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ORIGINAL ARTICLE

Identification of a novel heterozygous *SOX9* variant in a Chinese family with congenital heart disease

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Abstract

Background: Previous studies of individuals with hereditary or sporadic congenital heart disease (CHD) have provided strong evidence for a genetic basis for CHD. The aim of this study was to identify novel pathogenic genes and variants in a Chinese CHD family.

Methods: Three generations of a family with CHD were recruited. We performed whole exome sequencing for the affected individuals and the proband's unaffected aunt to investigate the genetic causes of CHD in this family. Heterozygous variants carried by the proband and her maternal grandmother, but not the proband's aunt, were selected. The frequencies of the variants detected were assessed using public databases, and their influences on protein function were predicted using online prediction software. The candidate variant was further confirmed by Sanger sequencing of other members of the family.

Results: On the basis of the family's pedigree, the mode of inheritance was speculated to be autosomal dominant with incomplete penetrance. We identified a novel heterozygous missense variant in *SOX9* in all affected individuals and one asymptomatic family member, suggesting an inheritance pattern with incomplete penetrance. The variant was not found in any public database. In addition, the variant was highly conserved among mammals, and was predicted to be deleterious by online software programs.

Li Gong and Chunyan Wang contributed equally to this work.

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Conclusions: We report for the first time a novel heterozygous missense variant in *SOX9* (NM_000346:c.931G>T:p.Gly311Cys) in a Chinese CHD family. Our results provide further evidence supporting a causative role for *SOX9* variants in CHD.

KEYWORDS

congenital heart disease, incomplete penetrance, missense variant, SOX9, whole exome sequencing

1 | INTRODUCTION

Congenital heart disease (CHD), which involves multiple structural and functional abnormalities in the heart during the beginning of the fetal stage, is one of the most common birth defects, affecting 6–13 in 1000 newborns (Abid et al., 2014; Bakker et al., 2019; Dolbec & Mick, 2011; Liu et al., 2019). CHD describes a variety of anomalies and malformations involving the heart and greater blood vessels, most notably patent ductus arteriosus (PDA), atrial septal defect (ASD), ventricular septal defect (VSD), and tetralogy of Fallot (TOF) (van der Bom et al., 2011). CHD can arise from genetic, epigenetic, and environmental causes; however, the pathogenesis of a most CHD cases remains unexplained (Blue et al., 2012).

In the past decades, several studies have suggested that genetic factors may play an important role in CHD (Morton et al., 2021; Shabana et al., 2020; Williams et al., 2019). Heart development is subject to a precise regulatory mechanism that involves a series of genes interacting at different biological processes. Abnormal expression of any of these genes can affect heart development, leading to malformation. Previous studies have confirmed that variants in numerous genes that are involved in heart development, namely those encoding cardiac proteins and those involved in signal transduction and transcriptional regulation, could lead to CHD. For example, *NOTCH1* (OMIM 190198), *NKX2-5* (OMIM 600584), *CITED2* (OMIM 602937), *GATA4* (OMIM 600576), *GATA6* (OMIM 601656), *MYH6* (OMIM 106710), *TBX5* (OMIM 601620), and *PRKD1* (OMIM 605435) are associated with CHDs, including TOF, ASD, and VSD (Dixit et al., 2021; Freylikhman et al., 2014; Maitra et al., 2010; Massadeh et al., 2021; Razmara & Garshasbi, 2018; Tomita-Mitchell et al., 2007; Yadav et al., 2021; Zhang et al., 2020). With the rapid development of whole exome sequencing (WES), more causal variants in CHD-related genes have been identified in patients with CHD (Page et al., 2019); however, the cause of many cases of CHDs remains unknown. Increasing evidence suggests that CHD has high genetic heterogeneity, and further studies are required to explore the genetic etiology of CHD and better understand the mechanisms underlying this condition. In addition, most forms of CHD are sporadic, and very few families with autosomal dominant CHD have been described and assessed by WES to identify the causative genes.

In the present study, we performed WES on a three-generation Chinese family with autosomal dominant CHD to identify novel genetic causes of CHD. We identified a novel heterozygous missense variant (c.931G>T) of *SOX9* (OMIM 608160), which encodes a member of the SRY-related HMG-box family of transcription factors, thus expanding the spectrum of CHD-causing genetic variants, which should be useful for further pathogenic studies.

