A Search for CDKN2A/p16INK4a Mutations in Melanocytic Nevi From Patients With Melanoma and Spouse Controls by Use of Laser-Captured Microdissection

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Objective: To determine the frequency at which the CDKN2A coding region is mutated in the atypical nevi of persons with sporadic melanoma.

Design: DNA samples, isolated by laser-captured microdissection of atypical nevi from 10 patients with newly incident cases of sporadic melanoma and their spouses as matched controls, were used as templates for nested polymerase chain reaction amplification of CDKN2A exons 1 and 2.

Results: No point mutations in the coding region of CDKN2A were observed in any of the melanocytic nevi.

Conclusions: Point mutations in CDKN2A are an uncommon event in the atypical nevi of persons with melanoma. As such, the data may support a hypothesis of melanocytic nevus histogenesis, in which the melanocytic nevus and malignant melanoma represent separate, pleiotropic pathways resulting from common stimuli, such as genomic damage from UV radiation.

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CONCEPTUAL MODELS OF melanoma development and progression incorporate common and atypical (dysplastic) nevi as potential stages in the evolution of the malignant phenotype in melanocytic systems.1 Support for this hypothesis can be found, among other lines of evidence, in the spatial coexistence of melanoma and nevi at both the clinical and histological levels and in the strong statistical correlation of increasing numbers and sizes of nevi with increasing relative risk for melanoma.2

A prediction of this widely held model system is that melanocytic nevi could share certain genetic alterations that are crucial to the initiation and/or evolution of malignant melanoma. The major melanoma gene thus far identified is CDKN2A, which is also known as p16INK4a. Point mutations or deletions at that 9p21 chromosomal locus are putatively initiating events in the transformation of melanocytic cells, and segregating p16 germline mutations account for up to 40% of all cases of familial melanoma.2 At present, there is no consensus as to whether melanocytic nevi, atypical or otherwise, sustain similar genetic alterations at a frequency sufficient to reflect a function as a significant factor in melanoma progression. For example, p16 protein and messenger RNA are expressed at seemingly normal levels in atypical as well as basal nevi, but at significantly reduced levels in many melanomas, including the earliest recognizable stage, melanoma in situ.3-5 Moreover, the phenotype of multiple and/or enlarged nevi does not genetically segregate with, nor readily link by polymorphic markers to, the CDKN2A locus.6,7 In addition, chromosomal loss, point mutations, and promoter methylation vicinal to the CDKN2A locus were not found in melanocytic nevi,8 nor were homozygous deletions or point mutations observed in dysplastic nevi.9 On the other hand, others have presented evidence that in atypical nevi, as in melanoma, there may be point mutations10 and hemizygous or homozygous allelic deletions at or near CDKN2A.11-14 Also, atypical nevi and melanoma express DNA mismatch repair genes at a lower level15 and have greater microsatellite instability at markers near CDKN2A than do common nevi.16

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See also pages 165, 193, and 225

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Financial Disclosure: None.
In the present study, we isolated the DNA of the most atypical nevi from a group of melanoma cases by laser-capture microdissection and screened the coding region (exons 1 and 2) of CDKN2A for point mutations. This study, which was a component of a larger case-control study of melanocytic nevus attributes in patients with melanoma, used spouses as matched controls. In doing so, we further tested the hypothesis that patients with melanoma, used spouses as matched controls. The patient population for entry into the study was approved by the University of Washington (Seattle) institutional review board. The patient population for entry into the study consisted of persons 18 years or older recently diagnosed as having melanoma, who were referred to the University of Washington Cancer Center for treatment and who had spouses willing to participate in the study. Complete skin examination identified the largest and most atypical nevus with a macular component for biopsy sampling from each case and control; although the most atypical nevus was selected, in some instances the lesions were not especially atypical. Ten case/control pairs were randomly culled from the source database of 87 matched pairs for the present analysis. The histological ratings of these nevi for microscopical criteria of melanocytic dysplasia are given in the Table.

The biopsy tissues were fixed in formalin and embedded in paraffin. Tissue sections of 10 µm were mounted on glass slides with polyethylene naphthalate (PEN) membranes (Carl Zeiss Inc, Thornwood, NY). The PEN membranes were attached to slides wetted with 70% ethanol and were fixed in place using Fixogum rubber cement. Before staining, the mounted sections were dried overnight at 40°C. The paraffin was removed with xylene, and the sections were stained with hematoxylin using standard methods in preparation for laser-capture microdissection.

**LASER-CAPTURED MICRODISSECTION AND DNA EXTRACTION**

The PALM (positioning and ablation with the laser microbeam) system (PALM Microlaser AG, Bernried, Germany) was used for microdissection of nevus-specific tissues. The dissected tissue fragments, which consisted of approximately 500 nuclei, were collected in 0.5-mL Eppendorf microfuge tubes. Then, 50 µL of 2 × magnesium-free polymerase chain reaction (PCR) buffer with 0.04% proteinase K was added to each tube. The samples were incubated at 37°C overnight. Heating the samples at 95°C for 6 minutes denatured the proteinase K. The samples were then kept at 4°C until used for PCR analyses.

