Screening for Large Mutations of the *NF2* Gene

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Neurofibromatosis 2 (NF2) is a genetic disorder caused by mutational inactivation of the *NF2* gene and is characterized by bilateral vestibular schwannomas, spinal tumors, and other benign tumors of the nervous system. Previously, we found intragenic *NF2* mutations in 99 of 188 unrelated NF2 patients by exon-scanning-based methods. Tumor analysis of 22 de novo NF2 patients led to the identification of 12 additional constitutive *NF2* mutations. The remaining 77 patients were further examined for large alterations using the newly developed gene dosage assay multiplex ligation–dependent probe amplification (MLPA). One deletion of a single exon, seven deletions of multiple exons, seven deletions involving the 3′ or 5′ end of the *NF2* gene, four deletions involving the whole *NF2* gene, and one duplication of three exons were detected. For 47 of the 77 patients, mRNA of adequate quality could be obtained, enabling transcript analysis, which confirmed eight alterations detected by MLPA. In addition, in one family, the mRNA analysis detected an insertion of two exons of another gene. Thus, deletions, duplications, and insertions affecting the *NF2* gene were found in 21 cases, which is 11% of the 188 unrelated NF2 patients studied, 16% of the 132 mutations identified, and 27% of the 77 cases in which no intragenic small mutations were detected by exon scanning. The combination of multiple screening techniques facilitated a mutation-detection rate of 100% for the 21 inherited cases in this study.

INTRODUCTION

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder characterized by bilateral vestibular schwannomas and spinal tumors (Evans et al., 1992a; Parry et al., 1994; Mautner et al., 1996). Meningiomas, other benign tumors of the nervous system, and ophthalmologic abnormalities also frequently occur in NF2 patients. The incidence of NF2 is about 1 in 30,000, and the genetic cause is a defect of a classic tumor-suppressor gene, *NF2*, on chromosome 22 (Rouleau et al., 1993; Trofatter et al., 1993). More than half of NF2 patients have no family history and thus carry de novo mutations (Evans et al., 1992b). *NF2* mutations have been found in up to 66% of clinically ascertained cases by using exon-scanning-based methods such as single-strand conformation polymorphism (SSCP) and temperature-gradient gel electrophoresis (TGGE; Kluwe et al., 1996; Parry et al., 1996; Ruttledge et al., 1996; Evans et al., 1998). Mosaicism, large alterations affecting single or multiple exons, and mutations in noncoding regions all may contribute to the failure to detect mutations for at least 34% of the NF2 patients screened. Indeed, recent studies revealed mosaicism in 24%–30% of sporadic NF2 patients and demonstrated that in nearly half the cases, failure to detect constitutional *NF2* mutations in the peripheral leukocytes of de novo NF2 patients was a result of mosaicism, as such mutations could be identified in corresponding tumors (Kluwe et al., 2003; Moyhuddin et al., 2003).

Large alterations affecting single or multiple exons, and mutations in noncoding regions as well as large genomic rearrangement cannot be detected by conventional exon-scanning-based methods because the wild-type allele will be coamplified. Two previous studies addressed this issue in NF2 by using Southern blotting, fluorescence in situ hybridization, and microarray-based quantitative comparative genomic hybridization (CGH), detecting 4 and 24 large deletions, respectively (Zucman-Rossi et al., 1998; Bruder et al., 2001). However, the first study included only 19 patients (Zucman-Rossi et al., 1998). The second study included 116 patients from a large number

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of collaborators in 12 countries (Bruder et al., 2001). However, some collaborators, including us, contributed patient material that had been screened by exon scanning, whereas others contributed patient material that was not first screened by exon scanning. Thus, an unbiased estimation of the frequency of large *NF2* deletions could not be obtained from these two studies. Furthermore, the methods used in both studies have a limited resolution, and therefore small alterations such as single-exon deletions could not be detected.

