

***Mxi1* Mutations in Human Neurofibrosarcomas**

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***Mxi1* is thought to negatively regulate Myc function and may therefore be a potential tumor suppressor gene. Little effort has yet been made to find alterations involving this gene in human solid tumors. We screened 31 human gastric cancers, 7 esophageal cancers, 85 bone and soft tissue tumors of various types, including 4 neurofibrosarcomas. We also examined 29 human tumor cell lines consisting of 12 esophageal cancers, 7 glioma/glioblastomas and 10 others for *Mxi1* mutations in exons 1, 2, 4 (HLH domain), 5 and 6. Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and subsequent sequencing revealed three distinct polymorphisms in the intron-exon boundary upstream from exon 6. We discovered a missense mutation, GCA to GTA (Ala 54 Val), in exon 2 in a neurofibrosarcoma patient (case 1), two missense mutations, AAA to CAA (Lys 118 Gln) and GAA to GGA (Glu 154 Gly) in exon 5 of another neurofibrosarcoma patient (case 2), and 3 amino acid substitutions, GTG to GCG (Val 179 Ala), GTT to GCT (Val 181 Ala) and TTC to CTC (Phe 186 Leu), in a third neurofibrosarcoma patient (case 3). In case 3, loss of heterozygosity was also demonstrated by informative (TTC)₃/(TTC)₂ polymorphism. Our data demonstrate that mutations occur in the *Mxi1* gene in neurofibrosarcoma. Missense mutations in the functional domain of *Mxi1* in these cases may be involved in the pathogenesis of neurofibrosarcoma.**

Key words: *Mxi1* — Neurofibrosarcoma — NF1 — *c-myc* — Mutation

Mxi1 antagonizes the functions of Myc by heterodimerizing with Max to compete with Myc for limited amounts of Max¹⁾ or with Myc/Max for the Myc/Max consensus recognition sequence, CACGTG.²⁾

The *Mxi1* gene has been mapped to chromosome 10, band q24-q25,^{3,4)} a region which shows frequent translocations, deletions, or rearrangements in a number of human tumors.^{5,6)} Induction of *Mxi1* expression in U87MG, a glioblastoma cell line lacking endogenous *Mxi1* expression, resulted in a decreased U87MG growth rate.⁷⁾ In another study, overexpression of *Mxi1* dose-dependently inhibited the expression of ornithine decarboxylase, which is believed to be a Myc target gene, both *in vivo* and *in vitro*.²⁾ The loss of *Mxi1* function is known to be associated with human glioblastoma development.⁷⁾ *Mxi1* knock-out mice exhibit progressive, multisystem abnormalities and increased susceptibility to tumorigenesis in response

to carcinogen exposure and also in the case of Ink4a deficiency.⁸⁾ Despite the accumulating evidence indicating that *Mxi1* plays a tumor suppressor gene role, to date, only a few studies of prostate cancer have demonstrated the *Mxi1* gene to be mutated or deleted.^{9,10)} Recently we demonstrated *Mxi1* alterations to be relatively common in esophageal squamous cancer cells from experimentally induced rodent tumors (Wang *et al.*, personal communications). Herein, we extended our search for *Mxi1* mutations to 31 human gastric cancers, 7 esophageal cancers and 85 bone and soft tissue tumors of various types including 4 neurofibrosarcomas. We also examined 29 human tumor cell lines derived from 12 esophageal cancers, 7 glioma/glioblastomas and 10 other tumors.

MATERIALS AND METHODS

Samples Specimens of 31 spontaneous gastric cancers, 7 esophageal cancers, and 85 bone and soft tissue tumors of various types, including 4 neurofibrosarcomas, 7 osteosarcomas, one fibrosarcoma, 8 chondrosarcomas, 4 malignant fibrous histiocytomas, 3 synovial sarcomas, 3 rhabdomyo-

