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# Toward a Survey of Somatic Mutation of the *NF1* Gene in Benign Neurofibromas of Patients with Neurofibromatosis Type 1

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## Summary

**Neurofibromatosis type 1 (NF1), a common autosomal dominant disorder caused by mutations of the *NF1* gene, is characterized by multiple neurofibromas, pigmentation anomalies, and a variety of other possible complications, including an increased risk of malignant neoplasias. Tumorigenesis in NF1 is believed to follow the two-hit hypothesis postulated for tumor-suppressor genes. Loss of heterozygosity (LOH) has been shown to occur in NF1-associated malignancies and in benign neurofibromas, but only few of the latter yielded a positive result. Here we describe a systematic approach of searching for somatic inactivation of the *NF1* gene in neurofibromas. In the course of these studies, two new intragenic polymorphisms of the *NF1* gene, a tetranucleotide repeat and a 21-bp duplication, could be identified. Three tumor-specific point mutations and two LOH events were detected among seven neurofibromas from four different NF1 patients. Our results suggest that small subtle mutations occur with similar frequency to that of LOH in benign neurofibromas and that somatic inactivation of the *NF1* gene is a general event in these tumors. The spectrum of somatic mutations occurring in various tumors from individual NF1 patients may contribute to the understanding of variable expressivity of the NF1 phenotype.**

## Introduction

Neurofibromatosis type 1 (NF1 [MIM 16220]) is an autosomal dominant inherited disease that is characterized by the occurrence of multiple neurofibromas and pigmentation anomalies (e.g., café au lait macules and Lisch nodules). Besides these diagnostic criteria for NF1, a variety of complications may arise, including an increased risk for certain malignancies. Another characteristic feature of the disease is the high degree of variable expressivity, which renders NF1 a model system for the study of modifying effects on hereditary diseases. The variable expressivity of the *NF1* gene defects abrogates the possibility of predicting patients' outcomes. The *NF1* gene has been mapped to chromosome 17q11.2, and, since its cloning in 1990, numerous constitutional mutations of patients have been described (Upadhyaya and Cooper 1998, NNFF International NF1 Genetic Mutation Analysis Consortium, Human Gene Mutation Database Cardiff). Point mutations, small insertions, and deletions are randomly scattered throughout the gene, with few positions where recurrent mutations have occurred. Tumorigenesis in NF1 has been assumed to follow the two-hit hypothesis for tumor-suppressor genes that was initially postulated by Knudson for the retinoblastoma gene (Knudson 1971).

LOH has been shown for a number of NF1-associated malignancies, as well as for malignant tumors or cell lines thereof from non-NF1 patients (Xu et al. 1992; Andersen et al. 1993; Johnson et al. 1993; Legius et al. 1993; Shannon et al. 1994). Recently, the somatic inactivation of the *NF1* gene has been described as occurring in benign neurofibromas also (Colman et al. 1995; Sawada et al. 1996; Däschner et al. 1997; Serra et al. 1997). One of these studies (Sawada et al. 1996) described a short deletion occurring in neurofibroma cells in a patient whose constitutional mutation was a deletion of the whole *NF1* gene. These authors were also able to show that only a subset of cells from the tumor harbored the somatic event. The other studies concentrated on a more indirect approach to searching for LOH in benign neurofibromas, but only few of these studies yielded positive results. Editing of the *NF1* mRNA has also been discussed as playing a role in tumorigenesis in NF1 (Cappione et al. 1997; Skuse and Cappione 1997). The analysis of the spectrum of mutations occurring in various neurofibromas of NF1 patients is an important task, because the type and frequency of these mutations could have an impact on the severity of patients' phe-

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notypes, as has been postulated for other hereditary cancer syndromes, such as familial adenomatous polyposis (Spirio et al. 1999). For the *APC* gene, the *RB* gene, and other tumor-suppressor genes, databases of constutional mutations and somatic mutations found in tumors have been set up as a survey of mutated alleles occurring in somatic and germ cells (APC Gene Database, RB1base). So far, only germline mutations and polymorphisms of the *NF1* gene are included in the databases (NNFF International NF1 Genetic Mutation Analysis Consortium, Human Gene Mutation Database Cardiff). The aim of our study was to start a systematic approach to searching for somatic inactivation of the *NF1* gene in neurofibromas. For this purpose, neurofibromas were screened for mutations causing premature translational termination with the protein-truncation test. In cases where no truncated peptide was found, tumor DNA was analyzed by LOH analysis with a series of polymorphic markers, two of which are newly described in this paper, in comparison to patients' blood DNA. This is the first description of a systematic search for somatic mutation of the *NF1* gene in neurofibromas from NF1 patients.

