Characterisation of germline mutations in the neurofibromatosis type 1 (NF1) gene

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Abstract

Neurofibromatosis type 1 is one of the most common inherited disorders with an incidence of 1 in 3000. The search for NF1 mutations has been hampered by the overall size of the gene, the large number of exons, and the high mutation rate. To date, fewer than 90 mutations have been reported to the NF1 mutation analysis consortium and the details on 76 mutations have been published.

We have identified five new mutations using single strand conformation polymorphism (SSCP) and heteroduplex analysis (HA) and three intragenic deletions with the microsatellite markers. Of the five new mutations, two were in exon 27a, two in exon 45, and one in exon 49 and these include 4630delA, 4572delC, R7846X, T7828A, and one in the 3' untranslated region (3' UTR). The two nucleotide alterations in exon 27a and the one in exon 45 are predicted to produce a truncated protein.

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Neurofibromatosis type 1 (NF1) is one of the most common inherited disorders with a birth frequency of approximately 1 in 3000. The disease is characterised by café au lait spots, neurofibromas, and Lisch nodules (hamartomas of the iris). NF1 has many complications which include complex neurocognitive difficulties, plexiform neurofibromas, and an increased risk of specific types of malignancy.¹

The NF1 gene is more than 300 kb in size, consists of 59 exons, and has been mapped to 17q11.2.²⁻⁵ The NF1 transcript encodes a protein, neurofibromin, which is related to a number of GTPase activating proteins (GAP) and is involved in the negative control of ras mediated signal transduction.⁶ Neurofibromin also appears to be associated with cytoplasmic microtubules. This association between ras mediated signal transduction and the cytoskeleton suggests that neurofibromin may play multiple roles in the regulation of cell division.⁷

The NF1 mutation rate, approximately 1×10^{-4} /gamete/generation,⁸ is the highest described for any human disorder, and is some 100 fold higher than that usually found at a single locus, with the result that almost half of all cases of NF1 represent new mutations. This high mutation rate could either be because of the large size of the NF1 gene or the presence of highly mutable sequences within the gene.

The identification and characterisation of disease specific mutations in the NF1 gene should enable one to detect such mutational "hot spots" for mutations and to study correlation between the genotype and phenotype.

The search for NF1 mutations has been hampered by the overall size of the gene and the large number of exons. To date fewer than 90 mutations have been reported to the NF1 mutation analysis consortium,⁹ and details of only 76 of these mutations have been published.¹⁰ We have previously reported the identification of 14 mutations within the NF1 gene.¹¹⁻¹⁴ We have since expanded our study to screen for NF1 gene mutations within exons 27a, 45, and 49 and here describe five new mutations identified by single strand conformation polymorphism (SSCP) analysis and heteroduplex analysis (HA). Three intragenic deletions have also been detected by analysing our families with intron specific microsatellite markers.

Materials and methods

We have studied a panel of 300 unrelated NF1 patients. The clinical details were available from the majority of the cases. Approximately 100 subjects represent sporadic cases, of which DNA from the parents was available from 11. Several patients also have unusual clinical features associated with their disease.

DNA was extracted from peripheral blood leucocytes. DNA digestion and Southern based hybridisation and analysis was carried out as previously reported.¹² DNA samples were amplified by PCR and the amplified products were then subjected to both SSCP analysis and HA.

Exons 27a and 45 were analysed by SSCP and the heteroduplex analysis was used for exon 49. Genomic DNA was amplified using (δ -³³P) ATP end labelled primers. The radio-labelled PCR products were resolved on 5% non-denaturing acrylamide gel containing 5% glycerol. PCR was routinely performed in a 6.25 μ l reaction volume, containing 5 ng genomic DNA, 20 pmol of each primer, 200 μ mol/l dNTPs, 50 mmol/l KCl, 10 mmol/l Tris (pH 8.3), 1.0-1.9 mmol/l MgCl₂, 0.01% gelatin, and 1 unit of *Taq* polymerase (Amersham). The annealing temperatures used for the amplification of exons 27a, 45, and 49 were 52°C, 54°C, and 53°C respectively, and the exon specific primer sequences used have been previously reported.⁹ DNA samples from 70 non-NF1 patients were also analysed as a control for each exon studied.