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Table I. Primers for PCR

Exon	Forward	Reverse
1	5'-ATGGAGCGGGTGAAGATGAT-3'	5'-GCACTGCCGAAAAAGATTAG-3'
2	5'-GGGTCAATGGATTTGGGTAC-3'	5'-TAAGCGTCCAGCTTGCTA-3'
4	5'-TAACCAGACTGTGCTGATTTG-3'	5'-ACCAGAACTGAGGGAATTGTG-3'
5	5'-TGTTTGTACTGGACTATACAC-3'	5'-ATGTTTAGTATTTTCATTAGAGAAG-3'
6	5'-GTTAGTTTTTGAAGGTGCGC-3'	5'-TGTTATGTCATGCTGGGTTC-3'

sarcomas, one alveolar soft tissue sarcoma, 4 Ewing sarcomas, 2 leiomyosarcomas, 2 liposarcomas, one odontogenic sarcoma, 22 giant cell tumors of bone, one giant cell tumor of the tendon sheath, one osteoblastoma, 2 chondromas, 3 chondroblastomas, one chordoma, one glomus tumor, one neurofibroma, 2 schwannomas, one synovial chondromatosis, one synovioma, 6 fibromas, and one fibrous dysplasia of bone, were obtained from Hamamatsu University School of Medicine, National Cancer Center, Tottori University, University of Tokyo and China Medical University. Twenty-nine human tumor cell lines including 12 from esophageal cancers, 3 glioblastomas, 4 gliomas, one neuroblastoma (GOTO), one cervical carcinoma (HeLa), one T-cell lymphoma (H9), one epidermoid carcinoma (A-431), one choriocarcinoma (BeWo), one hepatocellular carcinoma (HUH-6), 2 colon carcinomas (DLD-1 and 320), one osteogenic sarcoma (143B) and one fibrosarcoma (HT-1080), were obtained from the American Type Cell Collection and Japanese Cancer Research Resources Bank. Table II lists the neurofibrosarcomas.

DNA was extracted from fresh tissue by proteinase K digestion and phenol/chloroform.¹¹⁾ Using formalin-fixed, paraffin-embedded tissue, we scraped off areas where 90% or more cells were tumor cells under a microscope to extract DNA from multiple 10- μ m sections and put the scrapings in a tube. One milliliter of xylene was added to deparaffinize the tissue, employing gentle rotation overnight, then the mixture was spun for 5 min at high speed in a microcentrifuge. The xylene was removed, and another ml of xylene was added, followed by gentle rotation for 30 min. The sample was then spun for 5 min at high speed in a microcentrifuge. The pellet was washed with 1 ml of 100% ethanol for more than 30 min. This step was repeated twice more. The pellet was then washed with 1 ml of 80% ethanol and spun down for 5 min at high speed in a microcentrifuge. The pellets were dried, resuspended in 100 μ l of digestion buffer (50 mM Tris HCl, 1 mM EDTA pH 8.0, 0.5% Tween 20) containing proteinase K (0.04 mg/ml) and incubated at 37°C overnight. The suspension was then heated at 65°C for 7 min to inactivate the proteinase K, and finally centrifuged for 10 min. The supernatant was used as the DNA template in the subsequent polymerase chain reaction (PCR) amplifications.

PCR, single-strand conformation polymorphism (SSCP) and sequencing We designed primers for amplification of exons 1, 2, 4, 5 and 6 of human *Mxi1* by using flanking intronic sequences (Table I). A 100 ng DNA sample was diluted into a 24 μ l reaction solution containing 10 mM Tris-HCl (pH 8.5), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.2 mM of each deoxyribonucleoside triphosphate, 0.5 μ M of each primer, and 1 unit *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany). PCR was performed in a Progra Tem PC-700 (Astec, Tokyo). After an initial denaturation at 95°C for 5 min, the DNA was amplified in 30 cycles of 30 s at 95°C, 60 s at 55°C, and 60 s at 72°C, followed by a final extension step of 10 min at 72°C.

We added 5 μ l of loading buffer (95% formamide and dye) to 5 μ l of the PCR product, which was then denatured for 15 min at 96°C and loaded on 10% nondenaturing polyacrylamide gel with or without 5% glycerol at room temperature and 4°C. SSCP gels were stained with a silver staining kit (Wako, Osaka). Because PCR is notoriously error-prone and can introduce mutations with a frequency of approximately 8.0×10^{-6} per base pair per duplication of *Taq* DNA polymerase,¹²⁾ we independently repeated the PCR-SSCP three additional times on each sample that had an extra band on SSCP to rule out artificial mutation. We also used Pfu DNA polymerase (Stratagene, La Jolla, CA) which introduces mutations at a rate of 1.3×10^{-6} per base pair per duplication in cases showing a suspected mutation.¹²⁾

PCR fragments from the cases in which we found an extra band on SSCP, were cloned into pBS (Stratagene) after treatment with polynucleotide kinase or pGEM-T EasyVector (Promega, Madison, WI). Six clones were sequenced with Sequenase Version 2.0 (Amersham, Cleveland, OH), such that at least two clones exhibited the same sequence consistently. We also directly sequenced exon 6 of neurofibrosarcoma case 3 employing an "ABI PRISM" Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and an ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA) to confirm the mutations.