## Material and Methods

### Human Subjects

NF1 patients were diagnosed according to the criteria of the NIH Consensus Statement (Stumpf et al. 1988). An overview of the clinical data is given in table 1. Tumors were wide and deep-excised after injection of lidocaine. The two neurofibromas from patient NF260 were located on the abdomen, the neurofibromas from patient NF282 were located in the central area of the back, and the plexiform neurofibroma from patient NF284 affected the sciatic nerve. Neither the precise locations nor the distances from each other of the excised dermal tumors were documented by the surgeons.

### Nucleic Acid Preparation from Tumor Specimen

Neurofibromas were kept in Dulbecco's modified Eagles medium (DMEM) until the tumor tissue was separated from the surrounding skin under careful visual control. In some cases, the tumor tissue has been divided into several pieces to raise cultures of fibroblast-like cells. The other parts of the tumor or the tumor as a whole and the skin were immediately shock-frozen in liquid nitrogen and stored at −70°C. Frozen tissues were homogenized with a bead mill (Mikrodismembrator II Braun, Melsungen) before preparation either of total RNA with the RNA-purification kit from Qiagen or DNA with the Qiaamp Tissue kit from the same supplier. Both procedures were done according to the instructions of the manufacturer.

*Cultures of Fibroblast-Like Cells from Neurofibromas.*—Fresh tumor samples were minced into small pieces and cells were disintegrated by treatment with collagenase (1300 U) for 2 h. Cells were grown in DMEM supplemented with 10% fetal-calf serum.

*Reverse Transcription and PCR.*—Two micrograms of total RNA was reverse transcribed by random hexamer priming with Superscript II reverse transcriptase (Gibco BRL) in a final volume of 20 μl. PCR reactions contained the after components: 2 μl cDNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 mM of forward and reverse primer, and 2.5 U *Taq* Polymerase (Amersham Pharmacia Biotech). All forward primers for the protein truncation testing (PTT) approach included a leader sequence of a T7 transcription promoter plus eukaryotic translation initiation signals. Primer sequences for the PTT fragments were as follows; the numbers give the position of the first 5′ and the last 3′ base of the fragments amplified with the respective primers within the coding region of the neurofibromin mRNA (NNFF International NF1 Genetic Mutation Analysis Consortium). Leader: L=GGATCCTAATACGACTCACTATAGGGAGACCACCATG; P1: L-1-ATGGCCGCGCACAGGCCGGTGGAAT; P2: 1337-GTTTCACCAAACATATTTCGAA; P3: L-967-GCCTGTGTCAAACTGTGTAAAG; P4: 1868-TGACAGGAACTTCTATCTGCCTGCTTA; P5: L-1486-ATGGTGAAACTAATTCATGCAGAT; P6: 2675-CTGACAGGTGTATCTGCGTTT; P7: L-2308-ACTGCAGGAAACACTGAG; P8: 3583-TGTCAAATTCTGTGCCTTG; P9: L-3148-ATGGGAACATCAAACCAAGC; P10: 4393-AATCAGATGCTATATCAAGGA; P11: L-4024-ATGACTGAAAAGTTCTTCCATGC; P12: 5256-TAGGACTTTTGTTCGCTCTGCTGA; P13: L-4998-GGAGTACACCAAGTATCATGAG; P14: 6282-CGGACCTGTGGCTACTAAGAA; P15: L-5902-TACCCATCTATTCAATCAAA; P16: 6987-TATACGGAGACTATCTAAAGTATGCAG; P17: L-6574 ATGGAGGCATGCATGAGAGATATTC; P18: 7876-TGGCCTCTGCTAAGTATTCATA; P19: L-7531-GCCAACACTAAGAAGTTGCTTG; and P20: 8404-TCTGCACTTGGCTTGCGGAT. The first rounds of PCR reactions were done, for example, with primers 1 and 4; 0.5 μl of PCR products were then reamplified with primers 1 and 2 as well as 3 and 4, and so on.

