ARTICLE

Gross deletions in *TCOF1* are a cause of Treacher–Collins–Franceschetti syndrome

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Treacher–Collins–Franceschetti syndrome (TCS) is an autosomal dominant craniofacial disorder characterised by midface hypoplasia, micrognathia, downslanting palpebral fissures, eyelid colobomata, and ear deformities that often lead to conductive deafness. A total of 182 patients with signs consistent with a diagnosis of TCS were screened by DNA sequence and dosage analysis of the *TCOF1* gene. In all, 92 cases were found to have a pathogenic mutation by sequencing and 5 to have a partial gene deletion. A further case had a novel in-frame deletion in the alternatively spliced exon 6A of uncertain pathogenicity. The majority of the pathogenic sequence changes were found to predict premature protein termination, however, four novel missense changes in the LIS1 homology motif at the 5' end of the gene were identified. The partial gene deletions of different sizes represent ~5.2% of all the pathogenic *TCOF1* mutations identified, indicating that gene rearrangements account for a significant proportion of TCS cases. This is the first report of gene rearrangements resulting in TCS. These findings expand the *TCOF1* mutation spectrum indicating that dosage analysis should be performed together with sequence analysis, a strategy that is predicted to have a sensitivity of 71% for patients in whom TCS is strongly suspected.

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INTRODUCTION

Treacher–Collins–Franceschetti syndrome (TCS) is a rare autosomal dominant congenital disorder of craniofacial morphogenesis that occurs with an estimated prevalence of 1/50 000 live births. The syndrome is characterised by bilaterally symmetrical craniofacial abnormalities. The prominent clinical features are malar hypoplasia caused by hypoplasia of the zygomatic complex, mandibular hypoplasia, downslanting palpebral fissures, colobomata of the lower eyelids, and ear malformations often associated with bilateral conductive hearing loss. TCS has extreme inter- and intra-familial phenotypic variability, which ranges from perinatal death owing to a compromised airway to a phenotype that goes undetected by medical examination.¹ Penetrance is thought to be high but non-penetrance has been reported.²

Deleterious mutations in *TCOF1* (OMIM *606847) are associated with TCS type 1 (OMIM #154500).³ The longest transcript of *TCOF1* comprises 26 exons, which include the alternatively spliced exons 6A and 19.⁴ Other minor splice isoforms include transcripts lacking exon 6A, transcripts lacking exon 19 and transcripts that include a novel exon 16A.⁴ *TCOF1* encodes a low complexity, serine/alanine-rich protein called treacle, which has unique N and C termini and a large central repeat domain containing motifs shared with nucleolar trafficking proteins.⁵ Treacle is therefore thought to be a nucleolar phosphoprotein involved in ribosomal DNA gene transcription.⁶ The C-terminal region of treacle is important for localisation to the nucleolus^{7,8} and the N-terminal region contains a LIS1 homology

motif that may contribute to the regulation of microtubule dynamics, either by mediating dimerisation, or else by binding cytoplasmic dynein heavy chain or microtubules directly.⁹ As the structures affected in TCS patients arise from the first and second branchial arches during early embryogenesis,¹⁰ the abnormal development due to treacle haploinsufficiency may be caused by inhibition of production of properly modified mature ribosomal RNA (rRNA) as well as inhibition of rRNA gene transcription in the pre-fusion neural folds during the early stages of embryogenesis, consequently affecting proliferation and proper differentiation of these embryonic cells.¹¹ The recent identification of mutations in genes encoding subunits of RNA polymerases I and III (*POLR1D* and *POLR1C*) in a small subset of patients with TCS, confirm that TCS is a ribosomopathy and genetically heterogeneous.¹²

Mutations have been identified throughout the *TCOF1* gene and the vast majority identified to date are unique to the family studied and introduce a premature termination codon; missense mutations are rare and confined to the 5' end of the gene (http://genoma.ib.usp.br/TCOF1_database and references therein). The literature suggests that 62–82% of patients diagnosed with TCS have the diagnosis confirmed by sequence analysis of *TCOF1*, *POLR1D* or *POLR1C*,^{1,2,12,13} however, the aetiology of the remainder remains uncertain. Gross deletions or other rearrangements of *TCOF1* cannot be ruled out as they have only been investigated in one study by Splendore *et al*,¹⁴ who found no alterations in seven patients. This study looks at a larger cohort of 182 subjects using sequencing and MLPA.

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PATIENTS AND METHODS

Patients

The cohort of patients studied included 182 unrelated individuals with clinical features of TCS referred to the Oxford Regional Clinical Molecular Genetics laboratory between May 2005 and June 2011. A review of the referral information by an experienced dysmorphologist indicated that 119 of these cases were strongly suspected to have TCS, whereas 55 cases had a less secure diagnosis. In eight cases insufficient clinical information was available to make an assessment (Table 1). Patients were sequenced initially, and dosage analysis then carried out on those cases in whom no definitely pathogenic mutation could be found. Where available, parents were also analysed to determine if variants had arisen *de novo*.