2 | MATERIALS AND METHODS

2.1 | Study subjects

A three-generation Chinese family with CHD was recruited from Wuhan Children's Hospital. All family members were clinically evaluated by reviewing the patient history, performing physical examinations, and consulting the medical records. Two patients were diagnosed with CHD (I-1, III-1; Figure 2a). The proband's parents and aunt do not have CHD, and the proband's aunt has two healthy sons. The proband of this family was a 2-month-old girl (III-1). The diagnosis of the proband with complex CHD including VSD and ASD, and her maternal grandmother with ASD were confirmed using a Philips Epiq7C ultrasonic diagnostic instrument (S8-3 probe, frequency 3.0–8.0 MHz) according to American Society of Echocardiography criteria (Lang et al., 2015). The proband underwent two surgeries, including atrial septal defect repair (ASDR) and ventricular septal defect repair (VSDR). Her grandmother underwent ASDR at the age of 42. Both of the patients underwent open-heart surgery with cardiopulmonary bypass. Peripheral blood samples were collected from the patient and her family members.

2.2 | Whole exome sequencing and bioinformatic analysis

Genomic DNA was extracted from peripheral blood from the proband, her mother, her aunt, and her maternal grandmother using a QIAAMP DNA blood mini kit (Qiagen, Inc) according to the manufacturer's instructions. The quality of the DNA samples was then assessed using a NanoDrop2000 (Thermo Scientific, MA).

Exome sequencing was performed on the proband, aunt, and maternal grandmother. Briefly, the subjects' exomes were captured using an Agilent SureSelect Human All Exon V6 Enrichment kit (Agilent) and then sequenced on a NovaSeq platform (Illumina) according to the manufacturer's guide. All reads were mapped to the human reference genome (hg19) using Burrows–Wheeler Alignment version 0.7.9a (http://bio-bwa.sourceforge.net). Single nucleotide variants (SNVs) and indels were detected using Genome Analysis Toolkit version 3.5 (https://gatk.broadinstitute.org/hc/en-us) and annotated using ANNOVAR (https://annovar.openbioinformatics.org/en/latest/user-guide/download/) by consideration of splice site, intronic, exonic, 5′ UTR, 3′ UTR, intergenic, upstream, or downstream locations.

Variants that met the following criteria were retained for further analysis: (a) heterozygous missense, nonsense, frameshift, non-frameshift, or splicing site variants in the proband and her affected grandmother, but not in her aunt; (b) missense variants predicted to be deleterious by at least two of following online software programs: Sorting Intolerant From Tolerant (SIFT; http://sift-dna.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and Mutation Taster (http://www.mutationtaster.org); (c) variants that were absent or rare (variants with a minor allele frequency <0.1%) in East Asians and the total population in the GnomAD (https://gnomad.broadinstitute.org) dataset. Furthermore, the remaining variant were classified according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines as pathogenic, likely pathogenic, with uncertain significance and likely benign or benign. Benign and likely benign variants were excluded.

2.3 | Segregation analyses

The candidate variant was validated, and its segregation was analyzed, in the patient, her mother, her aunt, and her maternal grandmother by standard Sanger sequencing. Primer5 software was used to design the polymerase chain reaction (PCR) primers specific to the regions of the variants in *SOX9* for Sanger sequencing. The primer sequences are shown in Table 1.

TABLE 1 *SOX9*-specific primers used for Sanger sequencing

ID	Sequence	Length (bp)
SOX9-1F	GCCCAGTGCCACAATCCTC	1207
SOX9-1R	CCACCAGTCTTCGTCCATCCT	
SOX9-2F	GCCTGGAAGCTCAATCGG	1336
SOX9-2R	TCCCTCGCTGCTAAAGTGTAAT	
SOX9-3F	GACAGTTTGGCGGATTTCA	1915
SOX9-3R	GGGTACGAGTTGCCTTTAGC	

2.4 | Online prediction

The GnomAD database was used to determine the frequency of the missense *SOX9* variant in East Asians and in the total population. Online prediction programs, including SIFT, PolyPhen-2, Mutation Taster, and CADD were used to predict the effect of the missense variant on the SOX9 protein. CLC Sequence Viewer 8 software was used for conservation analysis.

