MUTATION IN BRIEF

Three Different Premature Stop Codons Lead to Skipping of Exon 7 in Neurofibromatosis Type I Patients

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Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder affecting one in 3,500 individuals. The mutation rate in the *NF1* gene is one of the highest known for human genes. Compared to other methods, the protein truncation test (PTT) provides improved efficiency in detecting *NF1* mutations which are dispersed throughout the gene which spans 350 kilobases of genomic DNA. We have applied the PTT and subsequent sequence analysis of cloned cDNA to identify mutations in NF1 patients. We report here the identification of two novel (W336X and Q315X), and one recurrent (R304X) mutation located in exon 7 and show that all three premature termination codons lead to skipping of exon 7 in a proportion of the transcripts derived from the mutated allele. Possible mutation-induced alterations of the RNA secondary structure and their impact on skipping of exon 7 of the *NF1* gene are explored and discussed. © 2000 Wiley-Liss, Inc.

KEY WORDS: *NF1*; neurofibromatosis type I; exon skipping; RNA secondary structure

INTRODUCTION

The neurofibromatosis type I (*NF1*) gene (MIM# 162200) is located on human chromosome subband 17q11.2, and the 8.5 kb coding region contains 60 exons including two that are subject to alternative splicing. Mutations in NF1 patients are dispersed throughout the entire gene. Thus, mutation detection is laborious and identification rates do not exceed 70 to 80 % (Park and Pivnick, 1998). The protein truncation test (PTT) provides the advantage over other methods to screen the entire coding region of the *NF1* gene for nonsense or frameshift mutations which represent about 80% of the characterised mutations in the *NF1* gene so far (Shen et al., 1996). This technique also allows the detection of aberrantly spliced transcripts (Heim et al., 1995; Messiaen et al., 1997; Hoffmeyer et al., 1998; Park and Pivnick, 1998). Usually, aberrant transcripts are indicative for mutations in splice or branching sites of exons. Recently, another mechanism, referred to as exon skipping, which associates premature termination codons (PTC) in DNA with mRNA that lacks the exon containing the PTC, has been shown to cause skipping of

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exons 37 and 7 of the *NF1* gene (Messiaen et al., 1997; Hoffmeyer et al., 1998). Different explanations how PTCs induce exon skipping have been described (for review see Maquat, 1995; Valentine, 1998).

We have screened 18 unrelated NF1 patients and families for mutations using the PTT. Among the mutations characterized, three were located in exon 7. One of the three stop mutations has previously been described (Hoffmeyer et al., 1998) and shown to be associated with exon skipping. The remaining two mutations in exon 7 were novel. We show that all three mutations lead to skipping of exon 7 in a significant proportion of the transcripts derived from the mutated allele in our patients. Further, we discuss possible mechanisms leading to the observed exon skipping.

MATERIALS AND METHODS

DNA and RNA were obtained from lymphocytes of freshly taken blood or from cultivated fibroblasts of NF1 patients.

The PTT was performed as described (Heim et al., 1995).

For identification of the mutations expected in the first half of segment S1, cDNA spanning exons 1 to 10a was amplified using the sense primer of fragment S1 and an antisense primer NF-335 (5´-gtt tca cca aac ata ttt cga a-3´) (Eisenbarth et al., 1999). PCR products were cloned into the AT cloning vector pGEM-T (Promega) and individual clones were subjected to sequence analysis. Sequencing was performed on an automated sequencer (LiCor) using the thermosequenase kit (Amersham).

For sequence analysis of genomic DNA exon 7 was PCR amplified using primers as described (Purandare et al., 1995).

To distinguish between transcripts either containing or missing exon 7, RT-PCR was performed using the sense primer (5´-cac aga ctg ata tgg ctg aat gtg-3´) together with the primer NF-335.

To specifically amplify transcripts lacking exon 7 the sense primer (5´-gaa acg atg ctg gtc aaa cag ttg c-3´) was used together with an antisense primer that comprises sequences from exon 6 at its 3´-end and from exon 8 at its 5´-end (5´-ctt gga tta aaa agc agg ttc tta ttc-3´). Nested PCR was performed with the primers that amplify exons 1 to 10a for the first PCR and the primers specific for the amplification of transcripts missing exon 7 in the second PCR.

RNA secondary structures were computed with the ***Vienna RNA Package***, version 1.3.1., (Hofacker et al., 1994) using on the energy parameters published in He et al. (1991) and Jaeger et al. (1989). Base-pairing probabilities are obtained in addition to the minimum energy structure using McCaskill´s partition function algorithm (McCaskill, 1990).