PCR and sequencing

The 27 coding exons (including exons 6A, 16A and 19)⁴ were amplified in 28 fragments (primer sequences and reaction conditions given in Supplementary Table 1). PCRs were purified using paramagnetic beads (Agencourt: Beckman Coulter plc, High Wycombe, UK), then sequenced using BigDye v3.1 chemistry (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Fragments were separated and analysed on an ABI3730 using Sequencing Analysis 5.2 software (Applied Biosystems). Mutation Surveyor software (Soft-Genetics, State College, Philadelphia, PA, USA) was used to identify sequence variants, and *in silico* splice prediction software used to predict alterations to splice sites (Alamut version 1.5 or 2.0, Interactive Biosoftware, Rouen, France). Nomenclature for sequence variants is in accordance with HGVS guidelines (www.hgvs.org) using GenBank NCBI reference sequence NM_001135243.1 (this includes exon 6A, but excludes exon 16A), as recommended by Splendore *et al.*¹⁴

MLPA

The P310-A1 or P310-B1 TCOF MLPA kit (MRC-Holland) was used according to the manufacturer's recommendations, and the reactions analysed using a 3730 automated analyser and GeneMapper software (Applied Biosystems). Statistical analysis of the data was carried out using a MLPA data analysis spreadsheet devised by the National Genetics Reference Laboratory, Manchester, UK (<http://www.ngrl.org.uk/Manchester>), with results presented as a bar chart. The P310-A1 and P310-B1 kits target exons 1–7, 9–18 (including exon 6A) and 21–26 (exon 26 is untranslated). Although several probes were designed for exons 19 and 20 none were robust enough for inclusion in these kits, however, there are only 2.8 kb between the flanking probes in exons 18 and 21. Exon 16A is also not targeted directly, but a probe-pair hybridises to sequence 760 nucleotides after the last base of this exon. The P310-A1 kit also contained a probe pair targeting exon 8, but due to problems with reliability it has been removed from the later B1 version of the kit.

Quantitative PCR

Quantitative PCR (qPCR) was performed using the SYBR Green detection method and an ABI Prism 7500 Sequence Detection System (Applied

Biosystems). All amplicons were 100–120 nucleotides in length (primer sequences given in Supplementary Table 1). Reactions were amplified in 20 µl volumes using 1x SYBR Green mastermix (Applied Biosystems), 0.2 µM of each primer and 10 ng genomic DNA. PCR conditions were as follows: 2 min initial step of 50 °C then denaturation for 10 min at 95 °C followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, ending with a final dissociation. The Sequence Detection System software (SDS version 1.2; Applied Biosystems) was used to analyse the qPCR data. The copy number was estimated using the comparative $\Delta\Delta C_t$ method where a normal control has a ratio of one.¹⁵

Array CGH (aCGH)

The TCOF1 gene region was targeted using a custom 180K oligoarray (Agilent Technologies, Wokingham, UK. Details on request). The array includes four probes in TCOF1 as well as probes in surrounding genes, including one in CD74. Analysis was carried out on a G2505B/G2539A microarray scanner (Agilent Technologies) that scans at 2 µm resolution.

RESULTS

Sequencing analysis

A total of 79 different pathogenic sequence variants (60 novel) were identified in 92 unrelated individuals, spread throughout the gene (Table 2). Of these, 59 are predicted to disrupt protein translation by introducing premature stop codons, or by abolishing the translation-start site. Four further missense changes in exon 1 (p.(Ile14Met), p.(His17Arg), p.(Ala21Pro), p.(Ala27Thr)) alter highly conserved amino acids in the LIS1 homology domain. In addition, two of these missense variants have arisen *de novo*. Fourteen further variants were predicted to disrupt splicing. Two variants, c.4366_4369delGAAA and c.4362_4365delAAAA, predict a frameshift that results in the replacement of the final 33 amino acids with 117 missense amino acids. No pathogenic sequence variants were detected in the alternatively spliced exon 16A; however, an in-frame novel deletion of 18 nucleotides (c.827_844del, p.(Gly276_Glu281del)) was detected in exon 6A in a case with extensive family history. The pathogenicity of this variant is currently uncertain.

A total of 27 other variants (20 novel) were identified during the screening of the cohort (Table 3). One of the novel variants could be classified as a non-pathogenic single-nucleotide polymorphism (SNP) on the basis that it was seen in multiple individuals. Five further unvalidated variants already recorded in dbSNP could be assigned as non-pathogenic from the data (Table 3). The other novel variants, however, could not be definitively classified as non-pathogenic SNPs, but were considered unlikely to be pathogenic. This was supported by parental studies in five cases.