The newly developed multiplex ligation-dependent probe amplification (MLPA) technique (Schouten et al., 2002) provides an easy-to-perform gene-dosage assay with high resolution. Using MLPA, deletions and duplications of exons have successfully been detected in *MSH2*, *MLH1* (Gille et al., 2002), *MECP2* (Erlandson et al., 2003), and *BRCA1* (Hogervorst et al., 2003; Montagna et al., 2003). In this study, we applied MLPA to detecting deletions and duplications of the 17 exons and two additional sequences in the 5′ region of the *NF2* gene in 77 NF2 patients in whom no intragenic *NF2* mutations had been found by exon scanning. Furthermore, transcript analysis was performed in a subset of these patients.

MATERIALS AND METHODS

Patients and Samples

All 188 unrelated patients included in this study had been diagnosed with NF2 using the presumptive NIH criteria (Gutmann et al., 1997). This study was approved by the local institutional review boards; and informed consent was obtained from all patients. Genomic DNA was extracted from peripheral blood leukocytes and tumor specimens using a Qiagen DNA extraction Kit. Exon scanning for small intragenic *NF2* mutations was carried out using SSCP, TGGE, or direct sequencing as described previously (Kluwe et al., 1996, 1998, 2003).

Multiple Ligation–Dependent Probe Amplification

The DNA concentration of the samples was measured using a Pico Green Kit from Molecular Probes (Leiden, The Netherlands), and approximately 100 ng was used for MLPA. The MLPA was performed in a thermal cycler with heated lid (Biometra, Goettingen, Germany) using the SALSA P044 NF2 MLPA Kit from MRC-Holland (Amsterdam, NL). This kit provides an optimized probe mixture for all the 17 *NF2* exons, two fragments from the 5′ region, and 11 control fragments from various chromosomes. Also included in this kit are one control probe specific for a chromosome 1 sequence for verification of successful ligation reaction and four ligation-independent probes for identification of samples with insufficient amounts of DNA. Information regarding the probe sequences and ligation sites can be obtained at www.mlpa.com. Hybridization, ligation, and amplification of the probes were carried out as described previously (Schouten et al., 2002). One microliter of the amplification product was analyzed using an ABI 310 automated sequencer, with Tamra 500 as the internal size standard. Samples with insufficient DNA were identified by prominent amplification of the four ligation-independent fragments, and the MLPA reaction was repeated with increased amounts of DNA. Data analysis was done by exporting the peak sizes, heights, and areas to an Excel file. To minimize any artifacts generated in the capillary electrophoresis, the peak height of each *NF2*-specific probe was normalized by dividing it by the combined heights of the five nearest control probes. The same procedure was then performed for the peak area of the probe. Finally, the relative peak height and area of each target probe was compared with those obtained from the control samples. Samples showing changes in both relative peak height and area of one or more NF2-specific probes were analyzed at least twice.

Transcript Analysis

Total RNA was extracted from peripheral leukocytes using a modified guanidinium thiocyanate–phenol method (Chomczynski and Sacchi, 1987). First-strand cDNA was synthesized using modified murine Moloney leukemia virus reverse transcriptase, SuperscriptII (Gibco-BRL), and oligo-dT as primer. The *NF2* transcript was amplified in four overlapping fragments, each covering exons 1–4, 5–8, 9–12, and 13–17, as described previously (Jacoby et al., 1996). The four amplified fragments were analyzed on polyacrylamide gels, and abnormal bands were excised. The extracted fragments were reamplified and sequenced.

RESULTS

A total of 188 unrelated NF2 patients were screened for *NF2* mutations, 21 of whom presented with a known family history of NF2 (Fig. 1). Initially, exons 1 through 15 of the *NF2* gene were amplified from genomic DNA from peripheral leukocytes and analyzed using SSCP, TGGE,

