Short Report

X-linked CHARGE-like Abruzzo–Erickson syndrome and classic cleft palate with ankyloglossia result from TBX22 splicing mutations


X-linked cleft palate (CPX) is caused by mutations in the gene encoding the TBX22 transcription factor and is known to exhibit phenotypic variability, usually involving either a complete, partial or submucous cleft palate, with or without ankyloglossia. This study hypothesized a possible involvement of TBX22 in a family with X-linked, CHARGE-like Abruzzo–Erickson syndrome, of unknown etiology. The phenotype extends to additional features including sensorineural deafness and coloboma, which are suggested by the Tbx22 developmental expression pattern but not previously associated in CPX patients. A novel TBX22 splice acceptor mutation (c.593−5T>A) was identified that tracked with the phenotype in this family. A novel splice donor variant (c.767+5G>A) and a known canonical splice donor mutation (c.767+1G>A) affecting the same exon were identified in patients with classic CPX phenotypes and were comparatively analyzed using both in silico and in vitro splicing studies. All three variants were predicted to abolish normal mRNA splicing and an in vitro assay indicated that use of alternative splice sites was a likely outcome. Collectively, the data showed the functional effect of several novel intronic splice site variants but most importantly confirms that TBX22 is the gene underlying Abruzzo–Erickson syndrome, expanding the phenotypic spectrum of TBX22 mutations.

Conflict of interest

None for any author.

Mutations in the TBX22 gene are well established as the cause of X-linked cleft palate (CPX) with ankyloglossia (MIM 303400) (1–3), as well as contributing to the prevalence of isolated cleft palate (4, 5). More rarely, other craniofacial anomalies including cleft lip and hypodontia have also been connected to TBX22 variants (6). Within multiple-affected families carrying the same mutation, the phenotypic spectrum can vary from asymptomatic females to males or females with a bifid uvula, a cleft of the soft palate, or a complete cleft of the hard and soft secondary palate, along with ankyloglossia (7, 8). The development of an animal model lacking Tbx22 highlighted the phenotype of submucous cleft palate, which is present in about half of male patients carrying a TBX22 mutation (9). Tbx22null mice also display choanal atresia, which suggests a possible developmental role in the formation of the choanae. This, along with the finding that normal Tbx22 gene expression specifically extends to the developing eye and otic vesicle (2, 10), led us to speculate...
about a possible overlap with CHARGE syndrome, where patients frequently have orofacial clefts and/or choanal atresia among other variable features (11). Approximately 60-70% of patients diagnosed with CHARGE syndrome are found to harbor mutations in the CHD7 gene (12, 13).

In this study, we investigated a CHD7-negative family that overlapped with a number of the characteristic features of CHARGE syndrome (14, 15). All three of the affected males exhibited secondary palate defects. One had a high arched palate and a bifid uvula, while the two brothers both received surgical repair for overt cleft palate. Other shared features indicative of CHARGE were large ears, flat malar configuration and sensorineural hearing impairment, while the two brothers also had unilateral iris coloboma (15). This family shows an apparent X-linked inheritance pattern, and their condition has been described as Abruzzo–Erickson syndrome (MIM 302905). Screening for TBX22 sequence variants identified a single, unique variant in close proximity to the TBX22 exon 4 splice acceptor site. Our ongoing screening program of unrelated families identified two further splice site variants, both within the putative TBX22 exon 4 splice acceptor site. Here, we investigated the likely functional impact of these mutations using in silico and in vitro splicing assays. We conclude that these mutations significantly affect correct splicing and are thus causative of the defects observed in both classic and atypical families.

### Methods

**Patient information, DNA samples and sequencing**

Blood and/or saliva samples were obtained from patients for DNA analysis with informed consent and ethical approval. DNA was isolated from peripheral blood leukocytes or saliva of the patients and consenting family members. Variants were compared to previously sequenced control DNA samples (4) and to an additional set of well-characterized, unaffected white European male and female samples, totaling 248 X-chromosomes. All eight TBX22 coding exons were amplified and sequenced as previously described (1).

**Splicing bioinformatics and in vitro analysis**

Bioinformatics analysis of splice variants was performed using NetGene2 (http://www.cbs.dtu.dk/services/NetGene2) (16), NNSPLICE 0.9 (http://www.fruitfly.org/seq_tools/splice.html) (17) and MaxEntScan (http://genes.mit.edu/burgelab/MaxEnt/XMaxEntScan_scoreseq_acc.html) (18). Constructs containing TBX22 exon 4, including 125 bp upstream and 250 bp downstream, with or without intronic variants were amplified from control and patient DNA samples, then cloned into the NdeI site of the pSVED minigene (19), a kind gift from Dr. Baralle. Constructs were transfected into HEK293T cells using FuGene reagent (Roche Diagnostics, Burgess Hill, UK) according to the manufacturer’s instructions. RNA was isolated after 48 h using Trizol (Life Technologies, Paisley, UK). Following reverse transcription polymerase chain reaction (RT-PCR) (19), cDNA was cloned into pGEM-T-easy (Promega, Southampton, UK) and sequenced using the T7 primer.

