Short Communication

Deletions of the *INK4A* Gene Occur in Malignant Peripheral Nerve Sheath Tumors but not in Neurofibromas

Helen P. Kourea,* Irene Orlow,*
Bernd W. Scheithauer,[†] Carlos Cordon-Cardo,*
and James M. Woodruff*

From the Department of Pathology,* Memorial Sloan-Kettering Cancer Center, New York, New York; and the Department of Pathology,[†] Mayo Clinic, Rochester, Minnesota

The *INK4A* gene, a candidate tumor suppressor gene located on chromosome 9p21, encodes two protein products, p16 and p19^{ARF}. p16 is a negative cell cycle regulator capable of arresting cells in the G1 phase by inhibiting cyclin-dependent kinases 4 (Cdk4) and 6 (Cdk6), thus preventing pRB phosphorylation. p19^{ARF} prevents Mdm2-mediated neutralization of p53. Loss of *INK4A* is a frequent molecular alteration involved in the genesis of several neoplasms, including tumors of neuroectodermal origin. This study investigated the frequency of *INK4A* gene alterations in a series of malignant peripheral nerve sheath tumors (MPNSTs) and neurofibromas (NFs). *INK4A* gene and the p19^{ARF}-specific exon 1 β were studied in 11 MPNST samples from 8 patients and 7 neurofibromas. Presence of *INK4A* deletions was assessed by Southern blotting hybridization and by a multiplex polymerase chain reaction (mPCR). *INK4A* point mutations were examined by single-strand conformation polymorphism (SSCP) and sequencing. The p16 promoter methylation status was determined by PCR amplification of bisulfite-treated DNA. Homozygous deletions of exon 2, thus affecting both p16 and p19^{ARF}, were identified in MPNSTs from 4 of 8 patients. Deletions, mutations, or silencing by methylation were not identified in the neurofibromas analyzed. Based on our results, we conclude that *INK4A* deletions are frequent events in MPNSTs and may participate in tumor progression. Silencing of p16 by methylation, which occurs often in several tumor types, is uncommon in MPNSTs. (*Am J Pathol* 1999, 155:1855–1860)

The *INK4* family of cyclin-dependent kinase inhibitors (CKIs) includes p16,¹ p15,² p18,³ and p19.⁴ *INK4A* gene, also known as *CDKN2A* gene, is located on the short arm of chromosome 9 (9p21),⁵ and its coding sequence consists of three exons with two alternative first exons, E1 α and E1 β . Splicing of E1 α with exons 2 and 3 encodes a 1.5845-kd protein known as p16. p16 complexes with and inactivates cyclin-dependent kinases 4 (Cdk4) and 6 (Cdk6), thus preventing phosphorylation of the retinoblastoma protein (pRB) and subsequent exit from the G1 phase of the cell cycle. If E1 β is used instead, the resulting protein product is entirely different due to alternative reading frame and has been designated p19^{ARF}.⁶ p19^{ARF} interacts with Mdm2 and prevents Mdm2-mediated degradation of p53.^{7,8}

INK4A gene alterations, namely point mutations, deletions, and gene silencing by methylation of the 5' CpG island of the p16 promoter region, have been identified in several tumor types,^{5,9–14} including neural crest-derived neoplasms such as melanoma,^{15–16} gliomas,^{17–20} and neuroblastoma.²¹

Malignant peripheral nerve sheath tumors (MPNSTs) are soft tissue tumors with a generally poor prognosis, thought to arise from Schwann cells and, therefore, to be of neuroectodermal origin.²² A frequent origin is malignant transformation of a neurofibroma (NF),^{23–25} which is the precursor of an estimated two-thirds of MPNSTs.²² The molecular alterations contributing to MPNST tumorigenesis are at present largely undetermined. Deletions and mutations affecting the p53 gene locus^{26,27} as well as overexpression of p53 protein^{28–30} have been identified in MPNSTs, but such alterations are absent in NFs.

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The first two named authors contributed equally to this work.

Address reprint requests to Carlos Cordon-Cardo, M.D., Ph.D., Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. E-mail: cordon-c@mskcc.org.

