J Hum Genet (2003) 48:545–549
DOI 10.1007/s10038-003-0066-7

SHORT COMMUNICATION

Ming-Tzen Liu · Jih-Shyun Su · Chun-Yu Huang
Shih-Feng Tsai

Novel mutations involving the NF1 gene coding sequence in neurofibromatosis type 1 patients from Taiwan

Received: 18 July 2003 / Accepted: 23 July 2003 / Published online: 26 September 2003

Abstract Neurofibromatosis type 1 (NF1) is a common cancer predisposition syndrome affecting the nervous system. The disease is one of the most common autosomal dominant diseases in all ethnic groups. Although the gene was mapped to human chromosome 17 and isolated in 1990, the detection of NF1 mutation is still considered to be a challenge as the gene is large and contains multiple exons. Here we report the detection of three genomic mutations in three Chinese patients living in Taiwan. A DNA diagnosis procedure was established to investigate the NF1 gene mutation at both the transcript and genomic DNA levels. Mutations causing transcript alteration were uncovered in three patients. In the first case, we detected a deletion involving exons 39–45 (nucleotide 7,260–8,167 in GenBank accession No. M89914). In the second case, a 2,199–2,448 deletion resulted in skipping of exon 13. The third case skipped the exon 3 in the mutant transcript. We further investigated what caused the cDNA deletion by PCR using genomic DNA as a template. In the first patient, we identified an approximately 17.5 kbp deletion in the *NF1* gene. In the other two patients, we identified a single-base substitution (IVS13 + 1G > A) at the splicing donor site in the second case, and an IVS3 + 1G > T substitution in the third case. We conclude that genomic deletion and alteration of splicing signal caused abnormal transcripts and truncated proteins in the three Taiwanese NF1 cases.

Keywords Neurofibromatosis type 1 · Gene · Mutation · Transcript analysis · Deletion

Ming-Tzen Liu and Jih-Shyun Su contributed equally to this article.

M.-T. Liu
Institute of Clinical Medicine,
National Yang-Ming University,
112 Taipei , Taiwan

M.-T. Liu · C.-Y. Huang
Department of Dermatology,
Veterans General Hospital-Taipei,
112 Taipei, Taiwan

J.-S. Su · S.-F. Tsai
Institute of Genetics, National Yang-Ming University,
112 Taipei, Taiwan

S.-F. Tsai (✉)
Division of Molecular and Genomic Medicine,
National Health Research Institutes,
128 Yen-Chiu-Yuan Road, Sec 2,
115 Taipei, Taiwan
E-mail: petsai@nhri.org.tw
Tel.: +886-226524120
Fax: +886-2-28200552

Introduction

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant diseases affecting all ethnic groups (Upadhyaya et al. 1998; Friedman et al. 1999). Mutations of the *NF1* gene are different among patients, making NF1 molecular diagnosis difficult. Detection of *NF1* mutation is generally considered to be challenging as the gene is large (about 350 kb) and it contains 60 exons and codes for at least three alternatively spliced transcripts (Shen et al. 1996; Viskochil 1999). Different types of mutations have been identified, and the mutation spectrum includes deletion, amino acid substitution, premature translation termination, insertion, splicing site mutation, and gross chromosomal rearrangement. Most *NF1* gene mutations are unique to a particular family. Only a few mutations are common, and few have been found in more than a few percent of patients analyzed (Fahsold et al. 2000).

To cover the wide range of *NF1* gene mutations, several diagnostic schemes have been proposed tailoring to the unique features of the *NF1* gene and variant sequences. Here we report the application of RT-PCR-based diagnostics for *NF1* transcript analysis and the detection of transcript alterations in three Chinese patients living in Taiwan. To our knowledge, this is the first report on *NF1* mutation analysis in this population.