### Results

Mutations in CHD7 were previously excluded in the family with Abruzzo–Erickson syndrome (unpublished results), prompting us to perform complete sequencing of the TBX22 coding and flanking intronic regions (Seq: NG_008998). This identified a single, unique sequence variant within intron 3 (c.593–5T>A: cDNA seq: NM_001109878). The variant was present in the two affected brothers, the affected uncle and was heterozygous in the mother (Fig. 1A). This sequence lies within the conserved splice acceptor consensus sequence for exon 4 and was not present in single nucleotide polymorphism database (dbSNP) or in control chromosomes ($n = 539$).

In a recent screen of new patients with classic CPX phenotypes, two further families were identified with putative splice site mutations involving the exon 4 splice donor sequence. One family carried a c.767+1G>A mutation, (IVS4+1G>A) that was also identified in a previously reported family (4), with later confirmation of a relationship between these two families (Fig. 1B). The proband of the new family branch has a submucous cleft palate, ankyloglossia, speech and language delay and left sided Eustachian tube dysfunction. The mother had ankyloglossia, which is widely seen in the extended family, in which the affected males also present with a submucous or soft palate cleft (4). The same sequence variant was independently identified in a Brazilian family that had been studied during the original identification of TBX22 in CPX (1). The third putative splice variant (c.767+5G>A) was identified in a sporadic patient where the male proband presented with a soft palate cleft and significant ankyloglossia (Fig. 1C). Both variants were not present in dbSNP or in control chromosomes ($n = 539$). These findings prompted us to perform bioinformatics and in vitro assays to investigate their likely functional effect on splicing, and to assess this mechanism as a likely cause underlying each patient’s phenotype.

The c.767+1G>A substitution occurs at the invariant G of the canonical splice donor site, therefore was expected to disrupt normal splicing and act as a positive control in these studies. Three different prediction algorithms working on slightly different principles were used. NetGene2 and NNSplice0.9 search a target sequence for potential sites and assign a strength score to those found (16, 17), while MaxEntScan, assigns a strength score to a given splice site sequence (18). As expected, the c.767+1G>A variant abolished the normal splice site. Interestingly, both NetGene2 and NNSplice0.9 predict a de novo splice site nearby at position c.767+5G (Table 1). MaxEntScan shows a 10-fold reduction to the assigned strength score.

### X-linked Abruzzo–Erickson syndrome

Approximately 60-70% of patients diagnosed with CHARGE syndrome are found to harbor mutations in the CHD7 gene. Mutations in CHD7 were previously excluded in the family with Abruzzo–Erickson syndrome (unpublished results), prompting us to perform complete sequencing of the TBX22 coding and flanking intronic regions (Seq: NG_008998). This identified a single, unique sequence variant within intron 3 (c.593–5T>A: cDNA seq: NM_001109878). The variant was present in the two affected brothers, the affected uncle and was heterozygous in the mother (Fig. 1A). This sequence lies within the conserved splice acceptor consensus sequence for exon 4 and was not present in single nucleotide polymorphism database (dbSNP) or in control chromosomes ($n = 539$).
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Fig. 1. Pedigrees of the affected families and identification of TBX22 sequence variations. (a) Abruzzo–Erickson syndrome family with sequence traces showing: top – c.593−5T>A hemizygous affected brothers and uncle, middle – heterozygous mother, bottom–unaffected control. (b) Multiplex family with CPX showing new branch (boxed), with sequence traces showing: top – c.767+1G>A affected son, middle – heterozygous mother, bottom – unaffected son. (c) Sporadic patient with CPX with sequence traces showing: top – c.767+5G>A affected male, bottom–unaffected control.

The c.593−5T>A variant was predicted to abolish the acceptor splice site by NetGene2, although the natural acceptor has a weak score in comparison to the donor site. The other programs reflect a modest change to the acceptor sequence. This correlates with the fact that while thymine is the preferred nucleotide in this position, the consensus sequence tolerates some variation. At the donor site, the c.767+5G>A variant has a strong negative effect with each of the programs used (Table 1).

