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Genetic analysis of the TBX3 gene promoter in ventricular septal defects

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ABSTRACT

Congenital heart disease (CHD) is the most common birth defect in humans. Genetic causes and underlying molecular mechanisms for CHD remain largely unknown. T-box transcription factor 3 (TBX3) plays a critical role in the developing heart in a dose-dependent manner. TBX3 represses chamber myocardial gene expression. Mutations in TBX3 gene have been associated to ulnar-mammary syndrome with multiple developmental defects, including cardiac defects. We hypothesized that the sequence variants within TBX3 gene promoter that change TBX3 levels may mediate CHD development. In this study, TBX3 gene promoter was genetically analyzed in large cohorts of patients with ventricular septal defect (VSD) ($n=325$) and ethnic-matched healthy controls ($n=359$). Seven sequence variants, including two single-nucleotide polymorphisms (g.3863 C>T and g.4095G>T), three novel deletions (g.4433_4435del, g.4672_4675del and g.4820_4821del) and two novel insertions (g.3913_3914ins and g.4735_4736ins), were identified. Five of the seven variants were identified in VSD patients and controls with similar frequencies. Two other variants were found only in controls. These variants, which were observed in high frequencies, did not modify or interrupt the critical binding site for basic transcription factors. Taken together, these results suggested that the sequence variants within the TBX3 gene promoter did not contribute to VSD etiology.

1. Introduction

Congenital heart disease (CHD) is the most common human birth defect that affects 1–2% of live birth (Hoffman and Kaplan, 2002). Although mutations in cardiac transcription factor genes, such as GATA transcription factor 4 (GATA4), T-box transcription factor 5 (TBX5) and NK2 transcription factor, locus 5 (NKX2-5), have been identified, genetic causes and underlying molecular mechanisms for CHD remain largely unknown. During the embryonic development, heart is the first organ to form, which is strictly controlled by transcription factors, signaling pathways, epigenetic factors and miRNAs (Bruneau, 2008; Kathiresan and Srivastava, 2012). Therefore, mutations and variants in any component gene in the cardiac gene regulatory networks may mediate development of CHD.

T-box transcription factors, containing a highly conserved DNA-binding domain, play regulatory roles in the embryonic development (Naiche et al., 2005). Six of TBX family members (TBX1, TBX18 and TBX20 of the TBX1 subfamily, and TBX2, TBX3 and TBX5 of the TBX2 subfamily) are essential for the developing heart. In formation of the heart chambers, TBX5 and TBX20 activate the chamber myocardial genes, whereas TBX3, together with closely related TBX2, represses same set of the genes (Greulich et al., 2011; Washkowitz et al., 2012). TBX3, a transcriptional repressor, is expressed and required for development of the heart, limbs, and mammary glands (Carlson et al., 2001; Chapman et al., 1996; Davenport et al., 2003). In mammals, TBX3 is expressed in non-chamber myocardium of atrioventricular canal, out flow tract, sinoatrial node and the cardiac conduction system in the developing heart (Christoffels et al., 2004; Hoogaars et al., 2004; Mesbah et al., 2008). In the human heart, TBX3 is observed in the floor of the atrium and atrioventricular canal myocardium (Sizarov et al., 2011). Many studies with germline-targeting and overexpression of TBX3 gene have revealed that TBX3 inhibits differentiation of the cardiac chamber myocardium by repressing myocardial gene expression, and induces formation of the cardiac conduction system (Bakker et al., 2008, 2012; Hoogaars et al., 2007; Mesbah et al., 2008, 2012; Mommersteeg et al., 2007; Singh et al., 2012; Wiese et al., 2009). TBX3 is also required for development of ventricular septum and outflow tract (Bakker et al., 2008, 2012; Hoogaars et al., 2007). In addition, TBX3 plays a role for self-renewal of the human embryonic stem cells and generation of induced pluripotent stem cells (Esmailpour and Huang, 2012; Han et al., 2010).

Mutations in TBX3 gene have been associated with ulnar-mammary syndrome (UMS), which is characterized by malformations of limb,

Abbreviations: CHD, congenital heart disease; CX40, gap junction protein connexin 40; GATA4, GATA transcription factor 4; MYH6, myosin heavy chain 6; NKX2-5, NK2 transcription factor, locus 5; NPPA, natriuretic peptide precursor type A; SNP, single-nucleotide polymorphism; TBX3, T-box transcription factor 3; VSD, ventricular septal defect; UMS, ulnar-mammary syndrome.

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mammary gland, genitalia, hair and teeth (Bamshad et al., 1997). Recent clinical studies report cardiac defects, including ventricular septal defect, in UMS patients (Linden et al., 2009). TBX3 gene mutations (such as missense, nonsense, deletion and frameshift) have been identified in UMS patients in different ethnic populations (Bamshad et al., 1997, 1999; Klopocki et al., 2006; Linden et al., 2009; Meneghini et al., 2006; Sasaki et al., 2002; Wollnik et al., 2002). TBX3-null deletion mice die in utero with phenotypes that closely mimic the human UMS manifestations (Davenport et al., 2003). Cardiac defects, such as double outlet right ventricle, transposition of the great artery and ventricular septal defects, are observed in the TBX3-deficeint model animals (Bakker et al., 2008; Hoogaars et al., 2007; Mesbah et al., 2008). Thus, TBX3 gene may be linked to non-syndromic CHD.

