TBX1 gene mutation screening in patients with non-syndromic Fallot tetralogy

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Fallot tetralogy (FT) is the most frequently observed conotruncal heart defect (CTHD) and accompanies 15% of the 22q11 deletion syndromes, DiGeorge/velocardiofacial (DGS/VCFS) syndromes. TBX1 is a gene located in the 22q11 region and has a role in neural crest migration and conotruncal development. The mouse Tbx1 locus shows 98% homology with TBX1. DGS/VCFS-like aortic arch abnormalities in the mouse were attributed to deletions in this locus. The T-box region, common to both mice and humans, is part of TBX1 with proven effects on heart outflow track anomalies. The role of TBX1 in non-syndromic CTHDs is still unclear. In this study, we screened the TBX1 gene T-box region exons in 50 FT patients without 22q11 deletion and in 50 healthy volunteers. Our study did not show any disease causing mutations, but one polymorphic change. These results do not support a major role of the T-box region in the etiology of isolated FT. Furthermore, this study also confirms that mouse cardiac-development study models do not always provide an explanation for human phenotype-genotype correlations.

Key words: tetralogy of Fallot, conotruncal cardiac anomaly, TBX1.

Congenital heart defects (CHDs) are a group of structural abnormalities that in humans have a combined incidence of approximately 1%. It is estimated that 4-5% of CHDs are associated with chromosome abnormalities, 1-2% are associated with single gene syndromes, and 1-2% are due to known teratogens, with the rest presumably determined in a multifactorial fashion[1]. Conotruncal heart defects (CTHDs) represent 15-20% of congenital heart diseases[2]. CTHDs share the morphological architecture of the presence of ventricular outflow tract anomalies with normally related great arteries and include tetralogy of Fallot (FT), pulmonary atresia with ventricular septal defect (PA-VSD), truncus arteriosus (TA) and interrupted aortic arch (IAA)[3].

Tetralogy of Fallot is the most prominent conotruncal defect and the most common form of cyanotic CHD, with an overall incidence of 10%. Approximately 15% of patients with tetralogy have a deletion of chromosome 22q11 (del22q11) in the setting of DiGeorge-velocardiofacial syndromes (DGS-VCFS)[4].

del 22q11 is the most frequent microdeletion found in man, with an incidence of 1 in 4,000 live births. Ninety percent of the patients have a ~ 3 Mb interstitial deletion, which is called the "typically deleted region"[5]. In 7% of the cases, deletion is 1.5 Mb shorter[6]. The studies with patients bearing the deletion and unbalanced translocation narrowed the critical region first to 250 kb (minimal DiGeorge critical region) and then to 150 kb (smallest region of overlap)[7]. The deletion can be seen sporadically along with 5-10% familiarity. Most of the patients share the same deletion without any change in size during inheritance. Nevertheless, various phenotypes among family members make it hard to establish a genotype-phenotype correlation. The deletion has been reported in association with >80 different birth defects and

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malformations occurring in many combinations and with widely differing severity. Mainly four distinct types are accepted: conotruncal anomaly dysmorphic face syndrome, DGS, VCFS, and strong syndrome. Cardiac anomalies are the most frequent (75%) manifestation followed by abnormal psychomotor development, otolaryngeal disorders, immunodeficiencies, hypocalcemia, craniofacial malformations, and some other rare anomalies[5,6]. In a series of 558 patients with 22q11 deletions, 409 (75%) had cardiac anomalies. The most common cardiac anomalies included FT (17%), VSD and IAA (14% each), pulmonary (10%), and TA (9%). Other anomalies included pulmonic stenosis, atrial septal defect, atrioventricular septal defect, and transposition of great arteries[8]. It was also reported that when the syndrome was described as conotruncal anomaly face syndrome, the most frequently associated heart defect was FT (74%)[9]. The abundance of conotruncal anomalies in del22q11 syndrome has led to the testing of this deletion in conotruncal cases. In two different studies – without separating cases as syndromic and non-syndromic – presence of conotruncal defects was reported as 17% and 17.6%, respectively[10]. In another study, 13% of all syndromic cases with conotruncal defects had del22q11[11]. There are other reported instances where fewer deletions were detected in the 22q11 region. One was a large study of isolated conotruncal anomaly patients in which of 628 individuals only one del22q11 was detected[12]. The other was 32 CHD cases with extracardiac anomalies, among whom 29 had conotruncal defects, with only two having del22q11[13].

