A search for evidence of somatic mutations in the NF1 gene

Alison M John, Martino Ruggieri, Ros Ferner, Meena Upadhyaya

Abstract

Neurofibromatosis type I (NF1) is an autosomal dominant disorder affecting 1 in 3000 people. The NF1 gene is located on chromosome 17q11.2, spans 350 kb of genomic DNA, and contains 60 exons. A major phenotypic feature of the disease is the widespread occurrence of benign dermal and plexiform neurofibromas. Genetic and biochemical data support the hypothesis that NF1 acts as a tumour suppressor gene. Molecular analysis of a number of NF1 specific tumours has shown the inactivation of both NF1 alleles during tumourigenesis, in accordance with Knudson's "two hit" hypothesis. We have studied 82 tumours from 45 NF1 patients. Two separate strategies were used in this study to search for the somatic changes involved in the formation of NF1 tumours. First, evidence of loss of heterozygosity (LOH) of the NF1 gene region was investigated, and, second, a screen for the presence of sequence alterations was conducted on a large panel of DNA derived from matched blood/tumour pairs. In this study, the largest of its kind to date, we found that 12% of the tumours (10/82) exhibited LOH; previous studies have detected LOH in 3-36% of the neurofibromas examined. In addition, an SSCP/HA mutation screen identified five novel NF1 germline and two somatic mutations. In a plexiform neurofibroma from an NF1 patient, mutations in both NF1 alleles have been characterised.

Keywords: neurofibromatosis; NF1 gene; somatic mutations
Materials and methods

PATIENTS
Patients were diagnosed as being affected by NF1 according to the diagnostic criteria formulated by the National Institute of Health Consensus Conference (NIH CC) 1987.

TUMOURS
Our tumour panel includes DNA isolated from 82 NF1 specific tumours (74 dermal neurofibromas, three plexiform neurofibromas, two pilocytic astrocytomas, and three schwannomas) from 45 NF1 patients. The corresponding lymphocyte DNA was also available from these patients. Plexiform neurofibromas were sent from clinics with specialist interest in neurofibromatosis.

DNA EXTRACTION
Tumour samples were transported in tissue culture medium and contaminating tissues, including skin, fat, and hair, was carefully dissected away under the supervision of a pathologist.

DNA was extracted by incubating tumour tissue for 1.5 hours at 65°C in 10% SDS, tissue extraction buffer (5 mmol/l NaCl, 1 mol/l Tris, 0.5 mol/l EDTA, 10% SDS) and proteinase K. Phenol/chloroform extraction was carried out and the DNA precipitated by ethanol and 3 mol/l sodium acetate. Constitutional DNA was extracted from peripheral blood cells by conventional methods.

PCR
Amplification of the DNA was carried out in a 12.5 µl volume containing 10 ng DNA, 12 pmol/l of each primer, 1.5 mmol/l MgCl₂, 1.25 mmol/l each dNTP, 1 U Taq polymerase, and buffer (Qiagen). Reactions were heated to 94°C for five minutes and then subjected to 35 cycles at 94°C for 30 seconds, 50-58°C for 30 seconds, 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. The NF1 primers used were as previously published.

LOH STUDIES
A panel of 13 markers (eight intragenic and five extragenic to NF1) were used to screen the panel of 45 tumour and blood pairs (table 1) to examine LOH in tumour tissue.

HETERODUPLEX ANALYSIS (HA)
A total of 15-20 ng of amplified DNA (two PCR products were pooled to increase sample throughput) was mixed with 1 µl 0.1 mol/l EDTA, heated to 94°C for three minutes, and slowly cooled to 40°C over 40 minutes to allow heteroduplex formation. The reannealed product was loaded onto 24 cm × 33 cm × 0.8 mm gels and electrophoresed at approximately 5 W/hour until the marker dye front was at least 15 cm from the origin.

SINGLE STRANDED CONFORMATION POLYMORPHISM (SSCP) ANALYSIS
Two µl of PCR product (8-10 ng DNA) was diluted 1:5 with loading buffer (95% formamide, 10 mmol/l NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) and heated to 94°C for three minutes. The product was loaded immediately onto 0.5 × MDE (Flowgen) gels and electrophoresed at room temperature for 50-150 Watt hours depending on the product size.

SILVER STAINING
Bands were visualised by silver staining. Gels were soaked in 0.1% silver nitrate for five minutes, rinsed briefly in distilled water, and then soaked in developing solution (1.5% sodium hydroxide, 0.01% sodium borohydride, and 0.15% formaldehyde) until bands appeared (five to 15 minutes). The bands were fixed by soaking in 0.75% sodium carbonate for two to five minutes and then rinsed briefly in water. The gel was transferred onto 3MM Whatmann paper and dried at room temperature. Aberrant bands were identified by their differing migration patterns.

