Exon 10b of the *NF1* gene represents a mutational hotspot and harbors a recurrent missense mutation Y489C associated with aberrant splicing

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Purpose: To analyze the spectrum and frequency of *NF1* mutations in exon 10b. **Methods:** Mutation and sequence analysis was performed at the DNA and cDNA level. **Results:** We identified nine exon 10b mutations in 232 unrelated patients. Some mutations were recurrent (Y489C and L508P), others were unique (1465–1466insC and IVS10b+2delTAAG). Surprisingly, at the RNA level, Y489C causes skipping of the last 62 nucleotides of exon 10b. Another recurrent mutation, L508P, is undetectable by the Protein Truncation Test. **Conclusion:** As exon 10b shows the highest mutation rate yet found in any of the 60 *NF1* exons, it should be implemented with priority in mutation analysis. **Genetics in Medicine, 1999;1(6):248–253.**

Key Words: Neurofibromatosis type 1, mutation, hotspot, aberrant splicing, polymorphism, MIR

Neurofibromatosis type 1 (*NF1*, MIM 16,2200) is one of the most common autosomal dominant disorders, affecting about 1:3500 individuals in all ethnic groups. The main characteristics are cutaneous or subcutaneous neurofibromas, café-au-lait (CAL) skin spots, iris Lisch nodules, and freckling.¹ Other features found in only a minority of patients include scoliosis, macrocephaly, pseudarthrosis, short stature, malignancies, and learning disabilities. As *NF1* clinically presents with great variability, even among patients in the same family, the pathogenicity underlying the phenotype must be complex.

The *NF1* gene has been mapped to 17q11.2 and was positionally cloned.^{2–4} The *NF1* gene is approximately 350 kb in size, contains 60 exons and codes for a 11- to 13-kb transcript with an open reading frame coding for 2818 amino acids.⁵ By RT-PCR, *NF1* mRNA appears to be ubiquitous, but by Western blot, neurofibromin is best detected in neural-crest-derived tissues.

The mutation rate in the *NF1* gene is one of the highest known in humans (reviewed by Huson and Hughes¹) with approximately 50% of all *NF1* patients presenting with new mutations. Despite the high frequency of this disorder in all populations, relatively few mutations have been identified at the molecular level, with most unique to one family. A limited number of mutational “hotspots” have been identified:

R1947X (C5839T) in exon 31 and the 4-bp region between nucleotides 6789 and 6792 in exon 37, both implicated in about 2% of the *NF1* patients (reviewed by Upadhyaya and Cooper⁶). In this study we report that another mutational hotspot resides in exon 10b. By analyzing altogether 232 unrelated *NF1* patients, we identified 9 mutations in exon 10b, indicating that this exon is mutated in almost 4% of *NF1* patients. So far this is the highest score obtained for a particular exon in the 9 years since the *NF1* gene was discovered.

MATERIALS AND METHODS

NF1 patients

For all patients, the diagnosis of *NF1* was based on the presence of two or more of the diagnostic criteria proposed by the NIH Consensus Statement in 1988⁷ and updated in 1997.⁸ The study was approved by the Institutional Ethical Committees and informed consent was obtained from the patients studied. Patients were recruited randomly without bias as they were seen for medical follow up and genetic advice. Patients were recruited as part of a general mutation study. Seventy-three patients were contributed by the Department of Medical Genetics of Gent and by the Service de Genetique, Hopital Erasme, Brussels; 159 patients were contributed by the Department of Pediatrics, Division of Genetics, University of Florida, Gainesville, and by the Departments of Neurology and Medicine, Center for Human Genetics, Duke University Medical Center, Durham.

Nucleic acid extraction

DNA and RNA samples were obtained from 37 unrelated *NF1* patients by extraction from EBV-transformed lymphoblastoid cell lines. In addition, DNA and total RNA samples

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Received: June 3, 1999.

