

# Genetic analysis of the TBX1 gene promoter in ventricular septal defects

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**Abstract** Congenital heart disease (CHD) is the most common birth defects in humans. The genetic causes for CHD remain largely unknown. T-box transcription factor 1 (TBX1), a dosage-sensitive regulator, plays a critical role in the heart development. Mutations in the coding regions of TBX1 gene have been associated to 22q11 deletion syndrome with cardiac defects and isolated CHD cases, including ventricular septal defect (VSD). To date, TBX1 gene promoter region has not been analyzed and reported in CHD patients. We hypothesized that the sequence variants within TBX1 gene promoter region may change TBX1 levels and mediate CHD development. In this study, the promoter regions of TBX1 gene were genetically and functionally analyzed in 280 VSD patients and 267 healthy controls. Two novel heterozygous variants, g.4353C>T and g.4510A>C, were found in two VSD patients, but in none of controls. The single-nucleotide polymorphism-rs41260844, g.4199T>C, was found more frequent in VSD patients than controls ( $P < 0.01$ ). Functional analyses revealed that these sequence variants significantly enhanced transcriptional activities of TBX1 gene promoter. Therefore, the sequence variants within TBX1 gene

promoter may contribute to the VSD etiology by altering the expression levels of TBX1 gene. Pharmaceutical or genetic manipulation of TBX1 gene expression may provide a novel personalized therapy to prevent and treat late cardiac complications for the adult CHD patients carrying these variants.

**Keywords** Congenital heart disease · Ventricular septal defect · TBX1 · Promoter · Sequence variant

## Introduction

Congenital heart disease (CHD) is the most common human birth defects affecting 1–2 % of live birth [1]. Despite advanced and effective surgical treatment, morbidity and mortality of CHD patients are significantly higher compared with the general population [2]. Adult CHD patients are predisposed to late cardiac complications, such as arrhythmias, coronary heart disease, and heart failure [3], likely due to the genetic defects. Although extensive genetic studies have revealed mutations in cardiac transcription factor genes in familial and sporadic CHD cases [4, 5], genetic causes and underlying molecular mechanisms of CHD remain largely unknown.

Heart is the first organ to form during the embryonic development. The gene regulatory networks strictly control the heart formation, involving signaling pathways, transcription factors, epigenetic factors, and miRNAs [6–10]. More than 20 T-box transcription factor (Tbx) genes have been identified. Tbx factors, containing a highly conserved DNA-binding domain, play critical regulatory roles in the embryonic development [11]. Six of Tbx family members (Tbx1, Tbx18, and Tbx20 of the Tbx1 subfamily, and Tbx2, Tbx3, and Tbx5 of the Tbx2 subfamily) are required

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in the developing mammalian heart [12]. *Tbx1* has been shown to be involved in the differentiation and proliferation of the second heart field, which contributes to the outflow tract and right ventricle [8, 12, 13]. In mice, *Tbx1* gene is expressed in the pharyngeal arches, pouches, and otic vesicle [14]. *Tbx1* in the mesoderm and endoderm of pharyngeal arches contributes to the morphogenesis of outflow tract and patterning of coronary arteries [13, 15, 16]. *Tbx1* in the pharyngeal mesoderm is also required for cardiac neural crest migration [17]. Therefore, *Tbx1* plays essential roles in the developing heart.

Human 22q11 deletion syndrome, including DiGeorge syndrome, conotruncal anomaly face syndrome and velocardiofacial syndrome, is featured with thymic hypoplasia, parathyroid hypoplasia, and cardiac defects, such as ventricular septal defects (VSDs), tetralogy of Fallot, and double outflow right ventricle [18]. Mutations in *TBX1* gene have been associated to major phenotypes in 22q11 deletion syndrome [18–21]. *TBX1* gene mutations that reduce its transcriptional activities have also been linked to tetralogy of Fallot and aortic arch anomalies [19, 22–24]. Mice with homozygous deletion of *TBX1* die before or at birth and display the major features in human 22q11 deletion syndrome [25–27]. Mice with heterozygous *Tbx1*-null allele develop hypoplasia of the distal outflow tract and mispatterning of coronary arteries [15].

Genetic studies indicate that *TBX1* is a dosage-dependent transcriptional regulator [18, 28]. Gain-function mutations in *TBX1* gene cause the same phenotype as human 22q11 deletion syndrome [21]. Transgenic mice overexpressing human *TBX1* had similar malformations and spectrum of the phenotypes is correlated with *TBX1* dosage [29]. Overexpression of *TBX1* in mice leads to VSD development [30]. Therefore, we hypothesized that reduced or increased *TBX1* levels, rather than mutations that change amino acids, may contribute to the VSD etiology. In this study, we genetically and functionally analyzed the promoter regions of *TBX1* genes in large cohorts of VSD patients and healthy controls.

