

## A Clinical Variant of Neurofibromatosis Type 1: Familial Spinal Neurofibromatosis with a Frameshift Mutation in the *NF1* Gene

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### Summary

Spinal neurofibromatosis (SNF) has been considered to be an alternative form of neurofibromatosis in which spinal cord tumors are the main clinical characteristic. Familial SNF has been reported, elsewhere, in three families—two linked to markers within the gene for neurofibromatosis type 1 (NF1) and the other not linked to *NF1*—but no molecular alterations have been described in these families. We describe a three-generation family that includes five members affected by SNF. All the affected members presented multiple spinal neurofibromas and café au lait spots, one member had cutaneous neurofibromas, and some members had other signs of NF1. Genetic analysis, performed with markers within and flanking the *NF1* gene, showed segregation with the *NF1* locus. Mutation analysis, performed with the protein-truncation test and SSCP/heteroduplex analysis of the whole coding region of the *NF1* gene, identified a frameshift mutation (8042insA) in exon 46, which should result in a truncated NF1 protein. The 8042insA mutation was detected in all five family members with the SNF/NF1 phenotype. To our knowledge, this is the first time that a mutation in the *NF1* gene has been associated with SNF. The clinical homogeneity in the severity of the disease among the affected members of the family, which is unusual in NF1, suggests that a particular property of the *NF1* mutation described here, a gene closely linked to *NF1*, or posttranscriptional events are involved in this severe neurological phenotype.

### Introduction

The neurofibromatoses are a group of neurocutaneous syndromes, characterized by growth abnormalities of the nervous system and of various organs, that show extreme clinical heterogeneity (reviewed in Riccardi 1992). Attempts to classify the neurofibromatoses have led to the consideration of two main clinical forms, type 1 (NF1) and type 2 (NF2), as well as several alternate and related forms (reviewed in Riccardi 1992 and in Viskochil and Carey 1994). Of these, only NF1 and NF2 are well-defined clinical entities for which genes have been identified (Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990; Rouleau et al. 1993; Troffater et al. 1993).

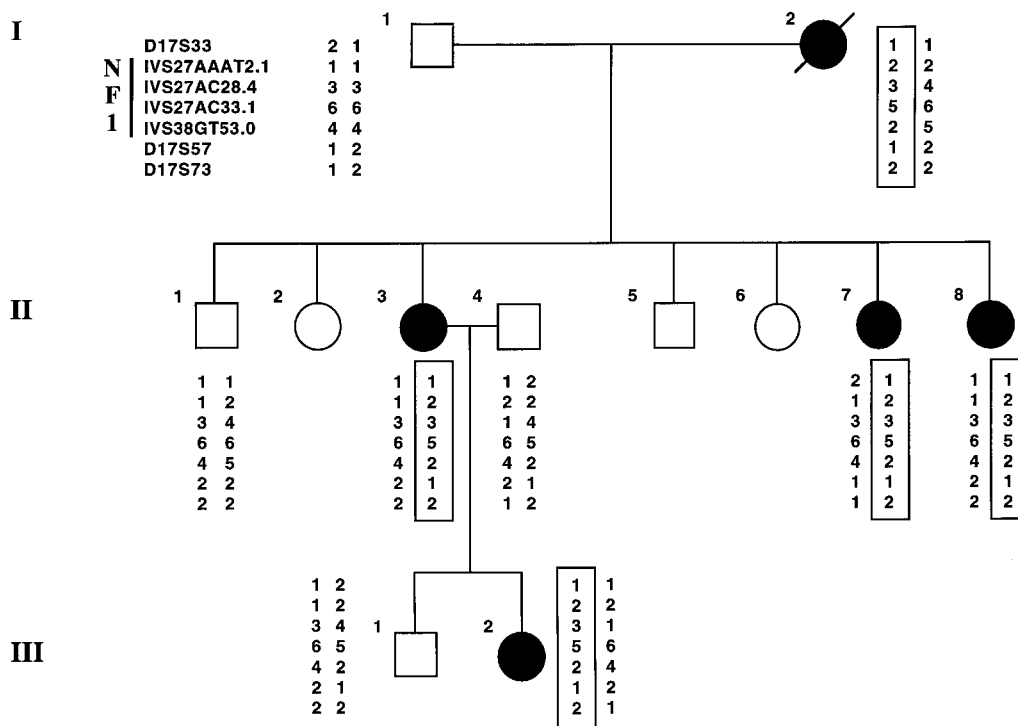
The gene responsible for NF1 maps to chromosome band 17q11.2, spans >350 kb of genomic DNA, and encodes an mRNA, of 11–13 kb, that contains at least 60 exons (Danglot et al. 1995; Li et al. 1995). The gene product of *NF1*, neurofibromin, has a central domain with sequence similarity to the GTPase-activating protein family, which can down-regulate p21-ras (Ballester et al. 1990; Martin et al. 1990; Xu et al. 1990a, 1990b). Moreover, the identification of somatic mutations in the *NF1* gene, from several NF1- and non-NF1-related tumors (Li et al. 1992; Xu et al. 1992; Andersen et al. 1993; Legius et al. 1993; The et al. 1993; Shannon et al. 1994; Colman et al. 1995; Sawada et al. 1996; Serra et al. 1997), supports the notion that *NF1* is a tumor-suppressor gene. The *NF2* gene, which is also a tumor-suppressor gene, maps to chromosome 22q12 and encodes a 595-amino acid protein, designated “merlin/schwannomin,” that has a sequence similar to that of a family of proteins that have been postulated to connect the cytoskeleton to the cell membrane (Luna and Hitt 1992; Rouleau et al. 1993; Troffater et al. 1993).

