MC1R Genotype Modifies Risk of Melanoma in Families Segregating CDKN2A Mutations

Neil F. Box,1 David L. Duffy,2 Wei Chen,1 Mitchell Stark,2 Nicholas G. Martin,2 Richard A. Sturm,1 and Nicholas K. Hayward2

1Centre for Functional and Applied Genomics, Institute for Molecular Bioscience, University of Queensland, and 2Queensland Institute of Medical Research, Brisbane, Australia

Mutations in the exons of the cyclin-dependent kinase inhibitor gene CDKN2A are melanoma-predisposition alleles which have high penetrance, although they have low population frequencies. In contrast, variants of the melanocortin-1 receptor gene, MC1R, confer much lower melanoma risk but are common in European populations. Fifteen Australian CDKN2A mutation–carrying melanoma pedigrees were assessed for MC1R genotype, to test for possible modifier effects on melanoma risk. A CDKN2A mutation in the presence of a homozygous consensus MC1R genotype had a raw penetrance of 50%, with a mean age at onset of 58.1 years. When an MC1R variant allele was also present, the raw penetrance of the CDKN2A mutation increased to 84%, with a mean age at onset of 37.8 years ($P = .01$). The presence of a CDKN2A mutation gave a hazard ratio of 13.35, and the hazard ratio of 3.72 for MC1R variant alleles was also significant. The impact of MC1R variants on risk of melanoma was mediated largely through the action of three common alleles, Arg151Cys, Arg160Trp, and Asp294His, that have previously been associated with red hair, fair skin, and skin sensitivity to ultraviolet light.

Introduction

To date, germline mutations in three different genes have been shown to influence risk of melanoma: cyclin-dependent kinase inhibitor 2A (CDKN2A [MIM 600160]) (Hussussian et al. 1994; Kamb et al. 1994a), cyclin-dependent kinase 4 (CDK4 [MIM 123829]) (Zuo et al. 1996; Soufr et al. 1998), and the melanocortin-1 receptor (MC1R [MIM 155555]) (Palmer et al. 2000). The basis of melanoma risk as determined by these genes probably revolves around at least two independent pathways (Whiteman et al. 1998), one being the CDKN2A/CDK4 cell-cycle and tumor-suppressor gene axis, and the other a pigmentation-associated predisposition axis implicated by the recent association of MC1R variants with red hair, fair skin, freckling, and melanoma (Palmer et al. 2000). Melanoma risk attributable to MC1R may arise through the action of solar ultraviolet (UV) light on lighter skin tones with diminished tanning capacity (Bliss et al. 1995; Breitbart et al. 1997) or, possibly, through a more direct intrinsic effect on melanocytic cellular transformation. On the other hand, linkage to the 9p21–22 region containing CDKN2A (Zhu et al. 1999) of a quantitative trait locus accounting for 33% of variance in flat mole count suggests that CDKN2A mutation may play a role in determination of mole density which, in turn, predisposes to later melanoma formation; this appears to be a risk factor quite distinct from the red hair and fair skin associated with MC1R variants (Garbe et al. 1994; Bliss et al. 1995; Grange et al. 1995; Grulich et al. 1996).

The p16INK4A protein encoded by the CDKN2A locus (Kamb et al. 1994b; Nobori et al. 1994) acts as a tumor suppressor that induces G1 cell-cycle arrest by binding to and inhibiting the kinase activities associated with cyclin D complexes with cyclin-dependent kinases 4 and 6 (CDK4 and CDK6). G1-S phase transition is usually dependent upon phosphorylation of the retinoblastoma protein (pRB) by the cyclin D1/CDK4 complex (Serrano et al. 1993; Lukas et al. 1995). Inactivation of CDKN2A via one of several mechanisms (homozygous deletion, mutation, and/or promoter methylation) is a frequent event in tumors of many types (e.g., see reports by Kamb et al. [1994a] and Nobori et al. [1994]; reviewed by Ruas and Peters [1998]). Furthermore, the presence of mutations at this locus in the germline of a small proportion of familial melanoma patients (e.g., see reports by Hussussian et al. [1994] and Kamb et al. [1994b]; reviewed by Hayward [1998]) indicates that p16 inactivation is an early and possibly an initiating step in melanoma tumorigenesis.