MLPA analysis

MLPA analysis identified partial gene deletions of TCOF1 in five patients (Figure 1). Patients 170 and 96 were shown to have deletion of a single MLPA probe, located in exons 1 and 26 (which encodes 3'-UTR), respectively. Reduced hybridisation due to SNPs under the probe-binding sites was ruled out by sequencing. Validation of these results for patients 170 and 96 was carried out by qPCR (see below). The three other patients showed reduced hybridisation to multiple adjacent probes consistent with a contiguous deletion of exons 1–15 (patient 452), exons 23–26 (patient 8) and exons 1–6 (patient 226).

Quantitative PCR

For patients 170 and 96, qPCRs were carried out to determine the extent of the deletion and to provide confirmation of the MLPA result (Figure 2). In-patient 170, data showed that the deletion encompassed the translation initiation signal, as well as exon 1 coding sequence. The deletion in patient 96 was shown to extend beyond the validated

Table 1 Numbers of unrelated patients tested broken down by clinical phenotype and mutation status

	Patients strongly suspected to have TCS	Patients with a less secure diagnosis of TCS	Patients with insufficient clinical information	TOTAL
Likely pathogenic variant identified by sequencing	80	11	1	92
Deletion identified by MLPA	4	1	0	5
Unclassified variant	1 ^a	0	0	1
No mutation identified	34	43	7	84
TOTAL	119	55	8	182

Abbreviation: TCS, Treacher–Collins–Franceschetti syndrome.

^aThe in-frame deletion found in exon 6A lies over the MLPA probe-binding site, so this also appears as a single exon deletion on MLPA. This patient has not been included in the mutation pick-up rate calculations.

Table 2 Pathogenic mutations identified in *TCOF1* by sequence analysis of the coding regions and exon–intron boundaries

Patient IDs	Location	<i>TCOF1</i> variant ^c	Predicted effect	No. of cases (inheritance)	Previous reports
423	Exon 1	c.1A>T	p.Met1?	1	
300	Exon 1	c.3G>A	p.Met1?	1 (<i>De novo</i>)	2
443	Exon 1	c.42C>G	p.Ile14Met	1 (Paternal)	
67	Exon 1	c.50A>G	p.His17Arg	1 (<i>De novo</i>)	
125	Exon 1	c.61G>C	p.Ala21Pro	1 (<i>De novo</i>)	
270	Exon 1	c.79G>A	p.Ala27Thr	1	
643	Exon 1	c.87delA	p.Val30*	1	
104	Intron 1	c.109-2A>T	Splice	1	
142	Exon 2	c.159G>A	p.Trp53*	1 (<i>De novo</i>)	
458	Exon 2	c.163C>T	p.Gln55*	1 (<i>De novo</i>)	19
1 and 44	Intron 3	c.304+5G>C	Splice	2	13, 18
291	Exon 4	c.357_358delAA	p.Ser120fs	1	
247	Exon 5	c.405_406delTG	p.Gly136fs	1	23
710	Intron 5	c.565+1G>A	Splice	1	
631	Intron 5	c.566-10C>A ^a	? Splice	1	
723	Exon 6	c.618delC	p.Ser207fs	1	
731	Exon 6A	c.827_844del ^b	p.Gly276_Glu281del	1	
123	Exon 7	c.1016C>G	p.Ser339*	1	
262	Exon 8	c.1096G>T	p.Gly366*	1 (Paternal)	
461	Exon 9	c.1303delC	p.Gln435fs	1 (<i>De novo</i>)	
573	Exon 9	c.1446dupA	p.Asp483fs	1	1, 23
132	Exon 9	c.1473dupC	p.Met492fs	1 (<i>De novo</i>)	
514	Exon 10	c.1557dupA	p.Gly520fs	1	3
198	Exon 10	c.1639_1640delAG	p.Ser547fs	1	1, 5, 34
24	Exon 10	c.1702C>T	p.Gln568*	1	
40	Exon 11	c.1729C>T	p.Gln577*	1	
194	Exon 11	c.1782_1788del7	p.Val595fs	1 (<i>De novo</i>)	
99	Exon 11	c.1813_1814delAT	p.Met605fs	1 (<i>De novo</i>)	
666	Exon 11	c.1837delG	p.Glu613fs	1 (<i>De novo</i>)	
393	Exon 12	c.1907delT	p.Leu636fs	1	
286	Exon 12	c.1952_1956delCTGCA	p.Thr651fs	1 (<i>De novo</i>)	
113	Exon 12	c.1993delG	p.Ala665fs	1	
400	Exon 12	c.1999dupC	p.Arg667fs	1 (<i>De novo</i>)	2
23	Exon 12	c.2065_2075del11	p.Pro689fs	1	
487	Exon 12	c.2098_2099delGA	p.Asp700*	1	35
476	Exon 12	c.2099_2102delATAG	p.Asp700fs	1	2
539	Exon 12	c.2103_2106delTGAG	p.Ser701fs	1 (Maternal)	1
515	Exon 13	c.2157dupG	p.Lys720fs	1 (<i>De novo</i>)	1
53	Exon 13	c.2164delC	p.Leu722fs	1	
74	Exon 13	c.2167C>T	p.Gln723*	1	
261	Exon 13	c.2285_2286delCT	p.Ser762*	1 (<i>De novo</i>)	
250	Exon 13	c.2287G>T	p.Glu763*	1 (<i>De novo</i>)	
586	Intron 13	c.2341-2A>G	Splice	1 (<i>De novo</i>)	
280	Exon 14	c.2426delC	p.Pro809fs	1 (<i>De novo</i>)	
569	Exon 14	c.2473_2476delTCCA	p.Ser825fs	1 (<i>De novo</i>)	
129	Intron 14	c.2478+5G>C	Splice	1 (<i>De novo</i>)	
78	Exon 16	c.2683C>T	p.Gln895*	1	2
263	Exon 16	c.2713G>T	p.Glu905*	1 (<i>De novo</i>)	
689	Exon 17	c.2962C>T	p.Arg988*	1	36
102	Exon 17	c.2969C>A	p.Ser990*	1	
363	Intron 17	c.3046+1G>A	Splice	1	
7	Intron 17	c.3047-2A>T	Splice	1	
345	Intron 17	c.3047-1G>A	Splice	1	
368	Exon 18	c.3112delG	p.Ala1038fs	1 (<i>De novo</i>)	1
693	Exon 18	c.3156C>T ^a	? Splice	1	
4 and 183	Exon 18	c.3163C>T	p.Gln1055*	2	
20	Exon 18	c.3183G>A ^a	? Splice	1	
160	Exon 20	c.3314delG	p.Gly1105fs	1	
203	Intron 21	c.3600+1delG	Splice	1 (<i>De novo</i>)	
381	Exon 22	c.3611C>G	p.Ser1204*	1	

