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A novel NR2F2 loss-of-function mutation predisposes to congenital heart defect

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ABSTRACT

Congenital heart defect (CHD) is the most common type of birth defect in humans and a leading cause of infant morbidity and mortality. Previous studies have demonstrated that genetic defects play a pivotal role in the pathogenesis of CHD. However, the genetic basis of CHD remains poorly understood due to substantial genetic heterogeneity. In this study, the coding exons and splicing boundaries of the *NR2F2* gene, which encodes a pleiotropic transcription factor required for normal cardiovascular development, were sequenced in 168 unrelated patients with CHD, and a novel mutation (c.247G > T, equivalent to p.G83X) was detected in a patient with double outlet right ventricle as well as ventricular septal defect. Genetic scanning of the mutation carrier's relatives available showed that the mutation was present in all affected family members but absent in unaffected family members. Analysis of the index patient's pedigree displayed that the mutation co-segregated with CHD, which was transmitted as an autosomal dominant trait with complete penetrance. The nonsense mutation was absent in 230 unrelated, ethnically-matched healthy individuals used as controls. Functional deciphers by using a dual-luciferase reporter assay system revealed that the mutant NR2F2 protein had no transcriptional activity as compared with its wild-type counterpart. Furthermore, the mutation abrogated the synergistic transcriptional activation between NR2F2 and GATA4, another core cardiac transcription factor associated with CHD. This study firstly associates *NR2F2* loss-of-function mutation with an increased susceptibility to double outlet right ventricle in humans, which provides further significant insight into the molecular mechanisms underpinning CHD, suggesting potential implications for genetic counseling of CHD families and personalized treatment of CHD patients.

1. Introduction

Congenital heart defect (CHD) is the most common form of birth malformation in humans, with an estimated prevalence of 1% in live births and as high as 10% in stillbirths (Benjamin et al., 2017; Fahed et al., 2013). Clinically, CHD is usually categorized into 25 different types, encompassing ventricular septal defect (VSD), double outlet right ventricle (DORV) and tetralogy of Fallot (Benjamin et al., 2017; McDermott et al., 2017). Although mild CHD can resolve spontaneously (Benjamin et al., 2017), severe CHD can lead to diminished quality of life (Neiman et al., 2017), decreased exercise tolerance (Chaix et al., 2016), brain development delay or brain injury (Marelli et al., 2016; Morton et al., 2017; Peyvandi et al., 2016), thromboembolism (Jensen et al., 2015; Masuda et al., 2017), infective endocarditis (Diller and Baumgartner, 2017; Kuijpers et al., 2017), pulmonary arterial hypertension (Müller et al., 2017; van der Feen et al., 2017), congestive heart failure (Budts et al., 2016; Hinton and Ware, 2017; Stout et al., 2016), arrhythmias (Holst et al., 2017; Khairy, 2016; Lüscher, 2016; McLeod and Warnes, 2016) and sudden cardiac death (Diller and Baumgartner, 2016; Engelings et al., 2016; Jortveit et al., 2016; Koyak

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Table 1
Primer pairs to amplify the coding exons and splicing boundaries of the *NR2F2* gene.

Coding exon	Forward primer (5′→3′)	Backward primer (5′→3′)	Product size (bp)
1	AACCAACCTCAACCAACCAGC	GCAGGCTAGCCAAACGTACC	684
2	GGGGACTGAGGCTGGTCATT	AGGAGCAGACTGGGTTTGCC	677
3	CGGTTGGGGCAGTTTGACAG	GCAAACAAACCAGTCTTTTGCCT	481

et al., 2017; Williams, 2016). Presently, CHD is still the most common cause of birth defect–related demises in infants, with nearly 24% of infants who died of birth defects having cardiac malformations (Benjamin et al., 2017). Although vast advancement in treatment of CHD during past decades has allowed over 90% of newborns with CHD to survive into adulthood, it results in an increasing number of adults living with CHD, and moreover, the morbidity and mortality in adult CHD cases are much higher than the general population (Bouma and Mulder, 2017; Mandalenakis et al., 2017). Despite significant clinical importance, the etiologies underpinning CHD remain largely elusive.