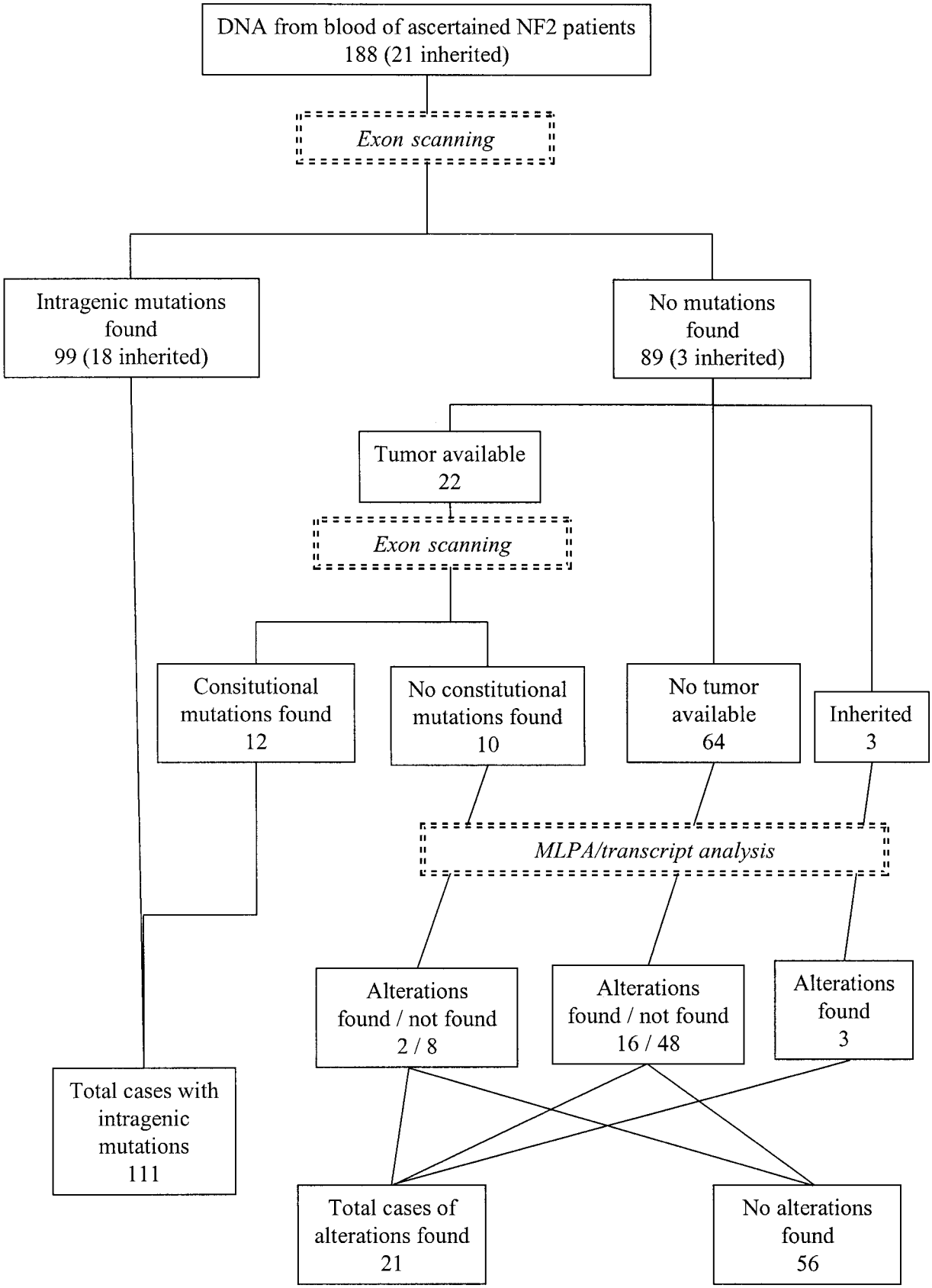

Figure 1. Results of mutation screening using various techniques and specimens in 188 unrelated NF2 patients.