**PCR ANALYSES OF p16 ALLELES**

Nested PCR was used to amplify exons 1 and 2 of the p16 gene. Primers HW1F, HW1R, HW2F, and HW2R were designed based on the published DNA sequence (GenBank Accession Nos. U12818 and U12819 for exons 1 and 2, respectively; National Center for Biotechnology Information [NCBI], Bethesda, Md). Primers X59F, X284R, X78F, and X482R were previously described. The following primers were used in the first and second round of PCR reactions to amplify exon 1:

**First round:**
- HW1F (5’ GTGGCTGCTTACACAGGAGTTG 3’)
- HW1R (5’ CCAATTTCTCGCAAACTTGC 3’)

**Second round:**
- X59F (5’ CGGCTGCTGAGGGAGGAGG 3’)
- X284R (5’ CTCAGAGCTCCTGACCAGTAC 3’)

The following primers were used in the first and second round of PCR reactions to amplify exon 2:

**First round:**
- X78F (5’ GGGCTGCTTACACAGGAGTTG 3’)
- HW2R (5’ TCTGTTGCTGGAATGAATGCTC 3’)

**Second round:**
- HW2F (5’ CCAATTTCTCGCAAACTTGC 3’)
- X482R (5’ TTTGAGCTCCTGACGAGTACA 3’)

The locations of all primers in the CDKN2A gene are shown in Figure 1. We did not analyze exon 3 because it encodes only 3 amino acids at the carboxyl terminus. The Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, Ind) was used for all PCR reactions. Using 10 µL of each sample from the laser-capture microdissection, the PCR reactions were carried out with 3 cycles at 95°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute, followed by 40 cycles at 95°C for 30 seconds, 64°C for 30 seconds, and 72°C for 45 seconds. The reaction mixture from the first round of PCR was purified with the Microcon spin columns (Millipore, Burlington, Mass). One tenth of the purified product was used for the second round reaction.

### Table. Histological Classification of the Melanocytic Nevi Analyzed for CDKN2A Genotype*

<table>
<thead>
<tr>
<th>Numerical Code</th>
<th>Status</th>
<th>Average Histological Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>834</td>
<td>Case</td>
<td>0.15</td>
</tr>
<tr>
<td>132</td>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>614</td>
<td>Case</td>
<td>NA‡</td>
</tr>
<tr>
<td>425</td>
<td>Control</td>
<td>NA‡</td>
</tr>
<tr>
<td>853</td>
<td>Case</td>
<td>0.92</td>
</tr>
<tr>
<td>538</td>
<td>Control</td>
<td>1.0</td>
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<tr>
<td>766</td>
<td>Case</td>
<td>0.15</td>
</tr>
<tr>
<td>022</td>
<td>Control</td>
<td>0.31</td>
</tr>
<tr>
<td>960</td>
<td>Case</td>
<td>1.23</td>
</tr>
<tr>
<td>091</td>
<td>Control</td>
<td>0.54</td>
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<tr>
<td>522</td>
<td>Case</td>
<td>0.15</td>
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<td>493</td>
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<tr>
<td>761</td>
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<td>Case</td>
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<tr>
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<tr>
<td>021</td>
<td>Control</td>
<td>0.61</td>
</tr>
</tbody>
</table>

*The histological slides were reviewed without knowledge of clinical case-control status by a 13-member panel of dermatopathologists from the North American Melanoma Pathology Study Group. Each slide was independently assigned a numerical value by the 13 dermatopathologists according to a 4-category scale, with 0 = no dysplasia, 1 = mild dysplasia, 2 = moderate dysplasia, 3 = severe dysplasia, and 4 = melanoma. The ratings for each lesion were averaged across the 13 reviewers in this column. Review by the 13-member dermatopathology panel was not available (NA‡) for these slides. One of us (M.P.) rated both lesions as mildly dysplastic nevi.

**METHODS**

### PREPARATION OF PARAFFIN-EMBEDDED TISSUE SECTIONS

Melanocytic nevi were collected from a series of patients with melanoma and matched spouse controls under a protocol approved by the University of Washington (Seattle) institutional review board. The patient population for entry into the study consisted of persons 18 years or older recently diagnosed as having melanoma, who were referred to the University of Washington Cancer Center for treatment and who had spouses willing to participate in the study. Complete skin examination identified the largest and most atypical nevus with a macular component for biopsy sampling from each case and control; although the most atypical nevus was selected, in some instances the lesions were not especially atypical. Ten case/
SEQUENCING OF PCR PRODUCTS

The reaction mixtures were purified with the Microcon columns and examined by 2% agarose gel electrophoresis prior to the sequencing reactions. Once the presence of the PCR products was confirmed by gel electrophoresis, the PCR products were labeled using the Big Dye Sequencing Kit (Applied Biosystems, Foster City, Calif) for sequencing. The PCR products were sequenced from both 5' and 3' ends to ensure the fidelity of the sequence data.

