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ANALYSIS OF NF1 GENE MUTATIONS IN NEUROFIBROMATOSIS TYPE 1 PATIENTS IN JAPAN

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Neurofibromatosis type 1 (NF1) is one of the most common inherited disorders and is characterized by abnormalities in multiple tissues derived from the neural crest. Here, we report two novel deletion mutations of the NF1 gene from two out of 25 unrelated Japanese NF1 patients. These mutations were detected using polymerase chain reaction (PCR) / single-strand conformation polymorphism (SSCP) analysis. Sequencing analysis revealed a 4 base pair (bp) deletion at 5679 (5679delACTG) in exon 30 in one patient and a single bp deletion at 5949 (5949delA) in exon 32 in the other patient. Both of these mutations resulted in frameshifts, followed by premature terminations of the mutant allele. Because only a few large rearrangements of the NF1 gene have been reported in NF1 patients, it is likely that subtle mutations such as these are common.

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen's neurofibromatosis, is an autosomal dominant disorder affecting about 1 in 4000 individuals in all ethnic groups. Common clinical features are peripheral neurofibromas, cafe-au-lait spots, and Lisch nodules of the iris, which may be associated with certain brain tumors, such as gliomas, glioblastomas and meningiomas (1).

The gene responsible for NF1 is located on chromosome 17q.11.2 (2,3) and has been cloned (4-7). The NF1 gene is estimated to be about 300 kilobase (kb) in length and the transcript is approximately 11-13 kb long. Considering the clinical heterogeneity even among patients in the same family (1), the pathogenesis of NF1 must be complex, although the main contributing factor must be the mutation of the NF1 gene. The mutation rate of the NF1 gene is approximately 100-fold higher than that of most genes (8), resulting in around 50% of NF1 cases representing new mutations. Although NF1 is one of the most common inherited diseases, relatively few cases of NF1 caused by abnormalities in the NF1 gene have been reported so far. Since translocations (4,9,10), large deletions and insertions (4,11-13) are rare in NF1 patients, more subtle mutations are likely the cause of this disorder (14).

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Abbreviations: NF1, neurofibromatosis type 1; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; bp, base pair; kb, kilobase; GRD, GTPase-activating protein-related domain; GAP, GTPase-activating protein.

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We and others have previously described the same point mutations at the first nucleotide of the codon for Arg-1947 in exon 31, "C", which is converted to "T", resulting in the generation of a stop codon at this position (6,15-17). This position is the only hotspot in NF1 gene so far identified in NF1 patients. In this report, we expanded our study for NF1 gene mutations to the neighboring exons 24, 28-30 and 32-36 by using polymerase chain reaction (PCR) / single-strand conformation polymorphism (SSCP) in 25 Japanese NF1 patients. We describe the identification of two novel small mutations, both of which created inappropriate termination codons.

MATERIALS AND METHODS

Subjects: Twenty-five unrelated NF1 patients were studied. All were Japanese and the diagnosis was made according to the criteria by Riccardi and Eichner (18). Fifteen had a family history and 10 were sporadic. DNA was prepared from peripheral blood as described previously (19).

PCR-SSCP analysis: DNA samples for SSCP were generated by using PCR (20) with the primer pairs previously described for exon 24 (21), exons 28-30 (22) and exons 32-36 (6). The numbering of exons follows the data presented at the National NF Foundation Consortium Meeting held at Salt Lake City in January, 1992. PCRs were performed with 30 cycles for 0.5 min at 95°C and 2 min at 60°C in PC-700 (Astec, Fukuoka, Japan) with Taq polymerase (Pharmacia, Uppsala, Sweden) and [^{32}P]dCTP (ICN Radiochemicals, Irvine, CA). The PCR products were subjected to analysis on 5% non-denaturing acrylamide gels with or without 5% glycerol at room temperature in 0.5xTBE (90mM Tris-borate, 2mM EDTA, pH8.3) at 13V/cm (23). DNA fragments were visualized by exposing the gels to Kodak XAR5 films.