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Figure 1 SSCP variant bands identified in NF1 patients; 5 ng genomic DNA was amplified using end labelled primers ($\delta^{32}\text{P}$) dATP. The radiolabelled PCR products were resolved on a 5% non-denaturing polyacrylamide gel with 5% glycerol. (A) Lane 3 contains a variant band observed in exon 45 of patient NF41. (B) Lane 3 contains a variant band in exon 45 in patient NF9. (C) Lanes 2 and 6 show variant bands in exon 27a in patients NF173 and NF59.

For heteroduplex analysis, 150 ng of amplified DNA (2 μl) either from an NF1 patient and a control, or from two different NF1

patients, was added to 8 μl water and 0.6 μl EDTA (0.1 mol/l). The mixture was incubated at 94°C for three minutes and then slowly cooled from 42°C to 37°C over a period of one hour to allow for the formation of a heteroduplex. A 0.5 μl quantity of loading buffer (Hoefer) was added to each sample. Samples were then electrophoresed on a 0.8 $\times$ MDE gel (40 $\times$ 30 cm) in 0.5 TBE buffer for 18 hours at 8 W.¹⁵ The DNA in the gel was subsequently visualised by silver staining.¹⁶

Eleven sporadic NF1 cases for which DNA samples were also available from both normal parents were screened with two microsatellite markers located in the intronic regions of the NF1 gene (the AAAT polymorphism in intron 27 and the 1VS 38 GT 53.0 in intron 38).^{17,18} Paternity in each family was assessed by screening with a panel of microsatellite markers located on several different chromosomes. To define the extent of the deletion identified, the DNA samples from the families with intragenic deletions were also studied for exon 5 and intron 41 polymorphisms.^{19,20}

Direct nucleotide sequencing was carried out on purified PCR product (Gene clean), using the dideoxy chain termination method (Sequenase version 2.0 USB). Sequenase reactions were analysed on a 6% non-denaturing sequence gel, vacuum dried onto filter paper, and then exposed for 24 to 48 hours to x ray film (Fuji) at room temperature.

Results

From a panel of 300 patients studied, five new mutations were identified and were fully characterised by direct sequencing. Of the five nucleotide alterations, four were identified by SSCP (fig 1). In addition, three de novo intragenic deletions were detected in three of the 11 sporadic cases. The details of each of these mutations are summarised in the table.

In patient NF59, the deletion of an adenine (A), at nucleotide 4630 in exon 27a, generated a shift in the reading frame, resulting in the alteration of eight amino acids before the introduction of an inappropriate stop codon at nucleotide 4655 (fig 2A,B). This mutation may result in the generation of a shortened non-functional protein of 1551 amino acids in com-

Details of the characterised mutations

Exon	No of patients or families screened	Analytical method	Patient ID	Age	Alteration at nucleotide	Amino acid change	Effect on protein	Type of NF1*	Clinical phenotype
27a	300	SSCP	NF59		A deleted at 4630		8 altered amino acids before termination at 4655	F	Cutaneous involvement, no complications
27a	300	SSCP	NF173	29	C deleted at 4572		27 altered amino acids before termination at 4655	S	Cutaneous involvement, plexiform neurofibroma
45	200	SSCP	NF9	80	C $\rightarrow$ T 7846	Arg to stop	Truncated protein	S	Cutaneous involvement, plexiform neurofibroma
45	200	SSCP	NF41	53	A $\rightarrow$ G 7828	Thr to Ala		S	Cutaneous involvement
49	284	HA	NF130	40	A $\rightarrow$ G 11 715	3' UTR		S	Mild cutaneous involvement
27-38	11	Microsatellite	NF931	2	Deleted intron 27 to intron 41		Truncated protein	S	Noonan-like features
38	11	Microsatellite	NF754	14	Deleted intron 38 to intron 41		Truncated protein	S	Learning problems
38	11	Microsatellite	NF637	11	Deleted intron 38		Truncated protein	S	Noonan-like features

* S = sporadic, F = familial.

Figure 2 (A) Sequence analysis on patient NF59. The deletion of adenine at nucleotide 4630 in exon 27a results in the alteration of the reading frame. (B) Adenine deletion results in eight altered amino acids different from the normal protein of the gene, which terminates prematurely at 4655.