Table 1. Deletion Analysis of *INK4A* Gene in MPNSTs from Southern Blot Hybridization and Comparative Multiplex Polymerase Chain Reaction Assay Data

Patient	Sample no.	<i>INK4A</i> gene deletion analysis		
		p16 Southern blot	Exon 2 mPCR	Exon 1 β mPCR
A	1	Del	Hom. Del	Hom. Del
B*	2I	Del	Hom. Del	N
B*	2II	Del	Hom. Del	N
C	3	N	N	N
D [†]	4	Del	n/a	Hom. Del
E	5	N	N	N
F [†]	6	N	N	N
G	7	Del	Hom. Del	Hom. Del
G	9	Del	Hom. Del	Hom. Del
G	10	n/a	Hom. Del	Hom. Del
G*	14I	Del	Hom. Del	Hom. Del
G*	14II	Del	Hom. Del	Hom. Del
H	8	N	N	n/a

mPCR, comparative multiplex polymerase chain reaction assay; Del, deletion; Hom. Del, homozygous deletion; N, Normal; n/a, not available.

* Same tumor samples.

[†] NF1 patient.

We recently studied p21^{WAF1} expression, a downstream factor of the p53 pathway, and found no significant differences between MPNSTs and NFs. We also examined factors involved in the RB pathway and, although no significant alterations of pRB were noted in MPNSTs, we observed loss of p27 and overexpression of cyclin E in MPNSTs.³⁰ The absence of such alterations in NFs suggests that these cell cycle regulators may participate in MPNST tumorigenesis. To further investigate the mechanisms involved in the G1-to-S phase transition, we conducted the present study to examine the frequency of the *INK4A* gene alterations in MPNSTs and NFs.

Materials and Methods

Patients and Tissue Samples

MPNST samples from eight patients operated on at Memorial Sloan-Kettering Cancer Center (MSKCC) between 1986 and 1997, as well as 3 and 4 NFs resected, respectively, at MSKCC and Mayo Clinic between 1988 and 1999, were examined. Two MPNSTs and four NFs were from patients with family history of neurofibromatosis (NF1). Criteria for the diagnosis of MPNST were the same as those specified in an earlier study.²⁴ All of the MPNSTs were spindle cell tumors and none were epithelioid in type. Seven tumors were high grade, and one patient (H) had a low grade MPNST arising in a NF (Table 1).

There were 11 tumor samples representing tissue from 8 primary MPNSTs and 3 recurrences (Table 1). Samples were embedded in a cryopreservative solution (OCT Compound, Miles Laboratories, Elkhart, IN), snap-frozen in isopentane precooled in liquid nitrogen, and stored at -70°C . Representative hematoxylin-eosin-stained sections of each frozen block were examined microscopically to confirm the presence of tumor, and only lesions with $>70\%$ neoplastic cells were included in the study.

Southern Blot Analysis

A 0.5-kb (kb) complementary DNA (cDNA) fragment containing the human p16 sequence was used as probe to assess deletion and rearrangement of the *INK4A* gene. A cDNA fragment containing glyceraldehyde phosphate dehydrogenase (GAPDH) sequences was used as a control probe for DNA quality and quantity. In general, Southern blot analysis was performed as described previously.¹⁰ Briefly, DNA was extracted by a non-organic method (Oncor, Gaithersburg, MD) from the tumor samples. Extracted DNA (7.5- μg aliquots) was digested with *TaqI* restriction enzyme, and the digested DNA was subjected to electrophoresis on 0.8% agarose gels and blotted onto nylon membranes. The membranes were prehybridized with Hybrisol I (Oncor) at 42°C for 1 hour and then incubated overnight at 42°C with probes labeled to high specific activity using $\alpha[^{32}\text{P}]\text{dCTP}$ (Dupont NEN Research Products, Boston, MA). Hybridized membranes were washed with $0.1\times \text{SSC}/0.1\% \text{SDS}$ at 70°C and subjected to autoradiography using intensifying screens at -70°C for 24 to 48 hours. The intensity of the specific *INK4A* gene and control bands was measured by a phosphorimager (Bas 1000-Mac, Bio Imaging System, Fujix, Fuji). Relative amounts of the *INK4A* gene present were determined by comparing gene-specific hybridization signals with those obtained using the control probe.¹⁰ Tumor samples presenting $<30\%$ of the relative *INK4A*/control ratio were considered as homozygously deleted.