## Methods

### Human subjects

All the VSD patients ( $n = 280$ , male 145, female 135, age range from 3 months to 40 years, median age 4.25 years) and unrelated controls ( $n = 267$ , male 214, female 53, age range from 1 month to 39 years, median age 3.67 years) were recruited from Jining Medical University Affiliated Hospital, Jining Medical University, Jining, Shandong, China. The VSD patients were diagnosed based on medical history, physical examination, electrocardiogram, and three-dimensional echocardiography, and then confirmed with cardiac catheterization or cardiac surgery. Both VSD patients and controls with familial CHD histories were excluded from this study. This study was approved by the Human Ethic Committee of Jining Medical University Affiliated Hospital and informed consents were obtained.

### Sequencing

Leukocytes were isolated from venous blood and genomic DNAs extracted. Two overlapped DNA fragments, covering the putative promoter region of *TBX1* gene (~1,200 bp upstream to the transcription start site), were generated with polymerase chain reaction (PCR). The PCR primers were designed based on the genomic sequence of human *TBX1* gene (Genbank access number, NG\_009229) and shown in Table 1. The DNA fragments were bi-directionally sequenced with BigDye<sup>®</sup> Terminator v3.0 reagents and a 3730 DNA Analyzer (Applied Biosystems, Foster city, CA, USA). The sequences were aligned and compared with the wild-type *TBX1* gene promoter.

### Functional analysis

The DNA fragments of wild-type and variant *TBX1* gene promoter regions (1,153 bp, from 4,034 to 5,185 in NG\_009229) were generated by PCR, and subcloned into

**Table 1** The PCR primers for the promoter regions of *TBX1* gene

PCR primers	Sequences	Location	PCR products (bp)
TBX1-F1	5'-TCCAAATAGGGCGCTGTAAG-3'	4,016	576
TBX1-R1	5'-CAGTCTTCGTAGTTGTGGGTGA-3'	4,591	
TBX1-F2	5'-AGAAATTGTGCCATTGATC-3'	4,493	703
TBX1-R2	5'-TCTCCGTCTTCGCCTATCAG-3'	5,195	
TBX1-F	5'-( <i>Kpn</i> I)-AGCAACTCCAGGCGGTCCATC-3'	4,034	1,153
TBX1-R	5'-( <i>Nhe</i> I)-CTCCTCTTCACCTGCCGCCAC-3'	5,185	

PCR primers were designed based on the genomic DNA sequence of *TBX1* gene (NG\_009229), in which transcription starts at the position of 5001

*KpnI* and *NheI* sites of luciferase reporter plasmid (pGL3-basic) to generate expression constructs, which were then examined by DNA sequencing. Designated expression constructs were transiently transfected into human embryonic kidney cells (HEK-293) and luciferases activities were measured using dual-luciferase reporter assay system on a Glomax 20/20 Luminometer (Promega, Madison, WI, USA). Expression construct pRL-TK expressing Renilla luciferase was used as an internal control. Empty construct pGL3-basic was used as negative control. The transcriptional activities were represented as ratios of luciferase activities over renilla luciferase activities. All experiments were performed three times independently, each experiment in triplicate. The transcriptional activities of wild-type TBX1 gene promoter in each experiment were set as 100 %.

### Statistical analysis

The quantitative data were represented as mean  $\pm$  SE and analyzed with a standard Student's *t* test. The sequence variant frequencies were compared between VSD patients and controls with  $\chi^2$  test using SPSS v13.0.  $P < 0.05$  was considered statistically significant.

## Results

The sequence variants of the TBX1 gene promoter were summarized in Table 2 and Fig. 1. Genotype frequencies of controls were in Hardy–Weinberg equilibrium ( $P > 0.05$ ). Two novel heterozygous sequence variants and two single-nucleotide polymorphisms (SNPs) were identified. The novel variants, g.4353C>T and g.4510A>C, were identified in two VSD patients, but in none of controls. The frequency of the SNP-rs41260844, g.4199C>T, in VSD patients significantly differed from that in controls ( $P < 0.01$ ). The SNP-rs41298629, g.4248C>T, was found in VSD patients and controls with similar frequencies.

In the NCBI SNP database, frequency of C allele of SNP-rs41260844 (g.4199C>T) is 52.1 % in the same ethnic population as this study. In this study, the frequency of C allele in controls was 43.8 %, which was similar to the SNP database.