Loss of heterozygosity (LOH) detection TG repeats in intron 3 and AAAAC repeats in the 3' non-coding region

of human *Mxi1* were detected. We performed PCR with primers 5'-GTAACACACTGTAGGATGGC-3' and 5'-TA-AAACCACAGGCGAGAGGA-3' for TG repeats, 5'-GG-TTACTCCAGTGCCAGTGT-3' and 5'-TTAAATACAGG-TCCTCTGACCC-3' for AAAAC repeats, to detect LOH in 31 gastric cancers, 7 esophageal cancers and 4 neurofibrosarcomas.

Polymorphism We first noticed (TTC)₂ in place of the (TTC)₃ in GenBank (U32515) in some of the tumors in our series. To determine whether or not this is a polymorphism, we surveyed peripheral blood DNAs of several populations. Peripheral blood DNA was extracted as

described previously.¹³⁾ The primers used for detecting (TTC)₃/(TTC)₂ were 5'-GTTAGTTTTTGAAGGTGCGC-3' and 5'-TGTTATGTCATGCTGGGTTC-3'.

RESULTS

We found an extra SSCP band in exon 6 in one case of chondrosarcoma. We also discovered extra SSCP bands in exon 2 in neurofibrosarcoma case 1, in exon 5 in neurofibrosarcoma case 2 and in exon 6 in neurofibrosarcoma case 3. The extra SSCP bands detected in 320 and HT-1080 were in exon 2 and the extra SSCP bands detected in