Sequencing analysis: The PCR products representing variants in PCR-SSCP analysis were prepared for blunt-ended ligation into plasmid vectors. Blunt-ending of samples performed with Taq polymerase were followed by phosphorylations at the 5' terminus by polynucleotide kinase. The samples were subjected to 1.2% agarose, and the gels corresponding to the bands were excised and purified by SUPREC -01 (Takara, Tokyo, Japan). Prepared PCR products for blunt-ended ligations were cloned into blunt-ended pUC18 plasmid. DNA sequences were determined by dideoxy chain termination method (24) with [^{32}P]dCTP using a sequencing kit (Sequenase ver. 2.0, USB, Cleveland, OH). The direct sequencing method was performed as described (25).

RESULTS AND DISCUSSION

The NF1 gene is a large gene, estimated to be 300 kb in length and composed of at least 49 exons. To date, only a small number of large rearrangements in this gene have been reported in NF1 patients. Since the publication of the flanking sequences of exons 24 and 28 to 36 (6,21) of the NF1 gene, a great deal of work has been carried out to detect more subtle mutations in this part of the gene in NF1 patients.

We have previously reported the investigation for mutations in exon 31 and the identification of a nonsense mutation at Arg-1947 in two unrelated familial NF1 patients in Japan (17). As the same mutation has been reported in three unrelated Caucasians (6,15,16), the first nucleotide in the codon for Arg-1947, "C", is a hotspot, regardless of ethnicity. Although small deletions, insertions and missense mutations have been identified in exon 24 (21,26), exon 28 (22), and exon 31 (16), the numbers are still few.

In the present study, we expanded our search for NF1 gene mutations into exons 24, 28-30, and 32-36 in 25 Japanese NF1 patients. The PCR-SSCP analysis revealed that one patient

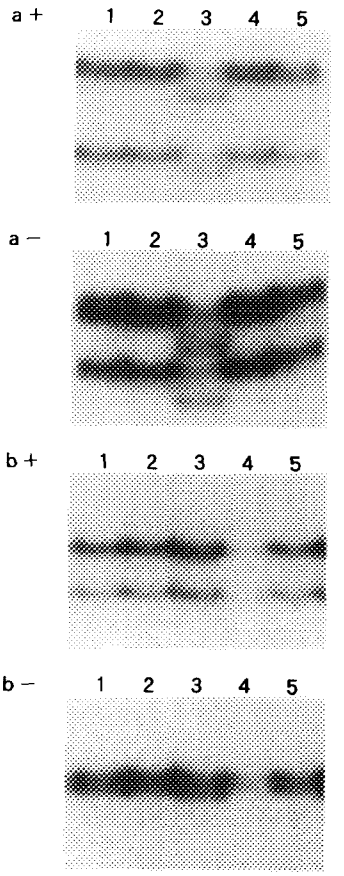

Figure 1. **PCR-SSCP analysis of exons 30 and 32 of NF1 gene.** The exon 30 or exon 32 of NF1 gene from each individual was amplified by PCR with intron-based primers (6,22). PCR products were heat-denatured and subjected to 5% polyacrylamide gel electrophoresis. Only a part of the gel of PCR-SSCP of exon 30 with **(a+)** or without **(a−)** 5% glycerol is shown. Lane 3 shows data from NF1-23 and lanes 1,2 and 4,5 are from other NF1 patients. PCR-SSCP of exon 32 with **(b+)** or without **(b−)** 5% glycerol is presented, again with lane 3 showing data from NF1-13.

(NF1-23) showed additional distinct single-stranded DNA fragments in exon 30, and another patient (NF1-13) showed DNA fragments with altered mobility in exon 32. NF1-23, a 33-year-old male, and NF1-13, a 29-year-old female, both had a family history of NF1 and its typical clinical features. The abnormality of the PCR-SSCP pattern in exon 30 was detected on a 5% polyacrylamide gel with or without 5% glycerol, while the aberration in exon 32 was detected on a 5% polyacrylamide gel without 5% glycerol, but not with 5% glycerol (Fig. 1).