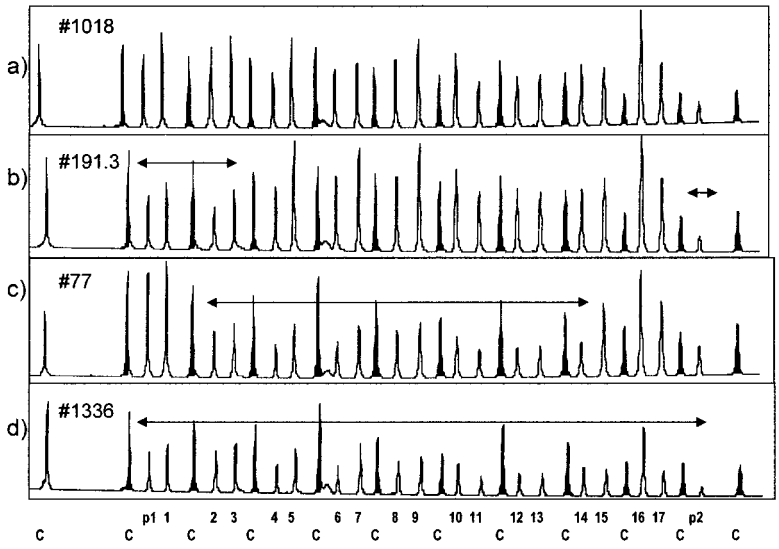

Figure 2. Deletions revealed by direct comparison of the MLPA electrophoresis peak patterns. Probe mix P044 contained probes for all 17 *NF2* exons (indicated as 1 through 17), 2 probes for the 5′ region of *NF2* (p1 and p2), and 12 additional control probes (C in the *x* axis) for other genes on various chromosomes: (a) no alteration in copy number of the 19 *NF2* fragments in patient 1018; (b) deletion spanning from the 5′ region to exon 3 in patient 191.3; (c) deletion covering exons 2–14 in patient 77; and (d) complete deletion of the *NF2* gene in patient 1336. Probes with reduced signal are indicated by bars.

or direct sequencing. Intragenic small *NF2* mutations were found in 99 unrelated cases (Kluwe et al. 1996, 1998, 2003, and unpublished data). In seven patients with sporadic disease, the ratio of mutant to normal *NF2* alleles was obviously less than 1, suggesting mosaicism.

For the other 89 patients—86 de novo and 3 familial cases—no mutations were detected by exon scanning in DNA from leukocytes. From 22 de novo patients, tumor specimens were available for genetic analysis. Identical *NF2* mutations in different tumors from one patient or two genetic alterations (e.g., one mutation and one loss of heterozygosity) in one tumor were identified in 12 cases. The failure to identify mutations in the corresponding leukocytes was most likely a result of mosaicism in these 12 patients (Kluwe et al., 1998, 2003).

In the remaining 77 patients—74 de novo and 3 inherited cases—no mutations were identified in DNA from either blood or tumor or no tumor specimens were available for further analysis (Fig. 1). To detect larger genomic abnormalities, DNA from these 77 NF2 patients was screened using the gene dosage assay MLPA. DNA from four patients with known intragenic *NF2* mutations were used as negative controls in all MLPA assays. An example of a sample exhibiting no alteration in copy number of any of the 17 *NF2* exons is shown in Figures 2a and 3a. The MLPA assay detected a decreased or increased copy number of exons in 20 cases (Table 1): 17 de novo and 2 inherited. In one case, a single exon deletion was identified. In seven cases, deletions were detected with one intragenic breakpoint and one breakpoint beyond the 3′ or 5′ end of the *NF2* gene (Figs. 2b and 3b), and seven cases were detected with intragenic deletions of multiple exons (Figs. 2c and 3c). Furthermore, four cases showed a deletion spanning all 17 exons and 2 segments in the 5′ region, indicating deletion of the whole *NF2* gene (Figs. 2d and 3d). One of these whole-gene deletions (in patient 65) was described previously in a study using array CGH (Bruder et al., 2001). Finally, in one case, the MLPA assay identified duplication of exons 2–4 (Table 1).

