

Confirmation of a Double-Hit Model for the *NF1* Gene in Benign Neurofibromas

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Summary

Neurofibroma is a benign tumor that arises from small or large nerves. This neoplastic lesion is a common feature of neurofibromatosis type 1 (NF1), one of the most common autosomal dominant disorders. The *NF1* gene codes for a protein called “neurofibromin.” It possesses a region that shares a high homology with the family of GTPase-activating proteins, which are negative regulators of *RAS* function and thereby control cell growth and differentiation. The evidence points to the *NF1* gene being a tumor-suppressor gene. NF1 patients also have an increased incidence of certain malignant tumors that are believed to follow the “two hit” hypothesis, with one allele constitutionally inactivated and the other somatically mutated. Recently, somatic loss of heterozygosity (LOH) has been described for neurofibromas, and mutations in both copies of the *NF1* gene have been reported for a dermal neurofibroma. The aim of our study was the analysis of the *NF1* locus in benign neurofibromas in NF1 patients. We performed LOH analysis on 60 neurofibromas belonging to 17 patients, 9 of them with family history of the disease and 8 of them sporadic. We have analyzed five intragenic *NF1* markers and six extragenic markers, and we have found LOH in 25% of the neurofibromas (corresponding to 53% of the patients). In addition, we found that in the neurofibromas of patients from familial cases the deletions occurred in the allele that is not transmitted with the disease, indicating that both copies of the *NF1* gene were inactivated in these tumors. Therefore, the recent reports mentioned above, together with our findings, strongly support the double inactivation of the *NF1* gene in benign neurofibromas.

Introduction

Neurofibromas are benign tumors that originate from a peripheral nerve sheath. They are primarily composed of Schwann cells and fibroblasts, but a mixture of other different cell types are also present (mast cells, endothelial cells, perineural cells, pericytes, and a small number of nerve cells) (revised in Riccardi 1992). Neurofibromas are also one of the most frequent features of neurofibromatosis type 1 (NF1), or von Recklinghausen disease, and they constitute an important diagnostic criterion of this complex genetic disorder. NF1 is a common autosomal dominant disease in man, with an incidence of $\sim 1/3,500$. It is a multisystemic disease, although the nerve system and the skin are the tissues mainly affected. Apart from the development of neurofibromas, café-au-lait spots, axillary freckles, and Lisch nodules of the iris constitute the most common clinical features of NF1. Approximately 2%–6% of NF1 patients develop malignant tumors (revised in Riccardi 1992). The *NF1* gene is located in the pericentromeric region of human chromosome 17 (17q11.2), contains 60 exons, and produces an 11–13-kb ubiquitously expressed transcript that encodes for a protein called “neurofibromin” (Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990; Li et al. 1995). Until now, only one clear functional domain has been attributed to neurofibromin (Martin et al. 1990). This domain consists of 360 amino acids of the predicted gene product and shows homology to the catalytic domain of the mammalian GTPase-activating protein (GAP) and the products of the yeast *IRA1* and *IRA2* genes, which can down-regulate p21^{ras} activity (Ballester et al. 1990; Xu et al. 1990a, 1990b). This region is called the “NF1 GAP related domain” (NF1-GRD) and is located in the central portion of the *NF1* gene, encompassing exons 21–27a (Li et al. 1995). Somatic mutations in the *NF1* gene have been described in several tumors, related to the disease or not (Li et al. 1992; Xu et al. 1992; Andersen et al. 1993; Legius et al. 1993; The et al. 1993; Shannon et al. 1994). All this evidence corroborates the hypothesis that *NF1* is a tumor-suppressor gene.

Contradictory genetic data have been presented relating to the molecular pathology involved in the genera-

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tion of neurofibromas. Preliminary studies failed to find loss of heterozygosity (LOH) in neurofibromas (Menon et al. 1990; Glover et al. 1991; Skuse et al. 1991; Lothe et al. 1995), whereas LOH was found in the analysis of malignant *NF1*-related tumors (Skuse et al. 1989; Legius et al. 1993). In addition, the presence of both alleles of the *NF1* gene has been described in Schwann cells derived from neurofibromas (Stark et al. 1995). However, recent investigations report the finding of (1) somatic *NF1* deletions in benign neurofibromas (Colman et al. 1995) and (2) mutations in both copies of the *NF1* gene in a dermal neurofibroma (Sawada et al. 1996). Furthermore, genetic instability in the analysis of several microsatellite markers has been reported in neurofibromas (Ottini et al. 1995).