In order to identify the sequence variations in these two patients, NF1-13 and NF1-23, we performed a sequencing analysis of exon 30 in NF1-23 and exon 32 in NF1-13. The regions corresponding to these two exons were amplified using PCR with a pair of intron-based primers (6,22), cloned into pUC18 plasmid and sequenced in their entirety. In NF1-23, two alleles of the NF1 gene were observed; one was a normal allele and the other was a mutant allele with a 4 base pair (bp) deletion at 5679 (5679delACTG) (Fig. 2a). In NF1-13, the sequence variation in exon 32 was also heterozygous. The mutant allele showed a single bp deletion at 5949 (5949delA)

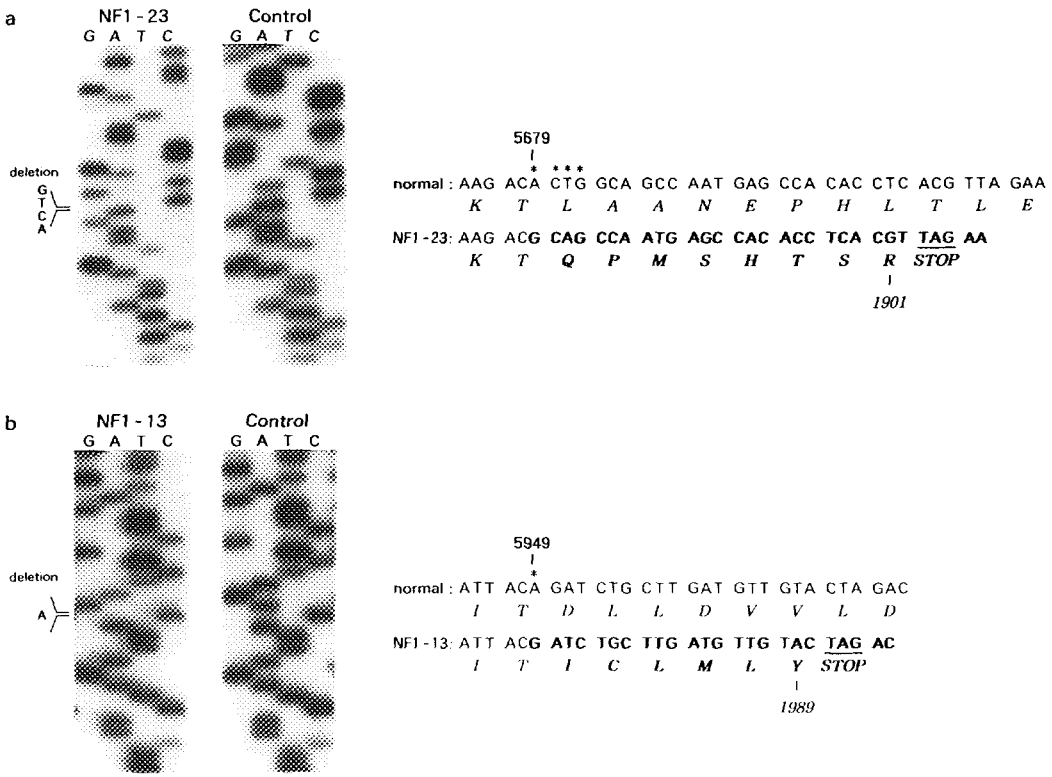

Figure 2. **Sequencing of exons 30 and 32 of NF1 gene.** PCR products of exons 30 and 32 of affected patients were cloned into pUC18 plasmids and sequenced as described in the Materials and Methods. DNA sequencings are shown on the left and the deduced nucleotide sequences and amino acid residues (*italics*) are shown on the right. Deleted nucleotides are indicated by asterisks. Frameshifts and altered amino acids in the patients are denoted by bold letters. **a.** Four bp (ACTG) were deleted from nucleotide 5679 to 5682 in exon 30 of patient NF1-23, resulting in an alteration of the reading frame, the generation of 8 amino acids different from normal neurofibromin and a premature termination at amino acid 1901. **b.** A one bp (A) deletion at nucleotide 5949 in exon 32 in NF1-13 changed the reading frame and caused the generation of 6 amino acids different from normal neurofibromin prior to the inappropriate termination at amino acid 1989.

