Constitutional and mosaic large NF1 gene deletions in neurofibromatosis type 1

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Abstract

A set of neurofibromatosis type 1 (NF1) patients was screened for large NF1 gene deletions by comparing patient and parent genotypes at 10 intragenic polymorphic loci. Of 67 patient/parent sets (47 new mutation patients and 20 familial cases), five (7.5%) showed loss of heterozygosity (LOH), indicative of NF1 gene deletion. These five patients did not have severe NF1 manifestations, mental retardation, or dysmorphic features, in contrast to previous reports of large NF1 deletions. All five deletions were de novo and occurred on the maternal chromosome. However, two patients showed partial LOH, consistent with somatic mosaicism for the deletion, suggesting that mosaicism may be more frequent in NF1 than previously recognised (and may have bearing on clinical severity). We suggest that large NF1 deletions (1) are not always associated with unusual clinical features, (2) tend to occur more frequently on maternal alleles, and (3) are an important mechanism for constitutional and somatic mutations in NF1 patients.

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Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition occurring in approximately 1 in 3000 people. NF1 is characterised by café au lait spots, neurofibromas, axillary freckling, and iris Lisch nodules. Subjects with this condition are also at an increased risk of malignancy.¹ The NF1 gene is large, spanning 350 kb of genomic DNA, and contains 59 exons.² The gene encodes a cytoplasmic protein called neurofibromin, believed to have a tumour suppressor role. The search for NF1 mutations has proven to be particularly challenging, owing to the large gene size, its high mutation rate, and the paucity of recurrent mutations. Over 100 mutations have been published and in only a few cases has the same mutation been identified in unrelated patients.³ The majority of published mutations are predicted to result in translation of truncated neurofibromin, and therefore presumably absence of a functional protein product. However, the protein truncation test recently developed for use in NF1 has been successful in identification of about two-thirds of mutations,⁴ leaving one-third of mutations undetected. Since large gene dele-

tions would escape detection by the protein truncation test as well as most other mutation identification methods previously used in NF1, we sought to identify large gene deletions in NF1 using loss of heterozygosity (LOH) analysis. We genotyped 67 typical patients and their parents at 10 intragenic NF1 loci (fig 1) to look for failure to inherit one parental allele (LOH), indicative of a deletion.

Patients, material, and methods

PATIENTS

Patients met accepted NF1 diagnostic criteria.²⁰ Forty-seven patients were new mutations; DNA was available from both parents in 35 of these patients, and from one parent in the remaining 12 cases (11 mothers, one father). Twenty affected parent-child pairs were also studied. This study was begun before any mutations had been found in this group, and patients were not excluded if a mutation was subsequently identified using other means.

PCR ANALYSES

Blood was obtained under IRB consent from patients and their parents, and DNA was extracted according to published methods.²¹ One patient's B cells were also transformed into a lymphoblastoid cell line with Epstein Barr virus.²² Ten markers (noted in fig 1) were genotyped for all patients; if the patient was apparently homozygous at a marker, the parents were also genotyped at that marker. When loss of a parental allele was identified, flanking markers were also studied. The markers were genotyped as described in the references, with the exception of a modified, non-radioactive approach for the *Alu* tetranucleotide polymorphism.¹¹ This PCR product was digested with *Bfa*I, which cuts the product into a constant 268 bp fragment and a polymorphic fragment varying in size from 127 to 139 bp. These fragments were resolved on 10% native polyacrylamide gels, stained with ethidium bromide, and visualised under UV illumination.

SOUTHERN BLOT ANALYSES

Samples in which PCR based analyses showed loss of a parental allele were used for Southern blot analyses to confirm deletions and to assist in identifying deletion endpoints. Southern blots were performed as described previously²³ using *Pst*I digested DNA from the five putative deletion patients and Hybond N nylon membranes (Amersham). The probes were labelled with α^{32} P-dCTP using a random prime system (Amersham Multiprime). Probes included

Figure 1 Results of LOH studies at NF1 and flanking polymorphisms in five deletion patients, with markers studied in all 67 patients marked with an asterisk. Black circles denote deleted loci, hatched circles denote uninformative loci, and white circles denote non-deleted loci. Reference numbers in square brackets. Estimated locations of flanking markers relative to NF1 are as follows: HHH202, 0.6 cM upstream; VAW212, 0.1 cM upstream; UT172, 1.5 Mb upstream; p11-1F10, 70 kb downstream; EW206/EW207, 4.8 cM downstream; Mfd15, 7-12 cM downstream. It was not possible to genotype the mother of 3514 for VAW212 and 1F10.