Accepted: September 9, 1999.

were obtained from 195 unrelated NF1 patients by extraction from peripheral blood lymphocytes. Total cellular RNA and genomic DNA was isolated as described.⁹

cDNA analysis and in vitro transcription/translation analysis

First strand cDNA was synthesized by random priming⁹ and cDNA was amplified using 5 primer pairs for amplification of the total coding region.¹⁰ 4 μ L PCR product was used in an optimized in vitro transcription/translation reaction as described.^{9,11} An identical truncated peptide fragment of 55 kD was observed in 2 of 37 patients by in vitro transcription/translation of the fragment spanning exons 1 to 12a and the corresponding cDNA was analyzed by cycle sequencing with and without subcloning using 0.15 μ M fluorescein isothiocyanate (FITC) labeled primers, designated by nucleotide positions: 5'-CTTCGGAATTCTGCCTCT-3' (400-418), 5'-CTGATATGGCTGAATGTG-3' (719-736), 5'-GCCTGTGTCAAACTGTGT-3' (967-984), and 5'-CACACCCAGCAATACGAA-3' (1367-1384), and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham, Arlington Heights, IL). Samples were loaded on a 6% LongRanger gel (FMC, Rockland, ME) containing 7 M urea and analyzed on an ALF automated DNA sequencer. To check for the presence of the missense mutation Y489C in a fraction of the cDNA, RT-PCR fragments were cloned using the pCR-TOPO cloning kit (Invitrogen, San Diego, CA) and 90 individual clones were further analyzed by cycle sequencing.

Genomic DNA analysis

Exon 10b was amplified using the primer pair as described¹² and PCR products were further analyzed by cycle sequencing without subcloning.⁹ Genomic DNA from 195 unrelated NF1 patients from which no EBV lymphoblastoid cell line was available was analyzed by heteroduplex analysis or single strand conformation analysis or both, and aberrant migrating PCR fragments were further analyzed by cycle sequencing.¹³ Mutations are reported according to the recommendations of the Nomenclature Working Group,¹⁴ with the start site of translation denoted as nucleotide 1 for both cDNA and genomic alterations.

RESULTS

The total coding region of the NF1 gene was analyzed by the protein truncation test in 37 unrelated NF1 patients from which an EBV lymphoblastoid cell line was available.¹⁰ In two patients, an identical shortened fragment of approximately 55 kD was discerned in the region encompassing the exons 1 to 12a. In both patients, in vitro transcription/translation for the other regions only showed normal sized fragments. By electrophoresis of the RT-PCR fragments from Patient 1 two discrete bands were discerned on a 1.5% agarose gel, i.e., a normal-sized band of 1868-bp and a band that was approximately 60 bp smaller. In Patient 2, however, only a normal-sized band was seen, indicating that the truncated protein of identical size was formed in a different way in this patient. cDNA sequencing

in this region indeed revealed a different mutation in both patients. In Patient 2, an insertion of C at nt 1465-1466 in exon 10b was found, immediately resulting in the creation of a stop codon at this site. In Patient 1, a deletion/skipping of the last 62 nucleotides of exon 10b was observed in RNA from both lymphocytes and the EBV-lymphoblastoid cells (Fig. 1B). Here too, the immediate result is formation of a stop codon at this site, explaining the identical picture seen by protein truncation analysis. Further analysis of exon 10b at the genomic level confirmed the presence of an insertion 1465insC in Patient 2. In Patient 1, however, a missense mutation was identified: A1466G, changing the codon for Tyr to Cys (Y489C) (Fig. 1A). Both parents of this sporadic patient did not carry this missense mutation. This missense mutation masquerades as a splicing defect: indeed substitution of A to G at position 1466 of the genomic DNA creates a new splice donor site (CT/GTAAG) (Fig. 1C). Analysis of the normal and mutant se-