(Fig. 2b). The sequence abnormalities were confirmed by using the direct sequencing method (data not shown). These two mutations generated frameshifts and inappropriate termination codons, resulting in the truncation from the carboxy terminal one third of the NF1 gene product, neurofibromin.

Neurofibromin is composed of 2818 amino acid residues containing a region of 360 amino acids called the GTPase-activating protein-related domain (GRD), which is structurally and functionally homologous to GTPase-activating protein (GAP) (27). Since the GRD as well as GAP stimulates GTPase and converts the GTP-bound active form of $p21^{ras}$ to the inactive form, neurofibromin might act as a tumor suppressor by inactivating the oncogene ras, although the functional properties of other parts of neurofibromin are still unknown. Exon 24 is located in the middle of the GRD and exons 28 to 36 reside downstream from it. If no other mutations reside in the coding region of the mutant allele, the mutant message will encode abnormal neuro-

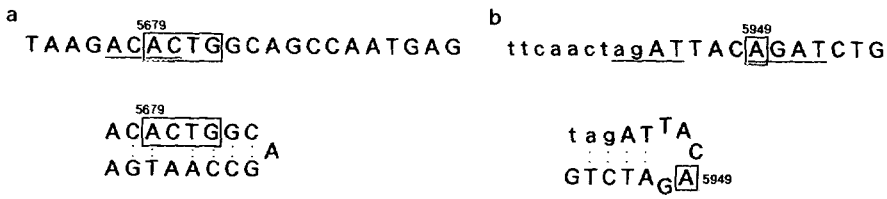

Figure 3. **Direct repeats and quasi-palindromic structures around the deletions. a.** Direct repeats of AC (underlined) are observed immediately around 5679 in exon 30. The deletion (boxed) starts at the first nucleotide of the second repeat. A quasi-palindromic structure flanking the deletion site (boxed) is shown below. Regions of self-complementarity are denoted by dots. **b.** Intron sequences are shown by small letters. Direct repeats of AGAT (underlined) are also identified around 5949 in exon 32. The deletion (boxed) occurs at the first nucleotide of the second repeat. A quasi-palindromic structure flanking the deletion site (boxed) is shown below. A region of self-complementarity is denoted by dots.

fibromin, which lacks one third of its carboxy end but still preserves the GRD. Our results should provide insight into understanding the structure-function relations of native neurofibromin.

The molecular mechanisms underlying gene deletions have been poorly understood, but the local DNA sequence environment is thought to be important in their pathogenesis. Direct repeats of between 2 and 8 bp located less than 4 bp apart are found in the vicinity of short (< 20 bp) gene deletions (28). The generation of these deletions is explained by the slipped mispairing model (29), which suggests that a misalignment occurs between direct repeats during replication. As shown in Fig. 3, the DNA sequence analysis revealed that direct repeats of 2 bp (AC) overlapped a 4 bp deletion in exon 30 and that direct repeats of 4 bp (AGAT) included a single bp deletion in exon 32. Palindromes (inverted repeats) or quasi-palindromes sometimes coincide with direct repeats in the immediate vicinity of the deletions. These structures have also been suggested to cause the generation of deletions in non-palindromic portions (28,30), through the endonucleolytic removal of unpaired bases followed by DNA repair. Both of the deletion mutations in our two cases were associated with quasi-palindromic sequences and overlapped with the unpaired region (Fig. 3). Although the mechanism(s) of human gene deletions are complex and much remains to be clarified, the collation of this data should contribute to the establishment of explanatory models for the locations of NF1 gene deletions.

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