It is thought that, at the cellular level, neurofibromas have a unicellular origin (Skuse et al. 1991). Classically, it has been considered that Schwann cells are the progenitors of neurofibromas (Waggener 1966; Kamata 1978; Stefansson et al. 1982), but recent studies have questioned this assessment, highlighting the crucial role of fibroblasts in the development of these benign neoplasias (Rosenbaum et al. 1995).

The aim of this study was to elucidate the role of the *NF1* gene in the pathogenesis of neurofibromas, by searching for deletions involving the *NF1* gene. Sixty peripheral neurofibromas belonging to 17 *NF1* patients, 9 of which had a family history of the disease, were studied. We analyzed 11 intragenic and extragenic *NF1* DNA markers located in chromosome 17, and we found LOH in 25% of the neurofibromas (corresponding to 53% of the patients). In addition, we found that deletions in neurofibromas from six patients with familial *NF1* occurred in the allele that is not transmitted with the disease, indicating that both copies of the *NF1* gene were inactivated. These data indicate that the inactivation of both copies of the *NF1* gene could be a crucial step in the development of benign neurofibromas.

Patients, Material, and Methods

Patient Identification and Sample Acquisition

Patients were diagnosed according to the accepted standard *NF1* diagnostic criteria. They were informed about the study, and consent was obtained from all of them. Neurofibromas were obtained from 17 patients; among these patients, there were 9 cases with family history, and 8 were due to *de novo* mutations. Sixty neurofibromas were obtained, and the diagnosis was confirmed by histopathological examination.

Sample Processing

Neurofibromas were obtained after surgery. A sample from each tumor was sectioned, stained, and examined by an experienced dermatologist, and the remainder was

immersed in liquid nitrogen. A part of each tumor was separated and completely defrosted. Tumor tissue (nearly always yellowish and well circumscribed), surrounding tumor tissue, and skin were carefully separated (according to the different morphology of each of these parts) with a scalpel. Each tissue was carefully sliced prior to digestion (Wu et al. 1995). DNA was extracted from each of these components, as described by Wu et al. (1995). Moreover, DNA from peripheral blood lymphocytes of each patient was extracted by the "salting out" method (Miller et al. 1988).

Analysis of LOH

To investigate LOH, tumoral DNA and nontumoral DNA from different tissues of the same patient were compared. PCR was performed, amplifying several intragenic *NF1* loci (RFLP exon 5, IVS27AAAT2.1, IVS27AC28.4, IVS27AC33.1, and IVS38GT53.0) and four markers flanking the *NF1* gene (*D17S33*, *D17S57*, *D17S73*, and *D17S250*). Tumors that showed LOH for *D17S250* (the most telomeric marker) were further analyzed with markers *D17S787* (17q22), *D17S789* (17q24.3), and *D17S785* (17q25.1) (Dib et al. 1996, pp. A102–A107), in order to determine the extent of deletions (data not shown). The genetic distances between these telomeric markers are approximately as follows: *NF1*–10 cM–*D17S250*–17 cM–*D17S787*–15 cM–*D17S789*–16 cM–*D17S785*. Two markers (introns 1 and 6) within the *P53* gene were also analyzed. Six markers corresponded to microsatellite polymorphisms, and the remainder were RFLPs (fig. 1). The analysis of all these markers was performed as described in the Genome Database (GDB), and the genetic distances between markers within the *NF1* region have been revised by Lázaro et al. (1996) (*D17S33*, *D17S57*, and *D17S73*) and by Friedman et al. (1995) and Rogers et al. (1995) (*D17S250*) and are also shown in figure 1. PAGE was performed with PCR products. Microsatellites were separated in 5% denaturing gels, and RFLPs were separated, after previous digestion, in 10% nondenaturing gels. DNA bands were detected by silver staining. Some of these markers (IVS27AAAT2.1, IVS27AC28.4, IVS27AC33.1, IVS38GT53.0, and *D17S250*) were also analyzed by fluorescence by use of an automatic genetic analyzer (Perkin-Elmer ABI PRISM™ 310).

