Rarity of Somatic and Germline Mutations of the Cyclin-dependent Kinase 4 Inhibitor Gene, CDK4I, in Melanoma

Masataka Ohta, Hirokazu Nagai, Masayoshi Shimizu, Debora Rasio, David Berd, Michael Mastrangelo, Arun D. Singh, Jerry A. Shields, Carol L. Shields, Carlo M. Croce, and Kay Huebner


Abstract

Evidence from cytogenetics, multipoint linkage analyses of familial melanoma, and loss of heterozygosity studies of familial and sporadic melanomas support localization of a melanoma susceptibility or tumor suppressor gene at chromosomal region 9p21–23. Recently, the inhibitor of cyclin-dependent kinase 4 (CDK4I; also known as p16INK4A, multiple tumor suppressor 1, or CDKN2 gene) has been mapped to 9p21 and shown to be mutated or deleted in a large fraction of cell lines derived from many tumor types, including melanoma, suggesting that this gene could be a melanoma suppressor gene. In order to test for somatic mutations in the CDK4I gene in tumors, DNAs from 30 surgically resected melanomas of both cutaneous and uveal origins were sequenced. No mutations were detected in the coding region of the CDK4I gene, while mutations or deletions were detected in 60% (9 of 15) of the endured melanoma cell line DNAs. Among presumptive familial cases, nine of which were members of families with one or two other documented melanoma cases, no germline mutations were detected by sequence analysis. A deletion in the second exon of the CDK4I gene was found in one germline allele of a familial melanoma patient from a family with eight affected first degree relatives. These results not only support the suggestion that the CDK4I gene is a familial malignant melanoma gene, they also suggest the presence of another suppressor gene locus within 9p21 which is the target of loss of heterozygosity in sporadic melanomas.

Introduction

The short arm of chromosome 9 was suggested as a locus of a melanoma tumor suppressor gene by earlier studies of cytogenetics and by LOH analyses (1–6). The subchromosomal localization of this putative suppressor gene was refined to the region 9p13–22 by molecular analysis of somatic or germline loss of 9p markers in melanoma patients (7–12). Additionally, assignment of a familial cutaneous melanoma gene (MLM) to 9p21–22 has been supported by three independent multipoint linkage analyses (13–15). Taken together, these studies strongly indicated the presence of a melanoma suppressor gene in chromosome region 9p21.

Recently, the CDK4I gene was mapped to the 9p21 region (16, 17) and suggested as a good candidate melanoma tumor suppressor gene because (a) the CDK4I gene is located within a small common region of homozygous deletion in melanoma cell lines (16, 17), a deletion encompassed by a single cosmId; (b) there was a high incidence of CDK4I gene mutation among melanoma tumor cell lines (16, 17); and (c) the function of CDK4I is relevant to a proposed mechanism of tumor suppression (16–18), since the CDK4I gene negatively regulates the cell cycle through a specific inhibition of cyclin-dependent kinase 4 (18).

Thus, we have examined the status of the CDK4I gene in a series of uncultered cutaneous and uveal melanomas, as well as lymphocytes from patients with familial melanoma. Additionally, DNAs from uncultered melanomas were tested for LOH on the short arm of chromosome 9. Uveal melanomas were included in this study because chromosome 9p abnormalities occur in up to 30% of these tumors. Lymphocytes of affected members of uveal melanoma families were examined in a parallel study.

Materials and Methods

CM1–13 were sporadic melanomas except for CM2 and CM4, which were familial cases by the definition given below. All the tumor DNAs of CM1–13 were derived from metastatic tumors. UM1–17 were sporadic primary melanomas which arose in either the choroid or the cilio-choroidal region; the composition of the cells within the resected tumor was more than 90% tumor cells by pathological diagnosis. Clinical features and karyotypes of melanomas of cases UM1, UM4, UM15, and UM16 have been reported previously (19). CF1–9 were presumptive familial cases for which 1 or 2 other first degree relatives had malignant melanoma. CF10 was a case of familial cutaneous melanoma in which melanomas occurred within 8 first degree relatives. Every case of CF1–10 belongs to an independent kindred.

Cutaneous melanoma cells were separated from lymphocytes by a gradient-density centrifugation. Uveal melanoma DNA was extracted from a portion of surgically resected tumor after cutting into small pieces. DNA from both cell lines and the tumors was extracted by standard methods (20). Some of the tumor DNA samples which contained visible pigments were purified through spin columns (Qiagen) according to the manufacturer’s instructions. Lymphocyte DNA was extracted by a blood DNA extraction kit (Qiagen).