Table 2 (Continued)

Patient IDs	Location	TCOF1 variant ^c	Predicted effect	No. of cases (inheritance)	Previous reports
110	Exon 22	c.3613G>A ^a	? Splice	1 (Maternal)	
727	Exon 22	c.3745_3747delinsA	p.Pro1249fs	1	
438	Intron 22	c.3781+2dupT	Splice	1 (<i>De novo</i>)	
606	Exon 23	c.3823delC	p.Arg1275fs	1	
507	Exon 23	c.3853dupC	p.Gln1285fs	1 (<i>De novo</i>)	
90	Exon 23	c.3983_3986delAGAA	p.Lys1328fs	1 (Maternal)	
358	Exon 23	c.4127_4128delCT	p.Ser1376fs	1	
416	Exon 23	c.4131_4132delTT	p.Ser1378fs	1	
401	Exon 23	c.4177_4178delGA	p.Asp1393fs	1	17
537 and 353	Exon 23	c.4218dupG	p.Ser1407fs	2	1, 2, 35
249	Exon 23	c.4219delT	p.Ser1407fs	1	
122	Exon 23	c.4231C>T	p.Gln1411*	1	
234	Exon 23	c.4321delG	p.Glu1441fs	1 (<i>De novo</i>)	
56	Exon 23	c.4339_4340delAA	p.Lys1447fs	1	13, 17
534	Exon 23	c.4342A>T	p.Arg1448*	1	
714	Exon 24	c.4362_4365delAAAA	p.Glu1456fs	1	
741	Exon 24	c.4363_4365delinsTAG	p.Lys1455*	1	
209, 437	Exon 24	c.4365delA	p.Glu1456fs	2 (1 Paternal)	1, 17
678	Exon 24	c.4366_4369delGAAA	p.Glu1456fs	1	
46, 167, 208, 347, 374, 378, 398, 469, 519, and 620	Exon 24	c.4369_4373delAAGAA	p.Lys1457fs	10 (4 Maternal, 2 <i>de novo</i>)	1, 13, 17, 18, 22, 35

^aClassified as potentially pathogenic owing to suggested effects on splicing predicted by *in silico* analysis, but not confirmed experimentally – see Discussion.

^bUnclassified variant of uncertain pathogenicity.