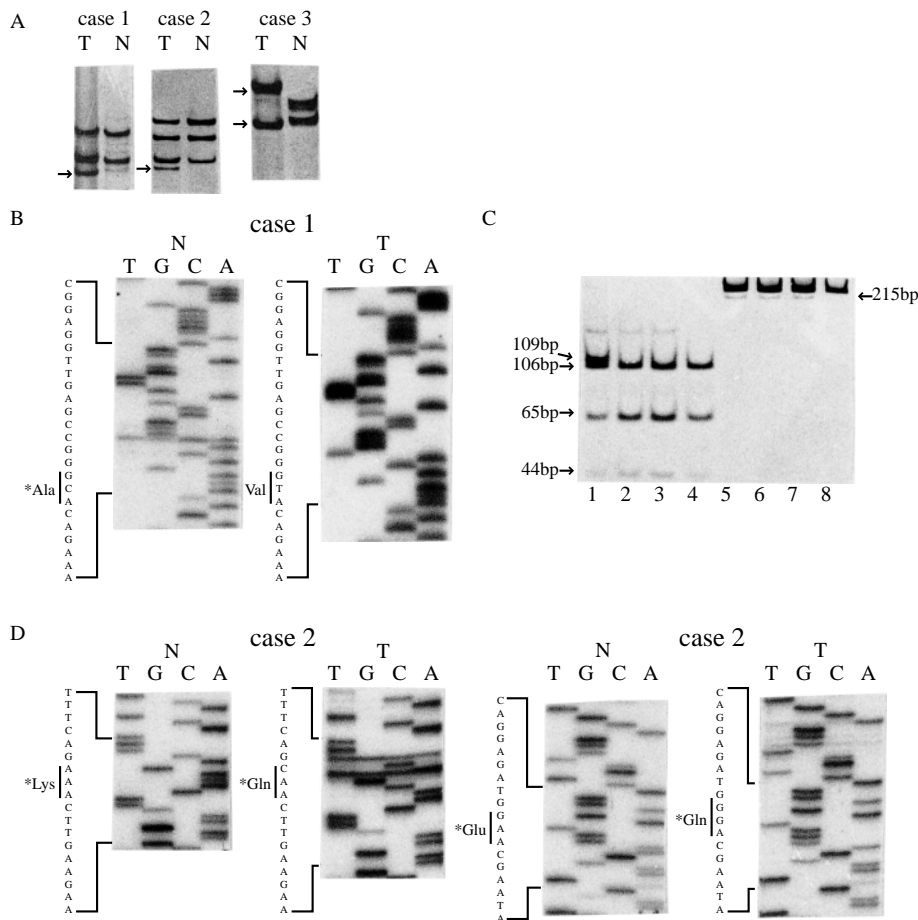


Fig. 1. The SSCP results of neurofibrosarcoma cases 1, 2 and 3 and the sequencing results of cases 1 and 2. (A) The SSCP results of neurofibrosarcoma cases 1, 2 and 3. Arrows indicate the extra bands. (B) The sequencing results of neurofibrosarcoma case 1. There is a missense mutation, GCA to GTA (Ala to Val), at codon 54. (C) The *Bsp1286I* digestion results of exon 2 PCR fragments from tumor and normal tissue DNA. The normal tissue is expected to generate two bands of 44 and 65 bp by *Bsp1286I* digestion, in addition to a constant band of 106 bp (lanes 2, 3 and 4). The mutation at codon 54 resulted in another 109 bp, presumably undigested, band as expected (lane 1, case 1). The bands of 44 and 65 bp in lane 1 may be from contaminating normal tissue or another wild-type allele. Lanes 5, 6, 7 and 8 represent undigested PCR products. D: The sequencing results of neurofibrosarcoma case 2. The left panel shows a missense mutation, AAA to CAA (Lys to Gln), at codon 118. The right panel shows the area containing another missense mutation, GAA to GGA (Glu to Gly), at codon 154.

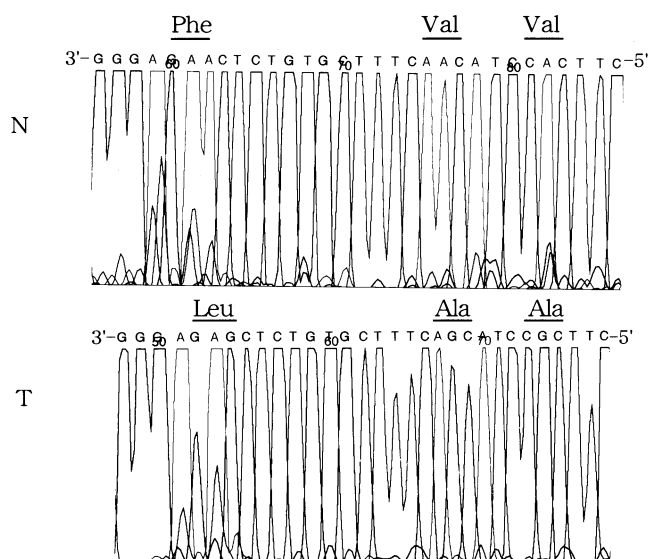


Fig. 2. The sequencing results of neurofibrosarcoma case 3. There are three missense mutations, GTG to GCG (Val to Ala), GTT to GCT (Val to Ala) and TTC to CTC (Phe to Leu), at codons 179, 181 and 186, respectively (Complimentary, reversed sequences are shown). N (upper) and T (bottom) indicate normal and tumor tissue, respectively.

Table II. Neurofibrosarcoma Cases

Case	Age	Sex	MxiI alteration			
			Exon	Codon	Nucleotide change	Amino acid change
1	73	M	2	54	GCA→GTA	Ala→Val
2	14	F	5	118	AAA→CAA	Lys→Gln
				154	GAA→GGA	Glu→Gly
3	59	M	6	179	GTG→GCG	Val→Ala
				181	TTG→TCG	Val→Ala
				186	CTT→CTC	Phe→Leu
4	43	M			—	—

DLD-1 and U87 were in exon 6. Fig. 1A shows the SSCP results of neurofibrosarcoma cases 1, 2 and 3. By sequencing, a $(TTC)_3$ to $(TTC)_2$ change was detected at the intron-exon boundary located 21 bp upstream from exon 6 in the chondrosarcoma case. In neurofibrosarcoma case 1, who had neurofibromatosis type 1 (NF1), we found one missense mutation in exon 2, GCA to GTA (Ala 54 Val) (Fig. 1B). According to the GenBank data, there are two *Bsp1286I* recognition sites, at codons 40 and 54, in exon 2. The PCR fragments of exon 2 originating from normal and tumor DNAs were digested with *Bsp1286I* to confirm that the normal exon 2 sequence is identical with the GenBank codon 54. As expected, the pattern shown in lane 1