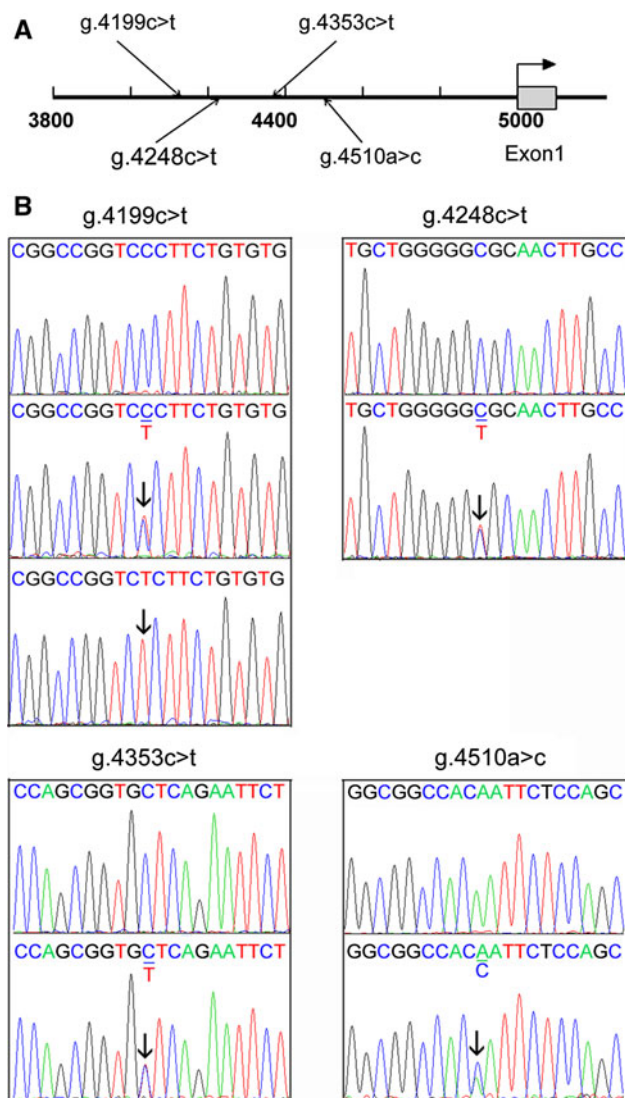
Heterozygous and homozygous SNP-rs41260844 (g.4199C>T) were found in VSD patients and controls, and frequency of the CC genotype in SNP-rs41260844 (g.4199C>T) in VSD patients was higher than that in controls. Accordingly, the TBX1 gene promoter carrying the SNP-rs41260844 (g.4199C>T) with T allele was considered as wild type in this study. The SNP was defined as rs41260844 (g.4199T>C), frequency of which in VSD patients was significantly higher than that in controls ( $P < 0.01$ ).

Analysis of the promoter region of TBX1 gene with transcription element search system (TESS, University of Pennsylvania) showed that potential transcription factor binding sites were disrupted or modified, and new transcription factor binding sites were created by the sequence variants, SNP-rs41260844 (g.4199T>C), g.4353C>T or g.4510A>C. The variant g.4353C>T disrupted the binding sites for GC binding factor (GCF) and E12 factors, and created the binding sites for glucocorticoid receptor (GR) and progesterone receptor (PR). The variant 4510A>C disrupted the binding site for E2F factor, and created the binding sites for insulin upstream factor 1 (IUF1), Yin and Yang 1 factor (YY1), and vitamin D receptor. The SNP-rs41260844, g.4199T>C, disrupted the binding sites for GR and T cell transcription factor-1 (TCF-1), and created the binding sites for histone transcription factor-2 (H4TF-2) and transcription repressor Tramtrack (Ttk). Therefore, the transcriptional activity of the TBX1 gene promoter may be affected by these sequence variants.

To examine the transcriptional activities of wild-type and variant TBX1 gene promoters, the expression vectors with wild-type (pGL3-WT) and variant promoters (pGL3-4199C, pGL3-4284T, pGL3-4353T, and pGL3-4510C) were constructed. HEK-293 cells were transfected and dual-luciferase activities were measured. The results showed that the transcriptional activities of pGL3-4199C, pGL3-4353T, and pGL3-4510C were significantly enhanced ( $P < 0.01$ ), compared with that of pGL3-WT (Fig. 2). The transcriptional activities of pGL3-4284T were not different from that of pGL3-WT. These results suggested that the sequence variants, rs41260844 (g.4199T>C), g.4353C>T, and g.4510A>C, may change TBX1 levels in VSD patients. Collectively, the SNP-rs41260844, g.4199T>C, g.4353C>T, and g.4510A>C,

**Table 2** Sequence variants within the TBX1 gene promoters in VSD patients and controls

Sequence variants	Genotypes	Location	VSD ( $n = 280$ )	Controls ( $n = 267$ )	$P$ value
g.4199C>T (rs41260844)	CC	−802	71	41	0.003
	CT		127	153	
	TT		82	73	
Locations of the variants upstream (−) or downstream (+) to the transcription start site (+1 at the 5001 of NG_009229)	g.4248C>T (rs41298629)	CT	−753	2	1.000
	g.4353C>T	CT	−648	0	
	g.4510A>C	AC	−491	0	