MC1R is a seven-pass transmembrane G-protein–coupled receptor, expressed in skin melanocytes, that activates adenylyl cyclase to elevate cyclic adenosine
monophosphate levels upon stimulation by the proopio-
melanocortin-derived peptides α-melanocyte-stimulating
hormone (α-MSH) and adrenocorticotropic hormone
(Thody and Graham 1998). Hormonal stimulation of
MC1R leads to eumelanogenesis and is central to the
tanning response of human skin after UV irradiation (Su-
zuki et al. 1999). During UV exposure of the skin, there
is an increase in melanin production that may occur, in
part, via an increase in tyrosinase gene transcription and
enzyme activity (Gilchrest et al. 1996; Sturm et al. 1998).
MC1R also regulates the balance of two distinct melanin
types, the red/yellow pheomelanin and black/brown eu-
melanin; this regulation, in turn, forms the basis of the
association between specific MC1R alleles and red hair
and fair skin (Valverde et al. 1995; Box et al. 1997; Smith
et al. 1998; Flanagan et al. 2000; Palmer et al. 2000;
Bastiaens et al. 2001). It has already been shown that
close common MC1R variant alleles, Arg151Cys, Arg160Trp, and Asp294His, are associated with an in-
creased risk of melanoma within the Queensland pop-
ulation; this risk is mediated, at least in part, by an effect
on pigmentation phenotype (Palmer et al. 2000).
Germline mutations in CDKN2A have been identified
in only a small proportion of the 5%–10% of melanoma
cases that occur in multiplex families. A recent popu-
lation-based assessment of the overall caseload of mel-
anoma in Queensland estimated that ~0.2% of all mel-
anomas may be attributed to germline mutation of this
gene (Aitken et al. 1999). Within the high-UV environ-
ment of Queensland, penetrance for melanoma is vir-
tually 100% by 80 years of age for those individuals
carring a germline CDKN2A mutation (D.T. Bishop,
F. Demenais, A.M. Goldstein, W. Bergman, J.N. Bishop,
B.B. de Paillerets, A. Chompret, P. Ghiorzo, N. Gruis,
J. Hansson, M. Harland, N. Hayward, E.A. Holland,
G.J. Mann, M. Mantelli, D. Nancarrow, A. Platz, M.A.
Tucker, the Melanoma Genetics Consortium [“Bishop
et al.”], unpublished data). Mutations within the CDK4
gene are even less frequent than those within CDKN2A
(Hayward 1999). On the other hand, MC1R variants
have been identified at a much higher frequency but
with much lower genotype relative risk for melanoma
(Box et al. 1997; Smith et al. 1998; Palmer et al. 2000).
Nevertheless, because of the high frequency of MC1R
variants in melanoma cases (Palmer et al. 2000), we
estimated that as many as one third of melanomas in
Queensland may be attributable to MC1R genotype.

The purpose of the present study was to determine
whether MC1R variants modify penetrance of CDKN2A
mutations that occur in a subset of multiplex melanoma
families. Affected and unaffected members of 15 multi-
plex families with melanoma were available for this anal-
ysis, with each family segregating one of a total of nine
different CDKN2A mutations (Walker et al. 1995; Flores

Subjects and Methods

Ascertainment of Samples and DNA Extraction

The fifteen melanoma pedigrees available for the pre-
sent analysis were identified as part of the Queensland
Familial Melanoma Project (Aitken et al. 1996). In brief,
extent of family history was determined in a total of
1,897 families ascertained as a subset of the 12,006 in-
cident cases of histologically confirmed cutaneous mel-
anoma that were diagnosed in residents of Queensland
during the period of 1982–1990 and were reported to the
Queensland Cancer Registry. The standardized fam-
ily risk index, described by Aitken et al. (1996, 1999),
was used to divide the total sample of 1,897 families
into three strata of familial melanoma risk, including
1,392 low-risk, 414 intermediate-risk, and 91 high-risk
families. Blood was collected and DNA extracted from
members of selected families, as described elsewhere
(Aitken et al. 1999). Extensive genotyping for CDKN2A
germline mutations has resulted in identification of a
total of 15 kindreds, all identified as high-risk, who con-
tain a total of nine different CDKN2A mutations: Gln50Arg, Arg24Pro, 46delC, Leu32Pro, Asp108Asn,
Leu16Pro, Gly35Ala, 9del24, 33ins24, or Met53Ile
(Walker et al. 1995; Flores et al. 1997; Whiteman et al.
1997; Aitken et al. 1999).

