NF2 gene in neurofibromatosis type 2 patients

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INTRODUCTION

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder that predisposes to nervous system tumors. The schwannomin (also termed merlin) protein encoded by the NF2 gene shows a close relationship to the family of cytoskeleton-to-membrane proteins linkers ERM (ezrin–radixin–moesin proteins). Even though penetrance of the disease is >95% and no genetic heterogeneity has been described, point mutations in the NF2 gene have been observed in only 34–66% of the screened NF2 patients, depending on the series. In order to generate tools that would enable an exhaustive alteration screening for the NF2 gene, we have deduced its entire genomic sequence. This knowledge has provided the delineation of a mutation screening strategy which, when applied to a series of 19 NF2 patients, has revealed a high recurrence of large deletions in the gene and has raised the efficiency of mutation detection in NF2 patients to 84% of the cases in this series. The remaining three patients who express two functional NF2 alleles are all sporadic cases, an observation compatible with the presence of mosaicism for NF2 mutation.

At least two clinical subtypes of NF2 have been described: the severe (Wishart) type has an early onset, a rapid course and is characterized by a marked predisposition to meningiomas and spinal tumors in addition to vestibular schwannomas (7), whereas the mild (Gardner) type has a late onset, and a comparatively benign course with a low incidence of meningiomas and spinal tumors (8). In general, NF2 manifestations are similar among members of a family, although some families with both mild and severe forms have been described (9). All linkage analyses are compatible with the existence of a single locus for NF2, that is localized on chromosome 22 (10,11).

The NF2 gene was identified on chromosome 22q12 by positional cloning strategies (12,13). The schwannomin (also termed merlin) protein encoded by the NF2 gene shows a close relationship to the family of cytoskeleton-to-membrane proteins linkers ERM (ezrin–radixin–moesin proteins). Schwannomin interacts with actin-based cytoskeletal structures (14–16) but its function remains poorly understood. However, recurrent observations of biallelic NF2 inactivation in schwannomas, meningiomas and mesotheliomas indicates that schwannomin acts as a tumor suppressor (12,13,17).

Identification of the NF2 gene enabled a search to be made for mutations in NF2 patients. Since its discovery, the application of single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) or the direct sequencing of exons has led to the identification of >100 different germline point mutations (12,13,18–22). Mutations have been described for all NF2 exons with the exception of exons 9 and the translated part of exons 16 and 17. Major hot spots for mutations have not been observed. The majority of observed germline NF2 mutations are nonsense, frameshift or splice site mutations which result in gene products with an altered or absent C-terminal domain. Such truncated proteins are generally unstable (16). A small number of mutations preserve this domain. They include missense mutations, in-frame internal deletions or splice site mutations causing exon skipping without frameshift (12,13,18–22). The search for genotype–phenotype correlations has revealed a link between mutations generating a truncated NF2 product and disease severity (21,22).

Point mutations have been observed in 34–66% of NF2 patients analyzed (12,13,18–22). Large size deletions have been described only rarely, and a single case of chromosomal transloc-
Figure 1. Schematic representation of the NF2 region corresponding to the 120 kb of genomic sequence. Restriction sites for EcoRI (E), BamHI (B) and HindIII (H) enzymes are indicated. Gray boxes represent repetitive sequences, numbered black boxes NF2 exons, dotted white boxes CpG islands, and triangles the ribulose-5′-phosphate-epimerase pseudogene region. Numbered black lines represent probes used in Southern blot analysis. FISH probes are indicated.

tion interrupting the gene has also been reported (12,13,23–25). The latter rearrangements, which escape detection by the techniques usually applied for the screening of point mutations, have never been searched for systematically because of the large size of the gene (90 kb). Furthermore, the search for mutations in non-coding, but functional, regions of the gene has been hampered by the lack of knowledge of their complete sequence. In order to provide tools that would enable exhaustive alteration screening for the NF2 gene, we have deduced its entire genomic sequence. This knowledge has enabled the design of a mutation screening strategy which, when applied to a series of 19 NF2 patients, has revealed a high recurrence of large deletions in the gene and has raised the efficiency of mutation detection in NF2 patients to 84% of the cases analyzed.