RESULTS AND DISCUSSION

Mutations

For three unrelated cases in our series of patients the PTT showed shortened polypeptides in the first segment S1 nucleotide 1 to 1868 (exons 1 – 12b) of the coding sequence of the *NF1* gene (Fig.1). The remaining segments S2-S5 revealed only wild-type polypeptides for these three cases. According to the sizes of the shortened peptides, the mutations were all suspected within the first half of the analysed fragment. Thus, RT-PCR was performed using primers that amplify exons 1 to 10a of the *NF1* gene to generate PCR products that were cloned. Subsequent sequence analysis of individual clones for each patient revealed a C to T transition at nucleotide position 943 in three of fourteen clones of patient P1. This mutation is predicted to change a glutamine at amino acid position 315 to a premature stop codon (Q315X). In two related patients, P2 and P3, three of six clones and two of eight clones, respectively, showed a G to A transition at nucleotide position 1007. This mutation is predicted to change the tryptophan at amino acid position 336 to a premature stop codon (W336X). Sequencing of fourteen clones of patient P4 identified a C to T transition at nucleotide position 910 in three clones, predicted to change the arginine at amino acid position 304 to a premature stop codon (R304X). All three mutations are located in exon 7. Thus, exon 7 was amplified from genomic DNA of the patients and sequenced to confirm the mutations. All four patients were heterozygous at the relevant nucleotide positions.

Mutation W336X and Q315X have not been described previously. Mutation R304X has been identified in another patient as disease causing (Hoffmeyer et al., 1998). The latter mutation occurs in a hypermutable CpG-dinucleotide and is thus compatible with the mechanism of methylation-mediated deamination of 5-methylcytosine (Cooper and Krawczak, 1993) which would explain its recurrence.

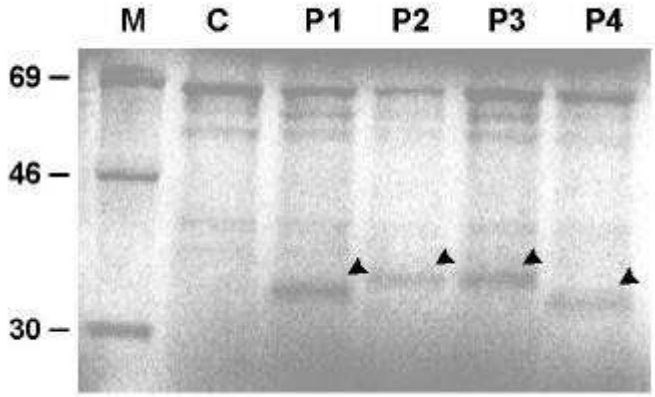

Figure 1: PTT results of the first segment S1of the *NF1* cDNA: The *in vitro*-synthesized polypeptides of a healthy control sample (C) and patients P1, the related patients P2 and P3 and patient P4 are separated in a 12% SDS polyacrylamide gel. The band sizes of the protein molecular weight standard (M) are indicated in kDa at the left of the gel. Shortened polypepties in the patients´ samples are indicated with arrow heads.

PTC-induced skipping of exon 7 analysed by RT-PCR

Although skipping of exon 7 has been reported to be associated with the PTC R304X (Hoffmeyer et al., 1998), the sequencing results did not reveal loss of exon 7 in transcripts of patient P4. On the other hand, we have previously demonstrated that illegitimate splicing of exon 7 of the *NF1* gene can be observed by RT-PCR even in healthy individuals if RNA was isolated from "aged" blood kept at room temperature overnight (Wimmer et al., in press). Therefore, we took particular caution to use RNA extracted from freshly taken blood for the investigation of exon 7 skipping by RT-PCR. For patients P2 and P3 no freshly drawn blood was available, therefore cultivated fibroblasts from patient P2 were used for RNA isolation. Analysis of the RT-PCR products showed a band corresponding to transcripts lacking exon 7 in addition to the full length PCR product for all patients but not the control lymphocytes (Fig.2A).