Fig. 1 (A) Cycle sequencing without subcloning of the genomic region of exon 10b in Patient 1 showing transition of A to G at nt 1466 resulting in the formation of a splice donor site CT/gtaag; (B) Cycle sequencing of the mutant cloned cDNA allele in Patient 1. The last 62-bp of exon 10b are skipped and followed by exon 10c, resulting in the formation of a stop codon at amino acid 489; (C) Schematic diagram of the genomic region surrounding exon 10b. Green boxes represent exons, red sequences denote splice donor sequences. By the transition of A to G at nt 1466 a novel splice donor is formed; (D) Cycle sequencing of a cloned RT-PCR fragment encompassing the exons 1 to 12b using sequencing primer 5'-CTTCGGAATTCTGCCTCT-3' (400-418). Between exon 4a and 4b the presence of the 31-bp MIR-sequence is shown; (E) Schematic diagram of the genomic region surrounding IVS 4a. Green boxes represent exons, red sequences denote splice donor sequences, green sequences denote splice acceptor sequences.

quence using the program for splice site prediction by neural network (available at URL: www-hgc.lbl.gov/projects/splice.html) showed a 0.86 score for the normal exon 10b donor site (GCTTTGT/gtaagtat) and a higher 0.97 score for the new donor site created by the missense mutation Y489C (AGAAGCT/gtaagtat). RT-PCR fragments from an EBV lymphoblastoid cell line of Patient 1 were cloned and 90 individual clones were further analyzed by cycle sequencing to check for the presence of the missense mutation in a fraction of the cDNA. In 50 cDNA clones showing a normal-sized band of 1868 bp on a 1.5% agarose gel, only the wild type sequence was found and in none of them the missense mutation was present. In 40 clones containing a slightly smaller insert (approximately 60 bp) as evidenced by agarose gel electrophoresis, the smaller size was due to the skipping of the last 62 nucleotides of exon 10b along with intron 10b in the mRNA (Fig. 1B). This indicates that the major outcome of the mutation Y489C at the cDNA level is skipping of the last 62 nucleotides of exon 10b. Although Y489C and 1465–1466insC are different mutations at the genomic DNA level, both result in the formation of a premature stop codon at exactly the same position well before the GAP domain of neurofibromin. As the finding of two different mutations at the same spot (i.e., 1465–1466insC and 1466A>G) is in itself indicative of a mutational hotspot,¹⁵ these findings prompted us to analyze exon 10b in a larger patient population consisting of 195 unrelated NF1 patients from which genomic DNA and total RNA was available but no EBV lymphoblastoid cell line. In Patients 3, 4, 5, and 6 the missense mutation Y489C was identified by heteroduplex analysis or single strand conformation analysis or both, followed by cycle sequencing of the aberrant migrating genomic fragments and further analysis at the cDNA level. In Patients 4, 5, and 6, the effect of the missense mutation Y489C at the mRNA level was identical to Patient 1, i.e., skipping of the last 62 nucleotides of exon 10b. Surprisingly, three different sized transcripts were seen after RT-PCR of total RNA extracted from fresh lymphocytes from Patient 3 (no EBV lymphoblastoid cell line was available from this patient). RT-PCR fragments were cloned and further analyzed by cycle sequencing. In the smallest sized fragments, the mutant exon 10b lacking the last 62 bp of the exon 10b was followed by the correct exon 10c but hereafter the exon 11 was neatly skipped out. Skipping of exon 11 was not seen in Patients 1, 4, 5, and 6. In the clones carrying the intermediate-sized transcripts, the mutant exon 10b lacking the last 62-bp was found. In the largest fragments, the normal exon 10b sequence was present as expected. Furthermore, between exon 4a and exon 4b, a 31-bp cryptic exon was inserted in *cis* with the skipping of the last 62 bp of exon 10b and exon 11 in some of the mutant clones (Fig. 1D). This 31-bp cryptic exon was also seen in some mutant clones lacking only the last 62 bp of exon 10b, but also in some normal clones. The same cryptic exon was recently found in a patient showing multiple splicing errors but an unknown constitutional mutation.¹⁶

In Patient 7, a deletion at the splice donor site of exon 10b was found (IVS10b+2delTAAG) and analysis of RNA ex-

tracted from a neurofibroma showed a strong shorter RT-PCR product in addition to the normal sized fragment. By cycle sequencing, it was shown that the shorter transcript was formed by the perfect splicing out of exon 10b. As this splicing alteration resulted in the production of a stable mRNA and the size of exon 10b is a multiple of three, the possibility remains that this allele produces neurofibromin lacking only the 45 amino acids encoded by exon 10b.