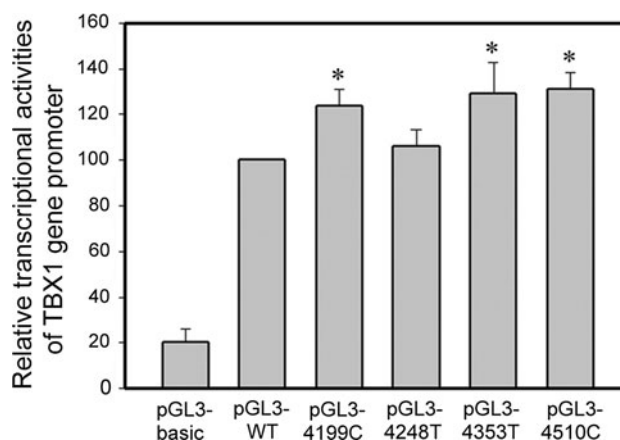


**Fig. 1** The sequence variants within the promoter regions of TBX1 gene in VSD patients and controls. **a** Schematic representation of the sequence variants within the TBX gene promoter regions. The numbers represents the sequence of TBX1 genomic sequences (Genbank accession number: NG\_009229). All the sequence variants were depicted. The transcription starts at the position of 5001 in the first exon. **b** Chromatograms of the sequence variants in forward orientations. *Top panel* shows wild type, *middle panel* heterozygous and *bottom* homozygous for SNP-rs41260844 (g.4199C>T). For other variants, *top panels* show wild type, and *bottom panels* heterozygous. All the variants are marked with *arrows*

may change TBX1 gene expression levels, contributing to the VSD etiology.

## Discussion

In this study, we genetically and functionally analyzed the promoter region of the TBX1 gene in large cohorts of VSD



**Fig. 2** Relative transcriptional activities of wild-type and variant TBX1 gene promoters. The expression vectors were transfected into HEK-293 cells and dual-luciferase activities were assayed. The transcriptional activity of wild-type TBX1 gene promoter was designated as 100 %. pGL3-basic was used as a negative control. The data were represented as mean  $\pm$  SE from three independent transfection experiments, each in triplicate. \* $P < 0.01$ , compared to pGL3-WT

patients and controls. Two novel heterozygous variants, g.4353C>T and g.4510A>C, and one SNP-rs41260844, g.4199T>C, were identified within the TBX1 gene promoter, which significantly enhanced the transcriptional activities of TBX1 gene promoter. Our results suggested that these sequence variants may upregulate TBX1 gene expression level in VSD patients, contributing to the VSD etiology.

Human TBX1 gene has been localized to chromosome 22q11.2 with 9 exons [31]. Expression of TBX1 gene is strictly controlled by signaling pathways and transcription factors, such as forkhead transcription factors, Wnt and Hedgehog signaling [32–34]. The promoter region of human TBX1 gene has not been characterized. In this study, the sequence variants within the putative TBX1 promoter region altered its transcriptional activities in cultured cells, suggesting that the transcription factors binding to its promoter may be altered.

In the heart development, TBX1 interacts with signaling molecules and transcription factors, such as Smad1 and serum response factor, to regulate its downstream target genes [12, 18, 35, 36]. A number of genes in the cardiovascular development are regulated by TBX1, including fibroblast growth factor 8 (Fgf8), Fgf10, forkhead box protein A2 (FoxA2), gastrulation brain homeobox 2 (Gbx2), hairy and enhancer of split 1 (Hes1), myocyte-specific enhancer-binding factor 2c (Mef2c) and paired-like homeodomain transcription factor 2 (Pitx2) genes [12, 13, 17, 18, 37–42]. Gbx2 gene is required for normal arch artery development [43]. Pitx2 gene, a bicoid-like homeobox gene, is expressed in the pharyngeal mesoderm.

Loss of function mutations of Pitx2 gene cause Rieger syndrome, which is associated with umbilical abnormalities, craniofacial, cardiac and ocular defects [44]. Fgf8 and Fgf10 signaling have overlapping function in maintaining second heart field [45]. Hes1 gene, encoding a mediator of Notch signaling, is expressed in the second heart field [46]. Therefore, changed TBX1 level may disrupt its interaction with other factors and alter the expression of its target genes, causing the abnormal cardiac morphogenesis.

In conclusion, we identified novel sequence variants within the proximal promoter of TBX1 gene in VSD patients, which significantly enhanced the transcriptional activities of TBX1 gene promoter. These variants may contribute to the VSD etiology by changing TBX1 levels. The responsible transcription factors and binding sites will be further investigated in our laboratory. Pharmaceutical and genetic manipulation of TBX1 gene expression may provide a novel personalized therapy to prevent and treat late cardiac complications for the adult CHD patients carrying these variants.

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