INTRODUCTION

Cardiac valvulogenesis, which occurs at mid-gestation in embryonic development, is a highly conserved process among vertebrates and lead to the unidirectional flow of blood in the heart (Wu et al., 2017). Formation of cardiac cushions in the atrioventricular canal (AVC) and outflow tract (OFT) regions of the primitive heart tube is the start of cardiac valvulogenesis. Primitive heart tube consists of three layers: outer layer of myocardium, middle layer of cardiac jelly (also known as extracellular matrix, ECX), and inner layer of endothelial cells. During heart valve formation, a cluster of endothelial
cells cover the sites of future valves and migrate into the cardiac jelly through a process named endothelial–mesenchymal transformation (EndMT). Expansion of local cardiac jelly coupled with mesenchymal cells together constitute the cardiac cushions. Then the cushions protrude from the underlying myocardium, forming thin, tapered leaflets with a single endothelial cell layer and a central matrix by cell differentiation, apoptosis, and ECM (extracellular matrix) remodeling (Chakraborty et al., 2010; Koenig et al., 2017; Wirrig & Yutzey, 2011).

Normal development and functional maintenance of the heart valves rely on coordinating interactions between signaling molecules and transcription factors in time and space during embryonic development. For example, VEGF pathway could regulate the proliferation of endothelial cells during valve development; decrease of VEGF (OMIM 192240) expression during development could affect the cushion formation by inhibiting endothelial cell migration into the cardiac jelly (Dor et al., 2001). NOTCH pathway, BMP/TGF-β pathway, and WNT pathway take part in cardiac valvulogenesis by regulating EndMT or activating mesenchymal program (Hurlstone et al., 2003; Nakajima et al., 2000; Timmerman et al., 2004). In addition, several transcriptional factors, such as TWIST1 (OMIM 601622), TBX20 (OMIM 606061), NFATC1 (OMIM 600489), and SOX9 (OMIM 608160) (Akiyama et al., 2004; Chakraborty et al., 2008; de la Pompa et al., 1998; Ferese et al., 2018; Kirk et al., 2007; Ranger et al., 1998), could regulate cell proliferation, differentiation, or leaflet remodeling during valve development.

Defects in any of the steps in valvulogenesis may lead to the valvular congenital heart disease. Tricuspid atresia refers to morphological deficiency of the valve and confined right atrioventricular traffic due to tricuspid development disorder. It accounts for 1.1%–2.4% congenital heart defects and is the main reason for cyanotic congenital heart disease that is still hard to treat (Frock et al., 2017). In this study, we reported a healthy couple who consecutively had two fetuses aborted due to tricuspid atresia. We tended to identify the cause of this disease in the family by whole-exome sequencing technology and functional experiments.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Skin tissues of odinopoeia fetus were obtained after getting the parents’ permission and DNA was extracted using TIANamp Genomic DNA Kit (TIANGEN). We also collected the peripheral venous blood of the parents and extracted DNA using the QIAamp DNA Blood Midi Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer’s instruction.

2.2 | Ethical compliance

The study followed the 2013 Declaration of Helsinki and was approved by local ethics committees of Xinhua Hospital.

2.3 | Whole-exome sequencing (WES)

DNA of the fetus was sent to a commercial company (Shanghai Biotechnology Co, Ltd), which provided sequencing service using the Hiseq 2000 platform. DNA was sonicated into fragments and hybridized to the array. Enriched DNA fragments were eluted and amplified using ligation-mediated polymerase chain reaction (PCR). The enriched DNA was then ligated with DNA ligase to fragments ranging in size between 2 and 5 kb randomly; resultant DNA fragments were cut into average 200 bp and were submitted to standard Illumina Hiseq 2000 platform. Image analysis and base calling were carried on with the Illumina’s Consensus Assessment of Sequence and Variation 1.8, using the default parameters. According to the quality of reads, the company analyzed the fragments with different tools.