RESULTS
Sequencing the NF2 locus
Genomic sequencing totaling 125 kb was centered around the NF2 gene. It contains the entire NF2 gene with 8.7 kb centromeric and 26.7 kb telomeric (Fig. 1). The 17 NF2 exons are distributed over 90 kb with a large first intron (32 kb). The search for known repeated elements in the entire sequence using the Censor software (censor@charon.girinst.org) identified the presence of 53 Alu, 15 LINE, 15 MIR, nine MER, three MLT1, one HERV9, one HERVR and one HRES element. In total, repeated elements represent 32% of the 120 kb sequence with an important predominance in the centromeric (5′) half of the sequence (repeated elements represent 60% of the first 30 kb). About two-thirds of the repeated sequences are organized in large clusters by concatenation of different elements. The largest cluster is 3 kb long and is localized in the first intron. It is composed of an ancient LINE element, fragmented by the insertion of nine Alu elements. Such a composite aspect of clusters is observed frequently in the genome. The 5′ to 3′ orientations of the different repeated elements are equally distributed in both directions. Finally, all Alu subtypes proposed by Jurka and Milosavljevic (26) are represented with a predominance of the ancient Alu J subfamily (36 Alu J for a total of 53 Alu elements).

Analysis of the 120 kb sequence with the GRAIL 1.2 program, using the definition of Gardiner-Garden and Frommer (27), indicated the presence of two CpG islands (Fig. 1). CpG1 is adjacent to the first intron, and should contain the NF2 promoter. However, unambiguous TATA and CAAT boxes were not observed. To date, the transcription initiation site of NF2 remains unknown. CpG2 is located 17 kb telomeric to the last exon of NF2. This region demonstrates two different homologies. A 0.5 kb region (coordinates 124 476–124 933) resembles a human telomere associated repeat sequence (TAR). Downstream, a region of 313 bp displayed homology with the retroviral sequence HERS1, an HTLV1-related sequence.

Apart from the NF2 exons, the GRAIL 1.2 software predicted no additional consistent protein-coding region. Search for homology with a BLAST request detected 1 kb in the first intron (coordinate 13 401–14 453) homologous to both ARSH1 (human autonomous replicating sequence H1) (28) and to the human...
putative ribulose-5′-phosphate-epimerase (90% identity in nucleotides). Computer translation of the homologous region identified two frameshifts indicating that the NF2 intron 1 contains a ribulose-5′-phosphate-epimerase pseudogene.

**Search for point mutations**

NF2 exons and their boundaries were scanned in 19 unrelated patients with a confirmed NF2 disease. Exons 1–15 and exon 17 were screened by DGGE, whereas exon 16 and the 5′- and 3′-non-coding regions were screened by direct sequencing. Mutations were identified in 10 patients and were localized in exon 2, 7, 8, 11, 12, 14 or 15 (Table 1). All mutations were private, half of them occurring in consensus splicing sequences. Four caused a frameshift and one a nonsense mutation. No mutation was found in CpG1, but four biallelic DNA variants were identified. Similarly, in the 1.1 kb 3′-non-coding region adjacent to exon 17, a biallelic variant was identified. However, genotyping of 28 independent CEPH individuals revealed the polymorphic nature of these variants (Table 2).