^cNomenclature for sequence variants is in accordance with HGVS guidelines (www.hgvs.org) using GenBank NCBI reference sequence NM_001135243.1 (this includes exon 6A, but excludes exon 16A), as recommended by Splendore *et al.*¹⁴ Variants reported previously may have used an alternative nomenclature system.

polyadenylation signal within exon 26,¹⁶ removing a large part of the 3'-UTR. However, the deletion did not appear to encompass the intron 25 splice acceptor site.

aCGH

Karyotyping and aCGH analysis were carried out on the affected brother of patient 8, in whom the exon 23–26 deletion was also demonstrated by MLPA. The deletion was not visible by karyotyping. Of the aCGH probes, only one is known to be within the deletion as it lies in intron 25. Results for this probe were consistent with it being deleted. There was no evidence for deletion of adjacent probes, indicating that the deletion does not extend into the adjacent gene *CD74*.

DISCUSSION

Of the 182 unrelated diagnostic referrals, 92 were found to have a pathogenic sequence variant and 5 a pathogenic gene rearrangement (Table 1). These are the first gene rearrangements described in *TCOF1*. The five deletions constitute 5.2% of all the pathogenic variants that were detected in *TCOF1* in this study. Of the patients with sequence changes, 57% had a frameshifting small deletion or insertion, or a mutation disrupting the start codon, 23% had a nonsense mutation, 16% had a variant predicted to affect splicing, and 4% had a pathogenic missense mutation (Table 4). This spectrum is similar to that described in previous studies that have shown that the majority of mutations in TCS1 lead to loss of protein function consistent with a mechanism of haploinsufficiency (http://genoma.ib.usp.br/TCOF1_database and references therein). The vast majority of mutations identified were only observed in one family consistent with previous studies that have shown that the majority of disease-causing mutations in *TCOF1* are private. The exceptions were c.304+5G>C, c.3163C>T (p.(Gln1055*)), c.4218dupG (p.(Ser1407fs)), and c.4365delA

(p.(Glu1456fs)), each found in two patients, and c.4369_4373delAAGAA (p.(Lys1457fs)) found 10 times. This last deletion has been reported several times previously (Table 1) and occurs within a repeat motif making it a mutational hotspot within the gene. Consistent with this hypothesis was the finding that this deletion had arisen *de novo* in two of the cases. Stratifying the mutation data by clinical phenotype (Table 1) indicated that overall pathogenic point mutations were identified in 51% of all referrals; this was increased to 68% if only those cases with a high clinical suspicion of TCS were included. Including MLPA data as well increased sensitivity to 71% in this group.

Of the missense variants that were identified four could be categorised as being likely to be pathogenic based on their location at highly conserved residues within the LIS1 homology domain, where other pathogenic missense changes have been reported.^{1,13,17–19} Patient 443, who presented with micrognathia, deficient zygomatic arches, minor downslant of palpebral fissures, only rudimentary condyles, but no obvious hearing loss, was found to have c.42C>G (p.(Ile14Met)). Another variant at this residue, p.(Ile14Phe), has been reported previously in association with TCS.¹⁸ The equivalent residue to p.Ile14 in the Lis1 protein is important in forming hydrophobic contacts between Lis1 homodimers.²⁰ Parental analysis indicated that the p.(Ile14Met) variant had been inherited from the clinically unaffected father, however, such phenotypic variability of mutations, even within families has been reported previously.² Patient 67, who has a clinical diagnosis of TCS, was found to have c.50A>G (p.(His17Arg)). The equivalent residue in the Lis1 protein is important for the forming of both hydrophobic contacts and hydrogen bonds between Lis1 homodimers,²⁰ and analysis of parental samples indicated that the mutation had arisen *de novo*. Patient 125, who had features suggestive of TCS, was found to have c.61G>C (p.(Ala21Pro)). Parental analysis indicated that it had arisen

Table 3 Novel unclassified variants, and variants considered likely to be non-pathogenic, identified in *TCOF1* during sequencing analysis