3 | RESULTS

3.1 | Clinical descriptions

A three-generation Chinese family including two CHD patients was enrolled in this study (Figure 2a). The proband (III-1) was diagnosed by echocardiography at 2 months and 2 days old as having as complex CHD. As shown on the ultrasonogram (Figure 1), the position of the heart, aortic ventricular connections, and aorta were normal, and the valve morphology, structure, and opening and closing were normal. However, interruptions occurred in both the atrial and interventricular septa. Furthermore, the results of the Color Doppler Flow Imaging showed that bidirectional shunt mainly left to right were detected at both the ventricular level and the atrial level. The peak flow velocity of the descending aorta was 1.3 m/s. There was a small amount of regurgitation at the tricuspid valve orifice, the peak flow velocity was 4.2 m/s, and the pressure gradient was 71 mmHg. There was a 1.8-mm left–right shunt signal between the descending aorta and the left pulmonary artery, with a peak velocity of 2.3 m/s, and the pressure gradient was 22 mmHg. Based on these results, the proband was diagnosed as having complex CHD, including ASD and VSD.

In addition, her maternal grandmother (I-1) was diagnosed as having ASD and underwent ASDR at the age of 42. None of the other family members exhibited CHD.

3.2 | Exome sequencing and cosegregation analysis

We performed WES on the affected subjects and the proband's unaffected aunt and excluded irrelevant or meaningless variants from the sequencing results by multiple bioinformatic analysis. Because the proband's maternal grandmother was affected, and her mother's phenotype was normal due to incomplete penetrance, we speculated that the inheritance pattern of the variant was dominant. Therefore, we selected rare heterozygous variants shared by the proband and her grandmother, but not carried by her aunt. In silico prediction revealed 11 deleterious variants, including 10 missense variants and one nonsense variant. The results are shown in Table S1. We ultimately identified missense variant c.931G>T (p.Gly311Cys) in *SOX9* and confirmed this variant by Sanger sequencing (Figure 2b). The results from the segregation analysis showed that the *SOX9* c.931G>T variant segregated in all affected individuals (I-1, III-1), as well as one family member who did not have any abnormal phenotypes (II-2). One unaffected family member (II-3) did

