Expression analysis of an FGFR2 IIc 5' splice site mutation (1084+3A→G)

R Kan, S R F Twigg, J Berg, L Wang, F Jin, A O M Wilkie


Sequence variations within splice sites may pose problems in the interpretation of their pathogenic effect, especially when these variations occur outside the highly conserved /gt (donor or 5' site) and ag/ (acceptor or 3' site) consensus dinucleotides that immediately flank most exons. A commonly used method to evaluate the probable effect of a sequence variation on splicing is to calculate the Shapiro-Senapathy (SS) score, which is based on the extended splice site consensus sequence.1,2 Here we present a sequence variation in a 5' splice site of the gene encoding fibroblast growth factor receptor type 2 (FGFR2) that maintains a consensus nucleotide at the variant position, but nevertheless causes a switch to the use of a cryptic 5' splice site within the upstream (IIc) exon. This variant is present in three generations of a family and manifests with mild features of Crouzon syndrome.

PATIENTS AND METHODS

Following informed consent blood samples were collected from members of the family pedigree shown in fig 1. DNA was isolated by proteinase K treatment and phenol chloroform extraction. RNA was also extracted from the blood of affected individual II-2 and cDNA synthesised by standard techniques.

Mutation screening of exons IIIa and IIIc of FGFR2 was undertaken in DNA from the proband using WAVE (3500HT; Transgenomic) denaturing high performance liquid chromatography.3 Sequencing was performed with Big Dye (version 3) on an ABI 3100 DNA sequencer.

Oligonucleotide hybridisation was carried out on cDNA derived from individual II-2. Reverse transcriptase PCR (RT-PCR) using primers 4F and 9R located respectively in exons 6 and 12 (exon numbering according to Kan et al4; see fig 2A), was performed as previously described.5 The RT-PCR product was separated by electrophoresis on a 2% agarose gel, blotted onto a nylon membrane (Zeta-Probe, Bio-Rad), and the presence of various potential splicing products was analysed by hybridisation to specific 32P radiolabelled oligonucleotide probes6 SPL7/8 (IIIa–IIIb), SPL7/9 (IIIa–IIIc) and SPL7/11 (IIIa-exon 12). An additional probe 9C/TM (5'-TGCTTGCGGGCCTGGAA-3') was used to detect splicing of the cryptic donor splice site in exon IIc to the downstream transmembrane exon 11.

RESULTS AND DISCUSSION

The family pedigree is shown in fig 1A and the facial phenotype is illustrated in fig 1B. The proband III-3 presented aged 3 ½ years with scaphocephaly and exorbitism. He was found to have sagittal and partial metopic synostosis, which was corrected surgically. His mother II-2, uncle II-3 (fig 1B) and grandfather I-1 (not illustrated) had a similar facial appearance but had not undergone any reconstructive surgery.

In view of the features of mild Crouzon syndrome we screened exons IIIa and IIIc of FGFR2. This revealed heterozygosity for a nucleotide transition, 1084+3A→G, located at the third (intronic) base of the 5' splice site lying downstream of the alternatively spliced exon IIc. The mutation was independently confirmed by demonstrating a new cleavage site for the restriction enzyme AccI (not shown). The same substitution was identified by DNA sequencing in three other clinically affected family members, I-1, II-2, and II-3, but not in two clinically unaffected individuals at 50% prior risk (II-4 and II-5). Although this nucleotide change has been described twice before, in a child with Pfeiffer syndrome whose parents were normal on DNA testing,6 and in a clinically unclassified patient with sagittal and lambdoid synostosis,7 these reports did not include verification of sample relationships, nor experimental data documenting the pathogenic mechanism of the mutation. We therefore sought to obtain direct evidence of an effect of this putative mutation on splicing.

The results of the oligonucleotide hybridisations are shown in figs 2B–E. Whereas the normal control sample only showed the expected exon IIc splice product (fig 2B, lane N), two additional shorter products were identifiable in the patient sample. DNA sequencing and hybridisation to specific oligonucleotides showed that one of these arose from direct splicing of exon IIIa to exon 12 (fig 2E), whereas the other utilised a cryptic splice site within exon IIc (5’-CGGgaatt-3’, new intronic nucleotides in lower case) (figs 2B, C). This cryptic splice site was previously known, both because it is

Abbreviations: FGFR2, fibroblast growth factor receptor type 2; RT-PCR, reverse transcriptase PCR; SS, Shapiro-Senapathy
activated by the pathogenic synonymous substitution 1032G→A in exon IIIc (Ala344Ala mutation), creating the donor splice site 5'-CAGgttaatt-3'. Because the wild-type cryptic sequence is utilised in a mutant allele with a 6 nucleotide insertion that destroys the normal 5' splice site, there was no evidence for ectopic expression of the alternatively spliced IIIb exon (fig 2D). The activation of the cryptic splice site by the 1084+3A→G mutation initially seemed surprising, because the consensus at +3 is either A or G, with A being slightly more prevalent than G in a large survey of 5' splice sites. However the SS score of splice site strength is somewhat lower for the mutated than the authentic splice site (75.39 wild-type against 72.25 mutated), and the difference is accentuated by several other methods used to calculate splice site strength (table 1). Interestingly, by two of these methods (SS and DG), the cryptic IIIc splice site is actually stronger than the authentic one. This suggests that only a mild reduction in strength of the wild-type site, as occurs in the case of the 1084+3A→G mutation, is sufficient to tip the balance towards use of the cryptic splice site. This analysis also shows that the cryptic site is further strengthened by the synonymous 1032G→A mutation (table 1).