TBX1 is a member of a phylogenetically conserved family of genes that share a common DNA-binding domain, the T-box. T-box genes are transcription factors involved in the regulation of developmental processes. Mouse Tbx1 has been previously shown to be expressed during early embryogenesis in the pharyngeal arches, pouches, and otic vesicle. Later in development, expression is seen in the vertebral column and tooth bud. Thus, human TBX1 is a candidate for some of the features seen in the del22q11 syndrome[14].

Researchers have focused on neural crest-related genes since the pharyngeal and aortic arches, which develop abnormally in DGS/VCFS, are neural crest-derived.

Jerome and Papaioannou[15] studied the mouse gene encoding the Tbx1 transcription factor in a genetically engineered mice haploinsufficient (Tbx1-/+) or null (Tbx1-/-) in Tbx1. Tbx1-/- mice died in utero with abnormal facies and thymus and parathyroid aplasia along with heart failure and malformed cardiac outflow tracts and aortic arches. Although Tbx1-/+ mice were viable and had no non-cardiovascular abnormalities, many had DGS/VCFS-like aortic arch abnormalities. Aortic arch abnormalities were also observed by Lindsay et al.[16] and Merscher et al.[17], who independently created mice hemizygous for large genomic segments including Tbx1 and other genes. Both groups showed that specific replacement of only the Tbx1 gene corrected aortic defects. Tbx1-/+ mice created by these groups also exhibited abnormal aortic arches. These findings are taken as evidence that human congenital conotruncal cardiac anomalies can be caused by alterations in gene dose of Tbx1.

The high incidence of FT in conotruncal heart anomalies and the suggested role of TBX1 in cardiac development - supported with mouse models showing developmental defects attributable to Tbx1 only - have set the basis of the design for this study. The gene-dosage changes in the T-box transcription factors can cause congenital defects[18]. Exons 4, 5, 6, and 7 of TBX1 are located in T-box region and show 98% homology to mouse Tbx1. Therefore, to clarify the role and to detect any possible causative relation of TBX1 mutations in cardiac malformations, we decided to screen this homology region in non-syndromic FT patients.

Material and Methods

Patient and control group selection and clinical evaluation: The patients were recruited among children that were under clinical investigation and therapy for FT in Dr. Sami Ulus Children's Hospital, Ankara, Turkey between January 2002 and September 2003. Fifty controls that were referred to the same hospital and otherwise free of any cardiac problems were also included in the study after going through tele-, electro- and echocardiographic evaluations. Informed consent was obtained from all parents either of the selected patients or control individuals. All patients were examined by pediatric cardiologists. Telecardiograms and electrocardiograms were performed for the

ascertainment of FT and possible additional cardiac anomalies, followed by 2B+colored Doppler echocardiography. Where indicated, cardiac catheterization and angiography procedures were applied (Table I).

Table I. Type of Fallot Tetralogy and Associated Anomalies in the Patients

Patient	Age/sex	Anomalies	Aortic arch position	Patient	Age/sex	Anomalies	Aortic arch position
P1	5/F	none	L	P26	2/F	PFO	L
P2	2.5/F	PA	R	P27	2/F	ACA	L
P3	1.5/F	none	L	P28	1.5/M	PA	R
P4	2/M	PAH	–	P29	2/F	ACA	L
P5	7/M	LPAH+ASD+ALCAPA	R	P30	1.5/F	PFO	L
P6	7/M	PFO	L	P31	1/M	–	–
P7	1.5/M	none	L	P32	4/F	PFO	L
P8	1.5/F	ACA	L	P33	1.5/F	none	L
P9	4/F	none	L	P34	1/M	none	L
P10	4/M	RPAOS	L	P35	0.3/M	none	L
P11	3/F	CH	L	P36	3/M	–	–
P12	3/M	PFO	L	P37	8/F	none	L
P13	1/M	none	L	P38	3/F	none	L
P14	8/M	none	L	P39	5/M	none	L
P15	3/F	sASD+BAA	L	P40	2.5/F	PA	R
P16	12/F	PAA+VPS+PD	L	P41	2/M	none	L
P17	4/F	LPAOS	L	P42	1.5/M	PFO	R
P18	2/M	ACA	L	P43	1.5/F	PFO	L
P19	1/F	PFO	–	P44	7/M	–	–
P20	8/M	PLSVC	R	P45	5/F	none	L
P21	2.5/M	PA+EVD+ASD	L	P46	3.5/M	none	L
P22	0.3/M	PFO	L	P47	7/F	–	–
P23	1/M	none	L	P48	2/F	none	L
P24	1/M	none	L	P49	1/F	PA+MAPCA	L
P25	5/M	–	–	P50	2/F	none	L