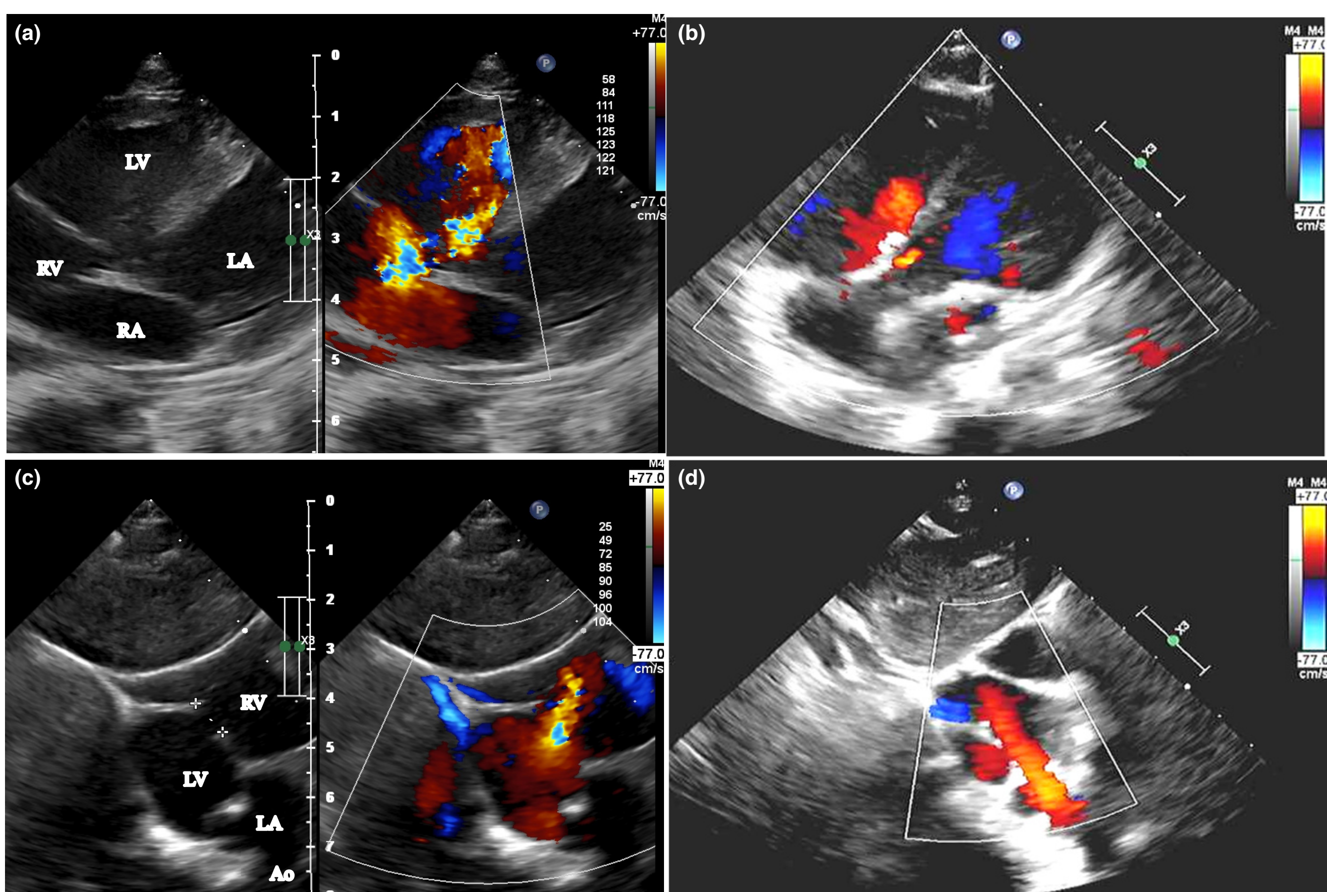
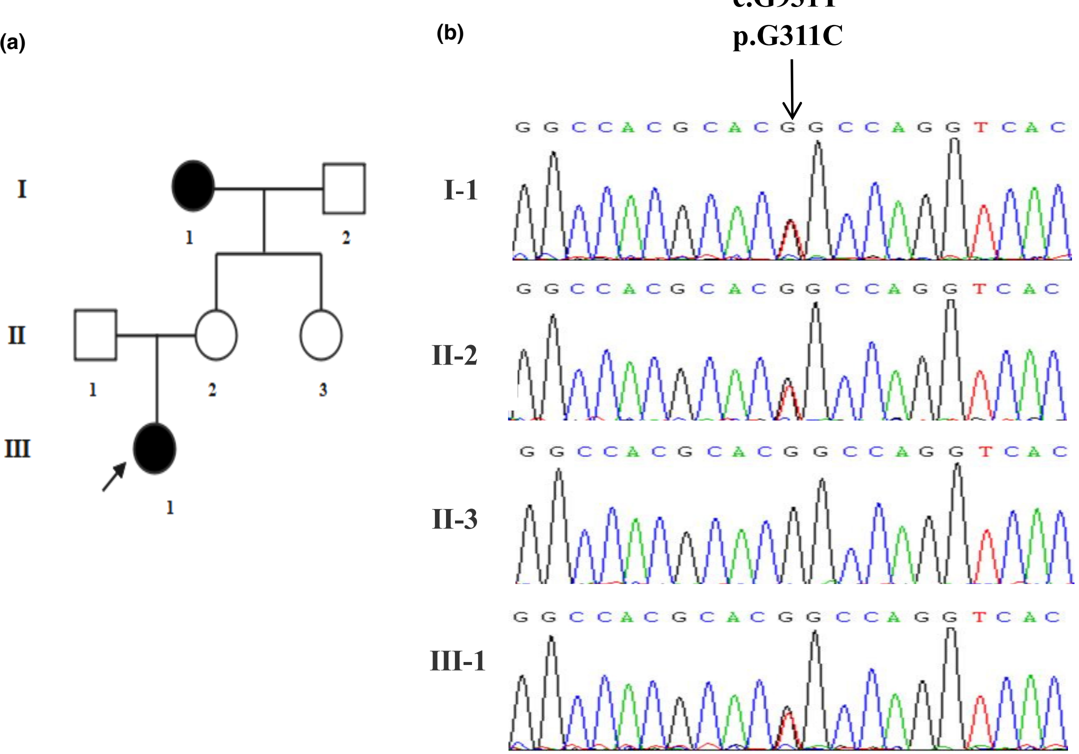
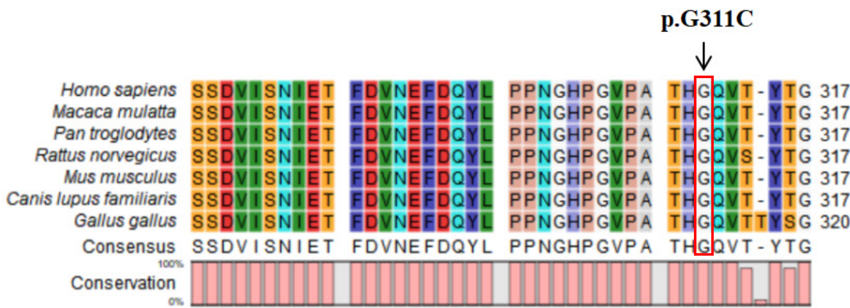
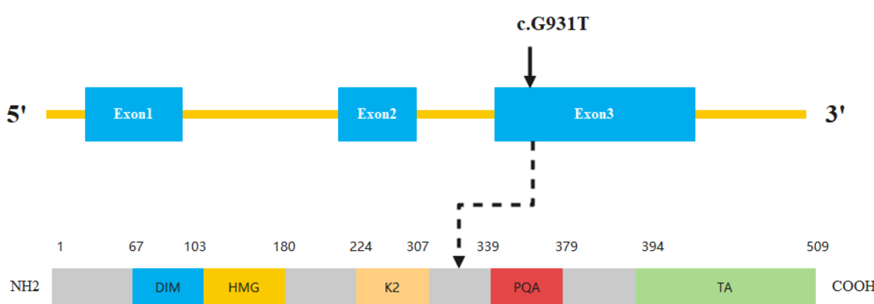


