Malignant Transformation of Neurofibromas in Neurofibromatosis 1 Is Associated with *CDKN2A/p16* Inactivation

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Patients with neurofibromatosis 1 (NF1) are predisposed to develop multiple neurofibromas (NFs) and are at risk for transformation of NFs to malignant peripheral nerve sheath tumors (MPNSTs). Little is known, however, about the biological events involved in the malignant transformation of NFs. We examined the *CDKN2A/p16* gene and p16 protein in NFs and MPNSTs from patients with NF1. On immunohistochemical analysis, all NFs expressed p16 protein. The MPNSTs, however, were essentially immunonegative for p16, with striking transitions in cases that contained both benign and malignant elements. None of the benign tumors had *CDKN2A/p16* deletions, whereas three of six MPNSTs appeared to have homozygous *CDKN2A/p16* deletions. Methylation analysis and mutation analysis of *CDKN2A/p16* in MPNSTs did not reveal any abnormalities. These results show that malignant transformation of NF is associated with loss of p16 expression, which is often secondary to homozygous deletion of the *CDKN2A/p16* gene. The findings suggest that *CDKN2A/p16* inactivation occurs during the malignant transformation of NFs in NF1 patients and raises the possibility that p16 immunohistochemistry may provide ancillary information in the distinction of NF from MPNST. (Am J Pathol 1999, 155:1879–1884)

Neurofibromatosis 1 (NF1; von Recklinghausen neurofibromatosis) is one of the most common autosomal dominant disorders, affecting approximately one in 3000 individuals.^{1,2} Diagnostic criteria for NF1 include two or more neurofibromas (NFs) of any type or one plexiform NF, café-au-lait macules, axillary or inguinal freckles, optic glioma, Lisch nodules, distinctive osseous lesions,

and/or a first-degree relative with NF1.³ Patients with NF1 have an increased risk of developing malignant tumors, in particular a tendency for some NFs to undergo malignant transformation to a malignant peripheral nerve sheath tumor (MPNST).^{2,4} At the present time, however, there are no clinical or pathological means of identifying or predicting which NFs will progress to MPNST.

Patients with NF1 have a defect in the *NF1* gene, which is located on the long arm of chromosome 17 (17q11.2).⁵ Given the predilection of NF1 patients to develop NFs, it is likely that inactivation of the *NF1* gene predisposes patients to the formation of benign NFs. Indeed, biallelic inactivation of the *NF1* gene has been documented in some NFs from NF1 patients.⁶ The subsequent genetic changes that underlie the transformation of NFs into MPNSTs in patients with NF1 are poorly understood. Cytogenetic analysis of MPNSTs in patients with NF1 have revealed complex but not always consistent karyotypic changes. Such changes sometimes involve chromosomes 17 and 22, where the *NF1*, *p53*, and *NF2* tumor suppressor genes are located.^{7–9} In particular, mutations of the *p53* gene have been documented in the progression of NF to MPNST in patients with NF1.^{10–17}

The p16 protein, encoded by the *CDKN2A/p16* gene on the short arm of chromosome 9 (9p21), is a tumor suppressor that inhibits the function of cdk4- and cdk6-cyclin D complexes. These cdk-cyclin complexes in turn regulate the retinoblastoma protein (pRb), thus controlling the G1-S phase checkpoint of the cell cycle. *CDKN2A/p16* inactivation thereby results in cellular proliferation.^{18–28} *CDKN2A/p16* inactivation is known to occur in a wide variety of human tumors, including carcinomas, glioblastoma, leukemia, and some sarcomas.^{29–39} Immunohistochemical studies have shown frequent aberrant p16 expression in various types of sarcomas.^{39,40} In addition, p16-null mice are prone to develop malignancies, particularly fibrosarcoma and lymphomas.⁴¹ Thus p16 alterations characterize some human and experimental sarcomas. Given the need to identify molecular events associated with the sarcomatous transition of NFs to MPNSTs, we therefore evaluated the role of the

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CDKN2A/p16 gene and p16 protein in NFs and MPNSTs from patients with NF1.