Previous studies have demonstrated substantial genetic basis for CHD, and in addition to chromosomal anomalies such as trisomy of chromosome 21 and chromosome 22q11 deletion, mutations in over 60 genes have been causally linked to CHD in humans (Andersen et al., 2014; Asadollahi et al., 2017; Blue et al., 2017; Boyle et al., 2016; Cao et al., 2016; Chen et al., 2016, 2017; Edwards and Gelb, 2016; Ellesøe et al., 2016; Fahed et al., 2013; Huang et al., 2016, 2017; LaHaye et al., 2016; Li et al., 2016, 2017; Li and Yang, 2017; Liu et al., 2016; Lu et al., 2016; Priest et al., 2016; Ramond et al., 2017; Reijnders et al., 2016; Rocha et al., 2016; Sifrim et al., 2016; Sun et al., 2016a, 2016b; Tong, 2016; Wang et al., 2017a, 2017b; Wells et al., 2016; Werner et al., 2016; Xu et al., 2017; Yoshida et al., 2016; Zaidi and Brueckner, 2017; Zhao et al., 2016; Zhou et al., 2016a, 2016b). Among these CHD-associated genes, most encode cardiac transcription factors, including NKX2–5, GATA4, HAND1 and TBX20 (Li and Yang, 2017). The expression profiles and functional roles in the heart of these transcription factors partially overlap during embryogenesis, indicating that they constitute a core regulatory network crucial for cardiovascular morphogenesis (Li and Yang, 2017). Nevertheless, CHD is of pronounced genetic heterogeneity, and the genetic determinants for CHD in most patients remain unclear.

Recently, terminal deletions of chromosome 15q, where several genes including the *NR2F2* gene are located, have been associated with syndromic CHD in humans, including VSD, atrial septal defect, and coarctation of the aorta (Nakamura et al., 2011). Furthermore, the expression of NR2F2 in the developing human fetal heart including the atria, coronary vessels, and aorta has been substantiated, and *NR2F2* mutations have been causally linked to isolated CHD, including atrioventricular septal defect, tetralogy of Fallot, aortic stenosis, VSD, coarctation of the aorta and hypoplastic left heart syndrome (Al Turki et al., 2014). These observational results make it reasonable to screen *NR2F2* as a preferable candidate gene for CHD in another cohort of patients.

2. Materials and methods

2.1. Study subjects

In this study, 168 unrelated patients with CHD and 230 healthy individuals used as controls were recruited from the Chinese Han population. The available family members of the index patient carrying an identified *NR2F2* mutation were also enrolled. All study subjects underwent a comprehensive clinical evaluation, including medical histories, physical examination and echocardiography with color Doppler. Patients with a genetic syndrome, such as DiGeorge syndrome and Turner syndrome, were excluded from this study. This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. The study protocol was approved by the local institutional ethical committee. Written informed consent was obtained from the study participants or their legal guardians prior to the study.

2.2. Genetic analysis of NR2F2

Peripheral venous blood samples were collected from the study participants and genomic DNA was isolated from blood leukocytes with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The referential genomic DNA sequence of the human *NR2F2* gene from the Nucleotide database (accession no. NC_000015.10) at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/Nucleotide) was used to design primers with the online program Primer-BLAT (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Detailed sequences of the primers used to amplify the coding exons and flanking introns of *NR2F2* by polymerase chain reaction (PCR) were given in Table 1. Amplification of genomic DNA was performed by PCR using HotStar Taq DNA Polymerase (Qiagen) on a Veriti Thermal Cycler (Applied Biosystems, Waltham, MA, USA) as previously described (Huang et al., 2016). The purified PCR products were used as templates for sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems). For a detected *NR2F2* variant, the single nucleotide polymorphism (http://www.ncbi.nlm.nih.gov/SNP), human gene mutation (http://www.hgmd.org), 1000 Genomes (http://www.1000genomes.org/) Exome Variant Server (http://evs.gs.washington.edu/EVS) and genomAD (http://gnomad.broadinstitute.org/) databases were queried to confirm its novelty.

2.3. Alignment of multiple NR2F2 proteins across species

The NR2F2 protein of human was aligned with those of chimpanzee, monkey, dog, cattle, mouse, rat, fowl, zebrafish, fruit fly, mosquito and frog using the online MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/).

2.4. Plasmids and site-targeted mutagenesis

Human heart cDNAs were prepared as described previously (Huang et al., 2016). The wild-type full-length cDNA of the human *NR2F2* gene (transcript variant 1; accession no. NM_021005.3) was amplified by PCR using the pfuUltra high-fidelity DNA polymerase (Stratagene, Santa Clara, CA, USA) and a pair of primers (forward primer: 5′-ACG GCTAGCGGCGCGCCGGAGCCCGAGAC-3'; reverse primer: 5′-ACGGCG GCCGCTCTGTTTCTCTCCCCCTTC-3′). The amplified fragments were doubly digested by restriction enzymes *Nhe*I and *Not*I (TaKaRa, Dalian, Liaoning, China). The digested products with a length of 1377 base pairs were inserted at the *Nhe*I-*Not*I sites of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to generate an expression plasmid NR2F2-pcDNA3.1. The sequence variant found in CHD patients was introduced into the wild-type NR2F2-pcDNA3.1 plasmid by site-directed mutagenesis using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) with a complementary pair of primers. The *APOB* promoter-driven firefly luciferase (APOB-luc) reporter plasmid was constructed as previously described (Al Turki et al., 2014). The expression plasmid GATA4-pSSRa and ANF-luciferase (ANF-luc) reporter were kindly provided by Dr. Ichiro Shiojima from Chiba University School of