Fig. 3. $(TTC)_2/(TTC)_3$ polymorphism in Caucasian DNAs. Lanes 1, 2 and 3 represent $(TTC)_3/(TTC)_3$, $(TTC)_2/(TTC)_3$ and $(TTC)_2/(TTC)_2$, respectively.

confirmed the mutation in codon 54 in neurofibrosarcoma case 1 (Fig. 1C). We also used *RsaI* to digest the PCR fragments of exon 2 originating from normal and tumor DNAs because the mutation induced an additional *RsaI* recognition site, and confirmed the result obtained with *Bsp1286I* (data not shown).

In neurofibrosarcoma case 2, who also had NF1, we detected two missense mutations in exon 5, AAA to CAA (Lys 118 Gln) and GAA to GGA (Glu 154 Gly), in the same clone (Fig. 1D). In neurofibrosarcoma case 3, we found 3 amino acid substitutions in exon 6, GTG to GCG (Val 179 Ala), GTT to GCT (Val 181 Ala) and TTC to CTC (Phe 186 Leu) (Fig. 2). In order to verify the mutations in case 3, we designed new primers, 5'-TGAAGGT-GCGCTATACTCGA-3' and 5'-AGGCTGCTGTGGTCAT-CAAT-3', for short exon 6 PCR fragments and confirmed the same base substitutions. Since the SSCP pattern contained residual wild-type allele in cases 1 and 2, some wild-type allele appears to remain in these tumors. On the other hand, no wild-type band was present in case 3, which probably reflects loss of the wild-type allele. The results of SSCP and sequencing of neurofibrosarcoma cases 1, 2 and 3 are summarized in Table II.

In exon 2 of the HT-1080, 320 cell line, we found a GGC-to-GGT alteration at codon 29. A $(TTC)_3$ to $(TTC)_2$ change, which is the same as in the chondrosarcoma, was detected in exon 6 in DLD-1 and U87MG.

We checked for LOH of *MxiI* using the TG repeats in intron 3 and AAAAC repeats in the 3' non-coding region, but found none in the two repeat regions of 31 gastric cancers, 7 esophageal cancers and 4 neurofibrosarcomas, which yielded corresponding normal DNA. The numbers of TG informative cases are 12, 2 and 0 for gastric cancers, esophageal cancers and neurofibrosarcomas, respectively. The numbers of AAAAC informative cases are 6, 4 and 0 for gastric cancers, esophageal cancers and neurofibrosarcomas, respectively.

As to the aforementioned alterations, $(TTC)_3$ to $(TTC)_2$ in intron 5 of DLD-1, U87MG and one of the chondrosarcoma cases, we could not determine whether these constituted somatic changes since there was no corresponding normal DNA in these cell lines and the sarcoma. We checked normal tissues from 50 Japanese, 50 Chinese and 44 Australian Caucasians and demonstrated 26:17:1 $\{(TTC)_3/(TTC)_3:(TTC)_3/(TTC)_2:(TTC)_2/(TTC)_2\}$ in the Cau-

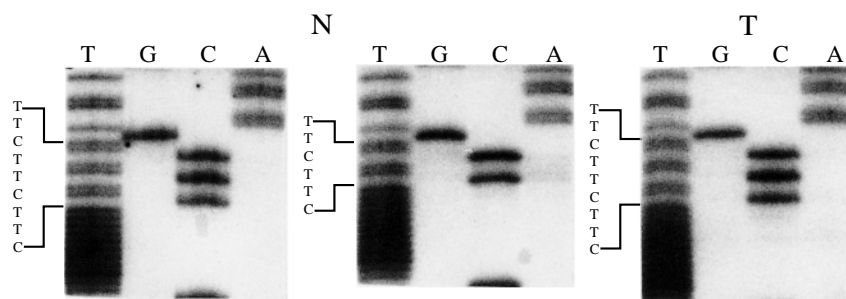


Fig. 4. The result of LOH in neurofibrosarcoma case 3. There were two different clones in normal tissues from neurofibrosarcoma case 3, $(TTC)_3$ and $(TTC)_2$, probably reflecting the informative heterozygotic state in this case. In contrast, the tumor contained only $(TTC)_3$ clone.