For LOH analyses, primers and PCR conditions were used as published (Weber et al. 1990; Ainsworth and Rodenhiser 1991; Lázaro et al. 1993*a,* 1993*b,* 1994; Hoffmeyer and Assum 1994; Purandare et al. 1996). The two newly identified polymorphisms locate to intron 1 (Pin1) and intron 28 (Pin28) of the *NF1* gene. The primer sequences for the new polymorphisms are as follows: Pin 1: CAGAGATTCTCCTCTCCTGCA, GAGTTCAACCTTTGAAGCCCA; Pin 28: TAGGTGTCCTACCCCTATAGT; AAAGGTCAAGAATCTTATAGAAG. For a few patients, it was possible to include the

## Table 1

**Summary of the Clinical Data of NF1 Patients, the Neurofibromas Analyzed, and the Mutations Identified by PTT or LOH**

| Patient, Sex | Age, Family History, Phenotype | Previously Known Constitutional Mutation | Neurofibromas Studied | Aberrant PTT Fragment | Mutation Identified[a] | Protein Level | LOH |
|---|---|---|---|---|---|---|---|
| NF56, F | 58 years, sporadic, >200 neurofibromas | C→T position 1246 | 1, dermal | None | ... | ... | No |
| | | | 2, dermal | None | ... | ... | Yes |
| NF260, F | 32 years, familiar, brain tumor, >100 neurofibromas | Unknown | 1, dermal | 5 | TS: C→T position 3721 | R→X position 1241 | NA |
| | | | 2, dermal | 6 | TS: not identified | | NA |
| NF282, M | 36 years, sporadic, >20 neurofibromas | Unknown | 1, dermal | 1, 5 | TS: C→T pos. 4021 | Q→X position 1341 | NA |
| | | | 2, dermal | 1, 5 | TS: C→T pos. 4084 | R→X position 1362 | NA |
| | | | | | C: G→A pos. 1260+1 13 bp IVS included (mRNA) | 11 altered amino acids, X at 428 | |
| NF284, M | 24 years, sporadic, skoliosis, >20 neurofibromas, 2 plexiform neurofibromas | Unknown | 1, plexiform | 6 | C: 14-bp duplication, position 4907–4920 | 40 altered amino acids, X at 1676 | Yes |

NOTE.—NA = not analyzed.

[a] C = constitutional; TS = tumor-specific.

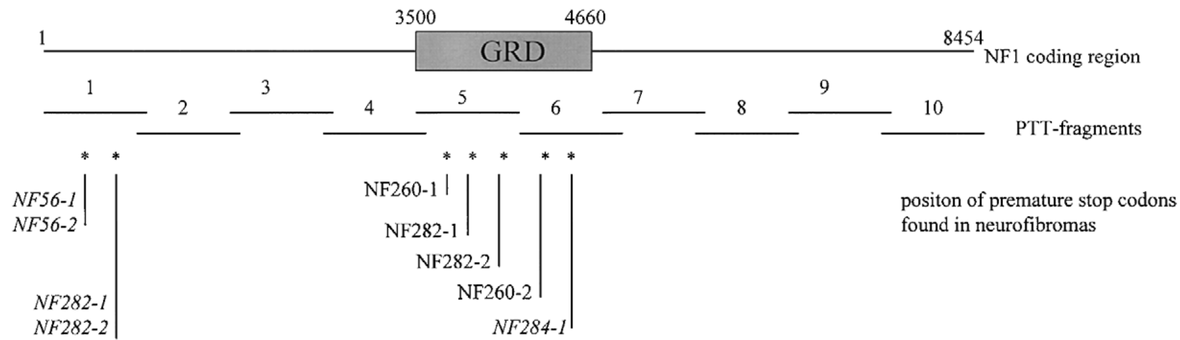


**Figure 1** Distribution of PTT fragments over the coding region of the *NF1* gene and relative position of premature stop codons that have been found in neurofibromas. Constitutional mutations are italicized.

germline mutations in LOH analysis of the genomic DNA isolated from blood and tumor specimen. In these cases, the following intron-based primers were used: PI8H: ACTTTTATCGGTATTTCTCAATATT; PI9R: TTAGCAATACCTTTTGGACTAA; for NF56, NFEx28H: TCTTTGTCTTTTTTGTCATTTTCC; NFEx28R: AGTCAAGAAAAGCAATGAATCGT for NF284.

Pin 1 was amplified at an annealing temperature of 62°C for 30 cycles. The forward primer was labeled with Cy5 and PCR products were run on the ALFexpress DNA Sequencer (Amersham Pharmacia Biotech) for typing of alleles. PCR products range in size from 206–218 bps according to the number of repeated units present in the alleles. The annealing temperature for the Pin 28 primers was 56°C and PCR products (957 bps) were digested with *Taq*I before agarose gel electrophoresis to obtain a better resolution of the products from the different alleles (±21 bp).

