Novel Mutations in the p16/CDKN2A Binding Region of the Cyclin-dependent
Kinase-4 Gene

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INTRODUCTION

A critical juncture in the regulation of the cell cycle occurs at the G₁-to-S transition (1–3). D-type cyclins and their kinase partners, CDK4 and CDK6, coordinately phosphorylate the Rb³ protein, thereby releasing the E2F restriction at G₁ and allowing for expression of S-phase genes and progression into the S phase (3). This sequence of events is regulated by a class of protein kinase inhibitors known as CDKIs (4). Most prominent among these inhibitors and the most relevant to human tumor suppression is p16/CDKN2A (5, 6). Originally identified through positional techniques applied to cell lines derived from sporadic tumors, p16/CDKN2A germ-line mutations have been found in the chromosome 9p21-linked melanoma-prone kindreds (7). Subsequent studies have shown that p16/CDKN2A is inherited cancer syndromes and sporadic tumors.

A second gene on the Rb pathway that has been implicated in the pathogenesis of various tumors encodes for the cyclin-dependent protein kinase inhibited by p16/CDKN2A, CDK4. At least two mechanisms have been described that interfere with the normal relationship between p16/CDKN2A and CDK4: alterations in the functionally critical exon 2 of CDK4: a lysine-to-glutamine substitution at codon 22 and the arginine-to-histidine mutation at codon 24. These findings document several novel changes in the p16-binding region of CDK4.

MATERIALS AND METHODS

DNA Material. Melanoma tumor cell lines (29) and DNA from cultured metastatic cutaneous melanomas and primary uveal melanomas (30) have been described previously. Primary invasive colon cancers were obtained from surgical specimens catalogued in the MGH Tissue Bank. Specimens were processed based on availability and not segregated by patient or tumor characteristics. The colon cancer cell lines tested included SW48, SW403, SW480, SW404, LS174T, WiDr, HT-29, and SK-CO-1 and were obtained from the American Type Culture Collection. The SK-N-SH neuroblastoma cell line was also obtained from the American Type Culture Collection; other neuroblastoma cell lines have been described previously (31).

Germ-line DNA from melanoma patients were obtained through the Pigmented Lesion Clinic at the MGH as described previously (19). These individuals had at least one first degree or second degree relative with biopsy-proven melanoma.

SSCP Analyses. High molecular weight DNA was extracted from tissue culture samples using standard phenol chloroform extraction protocols. DNA was subject to SSCP analysis for the detection of sequence alterations. Genomic primers for the second exon of the human CDK4 gene have been published previously (18): 2AF, GCTGAGGTCTACATCATCT; 2AR, CTCTAACACCTTGAGGC primers (40 pmol) were labeled using T4 polynucleotide kinase (New England Biolabs) and γ-³²P]ATP (Dupont/New England Nuclear) for 30 min at 30°C. PCR reactions were performed using...
end-labeled primers under standard conditions (1 - 0.1 M cold primer/0.09 M 32P-labeled primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.001% gelatin, and 200 pM deoxyoligonucleotide triphosphate); 10-μl reactions were performed in an MJ thermocycler: 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s (condition 1). Upon completion of the reactions, 25 μl of stop solution (95% formamide, 10 mM EDTA) was added. The reaction was heated to 95°C for 5 min, cooled immediately on ice for 5 min, and then allowed to come to room temperature. For analysis, 3 μl of each sample were loaded on an 0.5% MDE (FMC Bioproducts)/5% glycerol gel and subjected to 6.0 W at room temperature for approximately 12 h. Gels were then dried and exposed to X-ray film at room temperature or −80°C for 2-12 h.