Confirmation of Deletions

When LOH for a given marker was found, the analysis was repeated several times, in order to confirm the deletion. When results were not clear, a second DNA extraction from the same frozen tumor was performed. If the fluorescent primers were available, the analysis was performed both by fluorescence by use of an automatic genetic analyzer and by PAGE and silver staining.

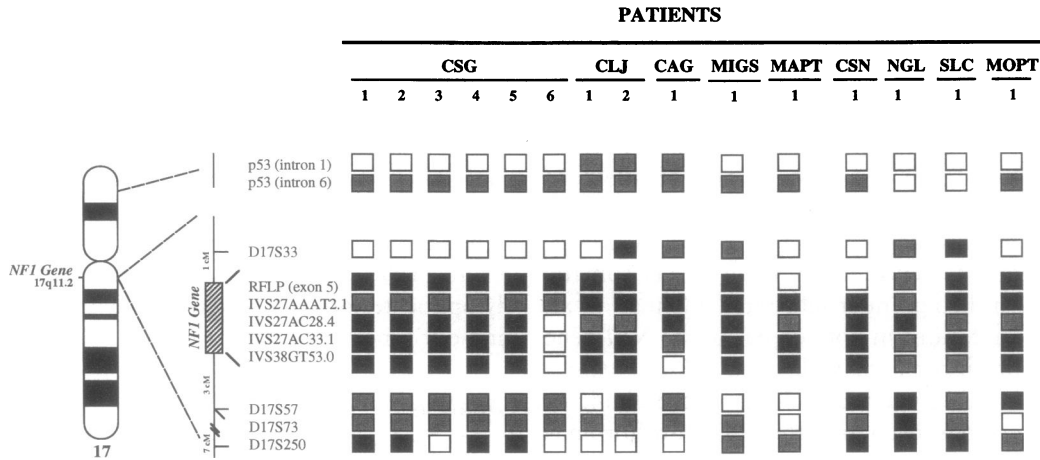


Figure 1 Ideogram of chromosome 17, showing DNA markers studied. Distance between markers located in the *NF1* region is given in centimorgans (cM). The nine patients showing deletions are indicated in uppercase letters (CSG, CLJ, CAG, MIGS, MAPT, CSN, NGL, SLC, and MOPT). The corresponding neurofibromas are indicated, by numbers, below them. Black squares denote LOH, gray-shaded squares denote uninformative loci, and white squares denote undeleted regions. Seven neurofibromas (CSG1, CSG2, CSG4, and CSG5 and CSN1, NGL1, and SLC1) lost the entire 17q arm (data not shown).

Results

A total of 60 peripheral neurofibromas were analyzed by use of 11 markers located on chromosome 17 (fig. 1); all the patients were informative for several of the markers used (two patients for nine loci, two patients for eight loci, five patients for seven loci, two patients for six loci, two patients for five loci, and four patients for four loci). LOH for one or more markers was detected in 15 neurofibromas from nine patients (figs. 1 and 2). Therefore, the percentage of somatic deletions in neurofibromas found in this study was 25% (corresponding to 53% of patients). LOH was not found in DNA from blood, skin, or surrounding normal tissue separated from neurofibromas. When DNA markers were visualized by PAGE, the intensity of the band showing LOH was variable among neurofibromas. This phenomenon was probably due to the different percentages of the cell type carrying the deletion in each neurofibroma. Table 1 depicts, for each patient with at least one neurofibroma with LOH, the number of informative loci, the total number of neurofibromas resected versus the number of neurofibromas with LOH, and the body location of both.

Among the 15 neurofibromas with LOH, 10 tumors (CSG1–CSG5, CLJ1 and CLJ2, MIGS1, SLC1, and MOPT1) seem to lose the whole *NF1* gene (or at least exons 5–38). In seven neurofibromas (CSG1, CSG2, CSG4, and CSG5 and CSN1, NGL1, and SLC1) LOH for *D17S250* (the most telomeric marker used in the present study) was detected. This marker is situated 10 cM distal to the *NF1* gene (Friedman et al. 1995; Rogers et al. 1995). In order to determine the extend of these deletions, three additional microsatellite markers span-

ning the whole long arm of chromosome 17 were analyzed (see Patients, Material, and Methods). LOH was observed for these three markers in the seven tumors analyzed, indicating the loss of the 17q arm telomeric to *NF1*. Moreover, for two neurofibromas (CLJ2 and SLC1), the deletion detected included several kilobases upstream of the *NF1* gene, indicated by loss of *D17S33*, which is situated 1 cM proximal to the *NF1* gene (revised in Lázaro et al. 1996). None of the neurofibromas that were informative for markers within the *P53* gene exhibited deletions in this region (fig. 1).