FIGURE 1 Echocardiogram images from patient III-1 showing the apex of the heart. (a, b) Apical four-chamber view showing a large ventricular septal defect (VSD) prior to surgery and the repaired VSD after surgery, respectively. (c, d) Apical three-chamber view showing an atrial septal defect (ASD) prior to surgery and the repaired ASD after surgery, respectively. Ao, aorta; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle

FIGURE 2 Genetic analysis of the missense variant in *SOX9*. (a): Pedigree of the family with CHD. The filled black symbols represent the affected members. The asymptomatic individual harboring the variant is indicated by the symbol with a central black spot. The arrow denotes the proband. (b): Sanger sequencing results from the proband, her mother, and her grandmother. The heterozygous c.931G>T variant in the *SOX9* gene was identified in the proband and her grandmother, but not in her mother. (c): Amino acid alignment of the SOX9 protein from several organisms. The position of Gly311 residue (highlighted by a red box) was highly conserved among different species. (d): The location of the variant in the intron-exon structure of *SOX9*. The arrow denotes the mutated site


(a)
(b)
c.G931T
p.G311C
I
II
III
I-1
II-2
II-3
III-1


(c)
p.G311C
Homo sapiens
Macaca mulatta
Pan troglodytes
Rattus norvegicus
Mus musculus
Canis lupus familiaris
Gallus gallus
Consensus
Conservation


(d)
c.G931T
5'
Exon1
Exon2
Exon3
3'
NH2
DIM
HMG
K2
PQA
TA
COOH

not carry the c.931G>T variant. These results confirmed that the inheritance pattern was autosomal dominant with incomplete penetrance.

3.3 | In silico analysis of the *SOX9* missense variant

The minor allele frequency of the observed variant was assessed in East Asian and global populations using the GnomAD database (see above; Table 2). The variant is reportedly absent in both the East Asian and global populations. The impact of this missense variant on protein function was predicted to be deleterious based on bioinformatics analysis using the SIFT, PolyPhen-2, Mutation Taster, and CADD databases. Furthermore, the *SOX9* variant was also found to be highly conserved among mammals (Figure 2c). These results suggest that this variant is highly pathogenic, and we hypothesized that this heterozygous missense variant of *SOX9* may be the cause of CHD in this affected family.