Spinal neurofibromatosis (SNF) has been considered to be an alternative form of neurofibromatosis. The presence of multiple spinal cord tumors, occurring in several members of the same family and segregating in an autosomal dominant fashion (familial SNF), has been reported, elsewhere, in three families. For two of these

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**Figure 1** Pedigree of a three-generation family with SNF. Segregation of markers flanking and within the *NF1* gene are shown (upper left). Blackened symbols indicate individuals with the *NF1*/SNF phenotype. The haplotype associated with the disease is boxed.

families, linkage to markers within the *NF1* gene was established; for the remaining family, no linkage to *NF1* was found (Pulst et al. 1991; Poyhonen et al. 1997). No molecular alterations have been detected in these families.

We have analyzed a family that includes five members severely affected by SNF. Mutation analysis, by means of protein-truncation testing (PTT), of the *NF1* cDNA allowed the identification of a frameshift mutation that cosegregates with the disease. This provides further evidence that it is unlikely that SNF/*NF1* is genetically distinct from *NF1*.

**Subjects and Methods**

*Subjects*

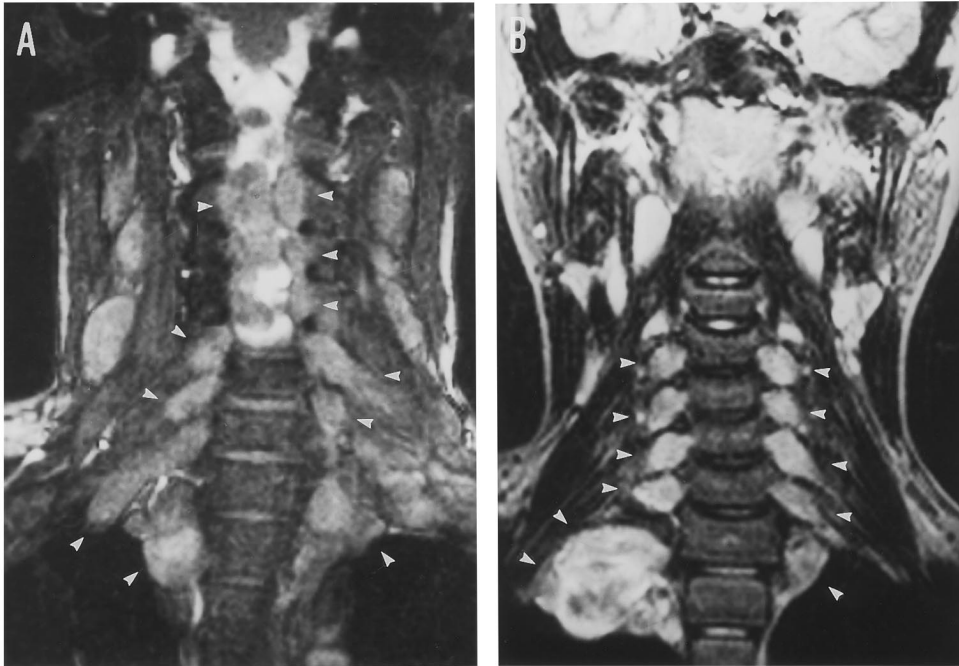
The family analyzed in this study had five members, in three generations, affected by SNF (fig. 1 and table 1). The five affected members were informed about the study, and consent was obtained from all of them. Patient I-2 was a 58-year-old woman who developed progressive paraparesis of her legs and right arm when she was 45 years old. She had multiple café au lait spots (CLS) but no cutaneous neurofibromas. Magnetic res-

**Table 1**  
Clinical Features of Family Members Affected with SNF

Feature	Patient				
	I-2	II-3	II-7	II-8	III-2
Age (years)	58 <sup>a</sup>	34	24	21	12
Spinal neurofibromas	C,D,L	C,D,L	C,D,L	D,L	C,D
Multiple CLS	Present	Present	Present	Present	Present
Cutaneous neurofibromas (n)	Absent	Present (3)	Absent	Absent	Absent
Lisch nodules	ND	ND	ND	Present	Present
Paraparesis (age [years])	Present (45)	Present (23)	Absent	Absent	Absent