Measures

An ongoing part of the family ascertainment proce-
dure involved submission of questionnaires about stan-
dard melanoma risk factors, including propensity to
burn in the sun, pigmentation (skin color at age 21 years
and eye color), total freckling in summer, and density of
melanocytic nevi. Data were also collected on age at
onset of melanoma, number of primary tumors, other
tumors of nonmelanocytic origin, and ancestry.

MC1R Genotyping

To obtain sufficient DNA product for sequencing and
allele-specific oligonucleotide (ASO) dot-blot detec-
tion of nine previously identified MC1R variants, in-
cluding Val60Leu, Asp84Glu, Val92Met, Arg142His,
Arg151Cys, Ile155Thr, Arg160Trp, and Asp294His, a nested-primer PCR strategy was used, as
described elsewhere (Box et al. 1997), for amplification
of extracted genomic DNA from the families with mel-
anoma. The first primer set, hMC1R N-outer and C-
outer (Box et al. 1997), for amplification
of genomic DNA, 1
× PCR buffer containing 20 mM
Tris-HCl and 50 mM KCl (Promega), 10% dimethyl-
sulfoxide (Dutton et al. 1993), 1.5 mM MgCl2, 25 pmol
of each primer (C-outer and N-outer), 200 μM of each
dNTP, and 1.25 U of Taq DNA polymerase (Promega). The reaction was initially denatured 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. Five microliters of the first-round reaction was used as a template to seed a second 25-µl reaction, which used 25 pmol each of the N-inner and C-inner primer pairs (Box et al. 1997). Amplification conditions were identical to those in the first round. The amplification product was used for either direct sequencing or ASO dot-blot analysis (Palmer et al. 2000; Box et al. 2001).

All of the family members who had developed melanoma by the time of this analysis, as well as a total of 50 unaffected individuals selected from the pedigrees, were genotyped by complete nucleotide sequencing to determine MC1R variant content within the families. Automated sequencing of the MC1R coding region was performed by the addition of 100–200 ng of DNA template to 8 µl of ABI Prism dye terminator premix (utilizing AmpliTaq DNA polymerase FS [Perkin Elmer]) and 3.2 pmol of primer. MilliQ water was added to make the final volume 20 µl, and the entire reaction mixture was then covered with paraffin oil. Cycling was performed on a Thermal Cycler Model 480 (Perkin Elmer) using standard cycling conditions: 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, were dried for 1 min at 95°C, and were submitted to an ABI 373 automated sequencer. Sequence data were analyzed utilizing the Sequencer program (Genecodes).

The remaining family members were MC1R genotyped using a dot-blotting procedure described elsewhere (Palmer et al. 2000). Denatured inner PCR reaction product was blotted onto Magna (MSI) nylon transfer membrane and was cross-linked under 150 mJ of UV energy. After this, the blot was prehybridized in 10 ml of Hybridization solution, and 0.1 mg/ml sonicated salmon sperm DNA for 1 h at 42°C. After the addition of 50–100 µl of the γ-[32P]-radiolabelled probe, dot blots were left to hybridize overnight at 42°C. Unbound probe was removed by washing for 20 min at 50°C in 10 ml of TMAC wash buffer, consisting of 3 M TMAC, 1 mM EDTA, 25 mM Na2HPO4, pH 6.8, 0.1% SDS, 5 × Denhardt’s solution, and 0.1 mg/ml sonicated salmon sperm DNA for 1 h at 42°C. After the addition of 50–100 µl of the γ-[32P]-radiolabelled probe, dot blots were left to hybridize overnight at 42°C. Unbound probe was removed by washing for 20 min at 50°C in 10 ml of TMAC wash buffer, consisting of 3 M TMAC, 1 mM EDTA, 25 mM Na2HPO4, and 0.1% SDS. Genotypes were visualized using autoradiography.

