Low Prevalence of Germline CDKN2A and CDK4 Mutations in Patients With Early-Onset Melanoma

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Background: In patients with cutaneous melanoma, early age at disease onset is characteristic in familial cases and in individuals with multiple primary melanomas. Both subsets of patients with melanoma are at risk for harboring germline CDKN2A or CDK4 mutations.

Objective: We set out to prospectively determine the prevalence of CDKN2A and CDK4 mutations in a group of young patients with melanoma.

Design: We prospectively screened 913 patients over a 6-month period and identified 519 patients with invasive melanomas. We invited 172 patients with melanoma who were younger than 40 years to participate in the study, and 49 patients consented and donated peripheral blood samples. Forty-nine percent (n=24) of our patients developed cutaneous melanoma before the age of 30 years.

Setting: A melanoma clinic in the Boston, Mass, area.

Main Outcome Measure: We used a combination of single-strand conformation analysis and direct sequencing of samples of peripheral blood leukocyte DNA to search for mutations in exons 1α, 1β, 2, and 3 of CDKN2A and in exon 2 of CDK4.

Results: The mean and median ages at diagnosis in our group were 30 and 32 years, respectively. Among a group of 49 patients, we detected 1 (2%; 95% confidence interval, 0.07%-10.8%) Met 53 Ile CDKN2A mutation, which was found in a patient with a strong family history of melanoma. This alteration has been previously shown to impair p16 function. One patient had an Ala 148 Thr change in CDKN2A, which has also been shown to be a polymorphism. We also detected a sequence polymorphism (in the 3′ untranslated region [3′UTR] of CDKN2A) in 27% of our patients. A similar incidence of this 3′UTR polymorphism was observed in a control population. We found no CDK4 mutations.

Conclusions: Germline CDKN2A and CDK4 mutations are not common in patients who develop melanoma at an early age. This finding contrasts with other cancer-predisposition syndromes, in which there is an increased incidence of germline mutations among young patients. Selection of patients with melanoma for genetic testing based solely on age at onset may not be warranted at the current time.

Arch Dermatol. 2000;136:1118-1122

CUTANEOUS melanoma has grown rapidly in incidence among fair-skinned populations over the past several decades. In 2000, an estimated 47 700 cases of cutaneous melanoma will occur in the United States, with 7700 deaths as a result.¹ Although the cause of cutaneous melanoma is unknown, both genetic factors and environmental carcinogens, such as sun exposure,² contribute to its development.

Greene and Fraumeni³ estimated that 8% to 12% of melanomas arise in a familial context. The recognition of atypical moles and multiple melanomas in a familial setting led to the first descriptions of a genetic cancer syndrome involving melanocytic tumors. Clark et al⁴ and Lynch et al⁵ independently described the B-K mole syndrome and the familial atypical multiple mole-melanoma syndrome, respectively. Over the past 5 years, CDKN2A has gained prominence as a melanoma-predisposition gene. In 1994, Hussussian et al⁶ demonstrated inactivating germline mutations of CDKN2A in a subset of chromosome 9p21–linked melanoma-prone kindreds.

The human CDKN2A encodes for 2 protein products, p16 and p14ARF, both of which can function as tumor suppressor genes.⁷,⁸ The p16 protein inhibits a protein kinase, CDK4, whose function is to drive cell-cycle progression by phosphorylating the retinoblastoma protein. CDK4 has also been shown to be mutated in sporadic melanoma tumors and in a subset of melanoma-
PATIENTS AND METHODS