p11-1F10 (D17S135),¹⁷ VAW212 (D17S117),⁶ and 17L1A.²⁴ While VAW212 and p11-1F10 detect polymorphisms, allowing LOH analysis to be performed, interpretation of the probe 17L1A required comparison to a control marker, a 0.6 kb *NotI/EcoRI* fragment of SNRPN from chromosome 15,²⁵ to normalise lane loading. Densitometry, used to analyse 17L1A (5' end of NF1) and the chromosome 15 control, used the SigmaGel (Jandel Scientific) program.

Results

NF1 deletions were identified in five new mutation patients (LC, 3514, UF153, UF113, and UF161). Clinical features in these patients are summarised in table 1. Results of LOH at NF1 and flanking polymorphic markers are shown in fig 1. Two patients had partial LOH (UF161 and UF113), indicative of somatic mosaicism for the NF1 deletion. Patient UF161 was previously reported as the first proven case of somatic mosaicism in NF1²⁶; the majority of her leucocytes have the deletion. In contrast, the lymphocytes of UF113 showed

the deletion in a smaller percentage of cells, such that the maternal allele is always visible but reduced in intensity. This held true in DNA from both a lymphoblastoid cell line and from fresh blood leucocytes. Fig 2 shows LOH in patients with constitutional deletions and partial LOH in UF113 at two markers.

Southern blot densitometry results suggested that the deletions in these five patients included the 5' end of NF1 (17L1A, a probe at the 5' end of intron 1) (fig 3). The visually evident decreased intensity of hybridisation at this marker was confirmed by densitometry.

Discussion

Using LOH analysis, we have identified five patients with NF1 deletions (7.5% of patients studied and 10.6% of the new mutation cases). The precise endpoints of these deletions have yet to be determined; however, the deletions appear to involve most of the NF1 gene. While UF153 was informative at only one locus, located in intron 27b, she was homozygous at all other NF1 markers and showed deletion by densitometry at 17L1A, located at the 5' end of intron 1. Since our primary detection strategy depends on marker informativeness, this percentage should be viewed as a conservative estimate of large NF1 deletions. These deletions would not have been identified by the protein truncation test or most other PCR based mutation detection methods, and thus LOH analysis is an important complementary mutation detection technique.

Previous studies testing for large NF1 gene deletions have used methods such as fluorescence in situ hybridisation (FISH),²⁷⁻³⁰ Southern based and somatic cell hybrid analyses,³¹ pulsed field gel electrophoresis (PFGE),³²⁻³³ and LOH at a smaller number of intragenic markers.³⁴⁻⁴⁰ LOH analysis has the advantage of being able to detect deletions encompassing even a single marker⁴¹ while using relatively small amounts of DNA. The disadvantages of this technique include its dependence on the informativeness of intragenic markers and the need for parental DNA. However, the high degree of heterozygosity for the microsatellite markers provides the opportunity to screen patients for these markers before obtaining parental DNA or performing dosage based analyses in patients with apparent homozygosity at all or most markers studied.

Other recent studies have found similar proportions (4-7%) of NF1 patients with large NF1 gene deletions.³⁷⁻⁴⁰ However, the patients with large NF1 deletions in our study do not appear to be more severely affected than

Table 1 Summary of clinical features in patients with NF1 deletions

	LC	3514	UF153	UF113	UF161
Age at examination (y)	20	11	9	10	31
Café au lait spots	Multiple	Multiple	Multiple	Multiple	Multiple
Cutaneous neurofibromas	Multiple	Several	None	Two	Multiple
Axillary freckling	Yes	Yes	Yes	Yes	Yes
Macrocephaly	Unknown	No	Yes	No	Yes
Mental retardation/learning disabilities	Normal to low normal IQ	"Learning disabilities"	Normal	Normal	Normal
Other features	Scoliosis	Right fibular pseudarthrosis	Mild scoliosis, hypothalamic glioma causing precocious puberty	Mild hemihypertrophy, bilateral sensorineural hearing loss	—