*In Vitro Transcription and Translation.*—PCR products were exposed to the coupled transcription and translation reactions, as recommended by the supplier (Promega). For labeling of peptides, $^{35}$S-methionine (Amersham Pharmacia Biotech) was included in the reactions. Peptides were analyzed by Laemmli SDS-PAGE, and gels were dried and exposed to X-ray films (Kodak X-Omat). Reaction products derived from tumor specimen were compared to products generated from fibroblast-like cells from healthy individuals in each experiment.

*Cloning and Sequence Analysis.*—The purified PCR products (PCR-Purification Kit [Qiagen]) were cloned in *Escherichia coli* JM109 applying the Sure Clone Kit (Amersham Pharmacia Biotech) as recommended by the manufacturer. After the isolation of the plasmid DNA with a commercially available kit (Amersham Pharmacia Biotech), sequencing reactions were performed with the Thermo Sequenase cycle sequencing Kit (Amersham Pharmacia Biotech) and run on an ALFexpress DNA Sequencer (Amersham Pharmacia Biotech).

## Results

### *Search for NF1 Gene Mutations in Neurofibromas with the Protein-Truncation Test*

Seven tumors from four NF1 patients were included in the screen for somatic mutations. Table 1 summarizes the data for NF1 patients and tumors that were analyzed by PTT. The constitutional *NF1* mutation was already known for one patient (NF56); the disease-causing mutations of the other three patients were unknown at that time, but two could be identified in the course of this study. PTT analysis in neurofibromas was done with a similar protocol to that used by Heim et al. (1995) in their screening for constitutional mutations in NF1 patients. The whole coding region of the neurofibromin gene was amplified in five overlapping RT-PCR fragments. Whereas most of the neurofibroma samples did not yield sufficient amounts of PCR product from these large fragments for the subsequent steps of the reaction, each fragment was further divided into two subfragments, each about 1 kb in size (fig. 1), and was reamplified in a nested PCR reaction (see PCR primers in "Materials and Methods").

Seven different shortened peptide fragments were detected in the tumor samples. An overview of the position of premature stop codons derived from fragments generated from the various neurofibromas is given in figure 1. Neurofibromas NF260-1 and NF260-2 revealed different altered PTT fragments; NF260-1 in fragment 5; NF260-2 in fragment 6. Neurofibromas NF282-1 and NF282-2 showed both an additional peptide of the same size derived from fragment 1 and each of them harbored a further aberrant peptide, compared to control cells in fragment 5. The peptide bands in fragment 5 generated from the two tumor samples were clearly different in size and therefore should reflect different somatic mutations in the neurofibromas of patient NF282, whereas the common mutation in fragment 1 should represent

the constitutional mutation. The last tumor that gave positive results with the PTT was the plexiform neurofibroma NF284-1, where an aberrant banding pattern was found for peptides derived from fragment 6. The result of a representative PTT-experiment is shown in figure 2. All other neurofibromas did not show any altered peptides compared to the pattern generated from fibroblasts of healthy individuals. Tumors NF56-1 and NF56-2 exhibited the additional band corresponding to the already known constitutional mutation of patient NF56.

The corresponding *NF1* gene regions, assumed to harbor the causal sequence alterations, were reamplified from the cDNA of neurofibromas with nested exon-based PCR-primers, PCR-products were cloned in *E. coli* and a number of recombinant plasmid clones were sequenced. In cases where we found alterations from the *NF1* cDNA sequence (M89914 [GenBank]), the presence of the mutation in the neurofibroma was first checked by PCR with cDNA as template and modified primers, that allowed to discriminate between the mutant and the wild-type allele by restriction digestion. In two cases (NF282 and NF284), the mutant and the wild-type allele could be distinguished simply by an altered size of the RT-PCR products (data not shown). All sequence variations initially identified at the cDNA-level were also found in genomic DNA samples isolated either from the corresponding neurofibromas in case of somatic mutations or from cultured fibroblast-like cells or blood cells of the patients for constitutional mutations.