Statistical Methods

Survival analysis was performed using the Survival 5 package of Therneau (1999), running on statistics package R, version 1.1.1. If MC1R and CDKN2A genotypes are the sole or overwhelming causes of melanoma in these families, then analyses including these variables will eliminate problems caused by the correlated (i.e., familial) nature of the data. However, if other familial factors are acting to increase risk, these may confound the detection of effects of MC1R. We therefore performed Cox proportional-hazards model analyses, stratifying on pedigree or nuclear family and also including family as a clustering variable in a gamma frailty model, in an attempt to control for residual familial correlation in risk of melanoma. Stratifying on nuclear families and restricting the analysis to only siblings is equivalent to the “sib-TDT” (Spielman and Ewens 1996) method of adjusting for population stratification. A Cox proportional-hazards model was also used to generate the survival curve showing age-specific probability of melanoma development for the Queensland population at large, using all cases of invasive melanoma reported to the Queensland Cancer Registry in 1996.

Results

There were 15 familial melanoma pedigrees available for the present study, in which nine different CDKN2A mutations were segregating (table 1). The families contained a total of 53 sibships and 270 individuals, with an average of 6.5 melanoma cases per kindred. The number of members for whom DNA or genotype information was available is shown in table 2. MC1R genotype was determined in 136 family members, and CDKN2A genotype was available for 162 individuals; however, only 131 of these individuals have both loci genotyped. There were 97 individuals with melanoma within the sample, 76 of whom were found to carry a CDKN2A mutation. Ten (10%) were designated as having sporadic melanoma, on the basis of the absence of any CDKN2A mutation; these 10 include one case each in pedigrees 40599, 40750, 40823, 41031, and 41162, two cases in pedigree 41105, and three in pedigree 60001. CDKN2A mutation data were not available for 11 cases (table 1). There was a total of 60 pedigree members with recorded skin color and 57 with recorded hair color.

Allele frequencies for the eight MC1R variants detected in family members are shown in table 3, together with frequency data from two samples of control individuals drawn from the general population (Palmer et al. 2000). The variant frequencies were similar to those observed in the southeastern Queensland population, except for the frequency of the Arg151Cys variant; this variant is present in ∼26% of family members but has a general population frequency of ∼10%. This difference is explained by the high degree of Celtic ancestry of the families (Aitken et al. 1999), since the Celtic population is one in which the Arg151Cys allele is known to be overrepresented (Smith et al. 1998). Only 14% of those genotyped did not carry an MC1R var-
Table 1

<table>
<thead>
<tr>
<th>PEDIGREE ID</th>
<th>NO. OF MEMBERS</th>
<th>CDKN2A MUTATION</th>
<th>NO. WITH MELANOMA STATUS</th>
<th>NO. OF SPOUSES</th>
</tr>
</thead>
<tbody>
<tr>
<td>40300</td>
<td>17</td>
<td>R24P</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>40582</td>
<td>24</td>
<td>33ins24</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>40599</td>
<td>30</td>
<td>Q50R</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>40750</td>
<td>20</td>
<td>G35A</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>40787</td>
<td>18</td>
<td>46delC</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>40823</td>
<td>14</td>
<td>L32P</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>40935</td>
<td>10</td>
<td>L16P</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>41001</td>
<td>27</td>
<td>M53I</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>41019</td>
<td>8</td>
<td>R24P</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>41031</td>
<td>19</td>
<td>M53I</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>41105</td>
<td>23</td>
<td>D108N</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>41119</td>
<td>12</td>
<td>33ins24</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>41154</td>
<td>8</td>
<td>M53I</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>41162</td>
<td>8</td>
<td>M53I</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>60001</td>
<td>38</td>
<td>M53I</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>Totals</td>
<td>270</td>
<td></td>
<td>97</td>
<td>162</td>
</tr>
</tbody>
</table>

* Determined by Monzon et al. (1998) to be a nonfunctional variant.

