone, the agonist for these receptors [28].

In the absence of evidence of any important involvement of *CDKN2A* and *MC1R* in the causation of ocular melanoma, future work on susceptibility to ocular melanoma should focus on other genes, such as *BRCA2* and genes that are functionally related to it.

Table 3 Relationship between the risk of ocular melanoma and *MC1R* genotype in 62 ocular melanoma cases and 726 controls of northern European descent

	Unadjusted odds ratio ^a	Adjusted odds ratio ^b	95% confidence interval
<i>MC1R</i> variants			
V60L	1.47	1.47	0.85–2.45
D84E	0.59	0.44	0.02–2.43
R151C	0.97	0.67	0.33–1.28
R160W	1.34	1.09	0.52–2.14
D294H	0.89	0.87	0.19–2.71
Any variant	1.08	1.05	0.69–1.59

^aAdjusted only for sex and age.

^bAdjusted odds ratios are from the logistic regression model including age, sex and the five *MC1R* genotypes coded as 0 (no variant at that site), 1 (heterozygote) or 2 (homozygote). The exception is the case of 'any variant', where a single dummy variable replaced the five individual variants.

Table 4 Relationship between the risk of ocular melanoma and *CDKN2A* genotype in 62 ocular melanoma cases and 131 controls of northern European descent

	Unadjusted odds ratio ^a	Adjusted odds ratio ^b	95% confidence interval
<i>CDKN2A</i> variants			
nt-191	1.04	0.80	0.40–1.55
nt442	2.19	3.38	0.43–26.73
nt500	1.03	0.86	0.35–2.08
nt540	0.92	0.86	0.37–2.00

^aAdjusted only for sex and age.

^bAdjusted for sex, age and *CDKN2A* variant. Because there is linkage disequilibrium between the different *CDKN2A* polymorphisms, we have included them as potential confounders.

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