Identification of a mutation that perturbs NF1 gene splicing using genomic DNA samples and a minigene assay

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Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disease. In recent studies on the neurofibromatosis type 1 (NF1) gene neurofibromin, splicing abnormalities were seen in 30-50% of cases when RNA taken from cell lines was analysed. Unlike mutations that alter critical amino acids or generate premature stop codons, splicing abnormalities can be very hard to predict from sequence analysis alone. Apart from the two base pairs 5′ and 3′ of each exon, few of the nucleotides in regions critical for splicing are absolutely conserved. As a consequence, it can be very difficult to conclude that a sequence variation found in a patient will alter splicing and so represents a pathogenic mutation.

This difficulty is well illustrated by a family with NF1 in which we recently identified a sequence variation. The three generation family is from the UK and meets NIH diagnostic criteria. The index case, at the age of 82, has classical features of NF1 including multiple café au lait macules, neurofibromas, and axillary and inguinal freckling. Her son was similarly affected and died in a road traffic accident. Her granddaughter additionally had macrocephaly and died aged 31 from a malignant nerve sheath tumour affecting the coeliac axis. Through sequencing all exons in the neurofibromin gene, as well as 50 base pairs of 5′ and 3′ intronic flanking sequence, only a single nucleotide change was found, consisting of a substitution of a cytidine for a guanosine 5 bp downstream of exon 3 (exon 3+5 G>C, fig 1). This variation was seen in the proband and the granddaughter and has not been found in 100 normal chromosomes (Mattocks et al, unpublished observations), consistent with it representing the disease causing mutation. The vicinity of the nucleotide change to the 5′ splice site of intervening sequence (IVS) 3 (figs 1 and 2A) suggests that it may interfere with the splicing of exon 3. However, there are examples of wild type sequences similar to the exon 3+5 G>C mutation in which the corresponding exon is spliced efficiently; for example within the normal NF1 gene IVS-1 and -7 share identical −1 to +5 sequence with the mutated IVS-3. We were therefore unable to predict on the basis of sequence analysis alone that this sequence change will...
interfere with splicing and cause NF1, and a direct analysis of exon 3 splicing assay was therefore required in this family.

**METHODS**

**Hybrid minigene constructs**

Human genomic DNA was amplified from normal and mutated (IVS 3 +5 G>C) NF1 exon 3 to generate a fragment that contained the exon 3 along with 309 bp of 5′ and 462 bp of 3′ intronic flanking sequence using the following oligonucleotides: 5′ GGAATTCCATATGTCTCAAGGTAACATCTATCC 3′ and 5′ GGAATTCCATATGTCAAGATTCTGGTACAGGTC 3′.

In the experiment designed to rescue splicing defects, we inserted this into the minigene. Following transfection and analysis of the construct in Hep3B cells, the mRNA produced by the cells was analysed for splicing pattern by RT-PCR. As shown in fig 2A, the exon 3 +5 G>C mutation produced by the cells was analysed for splicing pattern by RT-PCR. As shown in fig 2A, the exon 3 +5 G>C mutation dramatically affected pre-mRNA processing, causing exon 3 to be completely skipped.

To confirm the role of this mutation further, we constructed an alternate splicing assay. Two products are seen on agarose gel electrophoresis, with the 239 bp band representing an RNA product lacking exon 3 and the 323 bp band a product including exon 3, as indicated schematically on the right of the gel. Note that the C>G minigene excludes exon 3, while the WT gene includes this exon. Splicing is rescued (that is, exon 3 is included) by the coexpression within the minigene expressing cells of a U1 snRNA altered to be complementary to the mutation containing sequence (exon 3 G>C/C>G-U1) but not by expression of the WT-U1 snRNA (exon 3 G>C/WT-U1). In contrast, expression of WT snRNA (exon 3 G>C/WT-U1) does not rescue splicing (fig 2A), showing that this does not simply result from increased levels of U1 snRNA. This experiment proves that the exon 3 +5 G>C variation is a disease causing mutation that induces aberrant skipping of exon 3.

As techniques for the identification of sequence variation become faster and cheaper, the distinction between polymorphisms and pathogenic mutations will be an increasing challenge. The assay we have used here in the analysis of one NF1 family is a potentially valuable tool for the identification of those mutations that cause splicing defects. No RNA or further samples are required from the patient, making it feasible to carry out this further step in the molecular genetic diagnostic laboratory as part of the analysis of the DNA sample provided by the referring clinicians. In addition, the minigene system
effectively recreates within the cell line the splicing defect of each patient, so facilitating further studies on the relationship between genotype and phenotype in this disease.

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