Materials and Methods

Materials

Formalin-fixed, paraffin-embedded tumors were retrieved from the files of the Department of Pathology of the Massachusetts General Hospital. Eighteen tumors from 16 patients were examined; 13 of the patients carried a diagnosis of NF1. Of the 13 NF1 patients, seven had an MPNST arising in association with a NF; three had MPNST unassociated with identifiable NF; one had a NF and an MPNST resected at different times; and two patients had carried the diagnosis of "atypical" NFs. Three tumors (one schwannoma and two NFs) were from three patients who were not diagnosed with NF1. The study population consisted of 16 patients (seven males and nine females) that ranged in age from 9 to 57 (mean 29) years. DNA was extracted from formalin-fixed, paraffin-embedded tissue according to standard procedure.⁴² Before DNA extraction, all tumor tissues were examined histologically to determine benign and malignant areas and to ensure that they contained viable tumor.

Homozygous Deletions of *CDKN2A*

To assay for homozygous deletions of the *CDKN2A/p16* gene, we used a comparative multiplex polymerase chain reaction (PCR) technique that amplifies a 168-bp fragment of the 5' end of *CDKN2A/p16* exon 2 and a 187-bp sequence of the *APEX* nuclease gene on chromosome 14q, a site not frequently altered in sarcomas.^{27,28,38} The products were separated by electrophoresis on 2% agarose gels and visualized under ultraviolet light by ethidium bromide staining. This assay has been titrated to detect homozygous *CDKN2A/p16* deletions in tumors with less than 30% contaminating nonneoplastic cells and has been confirmed by Southern blotting of selected gliomas.^{27,28} Each PCR assay was repeated at least three times to confirm the ratios between the *CDKN2A/p16* band and the control band.

p16 Immunohistochemistry

p16 immunohistochemistry was performed with the JC8 mouse monoclonal IgG2a antibody, generated in the Massachusetts General Hospital Cancer Center. JC8 antibody is directed against the first ankyrin repeat (amino acids 1–32) of the p16 protein and has been characterized and described elsewhere.^{31,39,43}

Single-Strand Conformation Polymorphism Analysis

Single-strand conformation polymorphism (SSCP) analysis was performed on all three coding exons of the *CDKN2A/p16* gene, as previously described.^{44,45}

Methylation-Specific PCR of the *CDKN2A/p16* Promoter

The methylation status of the *CDKN2A/p16* promoter region was studied in two tumors that lacked *CDKN2A/p16* deletion but also showed loss of p16 expression. Methylation was assessed by methylation-specific PCR according to a published protocol,⁴⁶ with minor modification of the PCR cycle to include a touchdown of the annealing temperature from 67°C to 60°C, followed by 20 cycles at 60°C. The procedure entails bisulfite modification of DNA followed by amplification with primer pairs specific for methylated and unmethylated DNA. DNA samples from the colon carcinoma cell lines HT-29 and CaCo2 were used as positive controls for methylated DNA. The PCR products were run on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Results

Immunohistochemical staining for p16 protein in the two NFs from non-NF1 patients showed nuclear and faint cytoplasmic staining of most of the neoplastic cells. The one schwannoma showed an unusual staining pattern, in which staining was prominent in Antoni B regions and less extensive in the cellular Antoni A regions. Neurofibromas from the NF1 patients had prominent p16 immunopositivity, with most (60–100%) tumor cells displaying strong nuclear and lighter cytoplasmic staining (Figure 1, A and B). Two "atypical" NFs contained areas of increased cellularity and scattered cells with "degenerative" nuclear atypia and rare mitoses; these had been considered histologically benign. On immunohistochemical analysis, these two cases also showed nuclear and light cytoplasmic staining of most tumor cells, including the "atypical" cells (Figure 1, C and D).