To verify the results obtained from the MLPA assay, transcript analysis was performed on RNA from 47 patients. Nine alterations were found upon amplification and analysis of four overlapping fragments covering the 1.7-kb *NF2* transcript. Seven deletions and one duplication of exons 2–4 identified by MLPA were confirmed in the corresponding *NF2* transcript (Fig. 4A). Eight genomic deletions detected by MLPA were not found with transcript analysis (Table 1): three involving the whole *NF2* gene, four extending beyond the 5′ or 3′ end of the gene, and one covering 13 exons. No transcript could be examined for the other four deletions detected by MLPA (Table 1). In an additional inherited case (Table 1, case 57), transcript analysis revealed an insertion of two exons from another gene, on chromosome band 22q12, between exons 4 and 5 of the *NF2* gene (Fig. 4B). This insertion was barely visible in the father (57), whereas it was clearly present in the daughter (57.1), suggesting mosaicism in the father, who has no family history of NF2.

DISCUSSION

In this study, we screened 188 unrelated patients for *NF2* mutations using a combination of

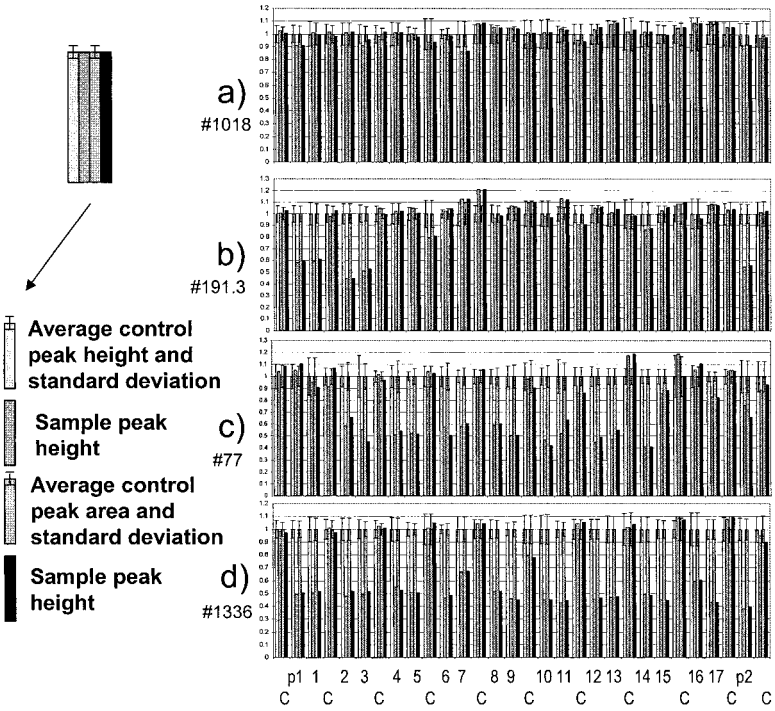

Figure 3. Deletions revealed by analyzed MLPA results. Relative peak height and area of each of the 19 *NF2* probes (p1, p2, and exons 1–17) were calculated by dividing them by those of the five closest signals of the control probes (C). The relative peak height and area of each target sequence were then compared with those obtained from four patients with known intragenic *NF2* mutations. (a) No alteration in copy number of the 19 *NF2* fragments in patient 1018. (b) Deletion spanning from the 5′ region to exon 3 in patient 191.3. (c) Deletion covering exons 2–14 in patient 77. (d) Complete deletion of the *NF2* gene including the 5′ region in patient 1336.

TABLE 1. Mutations Detected by MLPA and in mRNA

Patient ID	MLPA	Transcript analysis
268	Duplication of exons 2–4	Duplication of exons 2–4
107	Deletion of exons 5–8	Deletion of exons 5–8
71	Deletion of exons 8–9	Deletion of exons 8–9
68	Deletion of exons 15	Deletion of exons 15
225 (inherited)	Deletion of exons 15–16	Deletion of exons 15–16
229	Deletion of exons 15–16	Deletion of exons 15–16
230	Deletion of exons 15–16	Deletion of exons 15–16
249	Deletion of exons 15–16	Deletion of exons 15–16
18 (inherited)	Deletion of 5' region of exon 1	No alteration detected
62	Deletion of 5' region of exon 1	No alteration detected
91	Deletion of 5' region of exon 1	No alteration detected
77	Deletion of exons 2–14	No alteration detected
59	Deletion of exons 7–17	No alteration detected
25	Deletion of whole *NF2* gene	No alteration detected
65 (Bruder et al., 2001)	Deletion of whole *NF2* gene	No alteration detected
265	Deletion of whole *NF2* gene	No alteration detected
191,3	Deletion of 5' region of exon 3	Not examined
234	Deletion exons 5–17	Not examined
382	Deletion of exons 10–17	Not examined
1336	Deletion of whole *NF2* gene	Not exmained
57	No alteration detected	Insertion of two exons of a foreign gene on 22q12; slightly visible (Fig. 4B)
57.1 (inherited)	No alteration detected	Insertion of two exons of a foreign gene on 22q12; clearly visible (Fig. 4B)