Subjects and methods

NF1 patients were screened for the *NF1* gene mutations and all individuals donated blood samples under informed consent. Peripheral leukocytes were collected from 10 ml whole blood to extract RNA as well as genomic DNA, using 1 ml of Tri Reagent (Molecular Research Center, Inc., Cincinnati, Ohio, USA) per 10 ml whole blood collected. Two micrograms of total RNA were reverse transcribed with oligo-dT priming by AMV reverse transcriptase in a total volume of 20 µl at 37°C for 1 h. Five overlapping RT-PCR primer sets were designed according to a published method (Heim et al. 1995) to cover the *NF1* coding sequence of about 8.6 kbp in length. PCR reactions were carried out with the Expand Long Template PCR System (Roche Applied Science, Mannheim, Germany) using 2-µl cDNA as templates in a total volume of 50 µl. DNA amplification was achieved by an initial denaturation step at 94°C for 5 min, followed by ten cycles of (94°C for 15 s, 60°C for 40 s, 72°C for 2 min), 25 cycles of (94°C for 15 s, 60°C for 40 s, 68°C for 2 min and extend 20 s per cycle), and a final extension step at 68°C for 10 min. Genomic DNA was used as a template to amplify the target region by PCR with the Promega PCR kit (Promega, Madison, Wis., USA). The condition was 35 cycles of 94°C 30 s, 55°C 30 s, 72°C 1 min, and an extension step at 72°C for 7 min.

To pinpoint the mutation in the affected alleles, purified RT-PCR products (QIAquick Gel Extraction Kit; QIAGEN, Hilden, Germany) containing the variant sequences were rendered to direct sequencing. For manual sequencing, the reactions were performed with the Thermo cycle sequencing kit (Perkin Elmer) and labeled with ^{35}S dATP. For automated sequencing, ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit V2.0 was used and the reaction products were analyzed on a 3700 sequencer (ABI). For each patient, the entire coding region of the *NF1* gene was amplified with five overlapping RT-PCR segments each about 2 kbp in size. The amplified products were denoted as segment 1–segment 5(S1–S5), ordering from 5' to 3' of the *NF1* gene (Fig. 1).

Results

Two deletions were identified in the first case. The deletions were large enough that they could be readily detected by electrophoresis on 1% agarose gel. The first variant was an about 900 bp deletion in the segment 5 RT-PCR product. The second variant was in the segment 4 RT-PCR product and resulted in the loss of about 200 bp in length. The abnormal bands were excised from agarose gel and the DNA was extracted for DNA sequencing analysis. In the first variant allele, exons 39–45 deletion was identified (nucleotide 7,260–8,169, GenBank accession No. M89914) (Fig. 2a) and the mutation caused a frame-shift in the coding sequence. In the second variant allele, DNA sequencing confirmed a deletion of cDNA position 5,807–6,009, corresponding to skipping of exon 30 (Fig. 2b). The mutation caused premature termination of translation in exon 31. The two deletions effected the neurofibromin protein truncation in the C-terminus region and reduced from 2,818 a.a. full-length form to 2,334 a.a. and 1,859 a.a. abnormal forms, respectively.

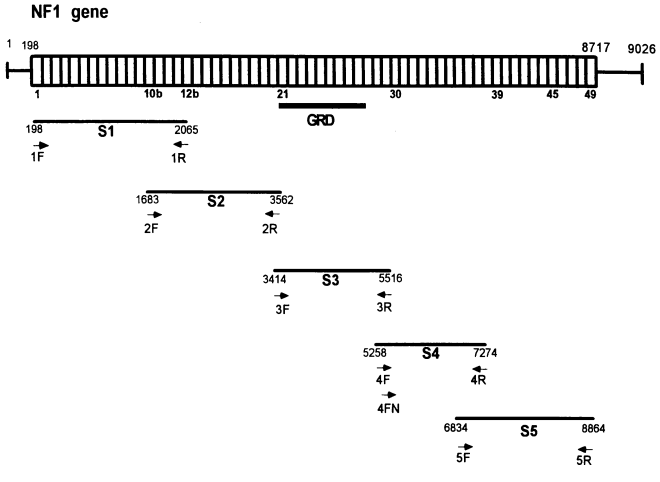

Fig. 1 Amplification of the *NF1* coding sequence. The entire coding region of the gene was amplified in five overlapping RT-PCR segments (S1, S2, S3, S4, and S5). The coding region starts at nucleotide position 198 and ends at 8,717 (GenBank accession No. M89914). The exon positions are depicted under *rectangles* and the *arrows* indicate PCR primers. *GRD* GTPase-activating protein related domain