SEQUENCING
Exons were amplified by cycle sequencing for 35 cycles using the thermo Sequenase cycle sequencing kit from Amersham. Primers were labelled using [³²P]dATP (Amersham) and T4 polynucleotide kinase (Promega). Approximately 3 µl (12-20 ng) product was diluted 1:1 with loading buffer and run on polyacrylamide gels for 1.5-3 hours at 75 W/hour.

Results
Evidence for LOH of the NF1 region was investigated in the 45 tumour/blood pairs

Table 1 Results of LOH studies at NF1 and flanking polymorphisms in four patients, screened with eight intragenic and five extragenic markers. Black circles denote LOH, black squares denote uninformative loci, and ND denotes samples that would not PCR for that particular marker

<table>
<thead>
<tr>
<th>Marker</th>
<th>T11</th>
<th>T14</th>
<th>T15</th>
<th>T19</th>
<th>T25</th>
<th>T33.1</th>
<th>T33.2</th>
<th>T68.1</th>
<th>T68.2</th>
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<td>NF1- EV120 (intron 27b)</td>
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<td>NF1- 24.8 (intron 27b)</td>
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<td>NF1- 28.4 (intron 27b)</td>
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<td>NF1- RdPF (intron 39)</td>
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A panel of eight intragenic and five extragenic DNA polymorphic markers was used. Ten out of 82 (12%) neurofibromas, isolated from seven separate patients, have shown evidence for LOH (fig 1). The end points of these deletions have not been defined and the constitutional NF1 mutations have still to be identified.

Exons 16-40 (29 exons) of the NF1 gene were targeted for investigation. This conserved region of the gene exhibits extended homology with the yeast IRA1 and IRA2 genes and is still the only domain of the protein to which any function has been ascribed and is involved in the downregulation of the p21 ras oncogene. The majority of exons (28/29) encompassing this region were screened in this study. Owing to problems associated with the variable quality and quantity of genomic DNA isolated from some tumours, approximately 1-5% of DNA samples proved to be refractory to mutational analysis. Five novel germline mutations and two somatic sequence alterations were found following the SSCP or HA mutational screen (table 2).

A band shift was identified by heteroduplex analysis in tumour tissue, but not in lymphocyte DNA, from patient T7 (fig 2). This somatic change, identified in a plexiform neurofibroma from patient T7, involved a CGA→TGA nonsense mutation at nucleotide 2446 of exon 16, resulting in the formation of a premature stop codon at residue 816 (fig 3A). The constitutional mutation in this family was a nucleotide substitution at the obligate donor GT dinucleotides (IVS4+1 G→A, intron 4), which was detected in the patient and two affected relatives (fig 3B). RNA was unavailable to confirm splice site abnormalities. A further, novel, somatic change (7168delGA) in

**Table 2 Five germline and two somatic mutations identified following a screen of exons 16–40 using SSCP/HA**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Somatic or germline</th>
<th>Mutation Exon/intron</th>
<th>Restriction site modification</th>
<th>Amino acid substitution/predicted protein changes</th>
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</thead>
<tbody>
<tr>
<td>BL7</td>
<td>G</td>
<td>IVS 4 + 1 Intron 4</td>
<td>No change</td>
<td></td>
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<tr>
<td>BL22</td>
<td>G</td>
<td>IVS17-2 Intron 17</td>
<td>No change</td>
<td></td>
</tr>
<tr>
<td>BL62</td>
<td>G</td>
<td>4024 del TA Exon 23-1</td>
<td>Gain Hpy178III site and lose Hpy188IX</td>
<td>Premature stop at residue 1344</td>
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<tr>
<td>BL17</td>
<td>G</td>
<td>5272 del C Exon 29</td>
<td>Gain MboI, DdeI</td>
<td>Premature stop at residue 1758</td>
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<td>BL23</td>
<td>G</td>
<td>7627 del AC Exon 41</td>
<td>Gain Del</td>
<td>Premature stop at residue 2400</td>
</tr>
<tr>
<td>T7</td>
<td>S</td>
<td>R816X Exon 16</td>
<td>No change</td>
<td>Premature stop at residue 815</td>
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<tr>
<td>T52.1</td>
<td>S</td>
<td>7168delGA Exon 40</td>
<td>Lose Hpy188IX, ApoI</td>
<td>Premature stop at residue</td>
</tr>
</tbody>
</table>

S = somatic, G = germline, T = tumour, Bl = blood.
Exon 40 was detected in one of two neurofibromas obtained from the same patient (T52). Although the corresponding blood DNA was not available from this patient, the mutation was assumed to be somatic as it was not detectable in the second tumour, as would be expected if it represented a germline mutation.

Discussion

Mutation analysis of the NF1 gene has so far identified constitutional mutations in 246 unrelated patients (NF1 Genetic Analysis Consortium, February 1999). Approximately 70% of these disease causing sequence alterations are frameshift or nonsense mutations that are all predicted to result in the synthesis of a prematurely truncated neurofibromin protein. To date, despite the extensive investigation of the NF1 gene by a number of different mutation screening methods, disease causing lesions have been identified in less than 40% of patients. The spectrum of NF1 germline mutations includes gross deletions, microdeletions, insertions, and base pair substitutions. The NF1 somatic mutational spectrum is, however, far less well defined, mainly because of the small number of studies carried out and the difficulty in detecting such changes. This study aimed to investigate the possible mutational mechanisms involved in the somatic inactivation of the NF1 gene in various NF1 related tumour tissues.

