Exome sequencing reveals novel *IRX1* mutation in congenital heart disease

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Abstract. Genetic variation in specific transcription factors during heart formation may lead to congenital heart disease (CHD) or even miscarriage. The aim of the present study was to identify CHD-associated genes using next generation sequencing (NGS). The whole exome DNA sequence was obtained from a stillborn fetus diagnosed with tricuspid atresia and complete transposition of the great arteries using high-throughput sequencing methods. Subsequently, genetic variants of CHD-associated genes were selected and verified in 215 non-syndromic CHD patients and 249 healthy control subjects using polymerase chain reaction combined with Sanger sequencing. Genetic variants of previously reported CHD-inducing genes, such as cysteine rich with EGF like domains 1 and cbp/p300-interacting transactivator with Glu/Asp rich carboxy-terminal domain 2, were discovered through the NGS analysis. In addition, a novel non-synonymous mutation of the iroquois homeobox 1 (*IRX1)* gene (p.Gln240Glu) was identified. A total of three non-synonymous mutations (p.Gln240Glu, p.Ser298Asn and p.Ala381Glu) of the *IRX1* gene were verified in 215 non-syndromic CHD patients, but not in 249 healthy volunteers. The results demonstrated that NGS is a powerful tool to study the etiology of CHD. In addition, the results suggest that genetic variants of the *IRX1* gene may contribute to the pathogenesis of CHD.

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Introduction

The incidence of congenital heart disease (CHD) varies between 4 and 8 in every 1,000 live births globally (1). However, it is considerably higher in the prenatal population; the percentage of miscarriages and elective abortions in pregnant women with structural CHD is reportedly 15 and 5%, respectively (2,3). The development and formation of the human heart is an intricate process. Unfavorable environmental and embryotoxic factors, genetic variations, numerical and structural chromosomal aberrations (e.g. trisomy of chromosome 21), as well as chromosomal microdeletions (e.g. DiGeorge syndrome), may all interfere with this process, thus leading to CHD or even miscarriage in some cases (4-6). The genetic etiology of CHD has been studied extensively over the last decade; a number of germ line mutations in cardiac transcription factors (7-13), including NK2 homeobox 5 (*NKX2-5*) (11,14), GATA binding protein 4 (*GATA4*) (15-17), T-box 20 (18), and Notch1 (19) have been validated. However, further research is required to better understand the underlying mechanisms of CHD. Next generation sequencing (NGS), in addition to its advantageous cost, accuracy and efficiency, has proven to be successful in identifying concordant variants in patients with the same disease (20). Genome-wide coverage may allow for a nonbiased approach, as it is not restricted to certain pre-selected regions. The conventional Sanger sequencing approach has been used to validate the candidate discordant variants obtained from NGS (21,22).

In order to obtain a comprehensive understanding of the effect of genetic variants on CHD, the present study used NGS to sequence the whole exome of a stillborn fetus diagnosed with CHD. In addition to a number of known CHD-inducing genes, genes with a poor association were additionally identified. The results provide a more complete understanding of the effect of specific genetic variants on CHD.

Materials and methods

Study population. A 0.5x0.5 cm section of tissue from the left ventricular of a male stillborn fetus (gestational age, 37 weeks), and 464 peripheral blood samples from 215 non-syndromic

patients with CHD (101 males and 114 females; mean age 8.84±12.98 years old) and 249 healthy control subjects (118 males and 131 females; mean age 47.56±16.62) were included in the present study (Table I). All the samples were obtained from individuals from Fujian Medical University (Fuzhou, China) and Shengjing Hospital of China Medical University (Shenyang, China) between 2009 and 2012. The stillborn fetus was diagnosed with tricuspid atresia and complete transposition of the great arteries (TGA) as confirmed by autopsy. Written informed consent was obtained from the parents and guardians of the patient and from the 464 additional subjects. The present study was approved by the ethics committee of Fujian Medical University (Fuzhou, China), and adhered to the tenets of the Declaration of Helsinki. Patients with CHD were routinely screened by performing clinical examinations, chest X-rays, electrocardiographs and ultrasonic echocardiograms. The pathological diagnosis of CHD was confirmed by open-heart surgery. The healthy control subjects were non-CHD adult outpatients from the same geographic area. Control subjects with congenital anomalies were excluded from the study.