In the second case, we discovered sequence variation in the segment 2 RT-PCR product. Similar procedures were applied to map the variant sequence, and *Nco*I digestion was used to verify the sequence alteration in the PCR product (data not shown). After electrophoresis in 1% agarose gel, the variant band was excised and the purified DNA was cloned into the pGEM-T vector for DNA sequence analysis. An exon 13 deletion was identified in the affected allele (Fig. 2c), and the variant sequence caused frame shift and resulted in premature stop codon in the exon 14.

In the third case, electrophoresis on 1% agarose gel revealed a lower band in the segment 1 RT-PCR product. A deletion of about 150 bp was suggested compared with the normal allele. Using the 1F primer, exon 3 deletion was confirmed by DNA sequencing (Fig. 2d). This mutation caused no frame shift in coding sequence but resulted in an in-frame deletion of 28 a.a. in the neurofibromin ORF.

To investigate the mechanism causing deletion in the cDNA, we designed several primers specific for genomic sequences in the boundary introns or exons of skipped regions. PCR was performed using genomic DNA as template and coupled with DNA sequencing analysis.

In the first case, using primers designed in exon 38 and 46, respectively, a 650-bp band was amplified from the patient but not from normal controls. Because the PCR condition is unlikely to generate the predicted size of 18,024 bp for normal sequence, the small band size may be due to a deletion in the genomic DNA. By DNA sequencing, we confirmed a genomic DNA deletion from intron 38–45 (nucleotide 52,187–69,635, GenBank accession No. L05367) (Fig. 3a). This genomic DNA deletion directly caused the mRNA deletion, as seen in RT-PCR analysis. Similarly, we designed primers to sequence the intron junction for the exon 30. However, we did not find genomic deletion or splicing signal mutation in the flanking region of exon 30.

For the second case, we used primers designed in intron 12 and 13 to amplify the exon 13 of the *NF1* gene. PCR products were then cloned into the pGEM-T