L: left, R: right, PA: pulmonary atresia, PAH: pulmonary artery hyperplasia, LPAH: left pulmonary artery hyperplasia, ASD: atrial septal defect, ALCAPA: anomalous left coronary artery from pulmonary artery, PFO: persistent foramen ovale, ACA: anomalous coronary arteries, RPAOS: right pulmonary artery out–track stenosis, CH: continuous hemi–azygous, sASD: secondary ASD, BAA: brachiocephalic artery anomaly, PAA: pulmonary artery aneurysm, VPS: valvular pulmonary stenosis, PD: pulmonary deficiency, LPAOS: left PAOS, PLSVC: persistent left superior vena cava, EVD: enlarged vertical ductus, MAPCA: major aortopulmonary collateral arteries.

Exclusion of syndromic cases: The patients that were diagnosed as FT were further investigated for the presence of extracardiac malformations and additional postnatal health problems. Presence of frequent infections, episodes of convulsions, family history, parental consanguinity, similar cardiac anomalies in the family, cleft lip/palate, developmental delay, and neurologic or psychiatric disturbances were questioned. Subsequently, body weight and stature of all the patients were evaluated via growth and development charts. A detailed examination was also performed for dysmorphisms. Serum calcium, phosphorus, and alkaline phosphatase levels were analyzed for hypocalcemia and hypothyroidism. Before excluding a patient from the study group, the tests were repeated in cases with pathologic results. A thorough neurological examination was done to determine the presence of accompanying findings. Patients were also tested for immunoglobulin (IgA, IgG, IgE, IgM), and CD3, CD4 and CD8 levels and ratios along with white blood cell (WBC) and total lymphocyte counts. These results were analyzed by pediatric immunologists for the presence of immunodeficiency. Immunoglobulin levels from case 8 and CD3/CD4/CD8 levels and ratios from case 9 could not be obtained. Since these patients had normal WBC and total lymphocyte counts and had no history of frequent infections they were included in the study group.

Cytogenetic testing: Blood samples were obtained from the patients for fluorescence in situ hybridization (FISH) analysis.

Phytohemagglutinin-stimulated lymphocyte cultures were harvested and spread onto slides with standard methods. Metaphase chromosomes were probed using TUPLE [DiGeorge region probe with control probe, direct labelled (Appligene, CP%146-DC)]. Five to 20 metaphases from each patient were examined for del22q11 (Fig. 1).

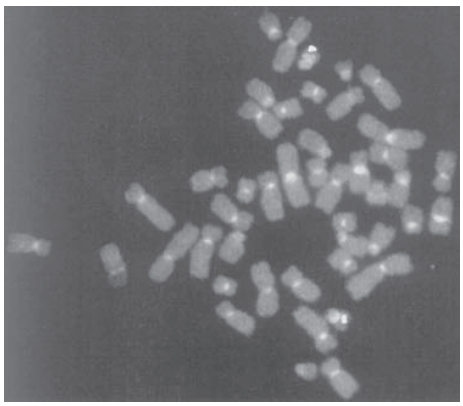

Fig. 1. Metaphase fluorescence in situ hybridization (FISH) imaging of a patient. Red signals: DiGeorge region; green signals: telomeric region.

During these evaluations, any patient with an additional pathology was excluded from the study. Fifty patients without del22q11 were included in the study as isolated FT (Table II).