RESULTS

The nuclei for mutational analysis of the p16 gene were obtained from the cells of melanocytic nevi by laser-captured microdissection, as described in the “Methods” section. Figure 2 depicts the histological appearance of a nevus before and after microdissection of its nuclei. Exons 1 and 2 and neighboring intronic regions of the amplified gene from each case and control sample were amplified using pairs of forward and reverse primers via a nested approach (Figure 1). An example of one experiment using laser-captured nevi is shown in Figure 3. All PCR pairs used for first and second (nested) PCR studies gave amplification products of the predicted size (data not shown).

The sequences for exons 1 and 2 obtained from nested PCR amplification of the 10 pairs of melanoma cases and spouse controls were aligned with the published genomic sequences of p16 (see “Methods” section) by use of the NCBI Blast program. No sequence mutations were identified in any of the atypical nevi or normal control tissue samples.

COMMENT

The failure to find any CDKN2A coding sequence mutations within this series of 20 atypical nevi indicates that point mutations or small deletions must be quite uncommon in the melanocytic cells of those lesions (ie, <5% prevalence). A caveat is that the study design would not have detected large hemizygous deletions involving one of the CDKN2A alleles, if such genetic alterations had been present. Nonetheless, our data suggest that mutations in genes other than CDKN2A are more centrally involved in the genesis of common nevi and their atypical variants.

Although atypical (dysplastic) nevi are much more prevalent than melanomas, and they are in most instances stable lesions, it is nevertheless thought that they may occasionally progress to melanoma.1,2,18 This hypothesis derives from such evidence as serial photographic examination of individual nevi and from the frequent spatial proximity of nevus remnants and melanoma within histological sections.1,19 Remnants of nevi in sections of melanoma are reportedly found at a frequency of approximately 10% to greater than 30%, and the association is not a random event, suggesting a precursor-product relationship.20 In a study of thin melanomas, histological evidence of an associated nevus was found in 51% of cases, and of these, 56% were atypical nevi and 41% were banal nevi.21

The hypothesis that atypical nevi are potential precursors to melanoma predicts that nevi could incur genetic alterations essential to the initiation and/or progression of melanoma. In some studies of DNA ploidy, the atypical nevus cells more frequently contained hyperdiploid DNA content than did banal nevi,22,23 but they were not characterized by the aneuploidy that is typical of melanoma.23 Other studies have failed to find abnormal DNA content in atypical nevi.24,25 In another experimental strategy, the tumor antigen profile of atypical nevus cells was determined to be intermediate between that of common nevus cells and melanoma, such that the expression levels of the tumor-related antigens, epidermal growth factor and nerve growth factor receptors, increased progressively from me-

Figure 1. Forward and reverse primers for amplification of exonic 1 and 2 sequences of the CDKN2A gene from the melanoma case and control nevi.

Figure 2. Histological appearance of a nevus before (A) and after (B) microdissection of its nuclei.
Figure 3. Exonic 1 and 2 (E1 and E2) fragments from the nested polymerase chain reactions. MW indicates molecular weight. Code numbers 2498493 and 2598404 corresponding to lanes 3, 6 and 4, 7, respectively, refer to the amplified exonic fragments obtained from samples of 2 separate nevus specimens.

lanocytes, banal nevus cells, atypical nevus cells, radial growth phase melanoma, vertical growth phase melanoma, and metastatic melanoma.26-28

Taken together with other lines of evidence3,6-9 the present data support the hypothesis that acquired or germ-line sequence variations in the coding or enhancing/promoting regions of the CDKN2A (p16) gene are not the major genetic determinants of the atypical nevus phenotype. This is not to say, however, that nevi do not share in genetic lesions at loci involved more distally in tumor progression. In this regard, recent interest has come to be focused on BRAF, a proto-oncogene functioning in the ras pathway of cell proliferation control. Initial and confirmatory reports have described activating mutations in the coding region of that gene within melanocytic nevi, as well as within melanoma cells, at a sufficiently high frequency to indicate a common role in melanocytic tumor progression.27-30 Kumar et al30 also reported activating ras mutations in several nevi, some in association with BRAF mutations, further implicating the ras/BRAF pathway in early genetic events that lead to melanoma. It is reasonable to expect that future molecular studies will identify additional genetic elements targeted for activating or deactivating mutations in both nevus and melanoma cells that could reflect shared pathways in oncogenic development, thus providing further insight into the molecular mechanisms of transformation and progression in melanocytic nevi.

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