STUDY PATIENTS

We prospectively screened (through chart review) 913 consecutive patients who were seen at the Massachusetts General Hospital (MGH) Melanoma Clinic over a 6-month period (December 1997 to August 1998) and identified 519 patients with invasive melanomas. The MGH Melanoma Clinic is a referral-based clinic for patients with a history of melanoma and high-risk individuals with a history of atypical moles or a family history of melanoma. We identified 172 patients who were or had been diagnosed as having an invasive or metastatic melanoma before they were 40 years old. The diagnoses did not have to have been rendered during the ascertainment period; the average interval between melanoma diagnosis and the study visit was 5.9 years (range, 0 [time at diagnosis] to 30 years). Only diagnoses that were histologically confirmed by a member of the MGH Dermatopathology Unit were included. Patients with diagnosis of melanoma in situ were excluded, given the occasional ambiguity between melanoma in situ and severely dysplastic nevus. At the time of the clinic visit, each of the 172 patients was provided with a detailed letter describing the study. Those patients expressing interest then underwent an extended discussion regarding the study content and risks. A total of 49 patients (28%) consented to the study and, in accordance with a protocol approved by the Dana Farber Cancer Institute and the MGH Institutional Review Board, donated 20 mL of blood for genetic analysis. The blood samples were coded, and the confidentiality of the patients was maintained. The results were not available to the patients. Medical and family histories and demographic details of participants were recorded through chart review and direct interview. All 49 patients were white and were unrelated to one another. Forty-three blood specimens from normal, healthy blood donors at the MGH blood bank were used for analysis of the polymorphism.

POLYMERASE CHAIN REACTION (PCP) AND SINGLE-STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

Samples of peripheral blood leukocyte DNA were isolated using a commercially available blood kit (QIAamp Blood Kit; QIAGEN Inc, Valencia, Calif). Exons 1α, 1β, 2, and 3 of CDKN2A and exon 2 of CDK4 were amplified using previously published primer pairs.42 In a 10-μl reaction volume, the final PCR mixture contained 10-mmol/L Tris-hydrochloride at a pH of 8.0, 50-mmol/L potassium chloride, 1.5-mmol/L magnesium chloride, 0.001% (wt/vol) gelatin, 0.5 U of Taq polymerase (Fisher Scientific, Pittsburgh, Pa), 1850 Bq (0.05 µCi) of [α-32P]-labeled dCTP (NEN Life Science Products, Boston), 1-mmol/L forward primer, 1-mmol/L reverse primer, 5% dimethyl sulfoxide (for CDKN2A exons 1β, 2, and 3) or 10% dimethyl sulfoxide (for CDKN2A exon 1α), and 100 ng of DNA from the patient. The samples were denatured at 95°C for 5 minutes, annealed for 30 seconds using a touchdown protocol (62°C×2 cycles, 60°C×2 cycles, 59°C×2 cycles, 58°C×3 cycles, 57°C×3 cycles, 56°C×3 cycles, and 55°C×15 cycles), and extended at 72°C for 30 seconds, with a final primer extension at 72°C for 10 minutes. The reactions were stopped with 4 volumes of stop buffer (95% formamide, 20-mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol).

Samples were denatured at 95°C for 5 minutes, chilled on ice immediately for 5 minutes, and loaded directly onto an 0.5X mutation detection enhancement gel (FMC BioProducts, Rockland, Me) in 0.6X Tris-borate-EDTA buffer. Five microliters of the eluate DNA was used as a template for secondary unlabeled PCR reactions carried out using the conditions described above. The unlabeled fragments were subjected to electrophoresis at 4 W overnight at room temperature. After electrophoresis, the gels were dried and exposed to autoradiographic film without a screen for 12 to 24 hours.

DNA SEQUENCING

Fragments demonstrating altered migration were directly excised from the SSCP gels after alignment with the autoradiograph and directly eluted in 50 µL of water at 37°C overnight. Five microliters of the eluate DNA was used as a template for secondary unlabeled PCR reactions carried out using the conditions described above. The unlabeled fragments were subjected to agarose gel electrophoresis, excised from the gel, and purified using a gel extraction kit (QIAquick Gel Extraction Kit; QIAGEN Inc). Direct sequencing was performed using a commercially available sequencing kit (AmpliCycle Sequencing Kit; Perkin-Elmer Corp, Foster City, Calif) according to the manufacturer’s directions.