Each variant was then functionally tested using an in vitro mRNA splicing assay (19). This was performed using wild-type (WT) and mutant variants within a 551 bp genomic fragment containing TBX22 exon 4 and partial flanking introns cloned into the minigene vector pSVED (Fig. 2A). Upon correct splicing, these constructs generate a 471 bp cDNA fragment when amplified with vector exon primers, whilst clones without the TBX22 exon generate a 296 bp fragment (Fig. 2B). As previously reported using this assay, this latter fragment was present at a lower intensity in all of the TBX22 exon 4 constructs, indicating that a proportion of transcripts skip the inserted exon regardless of sequence context (20). The WT construct generated the expected 471 bp fragment, as well as a predominant slightly larger and several less intense, smaller bands (Fig. 2B). Surprisingly, the band pattern for c.767+1G>T was broadly similar to WT, while the 471 bp fragment was greatly reduced in intensity or absent for the other mutant constructs.

To directly investigate the splicing products, RT-PCR was performed using primers designed to specifically interrogate either the upstream acceptor or downstream donor regions. For the acceptor splice site (Fig. 3A), three predominant fragments were detected in the WT and donor mutation constructs but only the two upper fragments for the acceptor mutation. Sequence analysis revealed that the smallest fragment corresponded to the correctly spliced exon 4. The two larger fragments (alt1 and alt2) represent alternate splicing products using two different vector-specific cryptic splice sites. The c.593−5T>A mutation leads to a significant increase in the most upstream cryptic splice site product (alt1). The frequency of the different splicing products recovered after clonal analysis is given in Table 2. It is therefore likely that the c.593−5T>A mutation weakens or abolishes the WT acceptor splice site of exon 4, as predicted by the bioinformatics analysis.
Table 1. Effect of sequence variants as predicted by web-based bioinformatics splice site prediction programs

<table>
<thead>
<tr>
<th>Sequence variant</th>
<th>Splice site</th>
<th>MaxEntScan</th>
<th>NetGene2</th>
<th>NNSplice 0.9</th>
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<td></td>
<td></td>
<td>Wild-type</td>
<td>Mutant</td>
<td>Wild-type</td>
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<tr>
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<tr>
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<td>8.98</td>
<td>-2.06</td>
<td>0.95</td>
</tr>
</tbody>
</table>

*a De novo splice site detected at c.767+5G with score of 0.88.

*b De novo splice site detected at c.767+5G with score of 0.68.

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**Discussion**

We report the identification of a *TBX22* mutation predicted to disturb normal splicing of a canonical splice acceptor site in a family with the CHARGE-like Abruzzo–Erickson syndrome (14, 15). Two other splice site variants involving the same exon were detected in new CPX families, one of which alters the canonical splice donor site (+1G>A) of the same exon. This variant abolishes normal splicing, providing an...
We previously reported a splice site mutation affecting TBX22 exon 6 (c.1006+1G>T; IVS6+1G>T) which positional cloning and segregation analysis showed to be the causal mutation in a large Icelandic kindred (1). It was speculated that c.1006+1G>T would cause aberrant splicing with the introduction of a premature stop codon, resulting in rapid removal of the transcript by nonsense-mediated decay. TBX22 transcripts were detected in a maternal carrier but not her affected son using lymphoblastoid cell lines (1). However, it is not known if adult transformed lymphocytes are representative of TBX22 expression during fetal palate development, which has a highly restricted spatiotemporal pattern (2). Here, we chose to use a combination of in silico and in vitro analysis, finding a strong correlation between the two independent methods. All three programs accurately detected the known natural splice acceptor and donor sites and all predicted that the +1G>A variant would abolish splicing. NetGene2 and NNSplice 0.9 also predicted
TBX22 mutations. The classic loss-of-function phenotype associated with possibly a non-functional protein, which gives rise to an altered ORF and therefore protein. The other mutation, possibly by using an alternative splice site, − be that the c.593 mutation with other features attributable to some other co-inherited mutation. It would therefore be helpful to be able to explain other key clinical features in relation to TBX22. One possible factor may be selective bias, where patients exhibiting CHARGE-like phenotypes are simply not investigated during embryonic development is supportive of a factor gene Tbx22null, such as those involving specific tissues. A better understanding of specific TBX22 mutations and their involvement in a wider phenotypic spectrum will have important implications for genetic counseling.

## Supporting Information

The following Supporting information is available for this article: Fig. S1. Expression of Tbx22 in E10.5 and E11.5 mouse embryos. Expression was investigated using wholemount in situ hybridization in the whole embryo (a) and the dissected head (b) using an antisense Tbx22 probe essentially as described in Pauws et al. (9). e, eye; man, mandible; max, maxilla; ov, otic vesicle.

Additional Supporting information may be found in the online version of this article.

## Acknowledgements

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## References