Polymerase Chain Reaction-Single Stranded Conformation Polymorphism (PCR-SSCP) and Comparative Multiplex PCR Analyses

PCR-SSCP assays were performed on all tumors using a slight modification¹⁰ of the method described by Orita et al.³¹ The primers used to amplify the exons 1 α (one fragment) and 2 (three overlapping fragments) of the

INK4A gene were published by Hussussian et al.¹⁵ For exon 1 β the following primer pairs were used: *INK4A*, exon 1 β (440-bp fragment) 5'-TCC CAG TCT GCA GTT AAG G-3' forward, 5'-GTC TAA GTC GTT GTA ACC CG-3' reverse; *INK4A*, exon 1 β (160 bp fragments) 5'-AAC ATG GTG CGC AGG TTC-3' forward, 5'-AGT AGC ATC AGC ACG AGG G-3' reverse; 3) *INK4A* exon 2 (set A) 5'-AGC TTC CTT TCC GTC ATG C-3' forward, 5'-GCA GCA CCA CCA GCG TG-3' reverse; *INK4A* exon 2 (set B) 5'-AGC CCA ACT GCG CCG AC-3' forward, 5'-CCA GGT CCA CGG GCA GA-3' reverse; *INK4A* exon 2 (set C) 5'-TGG ACG TGC GCG ATG C-3' forward, 5'-GGA AGC TCT CAG GGT ACA AAT TC-3' reverse. DNA was amplified in 28 to 30 cycles of PCR using a thermal cycler (Perkin-Elmer Cetus, Foster City, CA) and conditions that have been previously described.¹⁰ Briefly, these PCR reactions were performed in 10- μ l volumes containing 80 to 100 ng of template DNA, 2.2 μ Ci of α [³²P]dCTP (Dupont NEN Research Products) or α [³³P]dCTP (Amersham Life Science, Arlington Heights, IL), 3 mmol/L MgCl₂, 100 μ mol/L dNTPs, 3% dimethylsulfoxide, 0.6 U of *Taq*I polymerase, and 1 $\times$ PCR buffer (Promega, Madison, WI). The annealing temperatures ranged from 53° to 63°C. For the SSCP analysis of the exon 1 β (fragment 1), the PCR product was digested with *Nar*I and *Ehe*I restriction enzymes for 2 hours at 37°C. In general, both digested and nondigested products were analyzed by SSCP. The PCR products were denatured and loaded onto a nondenaturing 8% polyacrylamide gel containing 10% glycerol and subjected to electrophoresis at room temperature for 12 to 16 hours at 10 to 12 W. After electrophoresis, the gels were dried and exposed to X-ray film at -70°C for 4 to 24 hours.

An independent DNA amplification was performed for the comparative multiplex PCR assay. Simultaneous amplification of genomic DNA was performed using two sets of primers, one to the target gene sequence under study and the other to an internal control gene sequence. The GAPDH and ANDRR genes were used as internal controls for DNA quality and loading. The primers used for the GAPDH and ANDRR genes can be summarized as follows: GAPDH gene, 5' TGG TAT CGT GGA AGG ACT CAT GAC 3' forward, 5' ATG CCA GTG AGC TTC CCG TTC AGC 3' reverse; ANDRR gene, 5' GTG CGC GAA GTG ATC CAG AA 3' forward, 5' TCT GGG ACG CAA CCT CTC TC 3' reverse. The concentration and quality of each DNA template was determined by densitometry and by comparison with mass markers on an agarose gel (Gibco BRL). Each PCR reaction tube contained 50 to 100 ng of genomic DNA, 1 $\times$ PCR buffer (Promega), 3.2 mmol/L MgCl₂, 130 μ mol/L dNTP, 5% dimethylsulfoxide, 0.4 μ mol/L of each p16 exon 1 β primer, 0.4 μ mol/L of each ANDRR primer, 0.5 U *Taq* Polymerase (Promega), and 1 μ Ci of α [³³P]dCTP. Samples were preheated at 95°C for 5 minutes and amplified for 25 cycles with annealing temperatures ranging from 59° to 53°C, followed by an extension at 72°C for 10 minutes. PCR products were run in nondenaturing 6 to 7% polyacrylamide minigels at 1000V for 1 to 1.5 hours. Gels were dried and exposed to sensitive film and to a phosphorimage plate. The presence of the *INK4A* exons was expressed as the

ratio (target-band signal)/(control-band signal). All experiments were conducted at least twice, and to rule out false negative results due to partial DNA degradation, the control selected was longer than the target sequence. To establish potential *INK4A* allelic losses in the tumor DNA samples, tumor DNA samples characterized previously by the lack of *INK4A* sequences¹⁰ were used as control DNAs, validating the quantitative nature of the multiplex PCR method. Briefly, varying mixtures of tumor DNA and normal genomic DNA were co-amplified. These tumor-to-normal DNA mixtures represented a range of the *INK4A* exons content, varying from 0% of target (tumor sample control) to 100% of target (normal DNA counterpart). Samples presenting <20% of the control signal were considered homozygously deleted, and those presenting <50% as heterozygously deleted for exon 1 β .