Other	...	Mediastinal neurofibroma	Plexiform neurofibroma	...	...

NOTE.—C = cervical; D = dorsal; L = lumbar; CLS = café au lait spots; and ND = not determined.

<sup>a</sup> Deceased.



**Figure 2** Coronal T2-weighted spinal MRI scan of patients II-3 and III-2. *A*, Patient II-3: multiple bilateral neurofibromas, at cervical and dorsal levels, with the intra- and extraspinal components of a dumbbell neurofibroma growing into both the brachial plexus and the apical region of the lung. *B*, Patient III-2: multiple bilateral, paravertebral tumors, from C2–C3 to D3–D4, and two paravertebral masses, in the right- and left-lung apical regions. The arrowheads point to some of the spinal neurofibromas.

onance imaging (MRI) of the spine detected multiple intradural extramedullary masses, in the cervical, dorsal, and lumbar segments, corresponding to neurofibromas. Some of these masses had both intradural and extradural components, the so-called dumbbell tumors, with a large extraspinal component that produced foraminal enlargement. A surgical intervention to remove intradural neurofibromas from C1–C4 was performed in 1989. The patient had a very torpid clinical evolution that culminated in cardiac insufficiency and subsequent death, from a myocardial infarction, in 1996. Patient II-3 is a 34-year-old woman who had a surgical operation, when she was 16 years old, to remove a mediastinal neurofibroma. She has multiple CLS and three cutaneous neurofibromas. When she was 23 years old, this patient began to have difficulty walking and to show signs of progressive spastic paraparesis. The spinal MRI examination showed multiple tumors at C1–C7 (figs. 2A and 3A). A laminectomy was performed to remove neurofibromas from levels C1–C5. Shortly after surgery, the patient developed a complete transverse medullary syndrome, at the C2–C3 level, that required mechanical ventilation. Patient II-7 is a 24-year-old woman with multiple CLS and antecedents of surgical resection of a plexiform neurofibroma on the right arm. The spinal MRI examination disclosed multiple intra- and extra-