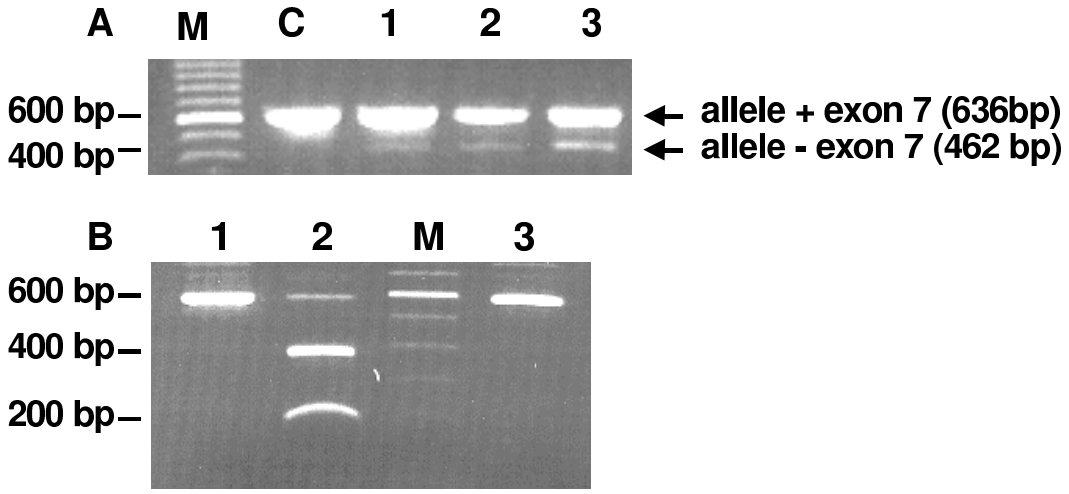

Figure 2: RT-PCR results of exon skipping: A: PCR products were derived from cDNA of lymphocytes from patients P1 (1), P4 (2) and a healthy individual (C) and of cultured fibroblast of patient P2 (3). B: PCR products obtained with primers that specifically amplify transcripts lacking exon 7. PCR products derived from cDNA of patient P4 undigested (1) and digested with *Rsa*I (2) and of a healthy control individual (3).

A polymorphic restriction site (Hoffmeyer and Assum, 1994) located in exon 5 of the mutant allele further allowed to determine whether the transcripts lacking exon 7 in patient P4 (R304X) were derived from the mutated allele. Nested PCR was performed to specifically amplify cDNA that lacks exon 7. The resulting PCR product was digested with *Rsa*I. Most of the PCR product was cut by *Rsa*I (Fig.2B, lanes 1 and 2), indicating that the observed exon skipping is mainly PTC induced. However, even after extensive overnight digestion a small amount of the PCR product remained undigested. The conclusion that a small proportion of transcripts lacking exon 7 is derived from the wild-type allele was further corroborated by the fact that using the same PCR approach we could also amplify a PCR product from cDNA synthesised from lymphocytes of freshly taken blood of a non-affected control individual (Fig.2B, lane 3).

RNA secondary structure analysis of wild-type and mutant exon 7

To study whether the alteration of the pre-RNA secondary structure is a possible mechanism leading to exon 7 skipping, as has been proposed (Hoffmeyer et al., 1998), we performed RNA secondary structure predictions for the wild-type exon 7 and the mutations described in this paper as well as the mutation 918delT. The latter mutation has been shown not to be associated with exon 7 skipping (Hoffmeyer et al., 1998). Since the length of the analyzed sequence has a strong impact on the predicted secondary structure we analyzed the exon 7 sequence in the context of differently sized (50 and 250 nucleotides) adjacent intronic sequences. We find that all four mutations are consistent with the structure depicted in Fig. 3 which is the most stable one for the wild-type sequence. Since no mutation leads to a non-pairing nucleotide combination and the deletion 918delT is located in an unpaired position the effect of the mutations is rather subtle at the level of secondary structures and we do not see any evidence for qualitative structural changes. However, there is a large number of structural variants, in particular at the intron-exon boundary that are energetically comparable to, and hence compete with, the minimum free energy structure (Fig. 3A). The frequencies of these individual structures in the diverse ensemble of structural alternatives may change substantially between the different mutants. However, a quantitative analysis of these effects is not meaningful, since it is of the same order of magnitude as the changes in the predicted base pairing probabilities resulting from the analysis of sequences of different lengths.