casians, while no such alterations were present in Japanese and Chinese, suggesting the $(TTC)_3/(TTC)_2$ sequences are clearly a polymorphism, but that the $(TTC)_2$ allele is rare in Japanese and Chinese (Fig. 3). In the normal tissue from neurofibrosarcoma case 3, the $(TTC)_3/(TTC)_2$ type was detected, while the $(TTC)_3/(TTC)_3$ type was present in tumor tissue, suggesting LOH (Fig. 4). We did not found LOH in this region of neurofibrosarcoma cases 1 and 2. In order to determine whether the alteration GGC to GGT at codon 29 of exon 2 of the HT-1080, 320 cell line is a polymorphism, we examined normal tissues from 30 Japanese and 40 Australian Caucasians. As no polymorphism was detected, we assumed this to be a silent mutation.

DISCUSSION

Screening a total of 123 primary tumors and 29 cell lines revealed the *Mxi1* mutation to be rare in human solid tumors, including one case of neurofibroma associated with NF1 and the neurofibroma portions of neurofibrosarcoma cases 1 and 2. The low frequency of *Mxi1* alterations is consistent with previous reports on lung and prostate cancer.^{14, 15} However, 3 of 4 neurofibrosarcomas, rare and highly malignant tumors, had mutations in functional exons. One missense mutation was detected at codon 54 in neurofibrosarcoma case 1, who had NF1. The mutation is situated between two critical regions, the Sin3-interacting domain and the basic domain. It would be intriguing to know whether this mutation influences the spatial interactions of Sin3 and *Mxi1*.

We found another missense mutation at codon 118 in the Zip region of neurofibrosarcoma case 2, who also had NF1. Since *Mxi1* and Max dimerize through their HLH and Zip motifs to prevent the formation of Myc-Max heterodimers and compete with these heterodimers for binding to target sites,¹⁶ the Zip region mutation observed here probably prevents the formation of *Mxi1*-Max het-

erodimers and may thereby impair the function of Myc inhibition.

We demonstrated another missense mutation at codon 154 in neurofibrosarcoma case 2 and missense mutations in exon 6 in neurofibrosarcoma case 3. These are at the C-terminal domain of *Mxi1*, which was shown recently to repress the transcription from the major *c-Myc* promoter, P2. The repression is independent of *Mxi1* binding to mSin3, but is dependent on the *Mxi1* HLH domain, Zip domain and C-terminal domain.¹⁷ The C-terminal domain mutations observed here may thus impair the *Mxi1* function. We also demonstrated LOH in neurofibrosarcoma case 3. SSCP analysis demonstrated loss of wild-type alleles in tumor DNA (Fig. 1A, case 3). A change, from “ $(TTC)_2$ -exon 6 wild type/ $(TTC)_3$ -exon 6 wild type” to “ $(TTC)_3$ -exon 6 mutation/- (missing)” is assumed to have occurred in this tumor. These findings in neurofibrosarcoma case 3 may be compatible with a two-hit mechanism.¹⁸ Although we could not find LOH in neurofibrosarcoma cases 1 and 2, other factors, for example methylation, that can not be detected by the method used here, may be involved in inactivation of the allele.

Neurofibrosarcoma is known to develop in patients with NF1 at a reported incidence of 2–4%, but little is known about the molecular genetic alterations that underlie this malignant transformation. Our findings suggest that the *Mxi1* mutation may play a role in malignant transformation of neurofibromas in NF1 patients. Neurofibromin, the product of *NF1* gene, contains a GTPase-activating protein (GAP)-related domain which negatively regulates Ras activity.^{19–22} Ras is involved in the regulation of *c-myc* gene expression.²³ Thus, multiple genes are involved in malignant transformation of neurofibroma in NF1 patients and *Mxi1* may be among the candidate genes.

Although *Mxi1* mutations were rare in our samples, the presence of *Mxi1* mutations in neurofibrosarcoma may justify a more extensive search given the possible involvement of this gene in other tumors. The codons involved

were conserved among human, rat, mouse and zebrafish, suggesting that they are important to the function of *Mxi1*. Although some of them changed only in the same neutral amino acid, these mutations were located in or near possibly functional regions, so the function of *Mxi1* may be affected. Further study of these mutations, especially in patients with NF1, is warranted.

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