The details of the mutations that were identified are summarized in table 1. Both tumors of patient NF282 exhibited the same G→A transition in position 1260+1, which is the splice-donor site of intron 9. The mutation leads to the inclusion of 13 bp of intervening sequence into the *NF1* messenger. The mutant allele is present in all tissues tested from patient NF282 (as indicated by an additional larger RT-PCR product or the presence of an altered restriction site of the genomic PCR-products by using modified intron-based primers; data not shown) and is therefore assumed to represent the germline mutation. Neurofibroma NF282-1 from this patient showed an additional C→T transition at position 4021, neurofibroma NF282-2 a further C→T transition at position 4084. Both alterations lead to premature stop codons in the corresponding *NF1* message.

The mutation of neurofibroma 1 from patient 260 (NF260-1) was a C→T transition at cDNA position 3721, resulting in a premature stop codon in the protein-coding region of the *NF1* gene. This mutation is specific for the tumor NF260-1 and is not present in the other tumor analyzed from this patient. The constitutional mutation of this patient is still unknown and we did not find any alterations that NF260-1 and NF260-2 had in common. Tumor NF260-2 showed a truncated peptide

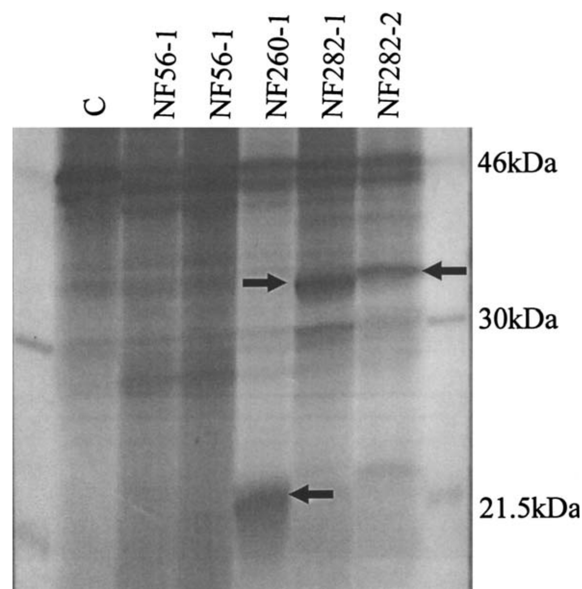


**Figure 2** Protein gel loaded with translation products of PTT fragment 5 derived from various neurofibromas of NF1 patients and cells of a healthy individual (C) NF260-1, NF282-1 and NF282-2 show a truncated peptide derived from this part of the *NF1* gene (*arrows*). PTT analysis was done in duplicate for NF56-1 to show the reproducibility of the peptide patterns.

in fragment 6, but we were not able to find the underlying sequence alteration of this tumor after sequencing of >10 recombinant plasmid clones. Possibly the mutant allele was greatly underrepresented and therefore has escaped detection.

The mutation that corresponds to the shorter peptide generated from the plexiform neurofibroma NF284-1, was a 14-bp duplication at cDNA position 4907, which leads to a disruption of the reading frame and introduction of a nearby stop codon (5027). This alteration is not a tumor-specific event, because it turned out to be present also in blood and cell cultures of patient NF284 and therefore represents the constitutional mutation. Although we failed to identify a second mutational event in this tumor, it exhibited LOH at the *NF1*-locus (see next section).

Regarding the somatic point mutations, we can not technically rule out the possibility that they may affect the gene copy carrying the germline mutation. It is not possible to link the somatic mutations with the regions harboring the respective constitutional mutations, given the fact that these mutations are too far away from each other to be analyzed in common with our test system. But, to us, this possibility seems rather unlikely, because in this case the progenitor cells of the lesions should have suffered from two independent somatic mutations in both alleles of the *NF1* gene, one affecting the con-

stitutional mutant allele and another affecting the wild-type allele.

### LOH Analyses in Neurofibromas with Negative PTT Results

The three neurofibromas with negative PTT results (NF56-1, NF56-2 and NF284-1) were included in the following investigations to search for loss of the wild-type allele in tumor samples (table 2). Seven of the nine genomic markers applied for this purpose are located in the *NF1* gene, whereas D17S33 and Mfd15 map proximal and distal to the gene, respectively. The two remaining markers reflect specific constitutional mutations of the NF1 patients under investigation and therefore were only tested with samples of the respective patient.

Pin 1 is a newly identified polymorphism (TGGA)n located in intron 1, lying about 10 kb 5′ of exon 2 (I. Eisenbarth, G. Assum, unpublished data). We found this polymorphism by searching for small nucleotide repeats in the GenBank clone AC004222, which comprises the proximal end of the *NF1* gene. The polymorphism reveals four frequent alleles with different numbers of the four-nucleotide repetitive unit.