Detection of homozygous deletions in the p16/CDKN2A gene was performed by PCR analysis of genomic DNA using primers described previously (7). A modified touchdown protocol was used for amplifying exons from the p16/CDKN2A gene: 94°C for 30 s; 60°C for 30 s; 72°C for 45 s; repeat heated to 95°C for 5 min, cooled immediately on ice for 5 min, and then

Table 1 Summary of CDK4 mutations from melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Alteration</th>
<th>Predicted effect</th>
<th>p16/CDKN2A Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-Mel 39</td>
<td>A 811 C</td>
<td>Lys 22 Gin</td>
<td>Wild type</td>
</tr>
<tr>
<td>SK-Mel 28</td>
<td>C 817 T</td>
<td>Arg 24 Cys</td>
<td>Wild type</td>
</tr>
<tr>
<td>SK-Mel 37</td>
<td>G 818 A</td>
<td>Arg 24 His</td>
<td>Wild type</td>
</tr>
<tr>
<td>MGH-BA-1</td>
<td>C 816 T</td>
<td>Polymorphism</td>
<td>Deleted</td>
</tr>
<tr>
<td>MGH-PO-1</td>
<td>G 818 A</td>
<td>Arg 24 Cys</td>
<td>Deleted</td>
</tr>
</tbody>
</table>

* Flores et al. (32) and this study.

By direct sequencing, SK-Mel 39 (pattern B) reveals a novel codon 22 mutation. At position 1 of codon 22, there is an A-to-C transition in one of the strands causing a consequent lysine to glutamine mutation (Fig. 2A, wild-type; Fig. 2B, mutant). This alteration has been demonstrated in only one of our lines.

We detected several alterations at codon 24. The wild-type sequence is shown in Fig. 3. SK-Mel 28 harbors a C-to-T transition (G-to-A substitution on the sequenced reverse strand) at position 1, leading to a heterozygous arginine-to-cysteine mutation. SK-Mel 37 (Fig. 3) contains a G-to-A mutation (C-to-T on sequenced reverse strand) at position 2 of codon 24, leading to a homozygous arginine-to-histidine change. The SSCP pattern for MGH-PO-1 (pattern E) is distinct from the pattern for SK-Mel 37 (pattern D), although certain shifted bands are shared. MGH-PO-1 contains G-to-A and T-to-A changes (C-to-T and A-to-T on reverse strand) at positions 2 and 3 of codon 24, respectively. The C-to-T transition at position 2 (Arg24His) on one strand most likely reflects the shared bands seen on SSCP between MGH-PO-1 and SK-Mel 37 (Fig. 1). A second A-to-T change at position 3 of codon 24 is a silent polymorphism that results in no amino acid alteration. Finally, on direct sequencing, MGH-BA-1 is due to a double CC-to-TT mutation that occurs at position 3 of codon 23 and position 1 of codon 24 (Fig. 4). These changes lead to a silent polymorphism at codon 23 and an arginine-to-cysteine change at codon 24.

**RESULTS**

**CDK4 Status in Neuroblastomas, Colon Cancers, and Paired Melanoma Samples.** In total, we examined 20 neuroblastoma cell lines, 8 colon cancer cell lines, 20 primary colon cancer samples, 17 cutaneous melanoma samples, and 23 uveal melanoma samples for mutations by SSCP analysis (data not shown). No mutations were detected in any of these samples.

**CDK4 Status in Melanoma Cell Lines.** We examined a total of 31 melanoma cell lines. Twenty-one of these lines are shown in Fig. 1. By SSCP, five distinct patterns, labeled A, B, C, D, and E, were observed in six cell lines. Shifts were confirmed on the reverse-strand SSCP (data not shown). The lines exhibiting SSCP shifts are MGH-BA-1 (pattern A, Lane 1), SK-Mel 39 (pattern B, Lane 3), SK-Mel 28 (pattern C, Lane 5), SK-Mel 37 (pattern D, Lane 14), and MGH-PO-1 (pattern E, Lane 17). Not shown in Fig. 1 is SK-Mel 29, which exhibits pattern C and is known to harbor the Arg24Cys mutation (17). A listing of all of the mutations detected is presented in Table 1.