4 | DISCUSSION

In this study, we recruited a Chinese family with CHD. The proband presented with complex CHD, including VSD and ASD. A novel heterozygous missense variant in *SOX9*, c.931G>T (p.Gly311Cys), was identified in the family by whole exome and Sanger sequencing.

Previous studies have reported that the majority of pathogenic genes in patients with congenital heart disease occur in genes encoding transcription factors such as GATA4 and NKX2.5 (Reamon-Buettner & Borlak, 2010; Zhang et al., 2017). Many of these cardiac-related transcription factors play important roles in the major developmental pathways. The Sry-related HMG box (*SOX*) gene family encodes transcription factors that play important roles in embryonic development and cell fate determination (Bowles et al., 2000). There are 10 subgroups of SOX proteins (A-J) (Pevny & Lovell-Badge, 1997). Emerging evidence suggests that the SOX family, and especially the SOXF subfamily (SOX7, SOX17, and SOX18), participate in normal cardiovascular development (Lilly et al., 2017). In addition, variants in *SOXF* genes have also been reported in human cardiovascular diseases. For example, Long et al. (2013)) found that the proband of a CHD family carried a *SOX7* (OMIM 612202) gene duplication. Zhu et al. (2018)) identified rare variants in *SOX17* (OMIM 610928) in the patients with pulmonary arterial hypertension and congenital heart disease and suggested that *SOX17* could be a new risk gene contributing to PH-CHD, as well as idiopathic/familial PH. These findings highlight how critical SOX proteins are to cardiovascular development.

SOX9 encodes a transcription factor that is a member of the E subgroup of SOX proteins (including SOX8, SOX9, and SOX10) (Stolt & Wegner, 2010). *SOX9* is located on chromosome 17q24, contains three exons, and encodes a 509–amino acid protein that is expressed in a variety of tissues, such as cartilage, testis, heart, glial cells, and inner ear (Lefebvre et al., 2019; Symon & Harley, 2017). Previous studies using multiple model animals and a series of functional experiments have investigated the expression of this protein and the important role it plays in heart development. Montero et al. (2002)) reported dynamic SOX9 expression during heart development in chick embryos. Sox9b is one of the two mammalian Sox9 homologs in zebrafish. Loss of Sox9b prevented the formation of epicardium progenitors and affected the formation and migration of the epicardial layer around the heart (Hofsteen et al., 2013) and Sox9b has also been reported to regulate the expression of the critical cardiac development genes such as nkx2.5, nkx2.7 (Gawdzik et al., 2018). Analysis of knockout mice has shown that Sox9 is essential for the pathway that controls the formation of the cardiac valves and septa, and upregulation of SOX9 expression was found to be associated with ventricular septal defects (Akiyama et al., 2004; Garside et al., 2015; Lincoln et al., 2007). In humans, heterozygous SOX9 variants have been reported to result in campomelic dysplasia (CD), a severe skeletal malformation syndrome characterized by congenital bowing of the long bones, hypoplastic scapulae, a missing pair of ribs, pelvic and vertebral malformations, clubbed

TABLE 2 Biological analysis of the missense variant c.931G>T in *SOX9*

Gene	Chromosome position	Variant	Frequency[a]	Online prediction				ACMG scoring	ACMG pathogenicity
				SIFT	PP2	MT	CADD		
SOX9	chr17:70119929	c.931G>T p.Gly311Cys	Absent/Absent	D	D	D	26.7	PM1 +PM2 +PP3	VUS

Abbreviations: MT, mutation taster (D, disease causing); PP2, polyphen-2 (D, damaging); SIFT, sorts intolerant from tolerant (D, damaging); VUS uncertain significance.

[a]Frequency in overall population/East Asian population in gnomAD.