Location	RefSNP ID	Unclassified variants ^a	Protein (predicted)	Comments
5'-UTR	ss411044913	c.-59G>A		Found in an unaffected parent of a TCS patient with a <i>de novo</i> pathogenic variant.
Intron 1	ss411046049	c.108+62_68del		Normal sequence is a tandem duplication of seven bases and this deletion removes the second seven bases. Not predicted to affect splicing.
Intron 1	ss411046377	c.109-28T>C		Not predicted to affect splicing. Patient also has the unclassified variants c.579G>A and c.2762C>T.
Exon 6	ss411046567	c.579G>A	p.Ala193Ala	Not predicted to affect splicing. Patient also has the unclassified variants c.109-28T>C and c.2762C>T.
Exon 7	ss411046734	c.1028G>A	p.Ser343Asn	Patient also has a pathogenic mutation in exon 4. Reported in another patient where it was maternally inherited. ²
Exon 8	ss411046903	c.1086G>A	p.Ala362Ala	Patient also has a pathogenic mutation. Parental testing indicated that this variant is <i>in cis</i> with the pathogenic variant.
Exon 8	ss411047058	c.1242C>T	p.Ser414Ser	Patient also has a pathogenic mutation. This variant was paternally inherited, whereas the pathogenic variant was <i>de novo</i> .
Exon 11	ss411047433	c.1854G>A	p.Ala618Ala	Not predicted to affect splicing.
Exon 12	ss411047596	c.1953C>T	p.Thr651Thr	Not predicted to affect splicing. Parental testing indicated that this variant is <i>in trans</i> with a pathogenic deletion also found in this patient.
Intron 12	ss411047777	c.2142+22C>T		Not predicted to affect splicing. Patient also has a pathogenic mutation. This variant was paternally inherited, whereas the pathogenic variant was <i>de novo</i> .
Exon 16	ss411047905	c.2762C>T	p.Pro921Leu	See discussion. Patient also has the unclassified variants c.579G>A and c.109-38T>C.
Intron 16	ss411048101	c.2859+26A>G		Not predicted to affect splicing. Patient also has the unclassified variant p.(Lys1460del).
Exon 16A	ss411048202	c.2919G>T	p.Arg973Ser	Found in a single patient who also has a <i>de novo</i> pathogenic mutation.
Exon 21	ss411048389	c.3528C>T	p.Pro1176Pro	Not predicted to affect splicing. Within same codon as the polymorphism rs1136103 (c.3527C>G, p.(Pro1176Arg)).
Intron 21	ss411048580	c.3600+64dupT		Not predicted to affect splicing.
Intron 22	ss411048723	c.3781+8A>G		Not predicted to affect splicing. Seen in two patients, one of whom has another pathogenic mutation.
Exon 23	ss411048875	c.3942A>C	p.Ser1314Ser	Not predicted to affect splicing.
Exon 23	ss411049004	c.4295_4296AT>GA	p.Asp1432Gly	Two nucleotide changes shown to be <i>in cis</i> . Residue not highly conserved between species.
Exon 24	ss411049198	c.4377_4379delGAA	p.Lys1460del	See discussion. Variant inherited from clinically unaffected mother. Patient also has the unclassified variant c.2859+26A>G.
Intron 24	ss411049364	c.4440+106G>T		Not predicted to affect splicing.
Location		Non-pathogenic SNPs ^a		Comments
Intron 3	ss411049542	c.305-52A>G		Found in three patients, including one with a pathogenic mutation; in this patient the variant was maternally inherited, whereas the pathogenic mutation was not (paternal sample not available). Maps to rs41287124 – minor allele frequency 0.8% (1000 genomes, North Americans).
Intron 6	ss411049643	c.640-69T>C		Seen in multiple patients.
Intron 7	ss411049778	c.1083+39G>A		Found in four unrelated patients including two with a pathogenic mutation. Maps to rs56113366 – minor allele frequency 12.5% (1000 genomes, North Americans).
Intron 8	ss411049884	c.1278+60G>C		Found heterozygously in five patients, including one with a pathogenic deletion on the other allele. Also reported as a polymorphism. ²
Exon 9	ss411047227	c.1281G>A, p.(Ala427Ala)		Reported previously in a patient with a pathogenic mutation ³⁵ . Maps to rs113299143 – minor allele frequency 3% (ESP cohort).
Intron 16	ss411050047	c.2860-27G>A		Found in three patients including one with a pathogenic mutation. Maps to rs112447402.
Intron 24	ss411050145	c.4440+108C>A		Found in three patients including two with a pathogenic mutation. Maps to rs111365835.

Abbreviation: SNP, single-nucleotide polymorphism.

^aNomenclature for sequence variants is in accordance with HGVS guidelines (www.hgvs.org) using GenBank sequence NCBI reference sequence NM_001135243.1 (this includes exon 6A, but excludes exon 16A), as recommended by Splendore *et al.*¹⁴ Variants have been submitted to dbSNP.

de novo. Finally, Patient 270, who had a clinical diagnosis of TCS, was found to have c.79G>A (p.(Ala27Thr)). Another variant at the preceding residue c.77C>T (p.(Ala26Val)) has been described previously and was apparently *de novo*.¹⁹ Parental samples were not available to determine if the newly identified variant was *de novo*.

A further variant, that was initially considered to be a missense change (c.3613G>A, p.(Gly1205Ser)), was classified as being likely to be pathogenic due to *in silico* predictions of aberrant splicing, combined with literature evidence. Patient 110 presented with symptoms suggestive of TCS comprising bilateral dysplastic ears and abnormal lower eyelids with sparse lower lid eyelashes. Clinical observations did not identify malar hypoplasia, and hearing is normal.