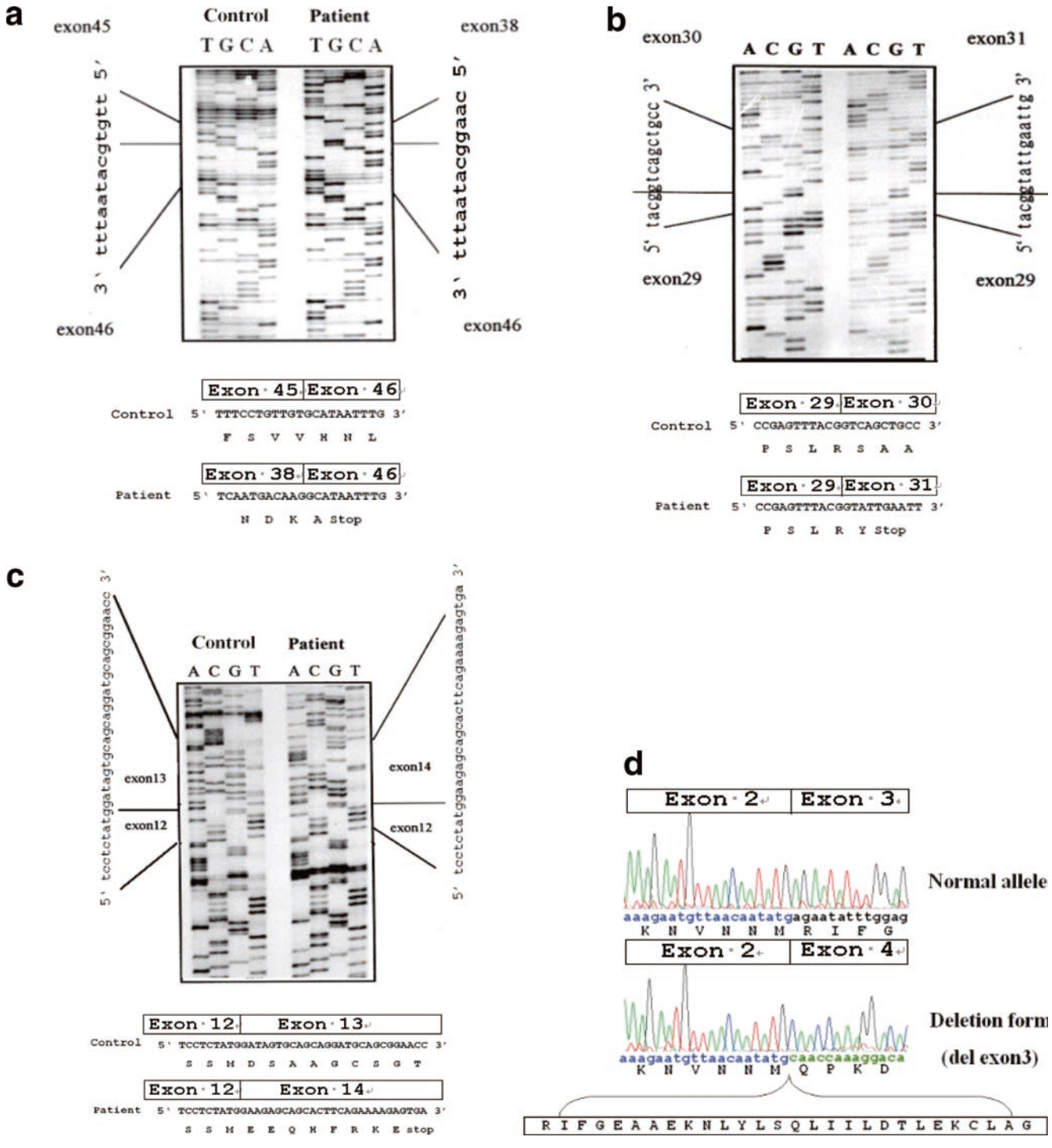

Fig. 2 a–d Abnormal transcripts detected by RT-PCR sequence analysis. **a, b** Case 1: DNA sequencing of the S5 and S4 RT-PCR products. Exon 46 was jointed by exon 38 and exon 31 by exon 29. The exons 39–45 and exon 30 deletions created a stop codon in exon 46 and exon 31, respectively. **c** Case 2: DNA sequencing of the S2 RT-PCR product of *NF1* cDNA. Exon 14 was jointed by exon 12. The exon 13 deletion created a stop codon in exon 14. **d** Case 3: The extent of the deletion was verified by DNA sequencing. The exon 3 skipping resulted in an in-frame deletion of 28 amino acids in the neurofibromin protein

vector. Two sequence alterations were identified: one was an A insertion in intron 13 (IVS13 + 52insA) and the other was G to A substitution in first base of intron 13 (IVS13 + 1G > A; nucleotide position 134 for GenBank accession No. Y07853) (Fig 3b). The latter variant may affect the splicing donor site and resulted in skipping of exon 13.