RESULTS

PATIENT CHARACTERISTICS

The features of our early-onset population are listed in Table 1. These 49 patients (20 men and 29 women) had a mean age of 30.6 years (range, 16-39 years) at diagnosis and a median age of 32 years at diagnosis. The mean and median ages at diagnosis for the entire pool of eligible patients (N=172) were 30.6 years and 31 years, re-
spectively, suggesting that there was no age bias in enrollment. However, since the family histories of patients who declined participation could not be verified, we cannot eliminate the possibility of enrollment bias based on familial melanoma. Two patients (4%) were younger than 20 years and 22 patients (45%) were between the ages of 20 and 29 years at the time of diagnosis. We thus had a representation of very young patients, as nearly half of our population was younger than 30 years at the onset of their disease. Although patients with melanoma were selected without consideration of family history, we did document the presence of cancer in the families. Overall, 17 patients (35%) acknowledged awareness of another relative with cancer. Fourteen patients described another relative with melanoma; 2 patients reported that they had a relative with pancreatic cancer; and 1 patient had a relative with Hodgkin disease. One of our patients had a metastatic melanoma with an unknown primary site, and 2 of the patients had multiple melanomas. The majority of patients (46/49 [94%]) had a single primary cutaneous melanoma.

**MUTATIONAL ANALYSES**

Using PCR-SSCP analysis, we detected an aberrantly migrating fragment in the 5’-end (Figure, top) of exon 2. Direct sequencing of the SSCP variant in Figure 1, top, revealed a G-to-C transversion in the third position of codon 53, causing a methionine-to-isoleucine missense mutation (Figure, bottom). The patient, a woman who was diagnosed as having a melanoma at the age of 32 years, had numerous atypical moles on physical examination and a strong family history of melanoma (father, sister, uncle, 3 aunts, and 2 cousins).

In addition to the inactivating mutation, we detected several polymorphisms. Direct sequencing of an SSCP migration shift in the 3’-end of CDKN2A exon 2 revealed the previously reported Ala 148 Thr polymorphism. Further- more, SSCP analysis of the CDKN2A 3’-untranslated region revealed a G/C polymorphism (+29 base pairs after the stop codon), which was shared by 13 of the 49 patients. The minor CCG (570/G) variant was present in 27% of our early-onset patients and 23% of a normal, healthy control population (9 of 43 patients [data not shown]). The mean age at diagnosis was 30.6 years for the CCG variant (570/C) and 30.8 years for the 570/G variant.

No mutations were detected in the p14ARF-specific exon 1β and exon 2 of CDK4 (data not shown). **Table 2** summarizes our findings.

**Table 1. Characteristics of 49 Study Patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (41)</td>
</tr>
<tr>
<td>Female</td>
<td>29 (59)</td>
</tr>
<tr>
<td>Age at diagnosis, y*</td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>2 (4)</td>
</tr>
<tr>
<td>20-29</td>
<td>22 (45)</td>
</tr>
<tr>
<td>30-39</td>
<td>25 (51)</td>
</tr>
<tr>
<td>Family history of cancer</td>
<td>17 (35)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>14 (29)</td>
</tr>
<tr>
<td>1 First-degree relative</td>
<td>9 (19)</td>
</tr>
<tr>
<td>1 Non-first degree relative</td>
<td>3 (6)</td>
</tr>
<tr>
<td>&gt;2 Relatives (first and non-first degree)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Hodgkin disease</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Primary melanoma</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>46 (94)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Multiple</td>
<td>2 (4)</td>
</tr>
</tbody>
</table>

*Median age, 32 years; mean age, 30.6 years.

**Table 2. Summary of Findings**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
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<tbody>
<tr>
<td>Mutations</td>
<td></td>
</tr>
<tr>
<td>Met 53 Ile</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Polymorphisms</td>
<td></td>
</tr>
<tr>
<td>Ala 148 Thr</td>
<td>1 (2)</td>
</tr>
<tr>
<td>570/G; 570/C Patients</td>
<td>13 (27); 36 (73)</td>
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<tr>
<td>Healthy controls</td>
<td>9/43 (21); 34/43 (79)</td>
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<tr>
<td>Mean age at diagnosis, y</td>
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<tr>
<td>570/G</td>
<td>30.8</td>
</tr>
<tr>
<td>570/C</td>
<td>30.6</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of 49 Study Patients