* Functional significance uncertain (see report by Monzon et al. [1998]).

* Pedigree contains one spouse who has developed melanoma.

* Functional consequence not assessed.

* Frameshift mutation expected to lead to a nonfunctional protein.

Table 2

<table>
<thead>
<tr>
<th>LOCUS AND GENOTYPE</th>
<th>NO. OF INDIVIDUALS</th>
<th>NO. WITH MELANOMA STATUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN2A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>68</td>
<td>10</td>
</tr>
<tr>
<td>Mutant</td>
<td>94</td>
<td>76</td>
</tr>
<tr>
<td>NA</td>
<td>108</td>
<td>11</td>
</tr>
<tr>
<td>MC1R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consensus</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>One variant</td>
<td>58</td>
<td>28</td>
</tr>
<tr>
<td>Two variants</td>
<td>58</td>
<td>34</td>
</tr>
<tr>
<td>NA</td>
<td>134</td>
<td>30</td>
</tr>
</tbody>
</table>

* NA = not available.

* Wild type for CDKN2A at each of Gln50Arg, Arg24Pro, 46delC, Leu16Pro, Asp108Asn, Gly35Ala, 9del24, 33ins24, and Met53Ile positions.

* Consensus genotype at each of the Val60Leu, Asp84Glu, Val92Met, Arg142His, Arg151Cys, Ile155Thr, Arg160Trp, Arg163Gln, and Asp294His positions.

iant, and, notably, all of the seven individuals with sporadic melanomas (CDKN2A-mutation negative) for whom MC1R genotype was available carried variants. To maximize the available information regarding skin and hair color, association with MC1R variants was tested with simple grouping of all study members, irrespective of pedigree or relationship. When all MC1R variants were grouped, there was a significant tendency for MC1R variants to increase in frequency as hair color lightened and became red (Mantel-Haenszel test for trend \( P = .01 \)). This was more marked when the three previously defined red hair color (RHC) variants, Arg151Cys, Arg160Trp, and Asp294His (Box et al. 2001), were pooled (\( P < .001 \)). A similar trend was observed with skin color, although all variants considered together were not significantly associated with skin color, and the association between skin color and RHC variants was marginal (\( P = .03 \)). CDKN2A mutations showed no association with skin or hair color in this data set.

The unstratified survival analysis that included MC1R and CDKN2A consensus homozygotes versus those individuals with one or more variants (table 4) showed that the presence of an MC1R variant allele significantly increased the risk of melanoma development in both CDKN2A carriers and noncarriers (\( P = .005 \)). The impact on melanoma risk of carrying both an MC1R variant and a CDKN2A mutation is demonstrated by a significant increase in raw penetrance (shown as a percentage of melanoma cases) from 50% to 84%, and a decrease in the mean age at onset from 58.1 to 37.8 years, when compared with family members who carry a CDKN2A mutation alone (\( P = .01 \)). There was little difference in risk of melanoma between family members carrying one versus two variant MC1R alleles (odds ratio [OR] 3.3, 95% confidence interval [CI] 1.25–8.59; OR 3.4, 95% CI 1.31–8.78, respectively, using MC1R consensus genotype as the reference category).

Multiple tumors were common among individuals...
Table 3  
Frequencies of MC1R Variants in 136 Typed Individuals