![Fig. 1. PCR-SSCP analysis of various melanoma cell lines.

A. MGH-BA-1; B. SK-Mel 39; C. SK-Mel 28; D. SK-Mel 37; and E. MGH-PO-1. Previously published cell lines include Lane 3 (SK Mel 39); Lane 4 (SK Mel 131); Lane 5 (SK Mel 28); Lane 7 (SK Mel 63); Lane 14 (SK Mel 37); Lane 16 (SK Mel 30); and Lane 19 (SK Mel 119).](image-url)
DISCUSSION

The Rb pathway has been shown to be frequently altered in human melanoma (17, 24, 32). Functional studies by Wolfel et al. (17) and Bartkova et al. (24) have established that the sensitivity of CDK4 to p16/CDKN2A inhibition is critically dependent upon an intact codon 24 in exon 2 of CDK4. However, to date, only two mutations in this region, an arginine-to-cysteine missense mutation at codon 24 (17) and an arginine-to-histidine mutation in the germ line of one French melanoma-prone kindred (25), have been reported. We have confirmed the Arg24His mutation in our melanoma cell lines and have also identified an additional mutation from this region: a lysine-to-glutamine missense mutation at codon 22. Three melanoma lines (SK-Mel 28, SK-Mel 37, and MGH-PO-1) contained mutations resulting from C-to-T transitions, whereas another (MGH-BA-1) revealed a CC-to-IT transition; these alterations are all suggestive of genetic insult from UV irradiation (33), a known risk factor for the development of melanoma (34). However, we cannot eliminate the possibility that these changes are present in the germ line and are not the result of somatic injury because we did not have access to germ-line DNA of patients from whom the melanoma cell lines were derived.

Among our melanoma cell lines, we detected five distinct alterations within this functionally critical region of CDK4. This incidence of CDK4 mutations is higher than has been reported previously and, however, their technique was based on allele-specific cleavage of the Arg24Cys mutation using digestion with SstI. We reanalyzed the samples using the SSCP analysis and also found no evidence of the Lys22Gln, Arg24Cys, or Arg24His mutations.

Fig. 2. Alteration at codon 22. Sequence analysis of SK-Mel 39 showing normal sequence (A) and an A-to-C change at the first position of codon 22 leading to a lysine-to-glutamine transition (B).

Fig. 3. Alterations at codon 24. Upper panel: NORMAL sequence of reverse strand; SKMEL28, a C-to-T mutation on the coding strand (0-to-A on sequenced reverse strand) leads to the previously described Arg24Cys mutation Lower panel: SKMEL37, a G-to-A transition (C-to-T on sequenced reverse strand) found at second position of codon 24 leads to an Arg24His missense mutation; MGH-PO-1, a similar Arg24His mutation and a T-to-A change (A-to-T on sequenced reverse strand) produces a polymorphism.
in particular, is higher than the incidence of mutations in primary tumors. We were unable to assess for the presence of these described mutations in the original metastases used to produce our cell lines; thus, we cannot address the question of whether these mutations were present in the patients' melanomas or arose in vitro. However, several lines of evidence suggest that this region of CDK4 is involved in melanoma tumor formation in vivo. The Arg24Cys mutation can be transmitted as a dominant oncogene in some chromosome 1p36-linked families (18); furthermore, preliminary evidence from Bres-sac-de Paillerets et al. (25) suggest that the Arg24His mutation is also heritable. Finally, Wolfel et al. (17) have demonstrated the Arg24Cys mutation in both the SK-Mel 29 line and in one uncultured melanoma tumor extracted from a paraffin-embedded sample. Thus, there is ample precedent supporting the involvement of CDK4 mutations in melanoma formation in vivo. The higher than reported incidence of mutations in our cell line samples may be explained if an advantage in in vitro cell line establishment or propagation is conferred by the mutated allele.