In the third case, the exon 3 of *NF1* was amplified using primers designed in intron 2 and 3. The PCR product was sequenced directly, and we observed mixed

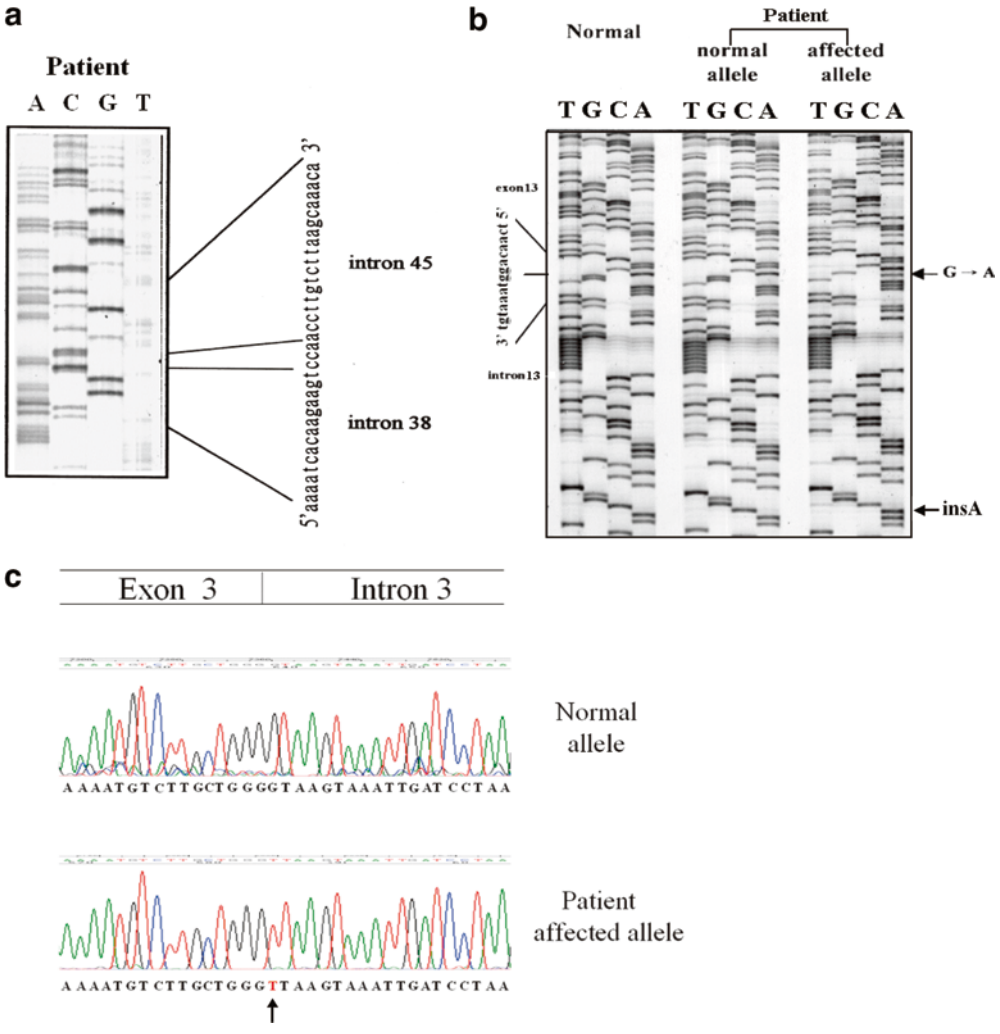

Fig. 3a–c Genomic DNA mutations in the three patients. **a** The 17,448 bp deletion (52,187–69,635; GenBank accession No. L05367) from intron 38 to 45 was identified in the first case. **b** IVS13 + 1G > A and IVS13 + 51insA alterations were identified in the second case. **c** IVS3 + 1G > T alteration was identified in the third case

sequences in the first base of intron 3 (data not shown). The PCR product was then cloned into pGEM-T vector to separate sequences from the two alleles. A G to T mutation in the first base of intron 3 was identified (IVS3 + 1G > T; nucleotide position 5 for GenBank accession No. U17658) (Fig 3c).