Pin 28, a polymorphism in intron 28 newly identified in this study, is characterized by a 21-bp duplication. Typing of 93 unrelated white individuals revealed allele frequencies of .41 for the duplicated sequence and .59 for the nonduplicated sequence.

The genomic polymorphisms were partly analyzed by PCR amplification with flanking intron-based primers and subsequent acrylamide gel electrophoresis of the products by using the ALFexpress DNA Sequencer (Pin1, AluI, 28.4, 53.0, Mfd 15), the other markers were analyzed by restriction digestion of amplification products and subsequent agarose gel electrophoresis (D17S33, RsaI, Pin 28, 10647). Because the constitutional mutations for the two patients (NF56 and NF284) had already been identified, it was possible to design an approach for both mutations that enabled discrimination between the *NF1* allele carrying the constitutional mutation and the wild-type allele.

For LOH analyses, the polymorphic markers were amplified in parallel from patients' blood DNA and DNA derived from neurofibromas. In each case, the amplification patterns obtained from the tumor samples of the patients were compared to the genotype of blood cells from the patients.

An overview of the results is presented in table 2. Tumor NF56-2 showed LOH with all informative genomic markers. Unequal allelic signal intensities were obtained with each of these markers from DNA of this tumor, whereas DNA from blood and from tumor NF56-1 yielded bands of equal intensities. By use of the constitutional mutation of patient NF56, we were able to show that the PCR product with the weaker signal intensity in NF56-2 derives from the wild-type allele, which can be cut with the appropriate restriction enzyme, whereas the mutant allele remains uncut in this assay (fig. 3). We did not find any hints of LOH at the *NF1* gene region in the other neurofibroma from this patient (NF56-1).

The plexiform neurofibroma NF284-1 revealed allelic loss for the wild-type allele, which was shown by PCR amplification of the genomic region of the *NF1* gene encompassing exon 28. Although blood and fibroblast-like cell cultures from the tumor exhibited two PCR products with the same intensity (wild-type allele and allele with the 14-bp duplication, which is the constitutional mutation of patient NF284), the signal representing the mutant allele was at least five times stronger than the signal corresponding to the wild-type allele when we used DNA directly prepared from the tumor tissue (data not shown). A reduction of messengers coming from the wild-type allele was also detectable in the tumor when RT-PCR was applied. This result implies that the fibroblast-like cells are not the primary affected cells in neurofibromas. Unfortunately, patient NF284

**Table 2**

**Summary of LOH Results with Polymorphic Markers of the *Nf1* Gene Region Applied to Patients' Blood and Tumor DNA**

| Patient and DNA Source | Status at | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | D17S33 5′ of NF1 | Pin 1 NF1 Intron 1 | RsaI NF1 Exon 5 | NF1 Mutation Exon 9[a] | AluI NF1 Intron 27b | 284 NF1 Intron 27b | NF1 14-bp Dupl Exon 28[b] | Pin 28 NF1 Intron 28 | 530 NF1 Intron 38 | 10647 NF1 3′UTR | Mfd 15 3′ of NF1 |
| NF56: | | | | | | | | | | | |
| Blood | NA | H | H | H | H | NI | NA | H | H | H | H |
| Neurofibroma 1 | NA | H | H | H | H | NI | NA | H | H | H | H |
| Neurofibroma 2 | NA | LOH | LOH | LOH | LOH | NI | NA | LOH | LOH | LOH | LOH |
| NF284: | | | | | | | | | | | |
| Blood | H | NI | NI | NA | NI | NI | H | NI | NI | NI | NI |
| Neurofibroma 1 | H | NI | NI | NA | NI | NI | LOH | NI | NI | NI | NI |

Note.—NA = not analyzed, NI = not informative, H = heterozygous, LOH = loss of heterozygosity.

[a] Specific for NF56.

[b] Specific for NF284.