DNA analyses of TBX1 gene: Genomic DNA was isolated with standard phenol-chloroform method[19]. TBX1 gene exons 4, 5, 6, and 7 were polymerase chain reaction (PCR) amplified from genomic DNA. Primers (Table III) were prepared according to suggestions of M. Budarf (personal communication with M. Budarf). Due to their lengths, exon 5 and exon 6 were amplified in two pieces. PCR amplifications were performed following an initial denaturation at 95°C for 5 min for 35 cycles at 95°C for 30 s, at Tm for 30 s, at 95°C for 1 min, and for 10 min at 72°C for final extension. Reaction samples were applied to agarose gel electrophoresis for testing the presence of amplification products. All PCR products were then screened by single strand conformation polymorphism (SSCP) along with a sample of a healthy control. EtBr stained SSCP gels were evaluated with a gel analysis

Table II. Clinical Evaluation and Laboratory Analyses of the Patients

Patient	Age/sex	Percentile body weight	Percentile stature	FISH	Patient	Age/sex	Percentile body weight	Percentile stature	FISH
P1	5/F	50	50-75	20	P26	2/F	75	3-10	20
P2	2.5/F	3	3	20	P27	2/F	3	3	20
P3	1.5/F	25	25	20	P28	1.5/M	10-25	10-25	15
P4	2/M	3	<3	20	P29	2/F	10	3-10	20
P5	7/M	50	50	20	P30	1.5/F	10-25	10-25	9
P6	7/M	10	10	20	P31	1/M	10	10	20
P7	1.5/M	10	3-10	5	P32	4/F	3-10	3-10	20
P8	1.5/F	10	10	5	P33	1.5/F	25	25	20
P9	4/F	10	10	20	P34	1/M	10	10	20
P10	4/M	90	50	20	P35	0.3/M	25	75	20
P11	3/F	3	<3	20	P36	3/M	10	10	20
P12	3/M	10-25	10-25	20	P37	8/F	25	25	20
P13	1/M	50	25-50	10	P38	3/F	10	10	20
P14	8/M	10	10	20	P39	5/M	10	3-10	20
P15	3/F	10-25	3-10	20	P40	2.5/F	50	50	20
P16	12/F	3	<3	20	P41	2/M	10	10	20
P17	4/F	25	25	20	P42	1.5/M	25	3-10	20
P18	2/M	50	25-50	20	P43	1.5/F	3-10	3-10	20
P19	1/F	10	10-25	20	P44	7/M	10	10	16
P20	8/M	25-50	25	20	P45	5/F	25	25	20
P21	2.5/M	50-75	50-75	20	P46	3.5/M	3	<3	20
P22	0.3/M	10	10	20	P47	7/F	3	3	20
P23	1/M	10	25	20	P48	2/F	3-10	3-10	20
P24	1/M	50	50	20	P49	1/F	10	10	20
P25	5/M	10	10	20	P50	2/F	10-25	10-25	20

FISH: Fluorescence in situ hybridization.

Table III. PCR Primers and Reaction Conditions Used to Amplify T-box Region Exons in Patients and Controls

Exon	PCR product (bp)	T_m	Primer
4	178	55°C	5'-TGCCTTCCACCAGCTAGG-3' 5'-CCGGTCCCTCACGCTTAC-3'
5A	194	60°C	5'-CTCGGGTTCACCTCCACAT-3' 5'-CAGCTTGAGCTTGTCGAAGG-3'
5B	179	60°C	5'-CGGACTCGCCTGCCAAGG-3' 5'-CAGGCCTCTTAGGGACAGG-3'
6A	197	60°C	5'-CTCCCACCCCACATCCTC-3' 5'-CCGCGGTGAATCGTGTCTC-3'
6B	175	60°C	5'-TCCATGCACAGATACCAGC-3' 5'-AATCCGCTCAGGTCCAGC-3'
7	149	65°C	5'-AGGCTGCAGGGCTCCAGC-3' 5'-CGCCCGGCGCTCACTCTC-3'

apparatus and software (Bass-Diana Imaging Software) against size markers (PCR DNA marker Amresco, E854). The fragments showing different mobility shifts were sequenced in a automated capillary electrophoresis system (using either corresponding forward or reverse primers) with BigDye Terminator™ according to the manufacturer's recommendations (ABI prism 310).

Results

A total of 50 patients (29 males, 21 females) ranging in age from 4 months to 12 years (mean age ±SD: 3.22±2.51) were included in the study. The control group was sex-matched with ages between 6 months and 12 years (mean age ±SD: 4.51±3.16). The TBX1 exons 4 to 7 were screened with SSCP analyses in both groups. There were no differences between the band mobilities of patients and controls for exon 4 (Fig. 2) and exon 6 (Fig. 3). SSCP analyses revealed three distinct electrophoretic bands (named as A, B and C) in exon 5 (Fig. 4). From the patients, 26 individuals were group A (52%), 3 were group B (6%), and 21 were group C (42%). In the controls, the distributions of the band patterns of SSCP for A, B and C were 25 (50%), 2 (4%), and 23 (46%), respectively. Each distinct band pattern was analyzed through sequencing in the bearers of the corresponding pattern groups both from patients and controls. The band pattern A was recognized as the normal allele, whereas for B and C there was C→T change in position 664. Group B was detected as homozygous (TT) and