Figure 3 (A) DNA sequencing of patient NF9 in exon 45. A transition from cytosine to thymine at position 7846 results in a stop codon. (B) Sequence analysis of patient NF41 in exon 45. A transition from adenine to guanine at position 7828 results in the substitution of threonine to alanine.

parison to the 2818 amino acids of the normal protein. Unfortunately, although detailed clinical information is not available, this patient appears to have classical NF1.

In patient NF173, a deletion involving a cytosine (C) at 4572, in exon 27a, also produces a change in the reading frame resulting in the alteration of 27 amino acids before the new

stop codon at nucleotide 4655. This nucleotide change also abolishes a restriction site for the enzyme *Bsa*II. This restriction site was present in DNA from her unaffected parents, indicating that the change in the patient is probably disease specific. This patient has typical cutaneous involvement and, in addition, macrocephaly, mild learning difficulties, and plexiform neurofibroma.

An alteration involving a base pair was identified within exon 45 in patient NF9; this involves a transition from C→T at position 7846 (fig 3A). This change occurs at a CpG dinucleotide and results in the conversion of an arginine to a stop at codon 2612. This woman has typical major defining features with a large plexiform neurofibroma and she represents a new mutation.

In patient NF41, a transition involving a change from A→G at nucleotide position 7828, which produces an amino acid substitution from threonine to alanine, was found (fig 3B). This alteration was not detected in any of the 70 normal controls or in 199 other NF1 patients. This mutational change also creates a new restriction site for the enzyme *Acc*I within a normally generated 269 bp fragment, which as a result is cleaved into two fragments of 117 bp and 152 bp, and the presence of this restriction site segregates with the disease. This is a non-conservative change and results in the substitution of a non-polar for a polar amino acid.

Only one new mutational change was identified by heteroduplex analysis on MDE gels. This was an A→G transition at nucleotide 11715 in exon 49, within the 3' UTR. This patient, NF130, has mild cutaneous involvement and no complications. She is the first case in her family. She has had three affected children from separate marriages and two of these children have NF1 and learning difficulties, but the samples from these were not available for analysis.

Eleven sporadic NF1 patients, where DNA was available from normal parents, were screened with two new microsatellite markers from within introns 27 and 38. In three of these families, the affected subjects were found to be hemizygous at these CA repeats owing to the presence of interstitial deletions involving intron 27 to intron 38 of the NF1 gene in one case and only intron 38 in the other two NF1 patients (fig 4). In all three families, the origin of new mutation was in the maternal germlines. To define the extent of these three deletions, the families were further analysed by screening with the exon 5 polymorphism.¹⁹ Unfortunately all the affected subjects were homozygous for this polymorphism; however, with the intron 41 polymorphism,²⁰ the NF1 patient from two families had not inherited the maternal allele. The precise extent of these three deletions remains to be determined.

Discussion

The detection of disease specific mutation in sporadic cases of neurofibromatosis type 1 (NF1) is crucial for the successful genetic coun-

Figure 4 Hemizygosity for a CA repeat IVS 38 GT 53.0 (intron 38) in three new mutation NF1 families. Panel A shows lanes 1–3 containing DNA from father, affected child, and mother. The child has not inherited a maternal allele. A similar pattern of non-maternal inheritance is observed in panels B and C.

selling and prenatal diagnosis of such families. The limited number of mutations so far reported in the NF1 gene include both large and small DNA rearrangements as well as the whole spectrum of point mutations, including splice site, missense, and nonsense mutations.¹⁰

We have identified five new mutations and three intragenic deletions (table). The two nucleotide alterations identified in exon 27a, as well as the one in exon 45, are all predicted to produce a truncated protein and are, therefore, expected to be disease causing mutations. The majority of NF1 mutations described to date have involved nucleotide alterations that would be predicted to result in a loss of neurofibromin production from the mutant allele. The protein truncation test (PTT) has recently been used to detect frameshift and nonsense mutations in both the Duchenne muscular dystrophy (dystrophin) and the adenomatous polyposis coli (APC) genes.^{21,22} Approximately 70% of previously identified mutations in the NF1 gene were also readily identifiable using the PTT.^{23,24} Thus the PTT appears to be a very promising assay system for mutational analysis of the NF1 gene and clearly has the potential to detect the majority of NF1 mutations.