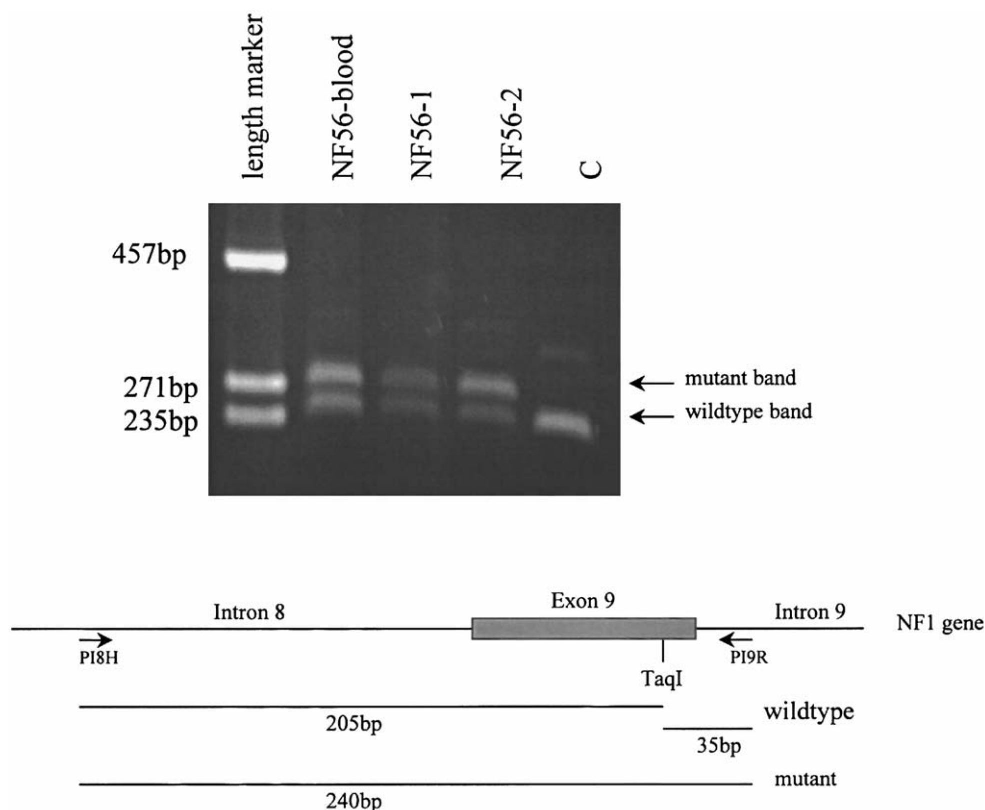


**Figure 3** Agarose gel electrophoresis of *Taq*I-digested PCR-products derived from patient NF56. The genomic region of the *NF1* gene harboring the constitutional mutation of patient NF56 was amplified with primers PI8H and PI9R. The wild-type allele can be completely digested, as shown in the control lane (C), the product from the mutant allele remains uncut. Although both products are visible with equal intensities in blood and tumor 1, tumor 2 reveals a stronger signal of the mutant allele, indicating loss of the wild-type allele in a portion of the tumor tissue. The difference in migration patterns between the length marker and PCR products is caused by the restriction buffer present in the samples.

was uninformative with all other intragenic markers tested, so that the extent of allelic loss remains unclear. D17S33, which locates proximal to the *NF1* gene in the tumor's DNA, retained heterozygosity, whereas the distal marker Mfd15 was not informative. The patient's state of homozygosity at eight of the nine markers tested can be considered as unusual. The reason for this remains unclear, since we do not have detailed information about the family history of the patient.

## Discussion

In this study, seven neurofibromas from four different NF1 patients were analyzed with a mutation-screening approach to search for somatic inactivation of the *NF1* gene. To our knowledge, this is the first report of an unbiased search for somatic inactivation of the *NF1* gene to determine the mutational spectrum and the frequency of second hits in neurofibromas. Seven aberrant peptides were identified by PTT, most of them deriving from cDNA fragments located in the GAP-related domain. We identified previously unknown constitutional mutations in two of our patients and three tumor-specific somatic mutations in our sample of neurofibromas. Four of these *NF1* variations are novel lesions (Human Gene Mutation Database Cardiff), whereas one of the tumor-specific mutations (R1362X) has been reported previously to occur in the germline of an NF1 patient (Upadhyaya et al. 1997). Interestingly, all three somatic mutations were C→T transitions, in a CpG- or CpApG-sequence context, indicating that the deamination of methylated cytosine residues may be one of the causes of the somatic mutations detected in neurofibromas. This may be true for all three mutations, since Woodcock et al. (1988) showed that not only CpG dinucleotides but also CpApG or CpTpG sequences are targets for cytosine methylation. Moreover, Andrews et al. (1996) directly showed that neurofibromin gene sequences are indeed methylated; at least in DNA derived from placenta and sperm, DNA methylation was almost exclusively restricted to CpG dinucleotides.