Searches for evidence of loss of heterozygosity (LOH) of the NF1 gene region have proved successful in identifying potential NF1 deletion mutations in various tumour tissues. Three of these studies have provided evidence for at least some degree of LOH of the NF1 gene region in neurofibromas from NF1 patients. Coleman et al found that 36% (8/22) of the neurofibromas analysed, derived from five unrelated NF1 patients, showed either partial or complete somatic deletions of one chromosome 17, and that these deletions always involved the NF1 region. These results were essentially corroborated in a recent study which showed evidence of LOH in 25% (15/60) of neurofibromas tested from 17 NF1 patients; again the deletions always included the NF1 gene region. In contrast to these reports, showing clear evidence for LOH in more than a third of all neurofibromas analysed, is the recent study from Daschner et al who found LOH in 2.6% of the 38 neurofibromas they investigated. Our study detected a higher level of LOH (12%) in neurofibromas than Daschner et al but it is still significantly less than the previous reports. In none of the above studies were the constitutional mutations identified.

In the present study, DNA was isolated from 82 neurofibromas obtained from 45 unrelated NF1 patients, the largest panel studied to date. Following a screen with a large panel of NF1 intragenic and extragenic polymorphic markers, we identified LOH in 12% (10/82) of the tumour tissues tested. Indeed, in one case, 12 individual neurofibromas were tested from one NF1 patient and none showed any evidence of genomic deletions with any of the markers screened. To date, the constitutional mutations in six out of seven of the NF1 patients that have shown LOH in their tumours have still to be determined.

Some of the reasons for the low mutation detection rate could be because the lesions are too small to identify, the alterations being located in the regions still to be screened, or, possibly, the mutation detection techniques used are not sensitive enough. Thorough SSCP/HA analysis of NF1 exons 16-40 for sequence changes has, to date, only detected two somatic mutations and five novel germline mutations (three deletions and two splice site

**Figure 3**

(A) Sequence analysis of the somatic alteration identified in patient T7, a nonsense mutation (R817X) at codon 2446 of exon 16. Lane (A) lymphocyte DNA from the patient, lane (B) DNA from patient’s plexiform neurofibroma, lane (C) lymphocyte DNA from patient’s mother. (B) Sequence analysis of germline mutation showing a donor splice site change in the obligate GT doublet (IVS4+1 G→A) observed in all affected members of the family of patient T7. Lane A is control DNA, lane B is patient T7 lymphocyte DNA, lane C is patient’s tumour DNA, and lane D is lymphocyte DNA from patient’s affected mother.
changes) (table 2). One of these alterations is located in the GRD region.

Neurofibromas are composed of a mixture of cell types (fibroblasts, mast cells, Schwann cells, perineurial cells) and it is not clear which cell types carry the genetic alterations underlying neurofibromas. Furthermore contamination of tumour DNA by normal cellular DNA will make LOH difficult to detect. Despite every effort to dissect any surrounding normal tissue carefully, it is always difficult to quantify the levels of contaminating normal cells present in any sample of dissected neurofibroma.

Ascertainment of the mutational events underlying the potential “second hit” leading to tumourigenesis in NF1 related tumours proved difficult, with only one study reporting the characterisation of both the germline and somatic changes of the NF1 gene in a dermal neurofibroma from a NF1 patient. The somatic nonsense mutation R816X detected in the plexiform neurofibroma from patient T7 in our study generates a premature protein at residue 816 and the same mutation was previously identified as a disease causing mutation in the lymphocyte DNA from an unrelated NF1 patient. The constitutional mutation in patient T7 identifies a splice site change IVS4+1 G→A, and this represents a novel mutation of the NF1 gene.

If we assume that our current mutational screening methodologies are sensitive enough to detect the majority of sequence alterations present, then these results would indicate that screening methodologies are sensitive enough to detect the majority of sequence alterations (table 2). One of these alterations is the methylation of gene promoter regions for the inactivation of a number of tumour suppressor genes. Furthermore, the methylation status of the NF1 gene may play an important role in the development of plexiform neurofibromas. Such hypermethylation of the NF1 gene will make LOH difficult to detect. Despite every effort to dissect any surrounding normal tissue carefully, it is always difficult to quantify the levels of contaminating normal cells present in any sample of dissected neurofibroma.

We would like to thank Professor David Cooper and Professor Peter Harper for their encouragement and Dr Nick Thomas for his comments on this manuscript. We are grateful to Dr Susan Huson and all other clinicians who have provided support and encouragement for this research and the patients and other families for their support. Financial support was provided by the Smiths Charny and the Neurofibromatosis Association.

30 NNF National NF1 Genetic Analysis Consortium. www.clem.edu.nf1gene/