Six of the patients showing deletions in the neurofibromas were familial cases and belonged to four independent kindred (fig. 3). In these families it was possible to assess the chromosome 17 segregating with the disease, and it was found that the deletions in the neurofibromas occurred in the non-*NF1*-associated chromosome (fig. 3).

Discussion

Data have emerged indicating that inactivation of both copies of a tumor-suppressor gene could be involved in the development of benign tumors (Ichii et al. 1992; Colman et al. 1995; De Vitis et al. 1996; Sawada et al. 1996). Our study strongly supports the idea that inactivation of both copies of the *NF1* gene also occurs in benign neurofibromas. This finding already had been presented by Colman et al. (1995), in a similar interesting study. They found somatic deletions in several neurofibromas from sporadic *NF1* patients. Because of both the impossibility of performing linkage studies in these patients and the failure to find their constitutional mutations, the double inactivation of the *NF1* gene in neuro-

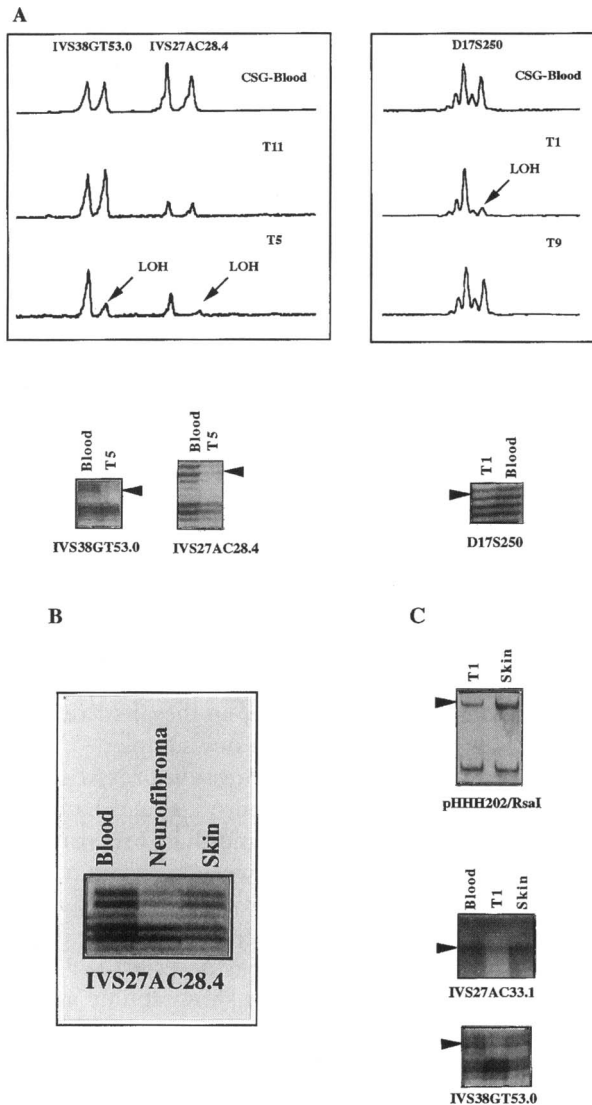


Figure 2 Allelic losses in several neurofibromas, for the markers indicated. *A, Top*, Fluorescence analysis of markers IVS38GT53.0, IVS27AC28.4, and D17S250 of patient CSG. In the left-hand panel, LOH is shown (arrow) in tumor T5 (IVS38GT53.0 and IVS27AC28.4), and T11 is shown as a tumor without LOH. In the right-hand panel, LOH is shown in tumor T1 (D17S250), and T9 is shown as a tumor without LOH. *Bottom*, Silver-staining detection of the same markers. Only blood and tumors with LOH (arrowhead) are shown. *B*, Silver-staining detection of marker IVS27AC28.4 from blood, skin, and neurofibroma of patient CAG. A loss of the larger allele is detected only in the DNA belonging to the neurofibroma. *C, Top*, Silver-staining detection of marker pHHH202/RsaI (D17S33) in patient SLC. *Bottom*, Silver-staining detection of markers IVS27AC33.1 and IVS38GT53.0 in patient MOPT. LOH is shown (arrowheads).

fibromas could not be completely demonstrated. Nevertheless, Sawada et al. (1996) have recently described the double inactivation of the *NF1* gene in one neurofibroma from an *NF1* individual who has a large germ-line *NF1*-gene deletion.