In PTT-negative neurofibromas we were able to describe allelic loss of the wild-type allele in two of three cases. Both tumors seem to harbor large genomic rear-

rangements. These may be deletions encompassing the whole *NF1* gene as well as flanking sequences. Alternatively, the elimination of the wild-type allele may be the result of somatic homologous recombination. Both mechanisms can act upon the *NF1* gene, as has been shown by Stephens et al. (1998). The authors found both interstitial deletions or isodisomy of 17q regions when they analyzed leukemic cells from children with NF1.

Taken together, three point mutations and two LOH events have been detected among seven neurofibromas. This accounts for a detection rate of 70% for somatic inactivation of the *NF1* gene. If we assume that the two screening methods applied can not uncover all possible mutations, like missense mutations, and that the power of the LOH approach is limited by the frequency of heterozygous genotypes of the polymorphisms employed, this rate seems to fit very well with the success rate described for the analysis of constitutional *NF1* mutations (Park and Pivnick 1998; Fahsold et al. 1999). Neurofibromas consist of a number of different cell types, and most of them exhibit a polyclonal X-inactivation pattern (Däschner et al. 1997). Therefore, it seems likely that only a subpopulation of cells from the tumors harbor the somatic mutation, as has been shown elsewhere by Sawada et al. (1996). Our data support this hypothesis, because we never found a complete disappearance of alleles in the LOH experiments; rather, the signals generated from the diminished alleles were about half as intense as those of the other alleles. In addition, fibroblast-like cells cultured from neurofibroma NF284-1 did not show the somatic mutation found within the tumor tissue analyzed without prior culturing. These results demonstrate that fibroblast-like cells are not affected by the second hit and therefore are not the cell type responsible for tumor growth, confirming the observation of Kluwe et al. (1999), who showed LOH in Schwann cell cultures raised from a neurofibroma of a NF1 patient but not in fibroblast-like cells.

It has been suggested that mRNA editing may have some impact on tumorigenesis of NF1 (Cappione et al. 1997). These authors have described a slightly increased level of mRNA editing, at position 3916C→U of the *NF1* cDNA, in tumors of NF1 patients, compared with nontumor tissue. This posttranscriptional modification leads to a premature termination codon in the respective mRNA and may result in expression of a truncated form of neurofibromin. Later studies of the same group revealed that this phenomenon is not a common feature of all tumors investigated (Skuse and Cappione 1997). All of the truncated peptides that we identified in our neurofibromas were caused by other mutations than the C→U exchange described by Cappione et al. We did not observe any conspicuous peptide pattern that would indicate a higher level of mRNA editing in the tumor samples compared to control cells.

Our results suggest that somatic mutation of the *NF1* gene is a general event in neurofibromas of NF1 patients. Although the number of identified mutations is relatively small, it seems likely that small subtle mutations occur with similar frequency to that of LOH in patients' tumors (three point mutations vs. two instances of LOH). All of the small mutations found in this study seem to cluster in the GRD of the *NF1* gene. However, because of the small sample size and the fact that all mutations are stop mutations, we believe that the apparent clustering may well have occurred by chance and does not point to a mutation-cluster region like that described for the APC gene, where 60% of somatic mutations are located in a narrow region of the gene (Béroud and Soussi 1996).

Despite the heterogeneous cell composition of neurofibromas, screening for somatic mutations is possible with the experimental approach described, and more-extensive studies using multiple neurofibromas from individual NF1 patients seem necessary to increase our knowledge about the somatic mutational spectrum in neurofibromas. It seems recommendable to include somatic mutations identified in neurofibromas in the NF1 database. These future studies may contribute to the unraveling of at least some features of variable expressivity in NF1. NF1 patients suffering from severe manifestation with several hundreds of neurofibromas should be studied for possible recurrence of the same mutation in neighboring tumors. If this is not the case, a possible involvement of genetically variant repair enzymes should be taken into account as modifiers for the NF1 phenotype.

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## Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

APC Gene Database, http://perso.curie.fr/Thierry.Soussi/APC_database.html

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for NF1 cDNA [M89914])

Human Gene Mutation Database Cardiff, http://uwcm.web.cf.ac.uk/uwcm/mg/hgmd0.html

NNFF International NF1 Genetic Mutation Analysis Consortium, http://www.nf.org/nf1gene.home.html

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for NF1 [MIM 16220])
RB1base, http://home.kamp.net/home/dr.lohmann/index.htm

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