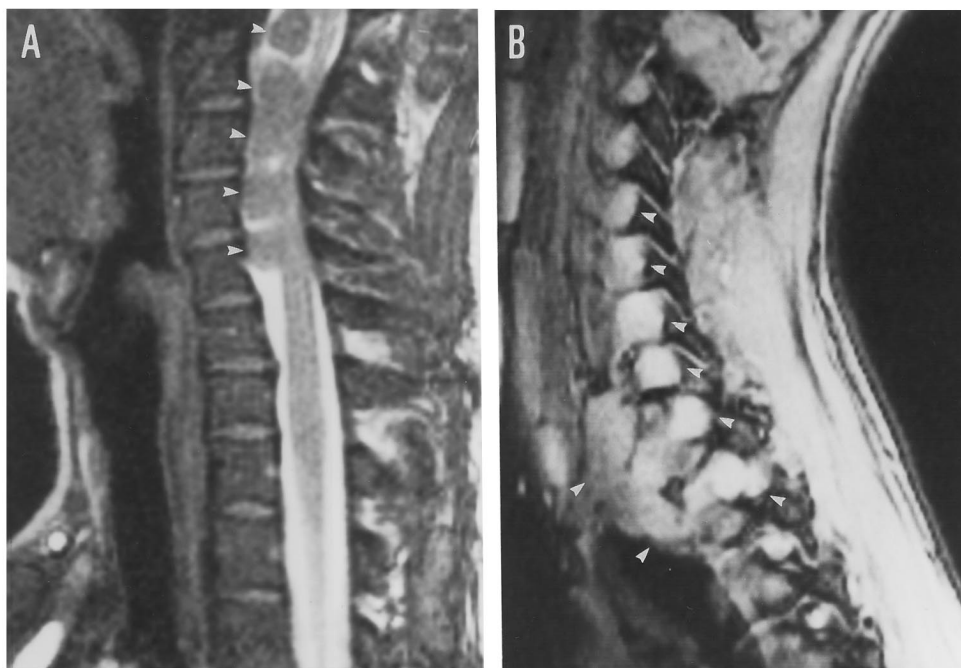
spinal neurofibromas in the cervical region, at the D6–D7 level, and in the lumbosacral region. Patient II-8 is a 21-year-old woman with multiple CLS and Lisch nodules. The MRI scans demonstrated bilateral lung apical tumors and a left paravertebral mass, at the L5 level. Patient III-2 is a 12-year-old girl with multiple CLS and Lisch nodules. The spinal MRI showed multiple bilateral tumors, from C2–C3 to D3–D4, and two paravertebral masses in the right- and left-lung apical regions (figs. 2B and 3B). The physical examination of the other members of the family revealed none of the major clinical features of NF1 (cutaneous neurofibromas, Lisch nodules, or CLS).

#### *RNA/DNA Extraction*

Total RNA was extracted, from peripheral blood lymphocytes, by use of the Tripure isolation reagent (Boehringer Mannheim), in accordance with the manufacturer's instructions. DNA was extracted by means of the "salting out" method (Miller et al. 1988).

#### *Reverse Transcription and PTT Analysis*

Two to five micrograms of RNA were reverse transcribed with 500 ng random hexamers and 200 U Superscript II reverse transcriptase (RT [Gibco-BRL]) in a



**Figure 3** Sagittal T2-weighted spinal MRI scan of patients II-3 and III-2. *A*, Patient II-3: intradural and extramedullary neurofibromas (*arrowheads*), with compression of the cervical cord and enlargement of the cervical spinal canal. *B*, Patient III-3: multiple cervical and dorsal neurofibromas (*arrowheads*), with extension outward from the spinal canal through the intervertebral foramen.