With the exception of one case, all 11 MPNSTs were conspicuously negative for p16 on immunohistochemistry (Figure 1, E and F). One tumor, which arose in association with a benign NF and did not demonstrate any unusual histological features, had scattered (<5%) p16-immunopositive malignant-appearing cells. For the cases in which benign and malignant elements were both present in a single section, the difference in staining was striking between the benign and malignant areas, with the p16-immunopositive NF highlighted against the p16-immunonegative MPNST (Figure 1, G and H).

DNA was extracted from 15 cases (the three tumors from the non-NF1 patients and 12 tumors (six NFs and six MPNSTs) from patients with NF1). None of the benign tumors (including the "atypical" NFs) had *CDKN2A/p16* deletions. Three of the six MPNSTs, however, had comparative multiplex PCR results that were consistent with homozygous *CDKN2A/p16* deletions (Figure 2). Although MPNSTs may have complex cytogenetic aberrations, chromosome 14q (the location of the *APEX* control gene) is not a common site of amplification⁴⁷; this argues against the possibility that the comparative multiplex results reflect multiple copies of chromosome 14q rather

Figure 1. **A** and **B:** Neurofibroma from a patient with NF1, showing bland-looking spindle cells separated by a myxoid and collagenous background (**A**). Immunohistochemical staining illustrates diffuse nuclear and faint cytoplasmic staining for p16 (DAB chromogen with hematoxylin counterstain) (**B**). **C** and **D:** "Atypical" neurofibroma showing increased cellularity and nuclear atypia (**C**). No mitotic figures are present. Immunohistochemical staining for p16 demonstrates nuclear and faint cytoplasmic staining of the neoplastic cells, including "atypical" cells (**D**). **E** and **F:** MPNST (**E**) showing marked increased cellularity, fascicular and herringbone growth pattern, nuclear atypia, and marked mitotic activity. Immunohistochemical staining shows no immunoreactivity for p16 (**F**). **G** and **H:** MPNST arising in association with a neurofibroma (**G**). An abrupt transition from the neurofibroma (**right**) to MPNST (**left**) is seen. Immunohistochemical staining of the same tumor (**H**) shows that the benign neurofibromatous component (**right**) stains for p16 protein, whereas the malignant component (**left**) is immunonegative for p16 protein.

Figure 2. Comparative multiplex analysis of the *CDKN2A/p16* gene. Homozygous deletion (HD) is present in three tumors (**lanes 3, 5, and 10**), as evidenced by preferential amplification of the control amplicon (**top band**) with minimal amplification of the *CDKN2A/p16* amplicon (**bottom band**). M, pUC18/*Hae*III digest size marker. **Lane 0:** Negative control ("no DNA"). **Lane 1:** Normal control DNA. **Lanes 2 and 3:** DNA from the same tumor, showing the benign (NF) component (**lane 2**) and the malignant component (MPNST; **lane 3**), the latter showing HD for the *CDKN2A/p16* gene. **Lanes 4 and 5** show findings similar to those of **lanes 2 and 3**, with DNA from the different areas in another tumor (**lane 4**, NF; **lane 5**, MPNST). **Lane 5** shows HD for the *CDKN2A/p16* gene. **Lane 6:** Neurofibroma from a patient with NF1. **Lanes 7–10:** MPNSTs from four different NF1 patients. **Lanes 7–9** do not show HD, whereas the MPNST in **lane 10** shows HD for the *CDKN2A/p16* gene.

than deletion of *CDKN2A/p16*. On the other hand, chromosome 14q can be lost in approximately 20% of MPNSTs,⁴⁷ raising the possibility that the three p16 immunonegative MPNSTs without demonstrable *CDKN2A/p16* homozygous deletions represent false negative results from coincident 14q and 9p loss. Methylation analysis of the *CDKN2A/p16* promoter and mutation analysis of the *CDKN2A/p16* coding region in two of the three MPNSTs without deletions did not reveal any alterations.

Discussion

Tumorigenesis in NF1 patients is a multistep process. Although changes in the *NF1* gene are likely initiating events, other genetic alterations must occur for benign tumors such as NFs to progress to malignancy. Because of the clinical importance of understanding the transition of NFs to MPNSTs, we have collected a series of NF-MPNST cases from NF1 patients to evaluate the genetic changes associated with this progression.