Whole exome sequencing and data analysis. Genomic DNA (gDNA) was extracted and purified using the FlexGen Blood DNA kit (CW0544A; CWBio Technology, Beijing, China). Purified gDNA (3 μg) was fragmented into 200 bp sequences. End repair, adenylation and adapter ligation were performed for library preparation using the NGS Fast DNA Library Prep set and following the manufacturer's protocol (CWBio Technology, Beijing, China). Library samples were pooled and hybridized to a customized capture array, including exons, splicing sites and immediate flanking intron sequences (5190-6216; SureSelectXT2 Human All Exon V5, 16; Agilent Technologies, Inc., Santa Clara, CA, USA). Sequencing was performed on an Illumina HiSeq 2500 instrument (Illumina, Inc., San Diego, CA, USA) to generate paired end reads. Adapter and low quality sequences (quality score ≤20 and sequencing depth ≤5) in the raw data were then removed using the Burrows-Wheeler Aligner (http://bio-bwa.sourceforge.net/bwa.shtml) (23). The sequencing reads were mapped to the human reference genome (hg19, http://genome.ucsc.edu) using the short oligonucleotide alignment program (SOAP) (http://soap.genomics.org.cn/soapsnp.html) and the Burrows-Wheeler Aligner (24,25). Single nucleotide polymorphisms (SNPs) and indels were detected using the Genome Analysis Toolkit and the SOAPsnp algorithm (http://soap.genomics.org.cn/soapsnp.html) (26), while annotation was performed according to the Consensus Coding Sequence of human GRCh37/hg19 (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19), the Human Genome Project (HGP, human genome build, 36.3), the Single Nucleotide Polymorphism Database (dbSNP; version, 130; www.ncbi.nlm.nih.gov/snp), the Haplotype Map Project (https://www.broadinstitute.org/data-software-and-tools) and the Sorting Intolerant From Tolerant prediction tool (27-29).

Sanger sequencing and protein structure prediction. Exon 2 of iroquois homeobox 1 (IRX1) was amplified in CHD patients and healthy controls by polymerase chain reaction (PCR), PCR reactions consisted of 20-30 ng of genomic DNA, 3 μl of PCR buffer, 3 μl of dNTPs, 0.3 μl of Hotstar Taq (Qiagen), 1.5 μl (20 pmol/μl) of each primer pair (forward: 5'-GGGTGACTTCCTGATCTGCC-3'; reverse: 5'-GAAGCAGGGATTAAGCGCAG3'), to a volume of 30 μl with distilled water. Reactions started with 15 min at 95˚C, followed by 30 cycles of 45 sec at 95˚C, 30 sec at 60˚C, 45 sec at 72˚C and finished with a 10 min extension period at 72˚C. using the forward primer, 5'-GGGTGACTTCCTGATCTGCC-3' and reverse primer, 5'-GAAGCAGGGATTAAGCGCAG3'. The PCR products were sequenced using the automated ABI 3730XL sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the forward primer, 5'-TCGAGTCCATTGAAGCGG-3' and reverse primer, 5'-TACCCTCCCGGCTCATGC-3'. Amino acid sequences of IRX1 in additional mammalian species were obtained from NCBI GenBank (www.ncbi.nlm.nih.gov/genebank), and sequence conservation analysis was performed using CLC Main Workbench version 7.7.3 (CLCbio; Qiagen Bioinformatics, Aarhus, Denmark).

Table I. Phenotype of 215 patients with non-syndromic congenital heart disease included in the present study.

Phenotype	No. of patients (%)
Atrial septal defect	48 (22.3)
Ventricular septal defect	52 (24.2)
Tetralogy of Fallot	24 (11.2)
Patent ductus arteriosus	24 (11.2)
Pulmonary stenosis	10 (4.7)
Other complex cardiac malformations	57 (26.5)

Table II. Main features of whole exome sequencing results.

Feature	Data
Raw data (Mb)	5,597.55
Clean data (Mb)	5,571.7
Aligned (%)	99.62
Initial bases on target	55,336,911
Bases covered on target	49,342,125
Coverage of target region (%)	98.00
Total effective yield (Mb)	5,133.05
Effective sequence on target (Mb)	2,756.55
Fraction of effective bases on target (%)	53.70
Average sequencing depth on target (%)	54.76
Fraction of target with at least 4x coverage (%)	96.30
Fraction of target with at least 10x coverage (%)	93.20
Fraction of target with at least 20x coverage (%)	84.50
Duplication rate (%)	6.1949
Total SNP	17,601
Missense sites	17,302
Indel sites	309

SNP, single nucleotide polymorphism.

Table III. Mutation sites of the congenital heart disease-associated genes identified in the present study.