A sister is also affected. The exon 22 variant is predicted to alter a residue conserved across many vertebrate species, but is in the central repeat region of the protein where pathogenic missense changes have not been reported. However, a *de novo* variant at an adjacent nucleotide (c.3612A>C) has been reported²¹ that caused precise skipping of exon 22, causing a frameshift and predicted premature termination of translation. *In silico* analysis showed that the aberrant splicing could be due to disruption of an exonic splice enhancer.²¹ Analysis using splice site prediction software suggested that the c.3613G>A variant in patient 110 might also disrupt this exonic splice enhancer and therefore could also lead to aberrant splicing of exon 22. Unfortunately, an RNA sample was not available to test this

Figure 1 Dosage results for the five patients with a partial *TCOF1* deletion. A 50% relative bar height indicates a heterozygous deletion. MLPA detected a heterozygous deletion of: (452) exons 1–15; (170) exon 1; (96) exon 26; (8) exons 23–26; (226) exons 1–6. Grey bars on the left represent control probes, white bars represent probes located within or nearby to all *TCOF1*-coding exons except exons 19 and 20. The grey bar on the right represents the untranslated *TCOF1* exon 26 (3'UTR).

experimentally. Family studies indicated that the variant was also carried by the affected sister and had been inherited from the clinically unaffected mother.

Two further exonic variants were also considered likely to affect splicing. The first variant (c.3183G>A) affects the last base of exon 18. Substitution of this G with a T nucleotide has been reported

Figure 2 Schematic illustrating results of MLPA and quantitative PCR analysis of *TCOF1* in (a) 5'-UTR region in case 170 and (b) 3'-UTR region in case 96. Coding sequence (black boxes), UTR (hatched boxes) and introns (thin black lines). Black lines above the figure indicate the target sites for the MLPA probes and the amplicons for qPCR relative copy number calculated from the quantitative PCR assay in the respective patient and a deletion control are shown in the graphs above each amplicon. ATG is the translation initiation signal, TGA is the translation termination signal and AATAAA is the polyadenylation signal. The dark grey line below the figure indicates deleted sequence where probes/amplicons show 50% dosage; the light grey line indicates sequence that is not deleted where probes/amplicons show normal dosage. Grey dashed lines show regions of uncertainty. Not to scale.

Table 4 The number and proportion of cases with each pathogenic mutation type identified in *TCOF1* by sequencing

Mutation type	Number of patients	Frequency (%)
Frameshift/start codon	52	56.5
Nonsense	21	22.8
Splice	15	16.3
Missense	4	4.3
Total	92	

to cause skipping of exons 18 to 19 in another patient,²² and analysis using splice-site predictor software indicates that the G>A substitution would also reduce the splicing efficiency of the intron 18 splice donor site. The second variant (c.3156C>T), also within exon 18, is a synonymous change. However, splice prediction software indicates that it may create a cryptic splice donor site 29bp upstream of the actual intron 19 splice donor site. Unfortunately, RNA samples were not available to test these hypotheses.

An intronic variant, c.566-10C>A, was also identified in patient 631 that was considered likely to be pathogenic. *In silico* splice-prediction software indicated that this variant might abolish the usual intron 5 splice-acceptor site and replace it with a novel, cryptic site 8 nucleotides upstream. If used, this would lead to the inclusion of 8 nucleotides from intron 5 into the mRNA resulting in a translational frameshift. Although RNA and parental samples were not available to test this hypothesis a similar mechanism was proposed for another variant, c.2196G>A, that was confirmed by RT-PCR analysis.²³

Two other novel variants identified were considered less likely to be pathogenic, but could not be ruled out entirely as no other obviously pathogenic change could be identified. In the first case, patient 195 presented with microtia, atresia of the external ear canal, mild mandibular hypoplasia and a bifid uvula and was found to have c.2762C>T (p.(Pro921Leu)) in exon 16 of *TCOF1*. Pro921 is not highly conserved between vertebrate species and lies within the central repeat region of the protein, where missense mutations have not been reported. Splice-prediction software did not predict any effect on splicing, but without experimental evidence this cannot be ruled out. The second case, patient 18, was found to have a maternally inherited in-frame deletion, c.4377_4379delGAA (p.(Lys1460del)), in exon 24 of *TCOF1* within a lysine-rich region near the C-terminus of Treacle that is not highly conserved. This variant has been found in another TCS patient (*TCOF1* Mutation Database, http://genoma.ib.usp.br/TCOF1_database/) as have other similar variants considered non-pathogenic; p.(Lys1455_Lys1457del), found in a patient with another *de novo* pathogenic mutation,¹⁹ and p.(Lys1460dup), also found in a control sample.²

Of the patients with deletions, all had a clinical diagnosis of TCS. The full extent of each deletion is not known as they involved either the first or last exons and therefore it cannot be ruled out that the deletions extend beyond the *TCOF1* gene into other nearby genes. *TCOF1* maps to 5q32 in a gene dense region; 10 genes map within 0.5 Mb 5' of *TCOF1* and 9 genes map within 0.5 Mb 3' of *TCOF1* (<http://www.ensembl.org/>).