In two unrelated NF1 patients (8 and 9; one Caucasian and one African American) an identical missense mutation was identified changing the codon for Leu to Pro (L508P; 1523T>C). This missense mutation that would not be detected by the Protein Truncation Test, does not create/destroy a restriction enzyme site, but shows a specific reproducible shift in single strand conformation polymorphism analysis. Analysis of 153 unrelated Caucasian Americans and 20 unrelated Black Americans ruled out that the alteration is a polymorphism. Another 30 unrelated Caucasian Europeans were analyzed by direct sequencing of exon 10b. In none was the missense mutation L508P found. Table 1 summarizes the effects on RNA splicing of the different germline mutations found in exon 10b.

A novel polymorphism was identified at a CpG dinucleotide in intron 10a: IVS10a-32C>T. We have tested 61 unrelated NF1 patients and 72 normal control persons for this polymorphism and no statistically different values were obtained between the two populations. Taking both populations together, we obtained allele frequencies of 0.68 for the IVS10a-32C allele and 0.32 for the IVS10a-32T allele. In four NF1 patients heterozygous for this polymorphism, we analyzed by RT-PCR the outcome of the IVS10a-32T allele with respect to the splicing of exon 10b and did not observe an effect on the splicing. As the number of polymorphisms at the 5' end of the *NF1* gene is limited, this novel polymorphism, which can be detected by *Mae*II digestion, may be a useful tool in segregation analyses.

DISCUSSION

In this study we identified nine novel exon 10b mutations in 232 unrelated NF1 patients: Y489C or 1466A>G (5X), L508P (2X), 1465–1466insC (1X), and IVS10b+2delTAAG (1X). Our findings indicate that exon 10b is mutated in almost 4% of the NF1 patients, although so far, only one missense mutation has been reported in exon 10b, K505E.¹⁶ However, only two studies analyzed the complete coding region of the NF1 gene including exon 10b, and the total number of patients analyzed in both studies is small, i.e., 36 patients.^{10,16} No other studies specifically looked at exon 10b. Mutational analysis is hampered by the large number of exons, the wide variety of mutations, and the presence of several pseudogenes. The majority of mutations identified hitherto reside in the GAP-related domain, encoded by the exons 21 to 27a and in the exons downstream of this GAP-related domain. The exons lying at the 5' end of the gene have not been studied exhaustively by many groups. One reason may be that for most exons at the 5' end the available intronic sequence information was limited to the

Table 1
Summary of the germline mutations found in exon 10b and their effects on RNA splicing

Patient	gDNA mutation	Tissue(s) analyzed ● cDNA observations
P1	Y489C; 1466A>G	EBV lymphoblastoid cell line and fresh lymphocytes ● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P2	1465–1466insC	EBV lymphoblastoid cell line ● formation of a stop codon at AA 489
P3	Y489C; 1466A>G	Fresh lymphocytes ● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489 ● skipping of 62 nt at cDNA nt 1466–1527 AND skipping of exon 11 ● insertion of a cryptic 31 nt exon, between exon 4a and exon 4b resulting in formation of a stop codon at nt 516 of the cDNA, both on the normal and mutant allele
P4	Y489C; 1466A>G	Fresh lymphocytes ● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P5	Y489C; 1466A>G	Fresh lymphocytes ● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P6	Y489C; 1466A>G	Fresh lymphocytes ● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P7	IVS10b + 2delTAAG	RNA from a plexiform neurofibroma (blood RNA not available) ● skipping of exon 10b
P8	L508P (1523T>C)	No RNA available
P9	L508P (1523T>C)	Fresh lymphocytes ● no effect on splicing of exon 10b

intron-exon boundaries, thus hampering the search for optimal primer pairs that can be used to amplify these exons, in the absence of sequences derived from pseudogenes.