Gene	Accession no.	Exon	Position	Protein	Effect
CRELD1	NM_015513	1	c.37A>G	p.M13V	Nonsynonymous
TMEM43	NM_024334	7	c.536T>C	p.M179T	Nonsynonymous
TLL1	NM_012464	20	c.2872A>G	p.T958A	Nonsynonymous
CITED2	NM_001168388	2	c.148G>A	p.A50T	Nonsynonymous
IRX3	NM_024336	2	c.1265T>C	p.L422P	Nonsynonymous
IRX5	NM_005853	3	c.763C>A	p.P255T	Nonsynonymous
MYOCD	NM_153604	10	c.1941G>C	p.Q647H	Nonsynonymous
IRX1	NM_024337	2	c.718C>G	p.Q240E	Nonsynonymous
IRX4	NM_016358	3	c.381A>G	p.P127P	Synonymous
IRX4	NM_016358	2	c.90A>C	p.G30G	Synonymous
IRX1	NM_024337	2	c.1272T>C	p.N424N	Synonymous
NKX2-5	NM_001166176	1	c.63A>G	p.E21E	Synonymous
TBX20	NM_001166220	1	c.39T>C	p.S13S	Synonymous

CRELD1, cysteine rich with EGF like domains 1; *TMEM43*, transmembrane protein 43; *TLL1*, tolloid like 1; *CITED2*, cbp/P300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2; *IRX*, iroquois homeobox 1; *MYOCD*, myocardin; *NKX2-5*, NK2 homeobox 5; TBX-20, T-box 20.

Results

To comprehensively investigate the association between germline mutations and CHD susceptibility, the present study was completed in two consecutive steps. Whole exome sequencing of the stillborn fetus discovered 17,601 SNP sites, spanning 98% of the target region (Table II). These variants were subsequently annotated according to the dbSNP and HGP databases. 17,302 missense sites and 309 indel sites were then selected as the candidate genetic variants (Table II). In the subsequent analysis, genes associated with heart formation, development and cardiovascular disease were selected for further consideration. A number of known causative genes for congenital heart malformations, including cysteine rich with EGF like domains 1 (*CRELD1*) (30), tolloid like 1 (*TLL1*) (31), cbp/P300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2 (*CITED2*) (32,33) and myocardin (*MYOCD*) (33), were detected in the present study. However, no mutations in the exons of additional pivotal genes, including *GATA4, GATA6, NKX2-5,* T-box transcription factor and heart and neural crest derivatives expressed 2, were identified (Table III).

Out of the candidate genes identified, a variant of *IRX1* (c.718C>G, p.Gln240Glu), which is an important gene involved in early heart development and limb formation (34), was selected for further investigation. The c.718C>G variant and whole exon 2 of the *IRX1* gene were screened in 215 non-syndromic patients with CHD and 249 healthy control subjects by PCR and Sanger sequencing. The c.718C>G variant was detected in a male (age, 5 years) with TGA and an atrial septal defect (Fig. 1, Table IV). In addition, a novel variant, c893G>A, p.S298N, was detected in a 1-year-old male with total anomalous pulmonary venous drainage (Fig. 1; Table IV). An additional previously identified variant, c.1142C>A (p.A381E; dpSNP cluster ID, rs530506520) was identified in a 3-year-old male with a ventricular septal defect phenotype (Fig. 1; Table IV). However, no non-synonymous variant was detected in the 249 healthy control subjects. Conservation analysis demonstrated that glutamine 240 and serine 298 residues are highly conserved among different mammalian species, while alanine 381was only moderately conserved (Fig. 2).

Discussion

NGS has become a powerful tool for identifying concordant variants in patients with the same disease. In previous studies it has successfully identified the causative gene of monogenic diseases (35), as well as a number of cancers, autoimmune diseases (36) and neurodegenerative diseases (37). In the present study, NGS was used to sequence the exome of a stillborn fetus with tricuspid atresia and complete TGA. In total, 17,302 missense sites and 309 indel sites were selected as candidate genetic variants. Out of these, a number of known cardiovascular disease-associated variants were identified, including *CRELD1* (30), *CITED2* (32), *MYOCD* (33), transmembrane protein 43 (38), *TLL1* (31), *IRX-1, IRX-3* and *IRX-5*. Therefore, the authors hypothesize that *CRELD1, CITED2* and *TLL1* genetic variants may have been responsible for the development of CHD in the fetus. It is possible that the *IRX-1, IRX-3* and *IRX-5* variants may have additionally contributed to CHD development.