### Table 1. Localization of mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Mutation type</th>
<th>Codon position</th>
<th>Consequence</th>
<th>Age at onset</th>
<th>Transmission</th>
<th>Gravity</th>
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<td>GL9a</td>
<td>2</td>
<td>Aggt→Agtt</td>
<td>J8081</td>
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<td>M</td>
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<td>7</td>
<td>T deletion</td>
<td>216</td>
<td>frameshift</td>
<td>17</td>
<td>N</td>
<td>S</td>
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<tr>
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<td>7</td>
<td>acgg del</td>
<td>217–128</td>
<td>frameshift</td>
<td>19</td>
<td>N</td>
<td>S</td>
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<tr>
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<td>7</td>
<td>GGgt→Ggtt</td>
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<td>27</td>
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<td>S</td>
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<tr>
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<td>11</td>
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<td>341</td>
<td>nonsense</td>
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<td>S</td>
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<td>14</td>
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<td>J481/482</td>
<td>splice acceptor</td>
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<td>S</td>
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<td>1–595</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>32</td>
<td>N</td>
<td>S</td>
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</table>

*a Mutation previously described in Merel et al. (19).

*b Deletion previously described in Sanson et al. (24).

J, junction; F, familial; N, new mutant; M, mild; S, severe.

### Table 2. Biallelic polymorphisms

<table>
<thead>
<tr>
<th>Locus</th>
<th>Position in NF2 sequencea</th>
<th>Polymorphic sequence</th>
<th>% heterozygosity</th>
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<tr>
<td>nfprom1</td>
<td>8240</td>
<td>cccG/Cggf</td>
<td>58</td>
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<tr>
<td>nfprom2</td>
<td>8348</td>
<td>tgc6T/Tgca</td>
<td>54</td>
</tr>
<tr>
<td>nfprom3</td>
<td>8787</td>
<td>gggC/Accg</td>
<td>43</td>
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<td>nfprom4</td>
<td>8881</td>
<td>cgg/Gcgag</td>
<td>31</td>
</tr>
<tr>
<td>nfp3′nc</td>
<td>99632</td>
<td>tcaC/Tgt</td>
<td>39</td>
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</tbody>
</table>

a DDBJ/EMBL/GenBank accession no. Y180000.
Search for NF2 rearrangement

In order to enable the detection and location of the boundaries of chromosomal rearrangements implicating the NF2 gene, Southern blot analyses were performed of DNAs from nine NF2 patients with unidentified mutation and 10 controls. These blots were hybridized sequentially with 12 probes derived from the single copy regions of the NF2 gene as indicated in Figure 1. During this procedure, every region of the normal NF2 gene is explored with at least two different digestions, thus enabling an efficient search for the presence of abnormal restriction fragments. Observation of a variant fragment in DNA from several controls led to the identification of a new HindIII restriction fragment length polymorphism (RFLP) in intron 1 (frequency of heterozygotes: 58%). In DNA from two NF2 patients, unique additional bands were observed (Fig. 2). For patient GL13, unique bands were revealed by probes 5 and 6. Fine analysis of the data suggested a 1.5 kb deletion encompassing exon 4. RT–PCR analyses evidenced a small NF2 transcript which, after sequencing, confirmed the in-frame exon 4 deletion. For patient GL08, abnormal bands detected by probes 8 and 9 indicated a rearrangement of intron 8. Compared with the DNA from controls, the DNA from this patient systematically yielded a weaker signal with probes 1–7. This difference was not observed for probes 10–12, suggesting the presence of a large deletion that would have removed the entire 5’ region of the NF2 gene. A similar semi-quantitative analysis of the data collected with the 12 NF2 probes and with probes derived from D22S1 and EWS on chromosome 22 indicated that in four other patients (GL01, GL04, GL10 and GL21) with large suspected NF2 deletions, indicating that the entire NF2 gene was indeed deleted (Fig. 3B). For GL10, additional FISH experiments using cosmid 98C4 confirmed that the EWS gene is deleted. For a fifth patient (GL08), FISH analysis demonstrated that the deletion involved the 5’ end of the gene (FISH probes 1 and 2) but not the 3’ end (FISH probe 3). Since none of the FISH probes overlapped the small deletion observed for patient GL13, the two chromosomes 22 of this patient appeared normal. Finally, no deletion was identified for the last three patients (GL07, GL11 and GL15).