<table>
<thead>
<tr>
<th>VARIANT</th>
<th>Families with Melanoma</th>
<th>Population Sample 1 (n = 390)</th>
<th>Population Sample 2 (n = 1,627)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val60Leu</td>
<td>.109</td>
<td>.120</td>
<td>.124</td>
</tr>
<tr>
<td>Asp84Glu</td>
<td>.011</td>
<td>.015</td>
<td>.011</td>
</tr>
<tr>
<td>Val92Met</td>
<td>.109</td>
<td>NA</td>
<td>.097</td>
</tr>
<tr>
<td>Arg142His</td>
<td>.000</td>
<td>NA</td>
<td>.009</td>
</tr>
<tr>
<td>Arg151Cys</td>
<td>.255</td>
<td>.089</td>
<td>.111</td>
</tr>
<tr>
<td>Ile155Thr</td>
<td>.007</td>
<td>NA</td>
<td>.010</td>
</tr>
<tr>
<td>Arg160Trp</td>
<td>.084</td>
<td>.076</td>
<td>.071</td>
</tr>
<tr>
<td>Arg163Gln</td>
<td>.051</td>
<td>NA</td>
<td>.050</td>
</tr>
<tr>
<td>Asp294His</td>
<td>.011</td>
<td>.031</td>
<td>.028</td>
</tr>
</tbody>
</table>

a Frequency estimated in an unselected sample of 390 haplotypes from southeastern Queensland, presented by Palmer et al. (2000). NA = not available.
b Frequency estimated in a second unselected sample of 1,627 haplotypes from southeastern Queensland (D.L. Duffy, N.F. Box, W. Chen, A. Green, N.G. Martin, R.A. Sturm, unpublished data).
c P < .001.

with melanoma in these families who carried a CDKN2A mutation (70%), but they were seen in only one of seven who did not. There was a trend for case individuals without a CDKN2A mutation who carried the MC1R consensus genotype to have fewer tumors, but the numbers in this group (with information available) are small (1/4 vs. 35/50).

The Cox proportional-hazard method was also used to assess the influence of both the CDKN2A mutation and the MC1R variants, on propensity to develop melanoma (table 5). Family members carrying a CDKN2A mutation were 13.35-fold (95% CI 6.01–29.67) more likely to develop melanomas than those individuals who carried the wild type. Carriage of any MC1R variant significantly increased the likelihood of melanoma development in these pedigrees, 3.72-fold (95% CI 1.48–9.37). Stratification on sex caused the hazard ratios to demonstrate an unusual tendency for more females than males to develop melanoma, although this tendency was not significant. The analyses that included pedigree as a covariate obtained parameter estimates (MC1R OR 4.7, 95% CI 1.1–19.9) similar to those shown in table 5, as did those conditioning on pedigree (MC1R OR 3.3, 95% CI 0.70–16.0), though the latter led to a loss of power reflected in a nonsignificant P value. Stratification on sibship reduced the sample size and broadened the CI still further. The frailty models using pedigree and sibship as clustering variables estimated the frailty variance as not significantly different from zero, suggesting that there were no large residual familial correlations that might confound the estimates of the effects of CDKN2A and MC1R.

When the Cox proportional-hazard analysis was restricted to either the Arg151Cys variant (the most common allele in these kindreds) or to the combination of the RHC variants, similar parameter estimates were obtained, but they were less significant than when all MC1R variants were pooled (for Arg151Cys, OR 1.48, 95% CI 0.91–2.42; for the RHC variants, OR 2.02, 95% CI 1.16–3.12). Grouping of the MC1R variants other than the three major variants that are important in hair and skin color determination gave a hazard ratio of 1.32 (table 5), which was not significantly different from the hazard ratio for those individuals carrying a consensus MC1R genotype, indicating that most of the effect of MC1R genotype on melanoma risk is due to the Arg151Cys, Arg160Trp, and Asp294His alleles. There were only 14 MC1R heterozygous parents of affected children available for analysis, and the transmission/disequilibrium test result was not significant (9/14 variant alleles transmitted; P = .42).

Since we detected no significant confounding effect of pedigree membership, we have summarized the results from the unstratified Cox proportional-hazards model as the predicted survival curve for each of the four genotype categories (fig. 1). These predicted curves are parallel because of the assumed proportional-hazards relationship and the absence of an interaction term in the selected model. For comparison, the age-specific probabilities of melanoma development within the Queensland population at large are also shown; despite the small numbers in some subgroups, the estimates of risk associated with the MC1R and CDKN2A “wildtype” genotype are close to those for the general population.

