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Genetic analysis of an enhancer of the NKX2-5 gene in ventricular septal defects

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ABSTRACT

Congenital heart disease (CHD) is one of the most common birth defects in humans. Mutations in cardiac transcription factor genes, such as GATA4, NKX2-5 and TBX5 genes, have been associated to a small portion of familial and isolated CHD cases. NKX2-5, a highly conserved homeobox gene, is expressed in the developing heart. During embryonic development, NKX2-5 plays pivotal roles in specifying cardiac progenitors, cardiac morphogenesis, cardiomyocyte differentiation and conduction system development. Numerous mutations in NKX2-5 gene have been reported in CHD patients, including atrial septal defect, ventricular septal defect (VSD) and tetrology of Fallot. We have previously identified the sequence variants within the NKX2-5 gene promoter in VSD patients. As several studies have revealed that the NKX2-5 gene is regulated by a complex module involving promoter and multiple independent cardiac enhancers, one of which is located between −3500 bp and −2500 bp upstream to the transcription start site, we hypothesized that the variants within the cardiac enhancer may contribute to CHD. In this study, we genetically analyzed the enhancer of NKX2-5 gene in large cohorts of VSD patients (n=322) and controls (n=336). The results showed that three novel variants, g.1467G>A, g.1487Ins with a 13 bp insertion and g.1515Ins with a 6 bp insertion, were identified within the enhancer element in both VSD patients and controls with similar frequencies (P>0.05). Therefore, our data suggested that the enhancer of NKX2-5 gene may not be a contributor to the VSD etiology. Other regulatory elements of the NKX2-5 gene will be further analyzed in CHD patients.

1. Introduction

Congenital heart disease (CHD) is the most common type of birth defects in humans, with the prevalence of 4–50 per 1000 live births (Pierpont et al., 2007). Morbidity and mortality of CHD patients are significantly higher even after effective surgical correction, compared to that in general population (van der Bom et al., 2011; Verheugt et al., 2010). Late cardiac complications are the main causes, which are probably related to genetic defects. To date, extensive studies have associated mutations in a number of cardiac transcription factor genes, such as GATA transcription factor 4 (GATA-4), T-box transcription factor 5 (TBX5) and NK2 transcription factor, locus 2 (NKX2-5), to a small portion of familial and isolated CHD cases (Bruneau, 2008). Therefore, understanding the genetic causes and underlying molecular mechanisms for CHD will provide clinical benefits for the adult patients.

The heart is the first organ to form during embryonic development. The cells originating from the first heart field, the second heart field and the cardiac neural crest contribute to the cardiac morphogenesis (Buckingham et al., 2005; Srivastava, 2006). The events occurring during the heart development are strictly regulated by a gene regulatory network involving several signal transduction pathways and cardiac transcription factors, such as GATA4, NKX2.5, TBX5 and TBX20 (Dunwoodie, 2007; Rochais et al., 2009; Scholl and Kirby, 2009). NKX2-5, a highly conserved homeobox gene, is expressed in the developing heart. NKX2-5 plays pivotal roles in specifying cardiac progenitors, cardiac morphogenesis, cardiomyocyte differentiation and conduction system development (Bruneau et al., 2000; Habets et al., 2002; Harvey, 1996; Hiroi et al., 2001; Jamali et al., 2001; Jay et al., 2004; Moskowitz et al., 2007; Pashmforoush et al., 2004). In adult heart, NKX2-5 is expressed and required for homeostasis and survival of differentiated cardiomyocytes (Toko et al., 2002).

More than 40 heterozygous mutations in the NKX2.5 gene have been reported in CHD patients, including atrial septal defect, ventricular septal defect (VSD), tetralogy of Fallot and transposition of the great arteries, with the prevalence of about 2% in isolated cases (De Luca et al., 2010; Elliott et al., 2003; Gioli-Pereira et al., 2010; McElhinney et al., 2003; Rauch et al., 2010; Reamon-Buettner and Borlak, 2010; Schott et al., 1998; Stallmeyer et al., 2010). Genetic studies in experimental

Abbreviations: CHD, congenital heart diseases; VSD, ventricular septal defects; GATA4, GATA transcription factor 4; TBX5, T-box transcription factor 5; TBX20, T-box transcription factor 20; NKX2-5, NK2 transcription factor, locus 2; MEF2C, myocyte-specific enhancer factor 2 C; NFAT, nuclear factor of activated T-cells; IRX4, Iroquois family homeodomain transcription factor 4.

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animals further confirm the clinical investigation. Mice with germline-disruption of NKX2-5 gene die before birth with abnormal looping heart (Lyons et al., 1995; Tanaka et al., 1999a). NKX2-5 knockout at mid-embryonic stage causes premature death and defective cardiac morphogenesis (Terada et al., 2011). Animals with heterozygous mutations in NKX2-5 gene show different phenotypes of CHD (Biben et al., 2000; Kasahara et al., 2001). Like GATA-4 and Tbx-5, NKX2-5 is a dosage sensitive regulator in cardiac morphogenesis (Jay et al., 2005; Postma et al., 2008; Pu et al., 2004). Dosage sensitive interdependence between cardiac transcription factors and chromatin remodeling complex has been observed in the heart development (Takeuchi et al., 2011). Therefore, we hypothesized that the sequence variants within the regulatory regions of the NKX2-5 gene may cause CHD. Within the NKX2-5 gene promoter region, we have identified the sequence variants that significantly change the expression levels of the NKX2-5 gene in VSD patients (Pang et al., in press).