TABLE 3 Summary of *SOX9* variants identified in patients associated with heart disorders

Variants	Description	Patient phenotype	Reference
p.Gln458ArgfsX12	A frameshift variant of SOX9	Campomelic dysplasia with a secundum atrioseptal defect	Kim et al. (2011)
4.7 Mb deletion	A deletion including SOX9 coding region	Campomelic dysplasia, coarctation of the aorta, patent ductus arteriosus, ventricular septal defect, and persistent foramen ovale	Smyk et al. (2007)
translocation	A translocation breakpoint 375 kb upstream of SOX9	Campomelic dysplasia with an atrial septal aneurysm and patent ductus arteriosus	Leipoldt et al. (2007)
~1 Mb deletion	A deletion upstream of SOX9	Pierre robin sequence and congenital heart defects	Sanchez-Castro et al. (2013)

feet, Pierre Robin sequence (PRS), and facial dysmorphia (Lefebvre et al., 2019; Leipoldt et al., 2007; Unger et al., 1993). Interestingly, abnormal cardiac phenotypes have been reported in some patients with CD. Mansour et al. (Mansour et al., 2002) reported that CHDs including VSD and ASD were found upon necropsy in 22% of CD patients. In addition, as the shown in Table 3, Kim et al. (2011)) found a heterozygous frameshift variant (p.Gln458ArgfsX12) in *SOX9* in a patient with CD and ASD. Smyk et al. (2007)) identified a 4.7-Mb deletion including the SOX9 coding region in a patient with CD, PDA, VSD, and persistent foramen ovale. Leipoldt et al. (2007)) showed that a patient with CD, atrial septal aneurysm, and small PDA carried a translocation breakpoint 375 kb upstream of *SOX9*. In addition, Sanchez et al. showed that the deletion of upstream of SOX9 was found in an isolated CHD patient without the phenotype of skeletal dysplasia, suggesting that disruption of cardiac enhancers located upstream of SOX9 may be responsible for CHDs in human (Sanchez-Castro et al., 2013). Therefore, it is possible that variants in *SOX9* cause CHD in humans.

In this study, we performed WES on three members of a Chinese CHD family to identify potential pathogenic variants and genes based on rigorous bioinformatic analysis. Because the proband and her maternal grandmother were affected, but the proband's mother did not exhibit the abnormal cardiac phenotypes, we speculated that the inheritance pattern was autosomal dominant with incomplete penetrance (Figure 2a). Eventually,+++ by applying several filtering processes, we identified a previously unreported heterozygous missense variant, c.931G>T (p.Gly311Cys), in *SOX9* in the proband and her affected maternal grandmother. Furthermore, Sanger sequencing confirmed that, while the variant was present in the affected individuals and the proband's mother, who did not have any abnormal cardiac phenotypes, the proband's healthy aunt did not carry the variant. These results suggest that the inheritance pattern in this family was autosomal dominant with incomplete penetrance. The missense variant identified in *SOX9* is absent in East Asian populations and in the overall human population, based on the genome dataset archived in gnomAD (see above; Table 2). In addition, the mutated site is highly conserved among mammals, and the variant was predicted to be deleterious by three online software programs. Therefore, the heterozygous missense variant that we found may cause CHD by affecting SOX9 protein function.

In conclusion, we identified a novel heterozygous missense variant in *SOX9* (NM_000346:c.931G>T:p.Gly311Cys) as a possible causative variant for CHD. To the best of our knowledge, this is the first report of CHD caused by a variant in this gene in a Chinese family. It is unclear how the variant contributes to CHD phenotypes. Therefore, further studies are needed to clarify the exact mechanism of the association between the missense *SOX9* variant and CHD by performing functional experiments in vitro and in vivo, and studies with larger sample sizes are required to identify more cases of CHD associated with *SOX9* variants.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation was performed by Li Gong and Haiyang Xie; ultrasound images were provided by Jun Gao; reagents, materials, analysis tools, and data were contributed by Binbin Wang, Jing Wang, and Shenggui Qi. The experiments were performed by Tengyan Li; data analysis and interpretation were

performed by Binbin Wang, Jing Wang, and Chunyan Wang. The first draft of the manuscript was written by Chunyan Wang. All authors commented on early versions of the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets for this article are not publicly available due to concerns regarding participant/patient anonymity. Requests to access the datasets should be directed to the corresponding authors.

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SUPPORTING INFORMATION

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