Discussion

The present study extends our previously reported association of MC1R gene variants with melanoma (Pal-
mer et al. 2000), by demonstrating a significant impact of MC1R genotype on penetrance of CDKN2A mutations in melanoma-dense pedigrees. The presence of an MC1R variant in addition to a CDKN2A mutation significantly increased the raw melanoma penetrance, decreasing age at onset by up to 20 years compared with individuals carrying a CDKN2A mutation alone. At age 50 years, 81% of people with both a CDKN2A mutation and an MC1R variant had developed melanoma, whereas only 57% of those carrying a CDKN2A mutation alone had developed melanoma. The presence of an MC1R variant alone was estimated to account for a risk of 14% by age 50 years. Although there were no melanoma cases without either CDKN2A mutations or MC1R variants, the Cox proportional-hazards model predicted an overall melanoma risk for this genotype of ∼10% by age 70 years, close to the Queensland population lifetime melanoma risk, previously estimated at ∼1 in 7. Penetrance of CDKN2A mutations, without regard to MC1R status, has been estimated at ∼58% by age 80 years in the United Kingdom, at ∼76% in North America, and at ∼92% by the same age in Australia (Bishop et al., unpublished data). The presence of a substantially higher environmental UV level within Australia has been suggested to account for this increase in penetrance of CDKN2A mutations. Frailty-variance estimates were not significantly different from zero when both pedigree and sibship were used as clustering variables, suggesting that the effects of CDKN2A and MC1R together may account for the majority of melanoma cases within these pedigrees. No significant differences in trends in individual pedigrees were observed in the survival analyses, suggesting that individual CDKN2A mutations in these families tend to have similar effects on melanoma penetrance, although power to detect such differences is low. At present, there is a dearth of studies that assess differences in melanoma penetrance in families carrying different CDKN2A mutations, and it can only be assumed that each CDKN2A mutation is acting similarly to increase risk of melanoma. It is clear that future attempts to assess more accurately the individual and combined impact of CDKN2A mutations on melanoma incidence will require knowledge of MC1R genotype status, which is demonstrated here to act as a significant modifier of CDKN2A penetrance. Tumor types other than melanoma were not overrepresented in this cohort of pedigrees (N.K. Hayward and J.M. Palmer, unpublished data), although other cohorts of families have been shown to have a significant excess of pancreatic cancers (Goldstein et al. 1993; Vasen et al. 2000).

Unlike CDKN2A mutations, MC1R variants clearly do not show functional equivalence in their influence on melanoma risk. The RHC variants, Arg151Cys, Arg160Trp, and Asp294His, account for much of the MC1R effect in increasing CDKN2A penetrance, with the remaining variants not significantly associated with melanoma risk. The same three RHC variants are those identified in association with fair skin, freckling, poor tanning capacity, melanoma, and nonmelanocytic skin cancer risk (Box et al. 1997; Smith et al. 1998; Flanagan et al. 2000; Palmer et al. 2000; Bastiaens et al. 2001; Box et al. 2001). Limited information on pigmentation phenotype was available for the family members in-

Table 4
Mean Disease-Free Survival, by CDKN2A and MC1R Genotype

<table>
<thead>
<tr>
<th>Genotype (CDKN2A, MC1R)</th>
<th>No. of Individuals</th>
<th>No. of Melanoma Cases (%)</th>
<th>Mean Age at Onset ± SE (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0, 0</td>
<td>10</td>
<td>0 (0)</td>
<td>...</td>
</tr>
<tr>
<td>0, 1</td>
<td>45</td>
<td>7 (15.6)</td>
<td>82.4 ± 3.90</td>
</tr>
<tr>
<td>1, 0</td>
<td>10</td>
<td>5 (50.0)</td>
<td>58.1 ± 7.23</td>
</tr>
<tr>
<td>1, 1</td>
<td>68</td>
<td>57 (83.8)</td>
<td>37.8 ± 1.75</td>
</tr>
</tbody>
</table>

* “0” indicates wild type CDKN2A or consensus MC1R at all positions analyzed. An MC1R value of “1” indicates one or two MC1R variants. Each CDKN2A mutation is carried in heterozygote form. The P value for difference between CDKN2A p1 group with and without MC1R variant is .01