In previous studies, we have identified a number of sequence variants in the promoters of TBX1, TBX5 and TBX20 genes in CHD patients (Qiao et al., 2012; Shan et al., 2012; Wang et al., 2012). Genetic studies have demonstrated that TBX3 acts as a dosage-dependent transcriptional factor for development of the cardiac conduction system (Frank et al., 2012). Overexpression of TBX3 in the embryonic heart suppresses chamber formation in mice (Hoogaars et al., 2008). In mice, homozygous mutations of TBX3 gene are embryonic lethal (Davenport et al., 2003). TBX3 gene mutations identified in UMS patients are all heterozygous. Therefore, we hypothesized that changed TBX3 levels, rather than mutations, may contribute to CHD etiology. In the present study, we genetically analyzed the TBX3 gene promoter in large cohorts of VSD patients and healthy controls.

2. Methods

2.1. Patients and controls

All VSD patients ($n=325$, male 146, female 179, age range from 3 month to 41 years, median age 4.42 years) were recruited from Division of Cardiac Surgery, Jining Medical University Affiliated Hospital, Jining Medical University, Jining, Shandong, China. VSD patients were diagnosed based on medical records, physical examination, electrocardiogram and three-dimensional echocardiography. Ethnic-matched healthy controls ($n=359$, male 295, female 64, age range from 1 month-39 years, median age 3.75 years) were recruited from the Physical and Examination Center in the same hospital. VSD patients and controls with familial CHD history were excluded from this study. This study was approved by the Human Ethic Committee of Jining Medical University Affiliated Hospital. Informed consents were obtained from the guardians.

2.2. Sequence analysis

The peripheral leukocytes were isolated and genomic DNA was extracted. Two overlapped DNA fragments, covering the TBX3 gene promoter (~1200 bp upstream to the transcription start site), were generated with PCR. PCR primers were designed based on the genomic sequence of human TBX3 gene (NCBI accession number: NG_008315.1) and shown in Table 1. The DNA fragments were bi-directionally sequenced with BigDye® Terminator v3.0 reagents and a 3730 DNA Analyzer (Applied Biosystems, Foster city, CA, USA). The sequences were aligned and compared with wild type sequence of the TBX3 gene promoter. For heterozygous insertion and deletion variants, the DNA fragments were subcloned into T vector and directly sequenced. The frequencies of sequence variants were analyzed with SPSS v13.0. $P<0.05$ was considered statistically significant.

3. Results

The human TBX3 gene promoter is a TATA-less promoter. A SP1 binding element and two CCAAT boxes are essential to basic transcriptional activity of the proximal TBX3 promoter (Smith et al., 2011). We further analyzed the TBX3 promoter, ~1200 bp upstream to the transcription start site, with transcription element search software (TESS program, University of Pennsylvania, USA). The analysis revealed several specificity protein 1 binding elements, four E-boxes and two activator protein 1 sites within the TBX3 gene promoter. No binding sites for human GATA4, NKX2-5 and T-box factors were found.

Table 1
PCR primers for the human TBX3 gene promoter [a].

PCR primers	Sequences	Location	PCR products
sequencing			
TBX3-F1	5′-ACCCAATCGCCCACCCCTTAC-3'	3758	627 bp
TBX3-R1	5′-CCATCCGGCCACTCGGTTCTAC-3′	4384	
TBX3-F2	5′-TAGAACCGAGTGGCCGGATGG-3′	4364	789 bp
TBX3-R2	5′-CGCAGGGCAGGGAGGATTTAG-3′	5152	

a, PCR primers are designed based on the genomic DNA sequence of the human TBX3 gene (NG_008315.1) and the transcription start site is at the position of 5001. Locations of the PCR primers are described as in the genomic sequence.

The TBX3 gene promoter regions were bi-directionally sequenced in VSD patients ($n=325$) and healthy controls ($n=359$). Distribution of the identified sequence variants was summarized in Table 2. The locations and chromatograms were depicted in Fig. 1A and B. Seven sequence variants were identified in VSD patients and controls, five novel insertion/deletion variants and two single-nucleotide polymorphisms (SNPs). Two deletion variants (g.4433_4435del with AGA deletion and g.4672_4675del with GCGA deletion), one insertion variant (g.4735_4736ins with GC insertion) and two SNPs (g.3863 C>T, rs11347985 and g.4095G>T, rs3759173) were found in both VSD patients and controls with similar frequencies ($P>0.05$). Two novel variants (g.3913_3914ins with CATCCA insertion and g.4820_4821del with CA deletion) were only found in two controls.

The insertion variant (g.3913_3914ins) interrupted a non-canonical E-box (CAGGTG) by inserting another non-canonical E-box (CATCCA), which may not alter transcription activity of the TBX3 gene promoter. All other variants did not modify or interrupt the SP1 elements, AP-1 binding sites and E-boxes. Collectively, these results suggested that the variants within the TBX3 gene promoter is not a contributor to VSD etiology.

4. Discussion

Cardiac transcription factors function in a dose-dependent way during the embryonic heart development. We hypothesize that

Table 2
TBX3 gene promoter variants in VSD patients and controls.

Variants	Genotype	Location [a]	Controls ($n=359$)	VSD ($n=325$)	*P* value
g.3863 C>T (rs11347985)	CT	−1138 bp	2	4	0.431
g.3913_3914insCATCCA	-/CATCCA	-1089 bp	1	0	-
g.4095 G>T (rs3759173)	GG	-906 bp	303	280	0.223
	GT		55	41	
	TT		1	4	
g.4433_4435delAGA	AGA/AGA	-568 bp	347	309	0.323
	AGA/-		7	6	
	−/−		5	10	
g.4672_4675delGCGA	GCGA/GCGA	-329 bp	208	190	0.768
	GCGA/-		135	117	
	−/−		16	18	
g.4735_4736insGC	-/GC	-266 bp	17	14	0.400
g.4820_4821delCA	CA/-	-181 bp	1	0	-

a, Locations of the variants are upstream (−) to transcription start site of the TBX3 gene (at 5001 of NG_008315.1), which is set as +1.