techniques. Exon scanning led to the detection of mutations in 111 (59%) cases (Kluwe et al., 1996, 1998, 2003). Among the 77 cases in which no mutations were detected by exon scanning, large alterations were detected by MLPA in an additional 20 cases. In addition, transcript analysis of 47 of the

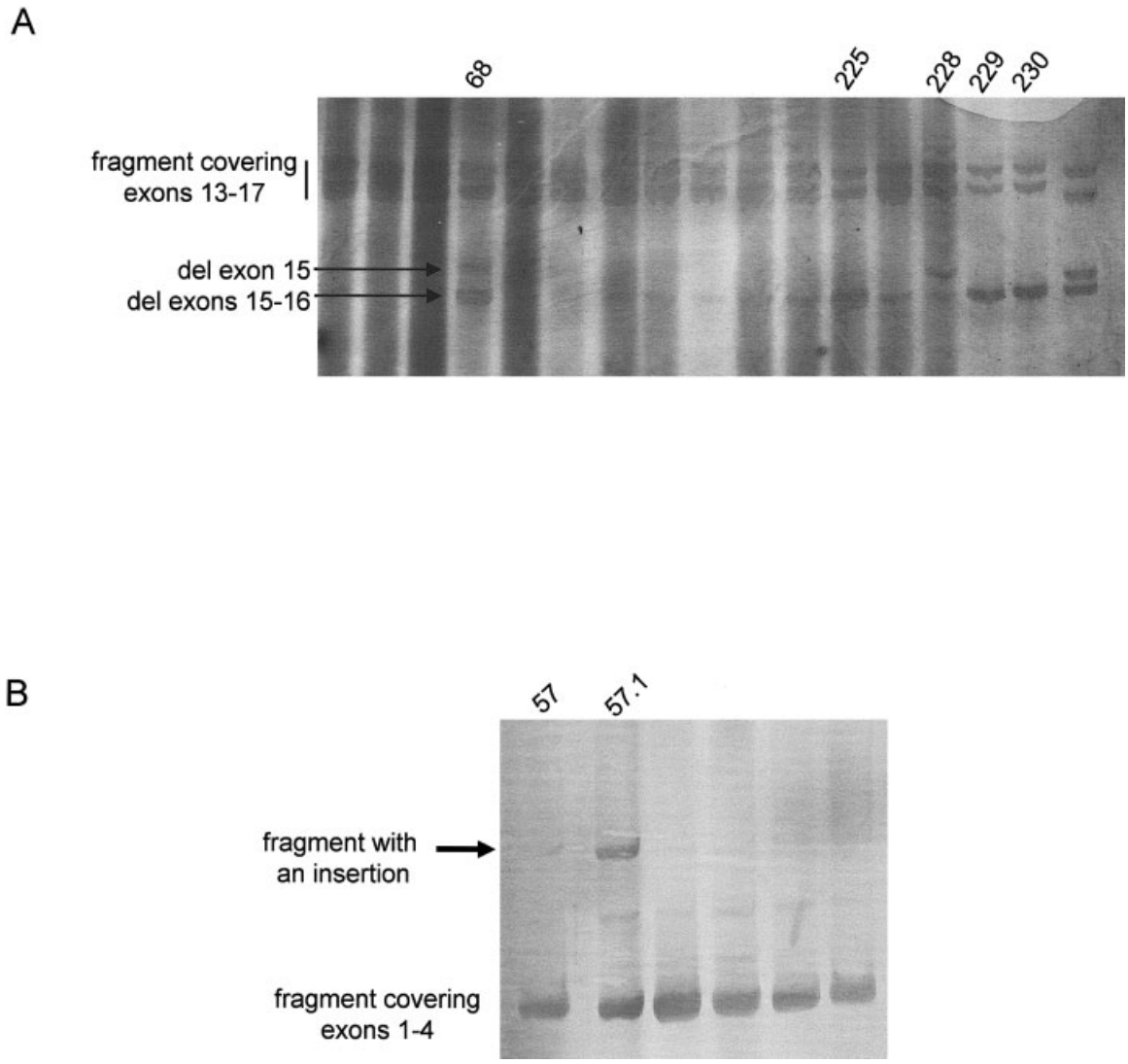

Figure 4. Transcript analysis. (A) Fragment covering exons 13–17 of the *NF2* transcript. Exon 16 is alternatively spliced, resulting in double bands. A fragment with an exon 15 deletion was found in patient 68 (upper array). A fragment with exons 15 and 16 deleted was found in patients 68, 225, 229, and 230. The alteration in patient 228 was caused by a mutation in a splice site in intron 14. The last lane contains size markers. (B) Fragment covering exons 1–4. A larger fragment was found in patient 57 and is faintly detectable in her affected father (array). Sequencing revealed an insertion of two exons of a 22q12 gene, between exons 4 and 5 of the *NF2* gene.

77 patients identified an insertion of a foreign segment between exons 4 and 5 of the *NF2* gene in an inherited case (57.1). The total number of 21 gross alterations affecting the *NF2* gene accounted for 27% of the 77 cases in which no intragenic small mutations were found by exon scanning and for 16% of the 134 mutations identified among the 188 NF2 patients screened.

With the identification of large alterations in three inherited cases, a 100% detection rate for *NF2* mutations was achieved for the 21 inherited cases in this study. For 56 patients (30% of the 188), the genetic causes are still unclear. Because they are all de novo patients, mosaicism may play a significant role. Large alterations may also exist in a mosaic manner, as in case 57 (Fig. 4B), making the detection more difficult. Furthermore, mutations in noncoding regions may also contribute to the failure of detection in these 56 cases.