The novel mutations 1465insC and Y489C are different at the genomic DNA level, but both result in the formation of a premature stop codon at the mRNA level at exactly the same position through different mechanisms. Y489C illustrates that, although the mutation is reported according to the recommendations of the Nomenclature Working Group, nomenclature can be misleading. Indeed, by nature Y489C belongs to the group of splicing error mutations and not to the group of missense mutations. This unexpected finding further stresses the importance of documenting the outcome of a mutation at the mRNA level.

It is intriguing that in 1 of the 5 patients carrying the mutation Y489C, analysis of cloned RT-PCR products showed that some mutant alleles skipped the last 62 bp of exon 10b as well as the complete exon 11. Exon 10c was retained in these clones. Skipping of exon 11 was not seen in the other patients. The cause of the exon 11 skipping in Patient 3 is unknown and may not even be due to a sequence change in the *NF1* gene, or it could be caused by a point change in an intron that renders this exon skipping more likely to occur. As we do not have an EBV lymphoblastoid cell line from this patient, we were not able to study whether this different splicing is tissue-specific. Noteworthy is that, by splice site prediction using neural networks

(available at: URL: www-hgc.lbl.gov/projects/splice.html), no splice acceptor sequence can be defined for exon 11, not even after using an acceptor score cutoff of 0.05. This might indicate that splicing of exon 11 is mediated through mechanisms different from splicing of those exons with a well-defined splice acceptor sequence.

The cryptic 31-bp exon between exon 4a and exon 4b was found on normal as well as on mutant cloned cDNA fragments of Patient 3. From ongoing experiments we know that this cryptic 31-bp exon is formed, albeit typically at a low level, in different cell types in *NF1* patients as well as in normal persons, and hence does not appear associated with a mutation in the *NF1* gene per se.¹⁷ In some neurofibromas, the cryptic 31-bp exon can be found on both alleles as well (unpublished results). A search of sequence databases showed that this 31-bp sequence was 100% homologous with a part of the *NF1* intron 4a. Using the splice site prediction by neural network program we found that this sequence is flanked by a splice acceptor sequence (score 0.51) ttgctgtctcctcctcacag/TAACCTTGATGATACTTCCC and a very strong splice donor sequence (score 0.99) TTTACAG/gtgagcaa (Fig. 1E). The mechanisms by which this splice acceptor/donor pair is up- or downregulated is unknown, nor is it known whether this transcript is translated in vivo. The 31-bp cryptic exon is part of a fragment of the ancient class of Mammalian-wide Interspersed Repeat

Table 2
Summary of the clinical findings in the patients carrying a mutation in exon 10b of the NF1 gene

Trait	P1	P2	P3	P4	P5	P6	P7	P8	P9
Sex	F	F	M	M	F	F	F	M	M
Age (Yr)	3	7.5	39	9	48	23	18	80	17
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Black American
CALs	>6 and >5mm	>6 and >5mm	>6 and >15mm	>6 and >5mm	>6 and >15mm	>6 and >5mm	>6 and >15mm	>6 and >15mm	>6 and >15mm
Cutaneous NFs	No	Yes (subcutaneous)	Yes	No	Yes	Yes	Yes	Yes	Yes
Plexiform NF	Yes: neck and left hemithorax	Yes: paravertebral	No	No	No	Several larger NFs that may be plexiforms	Yes: of left hand	No	Yes: periorbital
Freckling	Yes	ND	Yes	Yes	ND	Yes	Yes	Yes	Yes
Optic glioma*	No	ND	No	No	No	No	No	ND	ND
Lisch nodules	No	ND	Yes	Yes	Yes	No (no slit lamp exam)	Yes	ND	Yes
Osseous lesion	Hypoplasia of the left thoracic pedicles from thoracic vertebrae I–VII; thoracic scoliosis	Scoliosis requiring osteosynthesis	No	No	Scoliosis; no bone dysplasia	Scoliosis	No	No	No
First-degree relative with NF1	No	Yes	Yes	No	No	No	No	ND	No
Other manifestations	Macrocephaly	Macrocephaly	Complex partial epilepsy; mentally functioning at a lower level	Macrocephaly; learning disabilities	Seizures	Xanthogranulomas	Short stature, learning disabilities	None	Learning disabilities; macrocephaly

M = male, F = female.