Patients without identifiable NF2 alteration

Screening by DGGE, Southern blot, FISH and promoter sequencing of 19 NF2 patients failed to reveal any NF2 gene alterations in three cases. In these cases, RT–PCR analyses were performed. The amplified products exhibited a normal size, and the absence of a point mutation was confirmed by direct sequencing. In order to explore the putative silencing of one allele of the NF2 gene, polymorphisms in the 3’ and/or 5’ transcribed non-coding regions were analyzed at the DNA and RNA levels. Heterozygosity for at least one locus was evidenced in genomic DNA from these three patients. In all cases, sequencing of the cDNAs demonstrated that both alleles were expressed (Fig. 4).

DISCUSSION

This work provides the first exhaustive search for NF2 gene alterations in patients meeting the criteria for NF2. By a conventional search for point mutations in the coding region, we identified deleterious alterations in 52% of the cases, a percentage that is comparable with those previously published for other series (12,13,18–22). Strikingly, in two-thirds of the remaining...
sequencing of the entire exons (i.e. SSCP, DGGE and heteroduplex analysis). The present mutations that are based on PCR amplification of individual escape detection by the usual screening techniques for point evidenced by this FISH method in five out of 19 NF2 patients on metaphasic chromosomes. Large genomic alterations were fruitfully to develop probes that display intense specific labeling deletions (31).

In the present series, all 10 point mutations are predicted to cases, a deletion which encompassed at least one entire exon was evidenced. Among these six deletions, one was intragenic involving a 1.5 kb fragment including exon 4, another removed the 5′ half of the NF2 gene. Exon 4 deletion in patient GL13 is in-frame, indicating the possible localization of an important functional domain between amino acids 122 and 149. Recently the region between amino acids 122 and 185 has been identified as mediating the intermolecular association of the schwannomin with the N-terminal domain of the ERM proteins (29). Thus, the exon 4 deletion could contribute to delimit more precisely this domain of interaction in the schwannomin protein. The last four deletions encompassed the entire NF2 gene, indicating that the deletion was at least 90 kb long. In one of these cases, GL10, the deletion was shown to include the EWS gene, which is 350 kb centromeric to NF2. When intragenic, the boundaries of these large deletions appear to be localized at various positions. However, many boundaries are localized outside of the NF2 gene in regions that have not yet been sequenced. Thus, the mechanism underlying the high incidence of large deletions in NF2 patients remains currently unknown, contrasting this observation to that of multiple small mutations in the NF1 gene as the most common mutation mechanism (30).

In the present series, all 10 point mutations are predicted to generate exon skipping or translation frame shifts, and eight of these patients manifest a severe phenotype. In contrast, all six patients with a genomic deletion had a mild phenotype. Five patients carrying large deletions extending out of the NF2 gene did not manifest additional symptoms. In particular, analysis of patient GL10 suggests that hemizygosity for EWS may not have additional deleterious consequences. The observation of a mild NF2 phenotype associated with large NF2 deletions is in sharp contrast to the severe clinical features of patients with large NF1 deletions (31).

Since the NF2 gene is located on an autosome, large deletions escape detection by the usual screening techniques for point mutations that are based on PCR amplification of individual exons (i.e. SSCP, DGGE and heteroduplex analysis). The present sequencing of the entire NF2 gene has provided a reliable FISH method to search for genomic deletions. The identification of intronic regions devoid of repeated elements has been exploited fruitfully to develop probes that display intense specific labeling on metaphasic chromosomes. Large genomic alterations were evidenced by this FISH method in five out of 19 NF2 patients (26%), thus demonstrating its effectiveness and suggesting FISH as a powerful tool for the systematic screening of patients. Nevertheless, small intragenic deletions encompassing one or more exons, as observed for case GL13, were not detected by the FISH procedure. In such cases, if the deletion does not abrogate transcription of the mutant allele, an aberrantly sized transcript can be detectable by RT–PCR. Moreover, silent alleles or alleles that yield an unstable transcript can be detected by comparison of the homozygous/heterozygous status at the DNA and cDNA levels (Fig. 4).