An alteration involving A→G at position 7828 in patient NF41 was not seen in 70 control samples. Further investigations are required to identify and define the role of such missense mutations in the aberrant function of neurofibromin. Functional studies will be required to understand the underlying mechanism by which this threonine to alanine substitution causes disease.

An identical mutation, C5839T, at CpG dinucleotide has been described in exon 31 in six unrelated NF1 subjects who exhibit a spectrum of phenotypes.^{25–29} We have also previously reported identical changes in exon 17 in two unrelated NF1 patients whose phenotypic features were different.¹⁴ The clinical phenotype of NF1 is extremely variable, even within families, making a straightforward relationship between genotype and phenotype unlikely.

Easton *et al*²⁰ have presented evidence based on analysis of the variation of clinical features within family members for the role of modifying genes in determining the NF1 phenotype. The analysis, however, could not exclude that the NF1 genotype would be important for at least some disease features. The limited number of mutations reported to date has not allowed detailed analysis of this. The patients reported here will facilitate genotype/phenotype correlation but they provide no immediate obvious insights into this question.

To date, a total of 16 nucleotide substitutions have been reported in the NF1 gene.^{10,25–29,31} Indeed, eight of the 16 nucleotide substitutions so far reported in over 600 patients screened involve a C→T change at a CpG dinucleotide. The change in patient NF9 in the present study also involves a CpG dinucleotide. There is now clear evidence that the presence of CpG dinucleotides are associated with a high mutation rate, as it has been shown that alterations at these dinucleotides account for at least 35% of all point mutations causing genetic disorders.³²

In patient NF130, the A→G change at nucleotide position 11 715 is within the 3' UTR distal to exon 49 and is the only change so far reported in this region. She has three affected children from separate marriages and two of these children have NF1 and learning difficulties. It has been reported that the 3' UTR of the human NF1 gene is highly conserved in the mouse,³³ and therefore mutations within this region may be important for mRNA stability or translational efficiency.

Mutations have been found to occur non-randomly with respect to the surrounding DNA sequence in human genes.^{32,34} Certain sequences in the human genome are associated with the preferential formation of insertions and deletions. These sequence patterns include direct repeats, palindromes, quasi-palindromes, and runs of identical bases. This is consistent with a mutational mechanism based on the slipped mispairing model which suggests that misalignment can occur between such dir-

ect repeats during replication. The nucleotide environment for some of the NF1 gene deletions and insertions detected in the previous studies has also provided similar examples of flanking sequences containing direct repeats, palindromes, and quasi-palindromes.^{13,35,36} However, on examining the sequences flanking both the cytosine deletion in NF173, and the adenine deletion in NF59, no such direct or inverted repeats were observed (data not shown). However, a perfect copy of a deletion "hot spot" consensus sequence TGPPKMP was found in close proximity to del 4572C³² (M Krawczak, personal communication).

The three NF1 patients who showed hemizyosity with several microsatellite markers, owing to the presence of an intragenic deletion, all have learning problems. Interestingly, two of these deletion cases have NF-Noonan-like features which include hypertelorism and downward slanting palpebral fissures, posteriorly rotated ears, a short neck, and low posterior hairline. Hemizyosity with these microsatellite markers has previously been reported in several classical NF1 patients.³⁷ Unequal exchange between homologous chromosomes during meiosis may account for the high rate of intragenic deletions observed in this gene. In contrast to previous reports, where 95% of all new mutations appear to be of paternal origin,^{38,39} all three deletion cases in our study were of maternal origin.

The introduction of more sensitive analytical methods, such as the protein truncation test and direct RT-PCR of total cellular RNA, as well as a more comprehensive analysis of each of the exons of the gene, should help to identify the majority of NF1 mutations. The possibility that there is a clustering of mutations within the region of the gene that has not yet been analysed cannot be ruled out. As a growing number of causative germline mutations are identified, the overall mutational spectrum will become clearer. This will be useful for elucidating the normal gene function and would also provide us with valuable insights into the intricacies of underlying mutational mechanisms.

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