*Presence/absence of optic pathway glioma as evidenced after MRI evaluation; ND, not determined.

elements (MIR) (as defined in Genbank sequence AC004222, nt 22263 to 22346 and confirmed by A.F.A. Smit and P. Green, [personal communication, The Genome Center, Washington, DC, 1998] using the program RepeatMasker [Available at: <http://ftp.genome.washington.edu/RM/RepeatMasker.html>]). Further research into the direct outcome of this germline mutation at the mRNA level in different tissues and cell types in the different affected persons from this family, and comparison with the nonaffected family members as a control may shed more light on this event.

The missense mutation L508P was identified in two unrelated NF1 patients and absent in 203 unrelated control persons from different ethnic origin. Proline is a very rigid amino acid and its presence creates a fixed kink in a polypeptide chain. Although both amino acids are hydrophobic and the amino acid change is conservative, the introduction of a nonaliphatic imino group into the polypeptide may have dramatic consequences for protein structure. Moreover, leucine at this position is conserved in the mouse (L10370), rat (D45201), Fugu rubripes (AF064564), and drosophila (L26501). We presume that this missense mutation is pathogenic and may point to a region of the protein with a hitherto unknown function.

NF1 genotype-phenotype correlation studies have not been extensive or successful except for the consistent correlation between the deletion of the entire *NF1* gene and flanking region and a distinctive phenotype including severe developmental impairment, dysmorphic features, and large numbers of neurofibromas.¹⁸⁻²⁰ Detailed clinical data together with the genotypes of the patients were submitted to the NNFF International *NF1* genetic mutation database, to allow NF1 genotype-phenotype correlation studies in the future. In 4 of 9 patients scoliosis was present (Table 2). It is worth noting that in our patients with a mutation in exon 10b, a high proportion (5 of 9) harbored plexiform neurofibromas (Table 2). Patient 3, who does not have plexiform neurofibromas himself, has a child with a plexiform neurofibroma on the lip.

In conclusion, a novel mutational hotspot has been identified in exon 10b of the *NF1* gene: a mutation in exon 10b was found to be present in 9 of 232 (3.9%) unrelated NF1 patients. By cDNA-SSCP analysis of the total coding region in 71 Spanish NF1 patients, an identical skipping of nt 1466-1527 of exon 10b caused by the mutation Y489C was found in two patients (E. Ars and C. Lazaro, personal communication, Medical and Molecular Genetics Center-IRO, Barcelona, Spain). The finding of another missense mutation in exon 10b (K505E) in 1 of 14 unrelated American patients¹⁶ further underscores this finding. Taking these data together, 12 exon 10b mutations were reported in 317 unrelated NF1 patients (~3.8%) and is, thus far, the highest score obtained for an *NF1* exon. This is particularly significant given that exon 10b only encodes 1.7% of the open reading frame. Our results indicate that this exon should be implemented with priority in *NF1* mutation analysis.

Acknowledgments

This work was supported by the Fund for Scientific Research, Flanders, Belgium (L.M.M.) and by Het Bijzonder Onderzoeksfonds UG N°01107799 (L.M.M.), and by the National Institutes of Health (N53155), the U.S. Army, the Hayward Foundation and the Children's Miracle Network (M.R.W.). We also thank Dr. Francis Collins, Dr. Gene Jackson, Dr. Charles Williams, and Wendy Uhlmann for contributing some clinical samples and data.

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