Melanoma cell lines SK-MEL1 and G361 were purchased from the American Type Culture Collection (ATCC HTB 67 and ATCC CRL 1424, respectively; Rockville, MD). Melanoma cell lines WM8, WM164, WM983A, WM1158, WM373, A375, WM239A, WM1449B, and WM793 have been described (21, 22). Melanoma cell lines COLO38, MEWO, SB1, and M21 were established in the laboratory of Dr. Soldano Ferrone.

Primers for DNA amplification and sequencing were designed from the genomic DNA sequence of a cosmid clone, B4, which was isolated by hybridization to a CDK4I complementary DNA probe (18), from cosmid subclones of a yeast artificial chromosome clone, p531.2 Exon 1 of the CDK4I gene was amplified by PCR with primers 181F (GTCCCTCCAGGATT-TGAG) and 1108R (16), with the use of 5–10 ng of genomic DNA in a reaction volume of 20 μl, under standard conditions (20), except that 5% dimethyl sulfoxide was added, with denaturation of 95°C (5 min) followed by 20 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s. One μl of the product was subjected to a second round of PCR amplification by primers 2F (16) and 5R (16).

Received 8/12/94; accepted 9/1/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grants CA39860, CA51083, and CA39248 (D. B.).

2 To whom requests for reprints should be addressed, at Jefferson Cancer Institute, Room 1006 BLSB, 233 South 10th Street, Philadelphia, PA 19107.

3 The abbreviations used are: LOH, loss of heterozygosity; MLM, locus symbol for familial malignant melanoma syndrome; CDK4I, cyclin dependent kinase 4 inhibitor gene; MTSI, multiple tumor suppressor 1 gene; PCR, polymerase chain reaction; CM1–13, cases of cutaneous melanoma; UM1–17, cases of uveal melanoma; CF1–10, cases of cutaneous melanoma.

5 Unpublished results.


Received 8/12/94; accepted 9/1/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grants CA39860, CA51083, and CA39248 (D. B.).

2 To whom requests for reprints should be addressed, at Jefferson Cancer Institute, Room 1006 BLSB, 233 South 10th Street, Philadelphia, PA 19107.

3 The abbreviations used are: LOH, loss of heterozygosity; MLM, locus symbol for familial malignant melanoma syndrome; CDK4I, cyclin dependent kinase 4 inhibitor gene; MTSI, multiple tumor suppressor 1 gene; PCR, polymerase chain reaction; CM1–13, cases of cutaneous melanoma; UM1–17, cases of uveal melanoma; CF1–10, cases of cutaneous melanoma.
1108R under the conditions noted above. The PCR products were purified by Qiagen column, and 10 μg was subjected to sequencing reactions using the DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with the use of the 2F primer for the forward reaction and the 1108R primer for the reverse reaction. Products were analyzed by an automated sequencing apparatus (Model 373A, Applied Biosystems). Exon 2 was amplified with primers 178F (CTGAAAGATGGTATGGGAATC) and 179R (CTGAACTTCTCTGTGCTCCGA) under conditions noted above except for the annealing temperature of 62°C. One μl of the product was subjected to a second round of PCR reaction by primers 42F and 551R (16), and the product was subjected to sequencing analysis as described above with the use of the 42F primer for the forward reaction and 551R primer for the reverse reaction. Exon 3 was amplified and sequenced with the use of primers 175F (CACACTCCTTGACCTCAGGT) and 164R (GGGAAGCTTATATCCTACGTT) for the forward reaction, as described above, except that the template for the sequencing reaction was amplified with a cycle of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s. The DNA sequence of all exons of CDK4I from normal lymphocyte DNA was identical to the sequence reported by Serrano et al. (18), except that the twenty-seventh codon is GTG in the original report and is GGG in our sequence, resulting in the amino acid change from valine to glycine; no sequence variations of the coding sequences were observed among the DNA sequences of 5 normal individuals. Normal sequences were compared with those from tumor DNAs by aligning sequences for each case.

To look for similar mutations in uncultured melanomas, DNA sequence analysis of all exons of CDK4I from normal lymphocyte DNA was subjected to 30–35 cycles of PCR amplification as described above with an annealing temperature of 58°C in a volume of 10 μl containing 0.5 μM primers, 15 μM deoxynucleotide triphosphates, 5% dimethyl sulfoxide, 1 mM spermidine, 0.5 units of Taq polymerase (Boehringer Mannheim), and 0.1 μCi [α-32P]dCTP. Products from matched tumor and lymphocyte DNAs were loaded side by side and separated by electrophoresis through denaturing 8-M urea-polyacrylamide gels followed by autoradiography. For informative cases, loss of allele was scored when signal for a tumor allele was at least 50% reduced relative to the matched normal allele.