20- $\mu$ l reaction volume, under conditions recommended by the manufacturer. The entire *NF1* cDNA was then amplified (40 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C), with five pairs of overlapping primers that incorporate a T7 promoter and translation initiation site, as described elsewhere (Heim et al. 1995). After in vitro transcription/translation incorporating <sup>35</sup>S-methionine, the samples were electrophoresed on a 12% SDS-polyacrylamide gel. The gel was then dried and subjected to autoradiography for 6–48 h. When an abnormal fragment was detected, primers flanking the DNA sequence with the putative mutation were designed, and the RT-PCR product was sequenced by use of an automatic genetic analyzer (ABI PRISM 377 DNA Sequencer).

#### SSCP/Heteroduplex Analysis

The whole coding region of the *NF1* gene, amplified in 10 overlapping fragments (Hoffmeyer et al. 1994), was also analyzed by SSCP/heteroduplex analysis, as described elsewhere (Sala and Espinosa-Parrilla, in press).

#### Segregation Analysis

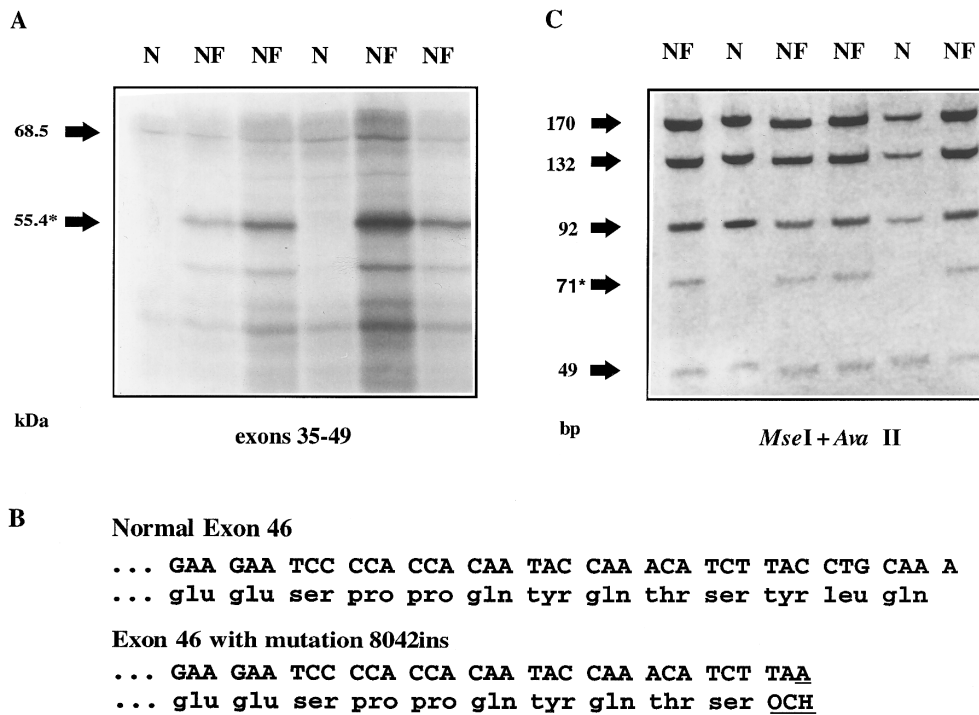
Haplotype analysis for seven *NF1* markers (four intragenic and three extragenic) was performed as described elsewhere (Lázaro et al. 1993). Haplotype analysis for four *NF2* markers (one intragenic CA-repeat and

three extragenic markers: *D22S268*, *D22S273*, and *D22S280*) was performed as described elsewhere (Marineau et al. 1993; Bourn and Strachan 1995). Restriction-enzyme analysis of cDNA, for detection of the 8042insA mutation, was performed as described elsewhere (Sambrook et al. 1989).

#### Results

Five family members affected with *NF1* shared a very severe neurological phenotype that included spinal neurofibromas (fig. 1 and table 1). Two of these five family members had paraparesis; the other three are still young (aged 12–24 years). All five members fulfilled the National Institutes of Health (NIH) diagnostic criteria for *NF1* (Stumpf et al. 1988). The SNF/*NF1* phenotype segregated with a seven-marker haplotype (four *NF1* intragenic and three extragenic markers) (fig. 1). *NF2* markers (one *NF2* intragenic and three extragenic) did not segregate with the disease (data not shown).