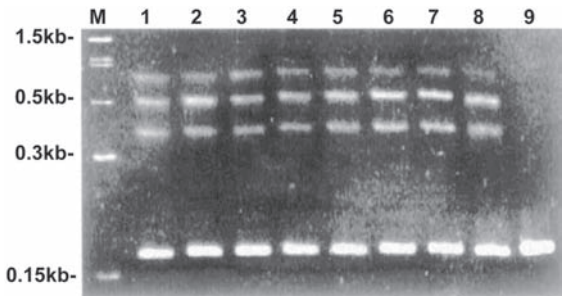

Fig. 2. Exon 4 PCR products in SSCP gel electrophoresis. M: marker, 1-2: samples from controls, 3-8: samples from patients, 9: non-denatured control PCR product. The fragments on the bottom are double-stranded reaction products.

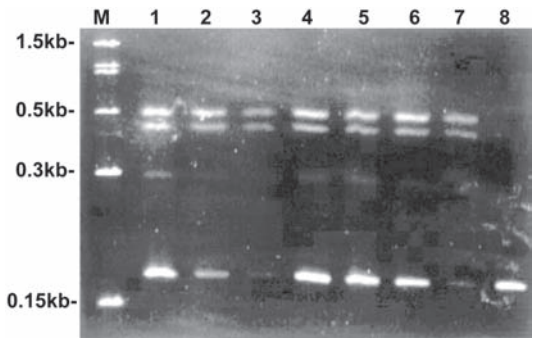

Fig. 3. Exon 6 PCR products in SSCP gel electrophoresis. M: marker, 1-7: samples from controls, 8: non-denatured control PCR product. The fragments on the bottom are double-stranded reaction products.

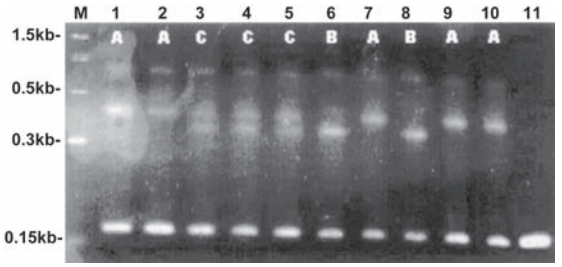

Fig. 4. Exon 5 PCR products in SSCP gel electrophoresis. M: marker, 1-8: samples from patients, 9-10: samples from controls, 11: non-denatured control PCR product. The fragments on the bottom are double-stranded reaction products. A,B,C: names referring to the different patterns of fragments.

group C as heterozygous (CT) (Fig. 5). When patient and control groups were compared, the frequency of the genotypes among the three groups was insignificant (p=0.85). In exon 7, two different mobilities were detected (Fig. 6); however, they both had the same sequence data, which were considered as normal.

Discussion

TBX1's role in neural crest migration is well known, and it is one of the major candidates for the clinical outcomes in del22q11 syndromes. A wide range of manifestations of this syndrome, mostly accompanied with cardiac outflow tract and aortic arch anomalies, suggested a causative role for TBX1 in isolated heart anomalies. The results of mouse studies indicate the major determining role of Tbx1 haploinsufficiency for the cardiovascular phenotype in del22q11 syndromes. A few human studies exist in the literature. Chieffo et al.[14] studied 16 patients without del22q11 to compare the sequence changes of TBX1 to Tbx1, and detected five changes, but with an amino acid difference in only one case, same as in controls. These findings are in accord with our data. In a total of 105 patients, 65 with isolated CTHDs and 40 with del22q11, Gong et al.[20] screened the complete set of