Figure 3 Southern blot results for control and deletion patients using 17L1A (at 5' end of the gene) and chromosome 15 control probe (SNRPN), showing deletion extending through 17L1A in all cases. Results of densitometric analysis are listed below each lane; the ratio in the control lane was assigned as 1.00, with ratios in the other lanes being compared to this control. The low level contribution of non-deleted cells in UF161 was indistinguishable from a non-mosaic deletion patient using this analysis.

patients with frameshift or point mutations, nor do they have other features (dysmorphism or mental retardation) suggestive of a contiguous gene syndrome,⁴² as has been seen in other NF1 patients with large deletions involving flanking DNA.^{27 29 31 39 43} Since the deletion endpoints have not been delineated, it is unclear if this difference is the result of a smaller deletion size or other modifying genes. In all cases, though, it is clear that most of the NF1 gene is lost. Previous studies suggest that a large proportion of cases with severe NF1 manifestations, dysmorphic features, and mental retardation/learning disabilities have large NF1 deletions.^{27 31} Our patients were not preselected for severity of NF1 features, but rather were chosen based on availability of parental DNA, and, in retrospect, all of the patients have NF1 features within the typical range of expression. While large gene deletions may be more frequent among dysmorphic and severe NF1 patients, this mutation mechanism also appears to be important among typical NF1 cases.

All five large gene deletions described here occurred on the maternally derived chromosome. Given the small numbers, this finding could be the result of chance, especially since samples from parent-child pairs more often included a mother than a father. However, other reports also suggest that large deletions

are primarily maternally derived.^{35 37 38 40} In a recent study, inheritance of intragenic and flanking markers was examined in a series of new mutation NF1 cases; two of 11 (18%) non-deletion cases were shown to be of maternal origin, while 16 of 21 (76%) deletion cases were maternally derived.³⁷ This predominance of maternal derivation of NF1 gene deletions is particularly significant when compared to previous studies which suggested that about 90% of new NF1 mutations are paternal in origin.^{44 45} These results suggest that in NF1, as in other genetic conditions (for example, haemophilia and Duchenne muscular dystrophy), the parental origin of new mutations may vary with mutation type, with deletions occurring more frequently in oogenesis.⁴⁶⁻⁴⁹ The significance of the maternal origin of germline and somatic mutations is unknown.

Two of five patients with NF1 deletions were identified as having somatic mosaicism. In one patient (UF161), the majority of her leucocytes showed the deletion (her results were indistinguishable from other constitutional deletion patients based on the Southern densitometry, and represented more than 95% of cells, based on subcloning of informative PCR products²⁶). In the other patient (UF113), a smaller proportion of leucocytes appear to show the deletion, as evidenced by Southern results. However, estimation of degree of mosaicism in leucocytes is not of particular significance since different tissues have been shown to have varying degrees of mosaicism.^{26 30}

The finding of mosaicism in two of five patients with NF1 deletions suggests that this phenomenon may be more frequent in NF1 than previously recognised. Any number of the remaining 62 patients could be mosaic as well, for mutations not detected by this method. In addition to these two cases, one of which we previously reported,²⁶ there have been two other reports of somatic mosaicism for large NF1 deletions.^{30 38} The finding that somatic mosaicism is not a rare event in NF1 is of clinical significance because: (1) an increased recurrence risk can be excluded in the unaffected parents of these patients; (2) the risk of transmission of NF1 from a parent with somatic mosaicism is unknown, but is probably less than 50% (this risk could be approximated in a study of sperm from mosaic males); (3)

Figure 2 Results of LOH studies at microsatellites IVS27AC28.4 in intron 27b (A, B) and (GATN)_n in intron 26 (C, D). (A) and (C) show complete LOH, indicative of deletion of the maternally derived allele. (B) and (D) show partial LOH, indicative of somatic mosaicism in UF113.

mosaicism may explain why some patients have limited NF1 features without meeting diagnostic criteria; and (4) the cellular distribution of the deletion may be such that blood (virtually the only tissue tested in diagnostic laboratories) may not show the mutation (especially with some techniques), which could lead to underdiagnosis. Finally, the only NF1 mosaic mutations detected so far have been large deletions; it is possible that mosaicism for other more subtle mutations occurs as well in NF1 patients, and thus this phenomenon must be routinely considered by geneticists.

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