In three cases the deletions identified could extend beyond the 5'-UTR into upstream genes. In-patient 170 the deletion in exon 1 was shown to encompass the translation initiation signal and at least part of the 5'-UTR. She presented as an isolated case diagnosed clinically, however, parental samples were not available to determine whether this is a *de novo* rearrangement. Patient 452, who has a deletion encompassing exons 1-15, has mandibular hypoplasia, cleft palate, down-slanting palpebral fissures, wide set eyes and abnormal palmar creases. Testing of the parents showed that the deletion is apparently *de novo*. Patient 226 has a deletion encompassing exons 1-6. He presented with a clinical diagnosis of TCS and has an affected son who also carries the deletion.

Of the nearest upstream genes, *CDX1* has been shown to have a phenotypic effect when the orthologous gene is knocked out in mice. *CDX1* lies ~173 kb upstream of *TCOF1* and encodes caudal-type homeobox transcription factor 1. The caudal-type transcription factors interact with *HOX* genes to generate the anterior-posterior axis, and viable homozygous *cdx1* knockout mice were shown to have anterior homeotic transformations of vertebrae concomitant with posterior shifts of hox gene expression in the somatic mesoderm.²⁴ The effect of haploinsufficiency of this gene in humans is not known but altered precise temporal and spatial expression of genes such as the *HOX* genes during embryonic development is required for accurate skeletal modelling.²⁵ Unfortunately, material was not available for aCGH to identify the true extent of the deletions beyond the first exon in these patients.

In the other two cases the deletion extended into the 3'-UTR, and may therefore extend into downstream genes. Patient 8 presented with dysmorphic features consistent with a diagnosis of TCS, and was shown to have a deletion of exons 23–26, that removes the C-terminal region of treacle important for localisation to the nucleolus. Array CGH analysis suggested that the deletion did not extend into the downstream *CD74* gene. His brother and mother were shown to be heterozygous for the same deletion, but neither individual expresses the characteristic phenotypic features associated with TCS. Such phenotypic variability has also been reported with *TCOF1* point mutations.^{1,2} The deletion in patient 96, who presented with mild TCS, and his more severely affected brother, does not encompass any coding sequence but removes part of the 3'-UTR that contains the polyadenylation signal for the major transcript. This is a sequence motif recognised by RNA-binding factors and is essential for transcriptional termination and efficient polyadenylation of mRNAs and release of the polyadenylated mRNA for export from the nucleus.²⁶ Mutations affecting a polyadenylation signal and the secondary structure of the 3'-UTR of mRNA have been shown to cause translation de-regulation²⁷ and have been reported in other diseases.^{28–31} It is therefore predicted that deletion of this signal in *TCOF1* would lead to reduced accumulation of treacle, consistent with a mechanism of haploinsufficiency in TCS. Parental samples were not available.

The two closest distal genes to *TCOF1*: *CD74* and *RPS14*, lie ~2 and 43 kb downstream from *TCOF1*, respectively. *CD74* encodes the major histocompatibility complex II-associated invariant chain (CD74) that functions as an MHC class II chaperone and is required for B-cell development in mice.³² *RPS14* encodes the ribosomal protein S14. In humans, heterozygous somatic deletions of *RPS14* are thought to be the major cause of 5q- syndrome;³³ an acquired myelodysplastic syndrome subtype, characterized by a defect in erythroid differentiation.

No pathogenic *TCOF1* gene alterations were identified in 84 remaining patients in the cohort (Table 1). It is possible that pathogenic variants in introns and regulatory regions not covered by the sequencing assay, or deletions and inversions undetectable by sequencing and MLPA may exist in *TCOF1* in these patients, particularly those 34 with signs strongly suggestive of TCS. The MLPA kits P310-A1 and P310-B1 (MRC-Holland) that were used do not contain probes for exons 19 or 20, however, while there remains the possibility that patients in this cohort have an undetected deletion or duplication of either or both of these exons, the chance of this is low as the exons are within a short 2.8-kb region between the adjacent probes in exons 17 and 21. It is also possible that mutations in one or more other genes are responsible for the TCS phenotype in these patients, such as the *POLR1D* and *POLR1C* genes recently reported.¹²

In conclusion, these findings suggest that gene rearrangements are responsible for a significant proportion (5.2%) of TCS-associated mutations. The proximity of nearby genes likely to have a phenotypic effect if haploinsufficient predicts that any deletions in patients without unusual additional symptoms would be submicroscopic. It is therefore recommended that dosage analysis by MLPA or a comparable method should be undertaken as part of a *TCOF1* gene screening service. These data suggest that combined sequencing and dosage analysis using MLPA has a test sensitivity of around 71% for patients that are referred because TCS is strongly suspected.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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