Results

Exon 1 or 2 of the CDK4I gene was deleted in 4 of 15 melanoma cell lines, as determined by PCR amplification of the cell line DNA by primers specific for exons 1 and 2 (Table 1). For 12 cell lines which retained exon 2, the sequence of exon 2 was determined. Six of these cell lines had seven different types of mutations as compared with the sequence of normal lymphocyte DNA. All of these mutations had some effect on the coding frame, such as frameshift or nonsense or missense mutations (Table 1, lower panel). In the SK-MEL-1 cell line, transition of G to A at nucleotide 436 was observed but was scored as a mutation according to the sequence reported by Serrano et al. (18). The primer sequences for the microsatellite markers D9S157, D9S171, D9S161, and D9S43 were obtained from The Genome Data Base (William H. Welch Medical Library, Johns Hopkins University). For LOH studies, 10 μg of genomic DNA was subjected to 30–35 cycles of PCR amplification as described above with an annealing temperature of 58°C in a volume of 10 μl containing 0.5 μM primers, 15 μM deoxynucleotide triphosphates, 5% dimethyl sulfoxide, 1 mM spermidine, 0.5 units of Taq polymerase (Boehringer Mannheim), and 0.1 μCi [α-32P]dCTP. Products from matched tumor and lymphocyte DNAs were loaded side by side and separated by electrophoresis through denaturing 8-M urea-polyacrylamide gels followed by autoradiography. For informative cases, loss of allele was scored when signal for a tumor allele was at least 50% reduced relative to the matched normal allele.

To look for similar mutations in uncultured melanomas, DNA sequence analysis of all exons of CDK4I gene was carried out using DNA from 17 cases of uncultured uveal melanoma and 13 cases of uncultured cutaneous melanoma in parallel with normal lymphocyte DNA (UM1–17 and CM1–13; Table 1). Optimal DNA sequences of tumors were obtained in 20 of 28 cases for exon 1, 27 of 30 for exon 2, and 30 of 30 for exon 3, respectively. These sequences were aligned with the sequences from the normal lymphocyte DNA. As compared with normal lymphocyte DNA sequences, the results for the tumors revealed neither mutated sequences nor altered fluorescing signals which might suggest the presence of mutated heterozygous alleles (Table 1). Also, DNAs from lymphocytes of familial cutaneous melanoma patients of 10 independent kindreds were subjected to sequence analysis for exons 1 and 2, and no evidence of heterozygous mutation was detected except for CF10, which showed intestinal deletion of 14 base pairs within exon 2, resulting in a frameshift in one of the alleles (Table 1).

To determine whether loss of 9p loci surrounding the CDK4I gene occurred in our panel of primary tumors, the status of four 9p-linked simple sequence repeat polymorphisms was determined in the matched normal/tumor pairs. Three of 12 cases of uveal melanoma and 2 of 4 cases of cutaneous melanoma exhibited LOH for at least 1 of the markers of this region, while the others failed to show evidence of deletion in this region (Figs. 1 and 2). The results of the LOH study in uveal melanoma are consistent with a recent result of Speicher et al. who reported 9p deletions in 3 of 11 uveal melanomas by comparative