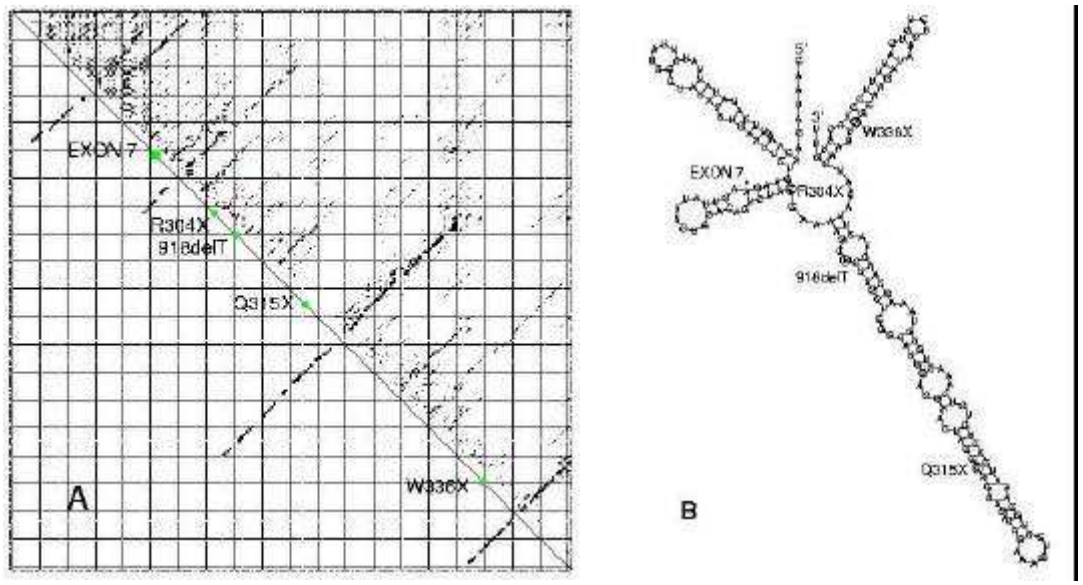

Figure 3: Secondary structure of exon 7 and the preceeding 50 bases. **A:** Each little square in the upper right half of the matrix corresponds to a base pair that is formed with a probability of more than 10^{-5} in thermodynamic equilibrium. The area of the little squares is proportional to the logarithm of the pairing probability. A 10x10nt grid is drawn to guide the eye. In the lower left half of the figure the minimum energy structure of the wild-type sequence is shown for comparison. Note that the same base pair appears twice, reflected about the diagonal, in this representation. Hence the upper and lower triangles can be used to convey different information about the structure. The position of the four mutations and the first position of exon 7 are marked on the diagonal. The corresponding rows and columns contain all possible base pairs that can be formed by these positions. **B:** Conventional drawing of the common minimum energy structure of all five sequences. The mutations are marked by circles, an asterisk indicates the beginning of exon 7.

Concluding remarks

Our results demonstrate that transcripts lacking exon 7 in NF1 patients are largely derived from the mutated allele. However, we show that in healthy individuals a small fraction of transcripts are also missing exon 7 and on the other hand a considerable amount of full-length transcripts in the patients (e.g. 3 of 14 clones in patients P1 and P4) is derived from the mutated allele. These observations indicate that the exon-intron boundaries of exon 7 are weakly defined, rendering this exon particularly prone to aberrant splicing. In contrast to Hoffmeyer et al. (1998), our secondary structure prediction does not indicate that any of the three mutations, including R304X, causes a qualitative change of exon 7 secondary structure. We propose that the mutations within exon 7 may lead to an increase of transcripts that lack exon 7 by two possible ways. (i) Assuming that one or more of the possible secondary structures induce skipping of exon 7, the mutations may quantitatively shift the frequency of individual structures in the diverse ensemble towards the structures leading to skipping of exon 7. (ii) Alternatively, the mutations may affect exonic splice site enhancer sequences (SES) which are necessary for appropriate splicing of weakly defined exons (Berget, 1995) . The mutation R304X is located within a sequence context that fits well with the hypothesis that the mutation alters the function of a splicing enhancer.

Recently, it has been shown that 50% of the mutations identified in NF1 patients resulted in splicing alterations although most of them did not involve the conserved AG/GT dinucleotides of the splice site (Ars et al., 2000). It has been proposed that variations in the RNA splicing mechanism may lead to differential expression of the splicing mutation and hence to different amounts of aberrantly spliced mRNA (Chiba-Falek et al., 1998). Thus,

Ars et al. (2000) speculate that mutations affecting splicing could account for part of the clinical variability that is observed in NF1 patients carrying the same mutation. The results we present here may lend further support to this hypothesis.

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