Table 2. Summary of Findings

In large clinics, where most patients with melanoma are seen, the family history is often inaccurate or unobtainable and other surrogate markers, such as early onset of...
The incidence of cancer is a common feature of genetic cancer syndromes, and germline breast and colon cancer have an increased risk of harboring mutations in other cancers, patients with early-onset colon cancer usually develop melanoma at an earlier age. Second, in other cancers, patients with early-onset colon cancer and breast cancer have an increased risk of harboring germline CDKN2A mutations. This finding was somewhat unexpected, for several reasons. First, early onset of cancer is a common feature of genetic cancer syndromes, and patients with melanoma who have a known increased incidence of CDKN2A alterations (eg, patients with a family history and patients with multiple primary melanomas) usually develop melanoma at an earlier age. Second, in other cancers, patients with early-onset colon cancer and breast cancer have an increased risk of harboring germline hMSH2 and hMLH1 and BRCA1 mutations, respectively.

We detected 1 female patient with a germline Met 53 Ile alteration among the 49 patients in our clinic-based population. The Met 53 Ile mutation has been previously described in various kindreds worldwide and has been shown to inactivate the function of p16. The strong family history in our patient (9 melanomas in the kindred) is consistent with a heritable predisposition. Of note, we detected no mutations among the 12 familial cases with a single other affected member. Similar findings of low CDKN2A mutation rates among families with only 2 affected individuals have been reported by others. These results support the notion that many familial cases represent clusters of sporadic melanomas among high-risk phenotypes or less penetrant susceptibility genes.

Two polymorphisms were detected among our patients. The 570/G polymorphism was not overrepresented in our study population compared with a control population, nor does it confer a risk for earlier age at onset (30.8 years vs 30.6 years). The Ala 148 Thr polymorphism has been previously described and shown to have no effect on p16 function.

Several possibilities exist to account for the low prevalence. The mechanism of melanoma induction is likely to be distinct from other hereditary cancers. Since strong epidemiological data implicate sun exposure in the pathogenesis of cutaneous melanoma, the genetic impact of a melanoma-predisposing mutation may be more susceptible to environmental modulation than breast or colon cancer. Alternatively, other less penetrant melanoma-susceptibility genes may contribute to early disease onset in addition to CDKN2A. Finally, our strategy will detect point mutations and overlapping biallelic loss. However, we cannot exclude monoallelic loss, nonoverlapping deletions, and hypermethylation as less common mechanisms of CDKN2A inactivation.

The frequency of CDKN2A mutations in the general population is presumed to be low. In an SSCP screen of germline DNA samples from 309 Montreal Canadians and Scottish controls, Sun et al found no mutations in CDKN2A exon 2, the predominant exon that is affected in melanoma (95% confidence interval, 0%-1.2%). Likewise, Aitken et al found no mutations in 200 unrelated control subjects from Queensland, Australia. Our study population departs from the general population, since the MGH Melanoma Clinic is a referral center for patients with melanoma and high-risk individuals with atypical moles or a family history of melanoma. Furthermore, those patients who opted to participate in a genetic investigation may have been prompted by family history. Since both of these potential sources of bias would inflate the observed prevalence, the true prevalence of CDKN2A mutations among young patients with melanoma in the general population may be even lower than 2%.

Whiteman et al analyzed the germline CDKN2A status of patients who developed melanoma in childhood and found 1 Leu16Pro alteration among 61 individuals. Childhood melanoma (before 12-15 years of age), however, is a rare entity that can be distinguished from early-onset melanoma. Up to 40% of childhood melanomas develop in giant congenital melanocytic nevi that may represent an altered hamartomatous substrate. In fact, Carr and Mackie demonstrated NRAS mutations in congenital nevus without melanoma, suggesting that these melanocytic hamartomas may be already genetically primed for subsequent tumor formation.

In summary, the prevalence of CDKN2A mutations among young patients with melanoma appears to be low (2%) but detectable. Other factors, such as amount of sun exposure during childhood, may influence age of melanoma onset. Currently, there is insufficient support for genetic testing of patients with melanoma based solely on early age at onset of disease.

Accepted for publication March 2, 2000.

This work was supported in part by an American Cancer Society Institutional Research Grant (Dr Haluska) and by the Marion Gardner Jackson Trust, a research fellowship from Warner Wellcome through the Dermatology Foundation, and a Clinical Research Training Grant from the American Cancer Society (Dr Tsao).


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