<table>
<thead>
<tr>
<th>Case</th>
<th>Exon 1</th>
<th>Exon 2</th>
<th>Exon 3</th>
<th>Cell line</th>
<th>Exon 1</th>
<th>Exon 2</th>
<th>Exon 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM1–13</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>COLO38</td>
<td>ND</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>UM1–4</td>
<td>–</td>
<td>–</td>
<td>N</td>
<td>WM08</td>
<td>ND</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>UM15, 6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>WM983A</td>
<td>ND</td>
<td>MUT</td>
<td>ND</td>
</tr>
<tr>
<td>UM47–11</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>WM437</td>
<td>ND</td>
<td>MUT</td>
<td>ND</td>
</tr>
<tr>
<td>UM12</td>
<td>–</td>
<td>–</td>
<td>A375</td>
<td>ND</td>
<td>MUT</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UM13, 14</td>
<td>N</td>
<td>N</td>
<td>ND</td>
<td>MEWO</td>
<td>ND</td>
<td>MUT</td>
<td>ND</td>
</tr>
<tr>
<td>UMM15, 16</td>
<td>ND</td>
<td>N</td>
<td>ND</td>
<td>WM239A</td>
<td>DEL</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>UMM17</td>
<td>N</td>
<td>N</td>
<td>ND</td>
<td>WM449B</td>
<td>ND</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>CF1–9</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>G361</td>
<td>DEL</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CF10</td>
<td>N</td>
<td>MUT†</td>
<td>N</td>
<td>WM793</td>
<td>ND</td>
<td>N</td>
<td>ND</td>
</tr>
<tr>
<td>SB1</td>
<td>DEL</td>
<td>DEL</td>
<td>ND</td>
<td>M21</td>
<td>DEL</td>
<td>DEL</td>
<td>ND</td>
</tr>
<tr>
<td>M21</td>
<td>DEL</td>
<td>DEL</td>
<td>ND</td>
<td>SK-MEL1</td>
<td>ND</td>
<td>N</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 1 DNA sequence analysis of the CDK4I gene

The DNA sequence results are summarized: CM1–13, metastatic lesions of cutaneous melanomas; UMM1–17, primary tumors of uveal melanomas; CF1–10, peripheral blood lymphocytes from patients with familial cutaneous melanomas. †, sequences were not optimal; N, sequences were identical to the normal and no mutated sequence signals from heterologous alleles were detected; ND, sequence analysis was not done; MUT, mutation was detected as shown in the lower panel; DEL, deletion was detected by PCR analysis using the primers for sequencing the exon.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mutation</th>
<th>Location†</th>
<th>Coding effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM164</td>
<td>C→CC</td>
<td>173</td>
<td>Frameshift</td>
</tr>
<tr>
<td>WM1158</td>
<td>C→T</td>
<td>232</td>
<td>Arg→stop</td>
</tr>
<tr>
<td>MEWO</td>
<td>C→TT</td>
<td>231–232</td>
<td>Arg→stop</td>
</tr>
<tr>
<td>A375</td>
<td>G→T</td>
<td>175</td>
<td>Glu→stop</td>
</tr>
<tr>
<td>UMM15</td>
<td>G→T</td>
<td>199</td>
<td>Glu→stop</td>
</tr>
<tr>
<td>WM373</td>
<td>GG→AA</td>
<td>255–256</td>
<td>Glu→Lys</td>
</tr>
<tr>
<td>WM983A</td>
<td>C→T</td>
<td>335</td>
<td>Pro→Leu</td>
</tr>
</tbody>
</table>

*b Deletion of 14 base pairs between position 234 and 249 in one of the alleles. 
*b G to A transition at nucleotide 436 was not scored as a mutation according to the report of Spruck et al. (23).

* The nucleotide positions are numbered according to the scheme of Serrano et al. (18).

Fig. 1. LOH on chromosome 9p21–23 in melanoma cases UMM8, UMM11, and CM3. The lymphocyte (L) and melanoma tumor (T) DNA were amplified by PCR with the use of the microsatellite primers for D9S157 (5157), D9S171 (5171), D9S161 (5161), and D9S43 (543). See Fig. 2 for the chromosomal localization of each microsatellite marker. Arrows, positions of the products which were scored as allelic loss.
genomic hybridization (24). The results with UM8 and UM11 indicate that the involved locus is telomeric to D9S43 and D9S161, respectively, while results with CM3 suggest a locus centromeric to D9S157 (Figs. 1 and 2). The region defined by these 3 markers contains the CDK4I locus. This result is consistent with the results of several LOH studies reported previously (6–12). Retention of both alleles flanking the CDK4I locus, D9S171 and D9S157, was observed in 6 cases.

Discussion

Our sequence analysis of the CDK4I gene in cultured melanoma cells showed a high incidence of mutation of exon 2. This result suggested that exon 2 of the CDK4I gene was a common target of mutagenesis, and confirmed the results of two recent reports (16, 17). However, the results of the DNA sequence analysis of the uncultured, surgically resected tumors of both cutaneous and uveal origin showed the absence of these mutations. These results coincide with the recent reports showing the rarity of CDK4I mutations in the uncultured tumors of various organs, not including melanoma (23, 25). It is possible that the DNA sequence for some of the tumors was derived from normal cells within the tumor due to homozygous deletion of the CDK4I locus in the tumor cells. However, homozygous deletion of the CDK4I locus could not have occurred at high frequency and cannot explain the complete absence of mutations in the uncultured melanoma cells in our cases. It is possible that the mutations observed frequently in melanoma tumor cell lines resulted from genetic changes during the proliferation of the cells in culture or that tissue culture selects for growth of cells with CDK4I mutations.