Discussion

NF1 is an autosomal dominant disease with variable clinical manifestations. Phenotype expression of individual patients in the same family can be uniquely different. The highly variable clinical features of NF1 are attributed to the fact that *NF1* mutations involve different functional domains of the protein and that other modifier genes could also affect the disease manifestation. In this series of case studies, we reported the molecular diagnosis of three Taiwanese patients who showed typical phenotypes of NF1. As an initial screen, we established a RT-PCR-based method to detect structural alteration of the *NF1* transcript. The five overlapping PCR fragments cover ~8.6 kbp of nonredundant coding sequence, corresponding to 73% of the transcript or 2.3% of the gene. Using this protocol, we could detect mutations in the patients. In the first case, two transcript anomalies involving the C-terminus of neurofibromin were identified. The C-terminus of neurofibromin contains several phosphorylation sites that may play a role in its function (Marchuk et al. 1991). In the second case, exon 13 deletion resulted in a stop codon after exon 12, causing the loss of the GAP-related domain (GRD). The GRD was thought to be critical for regulating the Ras signaling pathway (Xu et al. 1990; Basu et al. 1992). In the third case, an aberrant mRNA was found that caused 28 amino acids deletion in codon positions 69–96 of neurofibromin. Although there is no known functional domain in this region, the deletion might disturb the protein function and cause the disease.

Four aberrant transcripts were detected by this method. The high proportion of transcript anomaly in NF1 patients stresses the importance of screening mutations at the cDNA level. However, other mutation forms (for example, promoter and codon mutations) are not readily detectable by this approach. In order to improve the detection rate, several options are available.

Protein truncation assay has been applied successfully(Heim et al. 1995), based on the fact that deletion can cause visible difference in the length of encoded protein, to identify the NF1 gene mutations. However, the procedure involves multiple steps in the assay and the results are not always clear-cut. We suggest that direct DNA sequencing of the gene might be more straightforward. Given the high throughput of modern DNA sequencers, it is possible to design a resequencing scheme to detect nucleotide changes in the transcript or genomic sequence for the entire *NF1* gene.

Acknowledgements The authors wish to thank the NF1 patient families and their physicians for donating and collecting the blood samples for this study. Also, we greatly appreciate Dr. Tze-Tze Liu and Dr. Henry Sun for critically reviewing the manuscript and Ms. Chun-Hsia Chung for preparing the manuscript.

References

Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS, Downward J (1992) Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients. Nature 356:713–715

Fahsold R, Hoffmeyer S, Mischung C, Gille C, Ehlers C, Kucukceylan N, Abdel-Nour M, Gewies A, Peters H, Kaufmann D, Buske A, Tinschert S, Nurnberg P (2000) Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet 66:790–818

Friedman JM, Gutmann DH, MacCollin M, Riccardi VM (1999) Neurofibromatosis: phenotype, natural history, and pathogenesis, 3rd edn. Johns Hopkins University Press, Baltimore

Heim RA, Kam-Morgan LN, Binnie CG, Corns DD, Cayouette MC, Farber RA, Aylsworth AS, Silverman LM, Luce MC (1995) Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. Hum Molec Genet 4:975–981

Marchuk DA, Saulino AM, Tavakkol R, Swaroop M, Wallace MR, Andersen LB, Mitchell AL, Gutmann DH, Boguski M, Collins FS (1991) cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the NF1 gene product. Genomics 11:931-940

Shen MH, Harper PS, Upadhyaya M (1996) Molecular genetics of neurofibromatosis type 1 (NF1). J Med Genet 33:2–17

Upadhyaya M, Cooper DN (1998) Neurofibromatosis type 1: from genotype to phenotype. BIOS Scientific, Oxford

Viskochil D (1999) Neurofibromatosis 1. Introduction. Am J Med Genet 89:v–viii

Xu GF, O'Connell P, Viskochil D, Cawthon R, Robertson M, Culver M, Dunn D, Stevens J, Gesteland R, White R (1990) The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 62:599–608