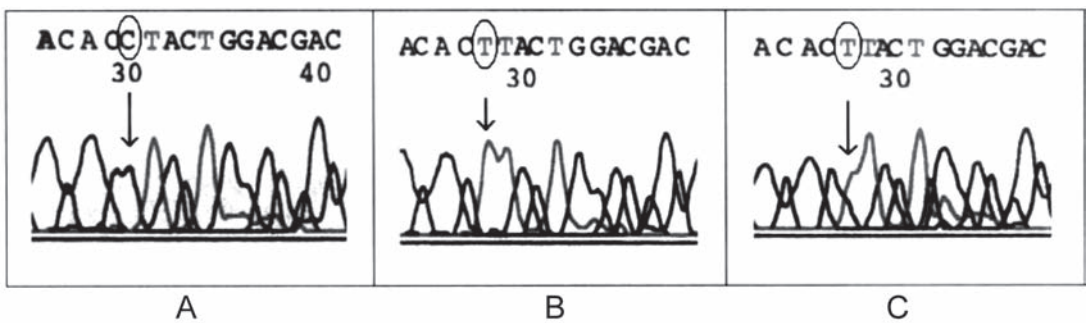

Fig. 5. Sequence analyses showing 664C→T change. A: homozygote CC, B: homozygote TT, C: heterozygote CT.

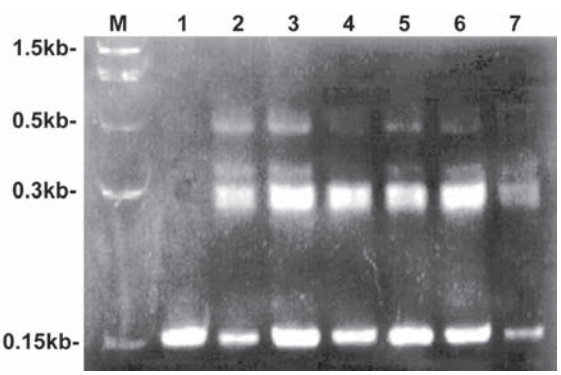

Fig. 6. Exon 7 PCR products in SSCP gel electrophoresis. M: marker, 1: non-denatured control PCR product, 2-7: samples from controls. The fragments on the bottom are double-stranded reaction products.

TBX1 exons for mutations. Their results were 8 common and 10 rare polymorphisms, and no disease-causing mutations. In a recent study, in 41 individuals with non-syndromic CTHDs, Conti et al.[21] observed no causative mutation other than 6 polymorphisms in TBX1. Taken together, these results weaken the role of TBX1 mutations in cardiovascular malformations. Since the study groups mentioned above contained a range of pathologies that could complicate the interpretation, we used strict phenotypic criteria for patient selection. Hence, our results did not reveal any novel mutations except a variant that was also detected in Conti's study [21]. Yagi et al.[22] also pinpointed the importance of patient selection. In their

study, clinical evaluation of the cases was done utilizing an elaborated scoring system and they included in the study only 13 patients with pharyngeal phenotype (craniofacial, velopharyngeal, ear, thymus, parathyroid and conotruncal anomalies) but without del22q11. In one of the cases with dysmorphic face, CTHDs (FT, PA, secondary ASD, major aortic pulmonary collateral arteries), thymus aplasia and parathyroid anomaly, 443T→A mutation was detected to change phenylalanine to tyrosine in the T-box region with a suggestive role of decreasing DNA binding activity and dimerization functions. In the same study, in another patient with facial and CTHDs (IAA, VSD, pulmonary hypertension), hypoplastic thymus, hypothyroidism, and deafness, 928G→A change in the well-conserved region of the gene was detected. A third and last mutation was detected in all three members of a family who had different clinical phenotypes. Here, 1223 delC causes frame shift and an immature stop codon resulting in the loss of C terminal region of TBX1 protein containing activator and repressor domains. This study is the only instance showing mutations in the T-box region.

The migration of cardiac neural crest cells into the pharyngeal arches around the arch arteries and finally into the outflow tract for conotruncal formation of the heart is a complex mechanism with the possible involvement of several other genes. The wide range of clinical forms of conotruncal anomalies also supports this idea. Categorical studies of related anomalies in large patient groups focusing on candidate loci would help in understanding the effects of genes on conotruncal anomalies in particular. However the relatively well-detailed functional role and its relation with other genes set Tbx1 apart, our results indicated that mouse-human homology region in the T-box does not provide an explanation for TBX1 function for human CTHDs. Yet, no mutation has been identified that is directly related to CTHDs. Several mutations and polymorphisms are capable of affecting the activities of the protein products and their expression patterns and causing a spectrum of clinical pictures. Therefore, investigating the complete exons and non-exon regions of TBX1 in humans may help in understanding the nature of CTHDs.

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