The molecular analysis of the 60 neurofibromas presented here revealed in 25% of them the existence of LOH for the region containing the *NF1* gene. This figure is significantly different from the percentage of germinal large deletions described thus far in the *NF1* gene (~15% of reported mutations in the NNFF Consortium [revised in Shen et al. 1996]). This difference could reflect a distinct mutation mechanism in germ-line versus somatic cells, the latter being more prone to deletion. Regarding the 75% of neurofibromas that did not show LOH, we believe that most of them will harbor, within the *NF1* gene, another somatic mutation, which is not detectable by the approach used in this study. Thus, we think that the majority of neurofibromas from *NF1* patients carry two mutated *NF1* copies. In support of this idea, we found that, in the neurofibromas of patients from the familial cases, the deletions occurred in the allele that is not transmitted with the disease (fig. 3), indicating that both copies of the *NF1* gene were inactivated in these tumors. These results suggest lack of neurofibromin function in these neurofibromas. When this statement is considered together with the fact that cutaneous neurofibromas essentially never transform to malignancy, it is reasonable to think that the absence of neurofibromin can deregulate cell proliferation in this tissue, albeit not to the extent of malignancy.

The size of deletions found varies, but, at least in nine neurofibromas, it seems to be large, probably removing the whole gene. Furthermore, in 7 of the 15 *NF1*-deleted tumors, LOH affects the whole 17q arm telomeric to *NF1*. This fact is in agreement with the results presented by Colman et al. (1995), in which four of eight neurofibromas with LOH had lost the telomeric markers analyzed. It is interesting to mention that, in most of cases, deletions seems to be restricted to the 5' region of the *NF1* and not to the 3' region. So, LOH appears not to involve the centromeric region but can affect the remaining part of the 17q arm. The region encompassing exons 28–38 is deleted in most of the tumors, at least in 11 of them. Three possible reasons could explain this finding. One reason is that this region has been saturated with polymorphic markers, making the detection of deletions easier. Another reason could be the existence of some specific sequences in these region, which facilitate the rearrangements found, already suggested by Upadhyaya et al. (1994). And, last, it could be that in this part of the gene a domain exists that plays an important role in neurofibroma development. It is worth noting that intron 27b is deleted in 13 of 15 tumors with *NF1* deletions. This intron spans >40 kb and has three genes (*EVI2A*, *EVI2B*, and *OMGP*) embedded within it (Viskochil et al. 1990). There is the possibility that these genes could be related to either the development of neurofibromas or the generation of these large deletions.

Other studies have failed to find LOH for the *NF1*

Table 1**Number of Tumors, Body Location, and Genetic Information for Patients with at Least One Neurofibroma Showing LOH**

Patient	No. of Informative Loci	No. of Neurofibromas with LOH/No. of Neurofibromas Studied	Neurofibroma Location(s)	Location of Neurofibromas with LOH
CSG	7	6/16	Trunk	Trunk
CLJ	7	2/9	Trunk, back, mammary aureole	Back
CAG	4	1/6	Back, right inferior extremity	Back
MIGS	7	1/1	Back	Back
MAPT	8	1/3	Left thigh, mammary aureole, trunk	Mammary aureole
CSN	9	1/1	Inferior part of the leg	Inferior part of the leg
NGL	6	1/1	Trunk	Trunk
SLC	7	1/3	Left and right forearm	Left forearm
MOPT	8	1/3	Right lumbar, left arm, left thigh	Left arm

gene in neurofibromas (Menon et al. 1990; Glover et al. 1991; Skuse et al. 1991; Lothe et al. 1995), but several reasons could explain this. Because neurofibromas are composed of an admixture of cell types, a complete absence of the deleted allele is not expected, and its detection will depend on the percentage of the specific cell type having the deletion, in comparison with the remaining cells that constitute the neurofibroma. Moreover, if either infiltration of normal tissues or incomplete removal during dissection occurs, the presence of a deletion will be greatly obscured. In addition, our findings suggest that ~25% of the neurofibromas have large deletions, making it necessary to screen a large number of neurofibromas with several informative markers, in order to identify somatic deletions.