In the complex process of splice site recognition, the RNA sequence in U1 small nuclear ribonucleoprotein plays an important role by binding to the 5' splice site at the -2 to +6 positions. The effect of the 1084+3A→G mutation is to reduce complementarity of the authentic 5' site to the U1 sequence, whereas the 1032G→A mutation enhances complementarity of the cryptic site (see ΔG values in table 1). The consequence of both mutations is similar: the preferential synthesis of FGFR2 mRNA lacking 51 nucleotides in the IIIc exon, thus encoding a protein that lacks 17 amino acids. It has previously been noted that the 1032G→A mutation is associated with a milder phenotype than most FGFR2 mutations and, based on the clinical phenotype of the present family, this also appears to be the case for 1084+3A→G.

Ohno et al proposed that +3A→G mutations at 5' splice sites are pathogenic when both the +4, and one or other of the +5/+6 positions, are not complementary to U1 in the wild-type sequence; the authentic FGFR2 IIIc 5' splice site indeed obeys this rule (+4 and +5 are non-complementary; table 1).

In summary, our data elucidate the mechanism of the 1084+3A→G mutation and confirm its pathogenicity. Although a G at +3 is frequently recognised as part of the

---

Figure 1 Clinical features of the family. (A) Pedigree showing transmission of the phenotype through three generations. (B) Facial appearance of family members (from left to right) II-3, II-5 (unaffected), III-3, and II-2 (Photograph with family's permission).
splice consensus, in a small number of documented cases +3A is nevertheless essential to accomplish normal splicing.

ACKNOWLEDGEMENTS

We thank the family for their help with this study and N Elanko and A O’Rourke for undertaking the mutation analysis.

Authors’ affiliations

R Kan, S R F Twigg, A O M Wilkie, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK

R Kan, L Wang, F Jin, Institute of Genetics and Developmental Biology and Laboratory for Human and Animal Genetic Studies, Chinese Academy of Sciences, Beijing, P. R. China

Figure 2. Analysis of FGFR2 splicing. (A) Part of FGFR2 showing various splice forms and their detection by different oligonucleotides; note the obligatory physiological alternative splice between either the IIIb or IIIc exons. (B–E) Blot hybridisation analysis of RT-PCR products using primers 4F (exon 6) and 9R (exon 12). The originating RNA was extracted from whole blood from II-2 (heterozygous for the 1084+3A→G mutation) (P), whole blood from a normal control (N), and control keratinocytes (K). (B) Oligonucleotide SPL7/9 matches the IIIa-IIIc boundary and normally detects a 640 bp product. In the patient, an additional smaller product is detected. (C) This product (589 bp) specifically hybridises to the 9C/TM oligonucleotide, showing that it is generated by use of the cryptic 5’ splice site within exon IIIc. The larger fragments are heteroduplexes. (D) An oligonucleotide (SPL7/8) diagnostic for the 643 bp IIIb splice form does not hybridise to the patient lane, but is positive in a keratinocyte sample that characteristically expresses this alternative splice form. (E) The patient sample also expresses lower amounts of a smaller 292 bp product that is identified using SPL7/10 as a IIIa-exon 12 splice form. This product was not present in control cDNA, but has been observed previously and might have a physiological function. Size markers are HaeIII-digested φX174 DNA.
Table 1  Comparison of 5′ splice sites

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>ΔG</th>
<th>MAX</th>
<th>MDD</th>
<th>MM</th>
<th>NN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Authentic</strong> IIIc</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>t</td>
<td>a</td>
<td>t</td>
</tr>
<tr>
<td>1084→+3A→+G</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>t</td>
<td>a</td>
<td>t</td>
</tr>
<tr>
<td><strong>Cryptic</strong> IIIc</td>
<td>C</td>
<td>G</td>
<td>t</td>
<td>a</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td><strong>Mutant cryptic</strong> IIIc</td>
<td>C</td>
<td>G</td>
<td>t</td>
<td>a</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td><strong>Authentic</strong> IIIb</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td>t</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>U1 snRNA</td>
<td>3′−</td>
<td>G</td>
<td>U</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
</tbody>
</table>

SS, Shapiro and Senapathy matrix; ΔG, RNA duplex stability with U1 snRNA. (Turner energy rules: www.bioinfo.rpi.edu/~zukerm/ma/energy)11; MAX, Maximum entropy model; MDD, Maximum dependence decomposition model; MM, First order Markov model; (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)14; NN, Neural network splice site predictor. (www.fruitfly.org/seq_tools/splice).19

REFERENCES

Expression analysis of an \( FGFR2 \) IIIc 5′ splice site mutation (1084+3A \( \rightarrow \) G)

R Kan, S R F Twigg, J Berg, L Wang, F Jin and A O M Wilkie

\( J \) Med Genet 2004 41: e108
doi: 10.1136/jmg.2004.018507

Updated information and services can be found at:
http://jmg.bmj.com/content/41/8/e108

These include:

References
This article cites 19 articles, 2 of which you can access for free at:
http://jmg.bmj.com/content/41/8/e108#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections

- Molecular genetics (1254)
- Calcium and bone (307)
- Drugs: endocrine system (107)
- Epidemiology (630)
- Ethics (220)
- Genetic screening / counselling (887)
- JMG Online mutation reports (168)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/