Genetic studies in mice have revealed that the NKX2-5 gene is regulated by a complex module of upstream and downstream regulatory elements, including promoter and multiple independent cardiac enhancers at different distances (Lien et al., 1999; Reecy et al., 1999; Searcy et al., 1998; Tanaka et al., 1999b). An enhancer of the NKX2-5 gene in mice is located between −3000 bp and −2500 bp upstream to the transcription start site (Searcy et al., 1998). Cross-species comparison reveals that this enhancer of the human NKX2-5 gene is located at a similar location (Lien et al., 2002). Therefore, we analyzed the enhancer of the NKX2-5 gene in large cohorts of VSD patients and healthy controls.

2. Materials and methods

2.1. Patients and controls

All the VSD patients (n = 322, male 160, female 162, age range from 1 month to 41 years, median age 4.42 years) were recruited from Jining Medical University Affiliated Hospital, Jining Medical University, Jining, Shandong, China. The VSD patients were diagnosed according to medical records, physical examination, electrocardiogram and three-dimensional echocardiography. The healthy controls (n = 336, male 270, female 66, age range from 1 month to 39 years, median age 3.92 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 and informed consents were obtained from the guardians.

2.2. Sequence analysis

Genomic DNA was extracted from the peripheral leukocytes. The enhancer regions of the NKX2-5 gene (−3711 to −2856 upstream to the transcription start site) were generated with PCR. The PCR primers were designed based on the genomic sequence of human NKX2-5 (Genbank access number, NG_013340). The forward and reverse PCR primers were 5′-ACAAGCGTGGAAATGAGAAGG-3′ and 5′-GACAAACAAACAGCCTTCAGC-3′. The PCR products of 856 bp were bi-directionally sequenced with BigDye® Terminator v3.0 reagents and a 3730 DNA Analyzer (Applied Biosystems, Foster city, CA, USA). For the insertion variants, the enhancer DNA fragments were subcloned in to T vector and directly sequenced to confirm the insertions. The frequencies of sequence variants were analyzed with SPSS v13.0. $P<0.05$ was considered statistically significant.

3. Results

As the first step, we first analyzed the 856 bp enhancer of the NKX2-5 gene (−3711 to −2856 upstream to the transcription start site) with TESS program (Transcription Element Search Software, University of Pennsylvania, USA, http://www.cbil.upenn.edu/tess). Within the enhancer element, there are an E-box (CATCTG), an NKX2-5 binding site (AAGTG), six GATA factor binding sites (GATA) and multiple Smad binding sites (GTCT/AGAC), consistent to the NKX2-5 enhancer in mouse (Lien et al., 2002).

The enhancer of the NKX2-5 gene was bi-directionally sequenced in VSD patients (n = 322) and healthy controls (n = 336). As shown in Table 1 and Fig. 1, three novel sequence variants, g.1467G > A, g.1483Ins with a 13 bp insertion and g.1515Ins with a 6 bp insertion, were identified in VSD patients and controls, with similar frequencies ($P>0.05$). No binding sites for GATA-4, Smad and NKX2-5, or E-box, were disrupted or modified. Collectively, the results suggested that the enhancer of NKX2-5 gene may not be a contributor to the VSD etiology.

4. Discussion

In a large cohort of isolated VSD patients, we genetically analyzed one of the cardiac enhancers of NKX2-5 gene, which contains multiple binding sites for cardiac transcription factors. Within the enhancer, three novel sequence variants (g.1467G > A, g.1483Ins and g.1515Ins) were found in both VSD patients and controls with similar frequencies, indicating that these variants did not contribute to the VSD etiology. The overall frequencies of mutations and variants in the NKX2-5 gene are about 2% in isolated CHD patients (Reamon-Buettner and Borlak, 2010; Stallmeyer et al., 2010; Pang et al., in press). Therefore, these results provided supportive evidence that mutations and variants within the NKX2-5 gene and its regulatory elements are not common in isolated CHD patients.

Human NKX2-5 is localized to chromosome 5q35 and consists of two exons encoding a protein of 324 amino acids (Shiojima et al., 1996; Turbay et al., 1996). The promoter region of NKX2-5 gene has been characterized for its cardiac expression (Shiojima et al., 2000). Genetic analyses in animals have demonstrated that expression of the Nkx2-5 gene is strictly regulated by a complex module of the promoter and multiple independent enhancers (Lien et al., 1999; Reecy et al., 1999; Tanaka et al., 1999b). In a large cohort of VSD patients, the promoter and encoding regions of the NKX2-5 gene have been genetically and functionally analyzed and reported by our group (Pang et al., in press). In this study, we further analyzed an enhancer of the NKX2-5 gene in VSD patients, and no variant identified was linked to VSD. Therefore, to completely elucidate the role of NKX2-5 gene in CHD patients, the enhancer of the NKX2-5 gene will be analyzed in other types of CHD patients and the other regulatory elements of the NKX2-5 gene will be analyzed.

Table 1
Sequence variants in the enhancer of the NKX2-5 gene.

Variants[a]	Genotypes	Location[b]	VSD (n = 322)	Controls (n = 336)	P value
g.1467G > A	GG	−3534	315	325	0.387
	GA		7	11	
	AA		0	0	
g.1483Ins	−/−	−3518	93	86	0.476
	GTGGCC(T)GGCCCCG/−		106	107	
	GTGGCC(T)GGCCCCG/ GTGGCC(T)GGCCCCG		123	143	
g.1515Ins	−/−	−3486	266	281	0.715
	GCGGAG/−		56	54	
	GCGGAG/GCGGAG		0	1	

Ins, Insertion. −/−, wild type.
[a] All the variants were named based on their locations in NG_013340, in which the transcription starts at 5000.
[b] Locations of the variants are indicated upstream to the transcription start site (+1).