PTT analysis of the fifth segment of the *NF1* cDNA (as indicated by Heim et al. [1995] for PTT analysis) detected a 55.4-kD polypeptide, in addition to the normal 68.5-kD product, for the 3' end of the *NF1* cDNA (exons 35–49) (fig. 4A), which suggests a truncating mutation between nucleotides 8000 and 8100 of the *NF1* coding sequence. Sequencing of the RT-PCR product re-



**Figure 4** Identification of mutation 8042insA in the SNF family. *A*, PTT analysis, showing a truncated polypeptide, of 55.4 kD(\*), in addition to the normal peptide, of 68.5 kD. The truncated NF1 polypeptide was detected only in the affected patients. *B*, Partial nucleotide and amino acid sequences of exon 46, showing mutation 8042insA, which creates a TAA stop codon at the mutation site. *C*, Detection of mutation 8042insA, which generates an *MseI* restriction site, by restriction-enzyme analysis. A 488-bp cDNA fragment was amplified, by use of primers flanking the mutation, and was then digested with the restriction enzymes *MseI* and *AvaII*. This resulted in six fragments, of 170, 132, 92, 49, 23, and 22 bp, in the unaffected members, and in two additional fragments, of 71 bp (asterisk [\*]) and 21 bp (not shown), in the affected individuals.

vealed the insertion of an adenine at position 8042, at codon 2681, in exon 46 of the *NF1* gene (fig. 4B). Mutation 8042insA creates a TAA stop codon at the mutation site, which leads to a truncated NF1 protein. PTT analysis showed that all affected members of the family produced the truncated polypeptide caused by mutation 8042insA. The mutation generates an *MseI* restriction site, which allows confirmation of the molecular alteration, at the cDNA level, by digestion (fig. 4C). The presence of the mutation in genomic DNA was also confirmed by amplification and digestion with the same enzyme (data not shown).

The PTT analysis of the other regions of the *NF1* gene did not reveal additional alterations. To uncover other potential nucleotide changes that could have escaped the PTT analysis (missense mutations and in-frame insertions and deletions), we studied the complete coding region of the *NF1* gene of the SNF family, by SSCP/heteroduplex analysis of 10 overlapping fragments of the *NF1* cDNA, and we found only the mutation 8042insA.

## Discussion

Because of the extreme clinical heterogeneity of the neurofibromatoses, there have been many attempts to classify these disorders into distinct categories (reviewed in Riccardi [1992] and in Viskochil and Carey [1994]). As yet, only NF1 and NF2 have been confirmed as genetically distinct diseases, as a result of the cloning of the respective genes. There are several related or alternate forms of NF1, such as Watson syndrome (Tassabehji et al. 1993; Ahlbom et al. 1995), Noonan syndrome (Tassabehji et al. 1993; Bahuau et al. 1996), autosomal dominant multiple CLS (Brunner et al. 1993; Abeliovich et al. 1995), and LEOPARD syndrome (Wu et al. 1996); however, there exists some controversy about their NF1 molecular basis.

SNF has also been described as an entity that is distinct from NF1 and in which the main clinical characteristic is symptomatic spinal cord tumors, which are present in only 5% of NF1 cases (Huson et al. 1988; Von Deimling et al. 1995) and are actually more common in NF2.

Recently, it has been suggested that spinal neurofibromas associated with *NF1* occur more often than initially thought, since they were found, by spinal MRI, in 36% of the *NF1* patients studied by Poyhonen et al. (1997) but were associated with clinical symptoms in only 7% of the patients. The infrequent detection, by other investigators, of spinal neurofibromas in *NF1* patients could be explained by the lack of routine spinal MRI studies. Although asymptomatic spinal neurofibromas are probably more common than previously thought, the occurrence of multiple spinal neurofibromas in all the affected members of the same family is exceptional.