Analysis of Methylation

The methylation status of the 5'CpG island in the promoter region of the p16 gene was determined with the CpG WizTM p16 Methylation kit (Oncor). Briefly, 0.5 to 1 μ g of DNA was denatured with NaOH 3 mol/L at 50°C for 10 minutes and treated with sodium bisulfite following manufacturer's protocol. After completion of the DNA modification, the DNA was purified by precipitation. The dissolved DNA was amplified by PCR using primers specific for the methylated (M) or unmethylated (U) sequences. Two to 3 μ l of template (corresponding to treated DNA, positive control for methylated DNA, positive control for unmethylated sequences, and dH₂O as negative control) were amplified in presence of 10 $\times$ Universal PCR buffer, 2.5 mmol/L dNTP mix, U or M primers, and AmpliTaq Gold (Perkin Elmer), under the following conditions: preheating at 95°C for 12 minutes, followed by 35 cycles at 95°C for 45 seconds, at 66°C for 45 seconds, and at 72°C for 1 minute. The PCR product was analyzed on a 2% agarose gel. DNA methylation was determined by the presence of a 145-bp fragment in those samples amplified with the M primers.

Results

Deletion Analysis of the *INK4A* Gene in MPNSTs and NFs

Table 1 summarizes the results of the deletion analyses. Homozygous deletions affecting the *INK4A* gene were identified in 6 of 10 (60%) MPNST samples from 4 of 8 patients studied by Southern blotting. Recurrent tumors from one case also showed *INK4A* homozygous deletions. Southern blot analysis of DNA extracted from seven NFs identified normal *INK4A* gene bands in all cases. Fig. 1 illustrates normal and altered *INK4A* band patterns in representative MPNSTs and NFs.

Comparative multiplex PCR analysis for exon 2 of the *INK4A* gene confirmed homozygous deletions of this locus in 6 of 10 different MPNST samples, corresponding to 3 of 4 patients with *INK4A* gene deletion by Southern blotting. The fourth sample could not be successfully

Figure 1. Southern blot analysis of the *INK4A* gene in MPNSTs. Genomic DNA was digested with the restriction enzyme *TaqI*. Southern blot was performed using a specific p16 complementary-DNA probe, as described in Materials and Methods. A control probe (GAPDH) for DNA loading was used in the analysis. The *INK4A* p16-specific signals shown in this figure correspond to bands of 3.7 kb (kb). The control probe signal shown corresponds to 3.7- to 5.0-kb bands. A NF sample (NFc) and a MPNST sample³ depict a normal band pattern. MPNST samples 4 and 9 illustrate a homozygous deletion of the *INK4A*-p16 gene. NF, neurofibroma; GAPDH, glyceraldehyde phosphate dehydrogenase.

analyzed due to limited tissue. The status of the p19^{ARF} exon 1 β was successfully analyzed by comparative multiplex PCR in 10 tumors from 7 patients. Homozygous deletion of exon 1 β was detected in 6 of 10 different MPNST samples, three primary tumors and three tumor recurrences (Figure 2). The samples were from tumors showing concomitant loss of the exon 2 of the *INK4A* gene, as well as p16 deletion by Southern blotting. Exon 1 β was preserved in one case displaying *INK4A* gene and exon 2 loss. One sample, showing no deletion of the *INK4A* gene or exon 2, could not be successfully analyzed by multiplex PCR for exon 1 β .

Figure 2. Deletion analysis of the *INK4A*-exon 1 β gene in MPNSTs and NFs. **A:** the standardization of the assay by incremental amplification of *INK4A*-exon 1 β with an increasing normal DNA target. To the right, the control curve was constructed with the relative *INK4A*/control ratios and the amount of normal DNA included in each sample. **B:** absence of *INK4A*-exon 1 β in MPNST samples 4, 9, 10, and 11-I, with retention in the three neurofibroma samples (NFd, NFe, NFF). To the right, the ratios (*INK4A*/control) obtained for the tumor samples are compared to those obtained for normal control DNA. Note the reduction in the ratios (expressed as a percentage of normal DNA) in the same cases. Samples 9, 10, and 11-I correspond to the same patient (G). NL, normal.

Figure 3. *INK4A* point mutations analysis in MPNSTs. The left panel illustrates the band pattern obtained for the tumor samples 2I and 2II (2I and 2II derived from the same tumor), by PCR-SSCP analysis. Additional *INK4A* bands (B*) were identified in the tumor samples 2I and 2II. Direct sequencing results of the PCR products obtained from the elution of normal (A) and abnormal (B*) bands from the same cases are shown in the middle panel, corresponding to the forward sequences. The asterisks indicate the base change. The single base substitution (G→A, **arrow**) produces the polymorphism (Ala→Thr), as indicated in the forward sequence.