Table 5
Hazard Ratios for CMM versus CDKN2A Genotype, MC1R Genotype, and Sex, Calculated by the Cox Proportional-Hazards Method

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN2A mutant</td>
<td>13.35</td>
<td>6.01–29.67</td>
</tr>
<tr>
<td>MC1R variant:</td>
<td>3.72</td>
<td>1.48–9.37</td>
</tr>
<tr>
<td>RHC variant</td>
<td>2.02</td>
<td>1.16–3.12</td>
</tr>
<tr>
<td>Other variant</td>
<td>1.32</td>
<td>.88–1.98</td>
</tr>
<tr>
<td>Female sex</td>
<td>1.48</td>
<td>.92–2.40</td>
</tr>
</tbody>
</table>

* Each CDKN2A mutation is carried in heterozygote form.
* Members carrying 1 or 2 variants considered together in this analysis.
* Other MC1R variants defined for this analysis as Val60Leu, Asp84Glu, Val92Met, Ile155Thr, and Arg163Gln.
Figure 1  Expected survival curves (Cox proportional-hazards model) for melanoma versus CDKN2A and MC1R genotypes. For clarity, the lines joining the points of the empirical survivor functions (black diamonds [•] denote CDKN2A mutation + MC1R variant; white diamonds [○] denote CDKN2A mutation + MC1R wt; white triangles [▼] denote CDKN2A wt + MC1R variant) have been omitted.

cluded in the present study, which prevented a more thorough analysis of this factor’s interactions with MC1R variants, CDKN2A mutations, and melanoma.

It is an interesting parallel that the presence of an MC1R variant shifts the age-specific CDKN2A mutation penetrance curve towards younger ages in a way similar to that observed for those families ascertained in a high-UV environment (Bishop et al., unpublished data). MC1R activity is crucial for an effective skin response to UV exposure, as assessed by visible tanning, and carrying a single RHC MC1R variant is enough to significantly diminish the skin’s capacity to respond in a protective way to UV exposure (Flanagan et al. 2000; Healy et al. 2000; Box et al. 2001). Our data suggest that carrying two MC1R variants adds no further risk than carrying one such variant, although a larger sample may reveal a difference. It is clear from earlier studies that the presence of a single RHC MC1R variant is enough to give a significant heterozygote effect on pigmentation and melanoma risk (Healy et al. 2000; Palmer et al. 2000) and may act here to shift the eumelanin/pheomelanin balance and to increase the amount of pheomelanin produced by the skin. Pheomelanin and related metabolites have been shown to be mutagenic and cytotoxic (Harsanyi et al. 1980; Sturm 1998), suggesting a dual UV sensitivity where increased levels of this type of melanin within the skin may not only have a diminished UV protective capacity but may actively promote generation of skin tumors. Alternatively, a single MC1R variant may be enough to exert a marked intrinsic and non–pigmentation-related effect on the propensity for melanocytic cellular transformation.

In conclusion, we have presented evidence that variant alleles at the MC1R locus significantly increase penetrance of mutations at the CDKN2A locus; we believe that this is one of the few good examples of effects of gene-gene interaction on disease risk documented to date, and it is supported by the accompanying study by
van der Velden et al. (2001 [in this issue]). One of the major goals for researchers attempting to understand the complexities of melanoma etiology is to identify host factors that influence age at onset, number of primary tumors, tumor site, and time to metastasis. Obtaining reliable predictors of risk and prognosis offers the promise of better management and prevention of many tumor types. This is nowhere more relevant than for melanoma which, if caught early, is eminently treatable but has very poor prognosis following metastasis.

**Acknowledgments**

This work was supported by the Queensland Cancer Fund and by Australian National Health and Medical Research Council grants 930223 and 961061. We are indebted to the study members for their cooperation. The Centre for Functional and Applied Genomics is a Special Research Centre of the Australian Research Council.

**Electronic-Database Information**

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

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

**References**


Sturm RA, Box NF, Ramsay M (1998) Human pigmentation genetics: the difference is only skin deep. Bioessays 20:712–716


Therneau T (1999) Survival 5 [computer program]. Mayo Clinic, Rochester