Although somatic mutations in the melanomas were not observed in our cases, it is possible that the CDK4I gene might be inactivated by homozygous deletion, as reported in melanoma cell lines (16, 17, 26). But it is unlikely that homozygous deletion occurred in a majority of cases, since the present results of the LOH study did not show any evidence of homozygous loss of markers surrounding the CDK4I locus (UM1–12 and CM1–4). These results do not exclude the possibility of a small homozygous deletion of CDK4I locus between the markers we have tested, and it is possible in a small fraction of cases.

Assignment of a familial cutaneous melanoma locus to the 9p21–23 region is indicated by the results of multipoint linkage analysis (13–15). Cannon-Albright et al. (13) reported the assignment of a melanoma locus to 9p21 in 5 of 11 melanoma kindreds by a multipoint linkage analysis. Although there is controversy as to heterogeneity of melanoma susceptibility loci, all three multipoint linkage reports agree on the involvement of the 9p locus in a large fraction of kindreds of familial cutaneous melanoma. Because CDK4I is localized to this region, it was suggested as a candidate familial melanoma gene. However, the DNA sequence analysis of lymphocytes of familial cutaneous melanoma of 10 independent kindreds showed the absence of germline mutations in the major coding exons of CDK4I, except for the CF10 case (Table 1).

The mutation in CF10 would be pathologically important because (a) CF10 is distinguished from CF1–9 by the occurrence of melanomas within multiple first degree relatives, while in kindreds of CF1–9, two or three melanomas were documented; (b) the effect of the deletion mutation on the coding region would be a frameshift eliminating more than one-half of the primary structure of the CDK4I protein, possibly resulting in dysfunction or inactivation of the protein; and (c) the mutation was detected from uncultured germline cells. The relationship between this mutation and melanoma in the kindred will be determined by examining vertical transmission of the mutation and occurrence of melanomas. A germline mutation of CDK4I was reported by Nobori et al. (17) in a lymphoblastoid cell line derived from a patient with dysplastic nevus syndrome; however, germline mutations of the CDK4I gene in uncultured cells have not been reported previously.

Our results suggest several important conclusions: (a) the germline mutation of the CDK4I gene in one member, CF10, of a familial melanoma kindred with multiple affected members, lends strong support to the CDK4I gene as a candidate MLM gene; (b) the majority of sporadic melanomas do not exhibit mutation or homozygous losses of the CDK4I gene, suggesting that another gene on 9p21 is the target of the frequent loss of heterozygosity in this chromosomal region in malignant cutaneous melanomas; (c) the majority of presumptive familial melanomas from families with two or three affected members do not involve germline mutation of the CDK4I gene; and (d) primary uveal melanomas, a fraction of which exhibit 9p LOH (UM8, UM11, and UM12; Fig. 2) and 9p cytogenetic changes (UM1 and UM15; Ref. 19), do not involve the CDK4I gene.

The localization of a melanoma LOH region between D9S157 and D9S161, which includes the CDK4I locus, is consistent with the tumor suppressor gene candidacy of the CDK4I gene. Absence or rarity of both germline and somatic mutations of this gene suggests that the CDK4I gene might be inactivated by homozygous deletion or by other mechanisms which do not affect the coding sequence of the gene (e.g., disruption of a functional noncoding region of the gene by rearrangement or modification of the DNA of the gene by an epigenetic mechanism). However, it is also possible that this chromosome region carries other gene(s), the somatic or germline alteration of which is responsible for initiation and/or progression of melanoma, in view of recent LOH studies which indicated the presence of at least two tumor suppressor loci in this region (9, 11).

Our observation of the absence of LOH within the 9p21–23 region in some cases is consistent with the result of LOH studies reported by
Acknowledgments

Although the CDK4G gene may be involved in the development of a fraction of melanomas, the results of the present study suggest the need to continue the search for genes on 9p other than the CDK4G gene; the region 9p21–23 remains a promising region as a target of these studies.

Acknowledgments

We thank Teresa Druck for help with the LOH studies and Soldano Ferrone for providing four of the melanoma cell lines.

References


Rarity of Somatic and Germline Mutations of the Cyclin-dependent Kinase 4 Inhibitor Gene, CDK4I, in Melanoma


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/20/5269