Instability in the analysis of microsatellite markers in neurofibromas has recently been reported (Ottini et al. 1995). Nevertheless, by analyzing six microsatellite markers in our sample consisting of 60 neurofibromas, we have not been able to detect any alteration. Furthermore, the usefulness of these microsatellite markers in the detection of instability was tested. Pairs of normal and tumoral colorectal tissue in which instability was known (provided by Dr. M. A. Peinado) were screened by use of these markers. Instability was detected with all of them (data not shown), indicating their reliability for this purpose, even though (1) these are not the same markers as were used by Ottini et al. (1995) and (2) there could be sensitivity differences between them. Thus, this molecular mechanism seems not to be involved in the origin of the neurofibromas screened in the present study. One possible explanation for this difference could be geographical genetic variances between the population studied by Ottini et al. (1995) and our population. Another possibility is that our samples were from fresh tumors, whereas the tumors used by Ottini et al. (1995) were extracted from paraffin-embedded sections. Inconsistencies in the analysis of microsatellite markers could

be easily produced, because of the low DNA amount usually obtained from paraffin-embedded samples, which could facilitate the generation of artifacts that can be confused with instability (M. A. Peinado, personal communication). We believe that, if 50% of neurofibromas had genomic instability, as they had reported, we would be able to detect it in our samples.

Studies on the variation in expression of *NF1* showed that the number of cutaneous neurofibromas had a high correlation between MZ twins and that this correlation decreased when the distance between relatives increased (Easton et al. 1993). This finding suggested both a minor role of the *NF1* type mutation and the existence of other specific trait-modifying genes (Easton et al. 1993). The finding that some neurofibromas show double inactivation of the *NF1* gene allows us to consider these tumors as partial knockouts for this gene. One remarkable finding of this study is that 66.6% of the familial cases of *NF1* show deletions of the *NF1* gene in the benign neurofibromas, always occurring in the *NF1* allele that does not segregate with the disease. This study strongly supports the hypothesis that most neurofibromas have both copies of the *NF1* gene inactivated. Therefore, they could be a good model for study of the differential expression of other genes involved in the formation and proliferation of neurofibromas, providing some insights into the mechanisms and functions of neurofibromin both in the disease and in the neoplastic processes.

The two-hit model for neoplasia (Knudson 1971) has been demonstrated in other benign tumors, such as adenomas (Ichii et al. 1992), meningiomas (De Vitis et al. 1996), and others. It is reasonable to assume that the number of genes involved in the generation of benign neoplasias would be less than that in malignancies, as is known for the development of colorectal tumor (Fearon and Vogelstein 1990). Thus, the study of benign tumors should facilitate the search for molecular mechanisms causing neoplasias. A great number of tumors