The presence of normal transcripts expressed from the two alleles, combined with the lack of alteration detected by FISH, RT–PCR and DGGE on all exons, provides a demonstration that both alleles of the NF2 gene are functional. Such a demonstration was obtained on lymphoblastoid cell lines from three patients with bilateral vestibular schwannomas. Thus, these patients meet the criteria for NF2, but are likely to have two functional NF2 alleles in their lymphoblastoid cell lines. This hypothesis was supported further by the direct sequencing of all NF2 exons. Although we cannot exclude that mutation in a gene distinct from NF2 may be responsible for the phenotype of these patients, the observation that all three patients are new mutants suggests the presence of a mosaic NF2 mutation which does not involve the B lymphocyte compartment. One such case has recently been reported (32). In familial cases, only the affected individual from the oldest generation is a putative mosaic patient, and mutations should be evidenced in the younger generations. In sporadic NF2 cases, such as those reported here, demonstration of mosaicism relies on the availability of samples from different tissues or from multiple independent tumors and is, therefore, more difficult to document.

MATERIALS AND METHODS

Patients and cell lines

Lymphoblastoid cell lines have been established of 19 independent NF2 patients from 1987 to 1993. Patients were collected by G. Fischer in neurosurgery departments of the Rhone-Alpes region in France. Clinical data were collected for all patients and met the diagnostic criteria for NF2 as defined by the 1991 NIH consensus conference statement. Patients were classified as mildly (M) or severely (S) affected according to the criteria described by Evans et al. (4) (Table 1).

NF2 sequence determination

Cosmids 72C, 101D, 142A and 57C were isolated from the LL22NCO1 library by a chromosome-walking procedure and overlap a 140 kb genomic region containing the entire NF2 gene (12). These four cosmids were sequenced entirely using a shotgun procedure as described previously (33). The whole NF2 sequence is deposited in the EMBL database (accession no. Y18000).

Point mutation screening

Mutation screening by DGGE was performed essentially as described in Mérel et al. (19). Screening for mutation in the 5′ UTR, 3′ UTR and exon 16 alternative was performed by direct sequencing of PCR products. PCR primer sequences and conditions are available on the Fondation Jean Dausset/CEPH web server (http://www.cephb/nf2deletion).
Southern blot and probes

Southern blotting was performed according to standard procedures after digestion by EcoRI, BamHI or HindIII (34). Probes were obtained by PCR (Fig. 1; http://www.cephb.fr/nf2deletion ). Prior to labeling by random priming, the amplified products were purified using a QIAquick PCR purification kit (Qiagen). Hybridization procedures were as described previously (34).

RT–PCR procedure

In all cases with unknown NF2 alterations, four RT–PCR amplifications were performed using the GeneAmp RNA PCR kit (Perkin-Elmer) with an oligo(dT) priming and primers described in Deguen et al. (35). Transcribed polymorphisms in the 5′- and 3′-non-coding regions were searched for by sequencing products amplified with primers NF5′UTRF- NF5′UTRR and NF3′UTF-NF3′UTR from the NF2 cDNA (http://www.cephb.fr/nf2deletion ). Polymorphic loci were characterized on the set of 28 reference CEPH individuals used by Weissenbach et al. (36).

Fluorescence in situ hybridization

In situ hybridization was performed using amplified PCR products described in Figure 1. For each probe, 1 µg of DNA was labeled with digoxigenin 11-UTP or biotin 16-UTP by nick translation using a Gibco BRL kit. The labeled products were hybridized at a final concentration of 2 µg/ml in the presence of 5 ng/l of sonicated salmon DNA, but without a pre-hybridization step. Slide preparation and hybridization steps were the same as 5 mg/l of sonicated salmon DNA, but without a pre-hybridization step. Seizinger, B., Kley, N., Klein-Szanto, A.J. and Testa, J.R. (1995) High frequency of inactivating mutations in the neurofibromatosis type 2 gene (NF2) in primary malignant mesothelioma. Proc. Natl Acad. Sci. USA, 92, 10854–10858.