In the present study, NFs from NF1 patients, including NFs that had given rise to MPNSTs and "atypical NFs," all expressed p16 protein. Benign nerve sheath tumors from non-NF patients also expressed p16 protein. Thus p16 is expressed in benign nerve sheath tumors, consistent with a role for p16 in controlling cell proliferation in these lesions. Significantly, however, with the exception of one tumor that showed staining of only scattered tumor cells, MPNSTs did not express p16 protein. For tumors in which NF and MPNST coexisted, the difference in staining between benign and malignant areas was striking, with loss of p16 noted only in the malignant components. These results demonstrate that loss of p16 expression is associated with the malignant transition of NF to MPNST in NF1 patients.

In half of the evaluable cases, the genetic mechanism for loss of p16 expression appeared to be homozygous deletion of the *CDKN2A/p16* gene. For the other cases, we did not detect *CDKN2A/p16* homozygous deletions, promoter methylation, or coding region mutations. Nonetheless, deletions not involving exon 2, methylation outside of the assayed region of the promoter, or point mutations in noncoding regions could all potentially affect protein expression and would not be detected with our analyses. Given the absence of p16 expression in the MPNSTs studied, the finding of gene abnormalities in half of the studied MPNSTs, and the chance of false negative comparative multiplex PCR findings (see Results section), it is possible that all of the MPNSTs harbor *CDKN2A/p16* gene alterations.

Only a few studies have examined the role of *CDKN2A/p16* in MPNSTs. Cohen et al⁴⁰ studied p16 and pRB expression immunohistochemically in 59 sarcomas, including six "neurofibrosarcomas," all of which showed normal p16 expression. Schneider-Stock et al⁴⁸ found loss of heterozygosity (LOH) at the *CDKN2A/p16* gene in only one of 14 "malignant schwannomas" (MPNSTs), but such LOH assays will often miss homozygous deletions. Importantly, neither of these studies indicated whether the MPNSTs were from NF1 patients. In a comparative genomic hybridization study⁴⁷ of sporadic and NF1-associated MPNSTs, three of seven NF1-associated MPNSTs and one of three sporadic MPNSTs showed allelic loss of chromosome 9p, consistent with inactivation of *CDKN2A/p16*. Recently Kourea et al⁴⁹ reported, in abstract form, deletion of the *CDKN2A/p16* gene in 60% of MPNSTs, with no cases demonstrating *CDKN2A/p16* methylation. These results are similar to the present findings, and it would be interesting to know if any of the patients studied by Kourea et al had NF1. These studies leave open the possibility that different mechanisms might be involved in the tumorigenesis of sporadic versus NF1-associated MPNSTs. The definition of sporadic MPNSTs also introduces a problem, because poorly differentiated spindle cell sarcomas may be diagnosed in different ways by different pathologists. The present study overcomes this potential problem by limiting the study to NF1-associated MPNSTs, by assuming that these tumors represent a homogeneous group of neoplasms that are derived from nerve sheath elements.

The association of *CDKN2A/p16* inactivation with the transition from NF to MPNST parallels that of *p53*, because *p53* alterations have been documented in MPNSTs but not in NFs. For *p53*, it has been suggested that immunohistochemical demonstration of p53, most likely indicating mutant protein, supports a diagnosis of MPNST.^{16,17} However, results for p53 staining have not been consistent in MPNSTs, and some p53 mutants do not lead to immunohistochemically detectable protein. Our findings raise the possibility that p16 immunostaining may provide ancillary information in the distinction of NF from MPNST. In this regard, it is interesting that the atypical NFs maintained p16 staining; if this proves true on larger series of cases, p16 immunopositivity would argue against a diagnosis of MPNST in difficult cases, perhaps

allowing a patient to avoid overly aggressive therapies. On the other hand, at the present time such assays are unlikely to provide information for predicting which NFs are likely to progress to MPNSTs, although the absence of staining may strengthen the argument that such a transition has already occurred.

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