A single-exon deletion was found in only one case and thus seems to be infrequent in NF2. Deletion of exons 15–16 and complete gene deletions were each found in four cases, making them the most frequent events found in our study. It is

unlikely that deletion of exons 15–16 is an artifact because both MLPA and transcription analyses were in concordance in all four cases. A possible cause may be paralogous recombination sites flanking the two exons, which may increase the probability of unequal crossover recombination, with the consequence of deletion of the intervening sequence. Further studies involving long-range PCR are needed to identify the precise breakpoints.

Nine large deletions detected by MLPA, involving at least 10 exons or the 5′ or 3′ end of the *NF2* gene, were not found in the corresponding transcript analysis. Indeed, deletions involving more than five exons or those involving the 5′ or 3′ end of the *NF2* gene were not detectable because the transcript analysis in this study was carried out on four overlapping fragments each covering only up to four exons.

For patient 57.1, in whom no mutation was found by MLPA, transcript analysis identified an insertion of a foreign fragment. The lack of detection in the MLPA assay is reasonable because this insertion did not cause a change in the copy number of any of the 17 *NF2* exons.

Our study revealed considerable frequency (11% = 21 of 188) of large alterations in the *NF2* gene and demonstrated that MLPA is a simple and effective method for detecting these alterations. According to our experience, MLPA is robust and the results are reproducible. Transcript analysis may enable detection of additional mutations, as demonstrated in case 57. However, to avoid unusual or illegitimate splicing and nonsense-mediated decay, RNA should be extracted from short-term cultured leukocytes in which protein synthesis is inhibited (Messiaen et al., 2000; Wimmer et al., 2000).

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