Mutation Analysis of Exons 1 α , 1 β , and 2 of the *INK4A* Gene

The analysis by PCR-SSCP for exons 1 α , 1 β , and 2 of the *INK4A* gene identified altered fragment mobility shifts involving exon 2 in one MPNST case. Sequencing disclosed the presence of a polymorphism associated with G→A transversion at codon 148 (Ala→Thr; Figure 3). In another MPNST case, a double silent transition at codon 83 CAC→CAT (His→His) and codon 84 GAC→GAT (Asp→Asp) was also observed.

Methylation Analysis of the *INK4A* Gene Promoter

In all samples analysis of the methylation status of the p16 promoter was performed. Methylation-specific fragments were absent in all MPNST and NF samples (data not shown).

Discussion

MPNSTs are rare neoplasms in the general population.²³ However, they develop with increased frequency in the setting of neurofibromatosis type 1 and are strongly associated with a pre-existing NF.²³⁻²⁵ This observation implies that NFs and MPNSTs participate in a model of multistage tumor progression analogous to the colonic adenoma-carcinoma sequence.

Study of the genetic alterations contributing to such evolution includes the analysis of the p53²⁶⁻³⁰ and RB pathways.³⁰ Mutations of the p53 gene^{26,27} as well as p53 overexpression²⁸⁻³⁰ have been described in MPNSTs, whereas they are absent in NFs. Underexpression of p27 and overexpression of cyclin E appear also to be involved in the MPNST tumor progression scheme.³⁰

In the present series, we identified homozygous deletions of the *INK4A* gene in 60% of the MPNST samples analyzed, derived from 4 of 8 patients. The Southern blot hybridization findings were confirmed by comparative multiplex PCR, identifying homozygous deletion of exon 2 of the *INK4A* gene in all of the successfully analyzed

cases. Findings in tumors from patients with and without NF1 did not differ. By contrast, none of the seven NF samples analyzed displayed *INK4A* gene deletion.

In these cases we also analyzed the p19^{ARF}-related exon 1 β . We identified homozygous deletions in 6 of 11 MPNST samples derived from 3 patients. All three cases displayed concomitant *INK4A* gene deletion. On the other hand, exon 1 β was preserved in one patient showing p16 deletion. Cases showing normal p16 gene status also displayed normal exon 1 β .

PCR-SSCP analysis and sequencing of *INK4A* gene exons 1 α , 1 β , and 2 did not identify tumor-specific point mutations, but revealed two polymorphisms involving exon 2. The first, in codon 148, has been already described,^{32–35} and the second is a double silent transition involving codons 83 and 84 of exon 2. Similarly, we did not detect *de novo* methylation of the exon 1 α promoter. Absence of tumor-specific point mutations and methylation of the *INK4A* promoter were also observed in a large cohort of soft tissue malignancies.¹⁴

Although the series is small, these data suggest that *INK4A* gene deletions are frequent events in MPNSTs, occurring in 50% of the patients. The frequency of *INK4A* gene deletion in MPNSTs is particularly high, because it exceeds the previously reported occurrence of p16 deletion in other tumor types such as soft tissue sarcomas.¹⁴ Notably, within that series of soft tissue malignant neoplasms the incidence of *INK4A/INK4B* deletions was highest in MPNSTs (40%).¹⁴ The significance of this finding is further emphasized by the absence of *INK4A* gene deletion in NFs. These findings in concert suggest that deletion of the *INK4A* gene is an important step in the process of malignant transformation of NF.

Moreover, the concomitant loss of exons 1 β and 2 suggests concurrent molecular abnormalities in p16 and p19^{ARF} proteins. p16 functions as a negative cell cycle regulator through the CDK4/cyclin D1/pRB pathway. On the other hand, p19^{ARF} has been reported to interact with Mdm2 protein and to interfere with Mdm2-mediated degradation of p53. Therefore, a single genetic event involving *INK4A* gene in chromosome 9p21 may simultaneously eliminate two tumor suppressor proteins, p16 and p19^{ARF}, affecting both pRB and p53 pathways. The concomitant inactivation of both pRB and p53 is considered critical in the process of tumorigenesis.^{14,36,37} In addition to p16 and p19^{ARF} deletion, we have previously identified p27 underexpression and cyclin E overexpression in MPNSTs but not in NFs.³⁰ These alterations likely act in concert to facilitate the G1-to-S progression of the cell cycle in MPNSTs and appear to participate in the tumor progression of the NF-MPNST sequence.

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