To date, only three other SNF families have been studied at the genetic level. Pulst et al. (1991) described two families, one of which satisfied NIH *NF1* criteria and showed linkage to the *NF1* locus. In the second family, all the affected members had only spinal cord tumors; this family was excluded by linkage from 17q11.2 and is probably linked to *NF2* (Pulst et al. 1991, 1997). Recently, Poyhonen et al. (1997) described a family with multiple CLS, spinal neurofibromas, and some other signs of *NF1* (dermal neurofibromas and axillary and inguinal freckles). Linkage to the *NF1* locus was established, and linkage to the *NF2* locus was excluded. No mutations in either *NF1* or *NF2* have been described in any of these families. All the affected members of the three-generation family we describe here have spinal neurofibromas, fulfill NIH *NF1* criteria, and have the frameshift mutation 8042insA in the *NF1* gene. This clearly links the *NF1* gene with familial SNF.

A hallmark of *NF1* is the high phenotypic variability between affected individuals, even among members of the same family. The only phenotype-genotype correlation described so far in *NF1* concerns the mental retardation and/or learning disabilities, mild facial dysmorphism, and large number of early-onset cutaneous neurofibromas associated with large deletions encompassing the whole *NF1* gene (Kayes et al. 1994; Wu et al. 1995; Leppig et al. 1996; Upadhyaya et al. 1996; Wu et al. 1997). One of the striking characteristics of the family reported here and of the SNF families reported elsewhere (Pulst et al. 1991; Poyhonen et al. 1997) is the absence or low number of cutaneous neurofibromas. This contrasts with the high number of early-onset cutaneous neurofibromas seen in patients with deletions of the whole *NF1* gene. The differences between these two phenotypes could be due to the presence, in the patients with SNF/*NF1*, of a partially functional, truncated *NF1* protein that is associated with different consequences at the neurological and dermatological levels.

The *NF1* family reported here is unusual because all the affected members present spinal neurofibromas. There are various possible explanations for the severe neurological phenotype in all the affected members of

this family. First, it is possible that the predicted 2,680–amino acid truncated neurofibromin of these patients has a negative residual function with a special effect in the development of the neural crest cells that are responsible for the SNF/*NF1* phenotype. However, we have found a frameshift mutation, in exon 48, that also gives rise to a stop codon in an *NF1* family with a classical *NF1* phenotype and without symptomatic spinal neurofibromas (E. Ars, E. Serra, A. Gaona, J. Garcia, H. Kruyer, C. Lázaro, X. Estivill, unpublished data). Second, SNF could be due to a second mutation, either in the normal *NF1* allele or in the same allele that contains mutation 8042insA. However, after an intensive analysis of the whole *NF1* coding region, we were unable to detect a second mutation. Furthermore, the second *NF1* allele of four of the patients reported here has been inherited independently, which excludes the presence of a common variant involved in the SNF phenotype. Third, it could be that a mutation in another gene, closely linked to *NF1*, is the cause of the SNF in this family. In support of this, one member of one of the SNF families described by Pulst et al. (1991) only had multiple CLS, had no spinal neurofibromas, but had inherited the *NF1* haplotype, which suggests that this case could be recombinant for a linked gene involved in the development of SNF. These families and others with concordant or discordant clinical data for SNF and *NF1* might be useful in the detection of modifier genes for this SNF/*NF1* phenotype. Finally, posttranscriptional events, such as alternative splicing or editing, that modulate the expression of the *NF1* gene (Skuse and Capione 1997) could contribute to the development of SNF.

Until now, the molecular genetics of families with spinal cord tumors and with clinical features of neurofibromatosis was unsolved. The identification of the mutation 8042insA in the *NF1* gene in the family reported here strongly suggests that other SNF families that satisfy NIH *NF1* diagnostic criteria might also have mutations in the *NF1* gene. The genetic characterization of these families should help us to understand the molecular alterations that cause this severe neurological phenotype.

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