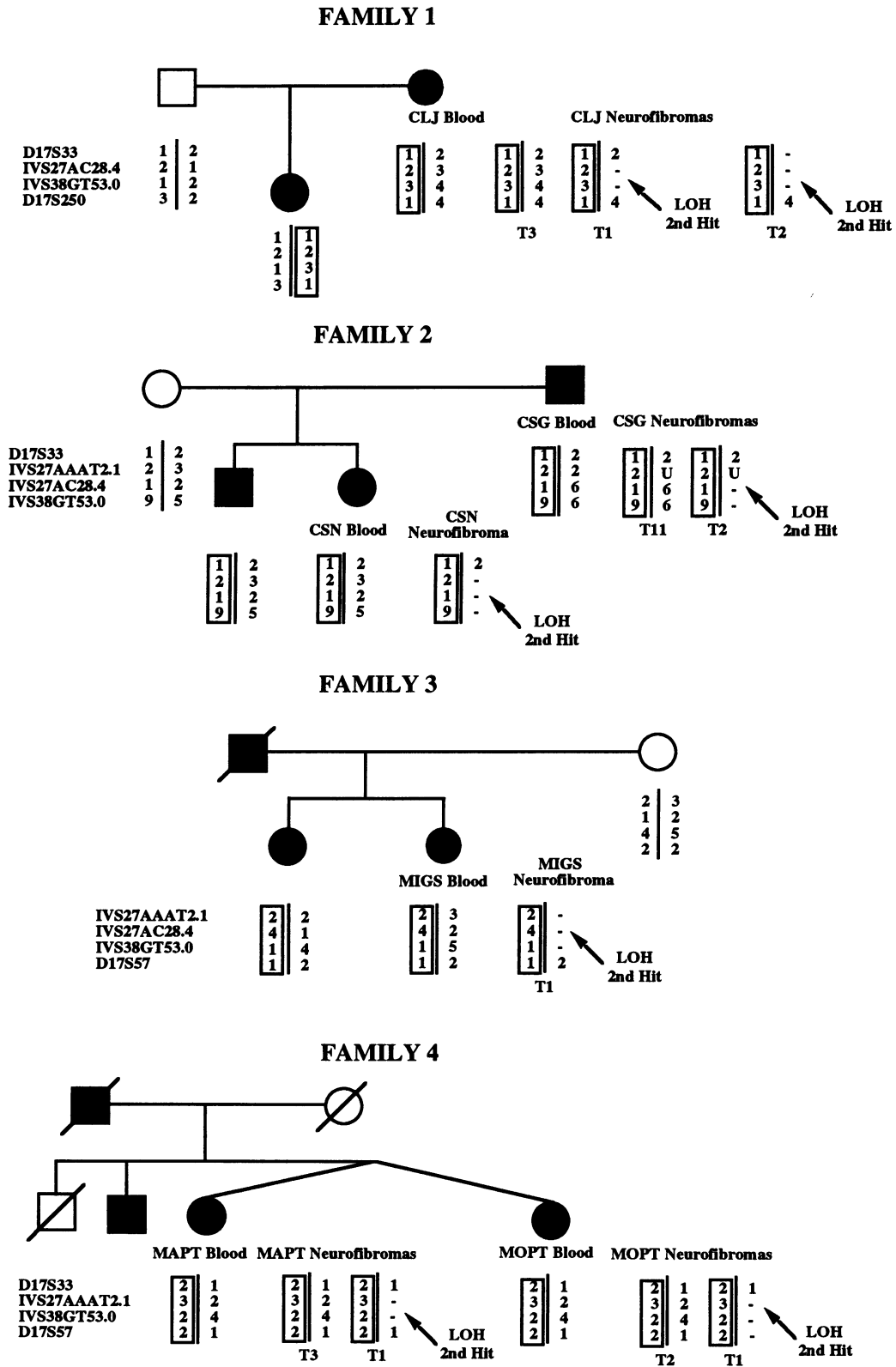


Figure 3 Pedigrees of the six familial *NF1* cases in which LOH was found in at least one neurofibroma. Haplotypes of markers within the *NF1* region are indicated below the symbol in the pedigree. Uninformative markers are denoted by “U.” The haplotype associated with the disease is boxed. The haplotypes of some neurofibromas of each patient are also shown. In all of the neurofibromas in which LOH was detected (arrows), the deletion was found in the non-*NF1*-associated chromosome.

arise from neural crest derivatives. The comparative study of such tumors could be useful to elucidate the genes and mechanisms controlling the development of these tissue-specific tumors.

Controversial data have emerged related to the contribution of the different cell types in the genesis of neurofibromas (Waggener 1966; Kamata 1978; Stefansson et al. 1982; Rosenbaum et al. 1995). It would be of great interest to find which cell type carries the genetic alterations causing neurofibroma formation. The fact that several neurofibromas show LOH for the *NF1* gene could be useful for this purpose. Once the different cell types are isolated from neurofibromas in which LOH has been detected, deletions can be used as internal markers to recognize which is the cell type harboring the LOH. It will also be interesting to study neurofibromas from patients without all the diagnostic criteria of *NF1*, to discover whether they follow the same molecular mechanism. In Gorlin syndrome, for instance, it has been found that LOH is significantly less common in sporadic cysts than in hereditary cysts (Levanat et al. 1996). Finally, further studies should be performed to elucidate the spectrum of *NF1* mutations present in neurofibromas, in order to assess whether the double inactivation of this gene is a general rule for the development of these tumors.

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