The *IRX* gene is highly conserved among vertebrates. A total of 6 *IRX* genes (*IRX1-IRX6*) are organized in two cognate clusters of three genes, *IRX1, IRX2, IRX4* and *IRX3, IRX5, IRX6*, respectively (39,40). The *IRX* gene exhibits restricted temporal and spatial expression patterns during murine neural and cardiac development (41). *IRX4* was the first cardiac transcription factor identified to be expressed in the ventricles alone at all stages of heart development (40). In chicken embryos, aberrant expression of *Irx4* affects heart chamber development (42). In mice, targeted inactivation of *Irx4* led to aberrant ventricular gene expression, including reduced expression

Table IV. Missense mutation sites in *iroquois homeobox 1* identified in 215 sporadic cases of CHD.

Position	Protein	Ref SNP number	Gender	Age (years)	Phenotype
c.718C>G	p.Gln240Glu	Novel	Male	5	TGA+ASD
c.893G>A	p.Ser298Asn	Novel	Male	1	TAPVD
c.1142C>A	p.Ala381Glu	rs530506520	Male	3	VSD

A total of three mutations were identified in three of the 215 patients diagnosed with sporadic CHD. CHD, congenital heart disease; TGA, transposition of the great arteries; ASD, atrial septal defect; TAPVD, total anomalous pulmonary venous drainage; VSD, ventricular septal defect.

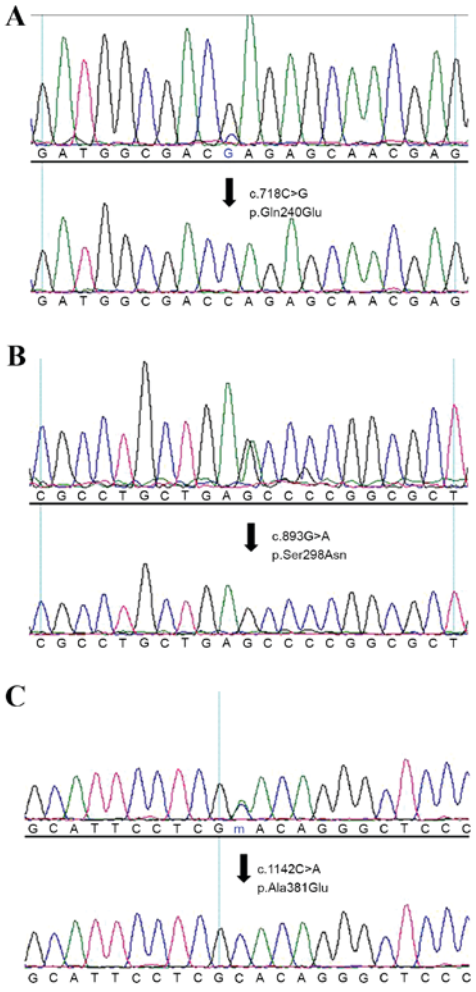

Figure 1. Missense mutation sites in *iroquois homeobox 1* detected in 3 cases of sporadic congenital heart disease. (A) c.718C>G; (B) c.893G>A; (C) c.1142C>A.

Figure 2. Conservation analysis of *iroquois homeobox 1* among different mammalian species performed using CLC Main Workbench software. P.240Gln and p.298Ser were highly conserved in mammalian species whereas p.381Ala was conserved only in *Pan*, *Homo*, *Bos*, *Rattus* and *Mus*, but not in *Gallus* and *Danio*. Pan, *Pan troglodytes*; Homo, *Homo sapiens;* Bos, *Bos taurus*; Rattus, *Rattus norvegicus*; Mus, *Mus musculus*; Gallus, *Gallus gallus*; Danio, *Danio rerio.*

of the basic helix-loop-helix transcription factor (40). In this previous study, adult Irx4Dex2/Dex2 mice developed cardiomyopathy characterized by cardiac hypertrophy and impaired contractile function (40). Cardiac expression of *Irx1, Irx2* and *Irx5* may partially compensate for loss of *Irx4* function (41).

In the present study, the coding sequence of the *IRX1* gene was screened in sporadic non-syndromic patients with CHD and healthy volunteers. The number of missense mutations identified was higher in CHD patients when compared with healthy volunteers (3 of the 215 CHD cases vs. 0 of the 249 controls). These results further support the notion that disrupted *IRX1* may be insufficient to induce a CHD phenotype, and that variants of the *IRX1* gene only contribute to an increased susceptibility of CHD.

In the present study, the whole exome of a stillborn fetus with tricuspid atresia and complete TGA was sequenced. A number of missense mutations in known CHD-associated genes, including *CRELD1, CITED2 and TLL1* were detected. In addition, the missense mutation rate of *IRX1* was observed to be higher in patients with sporadic CHD when compared with normal healthy volunteers. This suggests that genetic variants of *IRX1* may contribute to the development of CHD.

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