It is reasonable to assume that an Arg24His transition may also affect the sensitivity of CDK4 to p16/CDKN2A inhibition. In this setting, Arg24His would then be a dominant oncogene like Arg24Cys. A group from France has found an Arg24His germ-line mutation in a single French melanoma kindred (25). The details of this study have not been published. Our patients were obtained through the Pigmented Lesion Clinic at the MGH as described previously (19). All patients had a first or second degree relative with a biopsy-proven melanoma. Although we did not detect any Arg24His or Lys22Gln mutations in our patients, our population contains both inherited cases from dominantly transmitted cancer families and sporadic cases with a familial-associated high-risk phenotype (such as fair-skin) and/or family-oriented high-risk behaviors (such as concomitant exposure on vacations).

Given the rarity of CDK4 mutations reported in the literature, very little is known about the functional implications of these changes. Using in vitro site-directed mutagenesis, Coleman et al. (35) analyzed the critical CDK4 residues involved in p16/CDKN2A binding. Their results show that disruption of either codon 22 or codon 24 effectively abrogates interaction with cyclin D1 and p16/CDKN2A. In vivo, patients with the germ-line Arg24Cys mutation have no documented changes in p16/CDKN2A (18). Furthermore, in SK-Mel 29, p16/CDKN2A is unaltered (24). This pattern has led to speculation (24) that alteration in either p16/CDKN2A (through missense mutations, deletions, or hypermethylation) or the p16-binding domain of CDK4 has a similar functional consequence in the pathogenesis of malignancy. In our study, we have identified two cell lines that carry concurrent mutations in codon 24 of CDK4 and deletions of p16/CDKN2A. This finding is unexpected if the sole result of an abnormality in either of these proteins is abrogation of CDK4 inhibition; thus, the results raise several other possibilities: (a) in prior studies and in our lines, mutated CDK4 appears to function as an oncogene; only a single allele is abnormal. Loss of p16/CDKN2A in a cell with a single mutated CDK4 allele might release inhibition of the protein coded by the second, normal CDK4 allele; (b) p16/CDKN2A loss is likely to have several effects aside from interference with CDK4 inhibition. p16/CDKN2A binds to and inhibits other proteins, including CDK6. Loss of p16/CDKN2A may confer a selective advantage through dysregulation of other such molecules; (c) the CDKN2A gene encodes a second protein, p19ARF, that is also a negative regulator of cell cycle progression, but that functions through a mechanism independent of CDK6. Deletion of the gene may offer the cell a proliferative advantage through loss of the p19ARF protein; (d) CDK4 is also inhibited by other CDKIs, including p21, p27, p15 (2, 17), p57kip2 (37), and p19 (38). Although the in vitro data suggest that the Arg24Cys mutation in CDK4 does not affect binding to p21 or p27 (17), the effect of the Arg24Cys mutation on p57kip2 and p19 binding and the effects of the Arg24His and Lys22Gln mutations on other CDK1 interactions are unknown. Mutation of CDK4 in a cell carrying a p16/CDKN2A alteration may render the kinase refractory to inhibi-

![Fig. 4. Melanoma cell line MGH-BA-1 showing mutated sequence in Lanes 1–4 and normal sequence in Lanes 5–8. A CC-to-TT mutation at position 3 of codon 23 and position 1 of codon 24 leads to the previously described Arg24Cys mutation and a polymorphism.](image-url)
by other CDKIs as well. Investigation of these hypotheses awaits further studies. However, our data suggest that some proliferative advantage exists for melanoma cell lines carrying both CDK4 and p16/CDKN2A alterations.

In conclusion, we have carried out a genetic analysis of the functionally critical exon of CDK4 in neuroblastomas, colon cancers, and melanomas. Using an SSCP approach, we have detected several novel mutations in the exon 2 domain containing the p16/CDKN2A binding region. Whether these mutations play a role in the development of melanomas in vivo remains to be seen.

REFERENCES

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16. Whether these mutations play a role in the development of melanoma cell lines carrying both CDK4 and p16/CDKN2A alterations.
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