2.4 | Data screening and Bioinformatics analyses

We first screened the primary data following the criteria: Quality filter value was “PASS”; mutations located in exonic regions and gave priority to those in protein coding regions; mutation function excluded synonymous SNVs; max population frequency was lower than 0.001. General information of candidate genes was obtained from GeneCards (www.genecards.org) and PubMed and picked out those related to cardiac development or diseases. PROVEAN, PolyPhen, and MutationTaster were applied to predict the pathogenicity of mutations. MutationTaster was also applied to determine the homology of the mutation sites (www.mutationtaster.org). Alterations of protein properties and structures were analyzed by HOPE (www.cmbi.ru.nl/hope/).

2.5 | Plasmid construction and Site-directed mutagenesis

pCMV3.1-NFATC1 expression vector containing human NFATC1 coding sequence was purchased from Sino Biological (catalog: HG13963-CF). Mutant primers (F: 5’GGCTGAAACACCAAAGCACGCG3’; R: 5’CCAGGCTGCTGTTGGCCGC) were designed by online software Agilent (http://www.agilent.com/
home) and NFATC1 mutation was constructed by KOD-Plus-high fidelity PCR kit (TOYOBO). DEGS1 promoter region (approximate 1400bp) promoter region was amplified by PCR from Human genomic DNA and then cloned into the luciferase reporter pGL4.12 vector. All constructed plasmids were verified by sequence.

2.6 | Western blotting and cycloheximide chase assay

Total proteins of 293T or C2C12 cells were extracted by protein lysis buffer RIPA (Beyotime), and then separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Primary monoclonal antibody was anti-FLAG (1:1000, catalog: ARG62342, Arigo, China) and secondary antibody was anti-mouse (1:10000, Jackson). β-actin was chosen as a control. Detection of targeted protein bands was performed by ECL western blotting detection reagents according to the manufacturer's protocol.

For cycloheximide chase assay, C2C12 cells were transfected with wild-type NFATC1 and p.P.D322N plasmids. Thirty-six hours after transfection, cells were treated with 100 mg/ml of cycloheximide (MCE, HY-12320) for the indicated times (0, 30, 60, 90, and 120 min). Western blotting was used to analyze the protein expression.

2.7 | Cellular immunofluorescence

Wild-type NFATC1 and p.D322N plasmids were correspondingly co-transfected with C2C12 after 12 h of cell culture. Thirty-six hours after co-transfection, cells were treated with paraformaldehyde, and the primary antibody (1:1000, catalog: ARG62342, Arigo) was incubated overnight at 4°C. The next day after incubating the secondary antibody (Cy3-labeled goat anti-mouse IgG, Beyotime) for 1 h at room temperature, the cell was stained with DAPI staining solution. Finally the cells were observed under a fluorescence microscope.

2.8 | Dual-luciferase reporter assay

C2C12 cells were co-transfected with 25 ng wild-type NFATC1 or mutant plasmid and 100 ng DEGS1 promoter reporter plasmid. Ten nanograms of pGL4.74 plasmid was used as an internal control. Firefly and renilla luciferase activities were measured using the Dual-Glo luciferase assay system 6 kit (Promega) and the Centro XS3 LB 960 Microplate Luminometer (Berthold). The results represent the means of three independent experiments performed in triplicate.

2.9 | Statistical analysis

Analyses of western blotting and dual-luciferase reporter assay were performed using t-test by Prism GraphPad 6.02 software, and p < 0.05 was considered to be significant.

3 | RESULTS

3.1 | Clinical findings

I1: Father of the proband, a 25-year-old healthy male who mainly engaged in manual labor, smoking history with an average of 10 cigarettes per day. The father's family had no significant history of genetic disease or heart disease.

I2: Mother of the proband, a 25-year-old healthy female who played the role of housewife. The mother’s family also had no significant history of related diseases.

II1: Brother of the proband. Aborted due to the diagnosis of tricuspid atresia in the mother's uterus.

II2: Proband of this case. Prenatal examination of the mother showed that the fetus carried tricuspid atresia. Fetal echocardiography of the proband exhibited right ventricular dysplasia, left ventricular 11.8 mm, right ventricular 7.7 mm, RV/LV=0.65, perimembranous ventricular septal defect 3.8 mm, and tricuspid atresia.

In general, a healthy couple consecutively had two fetuses aborted because of tricuspid atresia. While in their family, no genetic or cardiac disease history was detected (Figure 1).

3.2 | Genetic analyses and candidate gene mutation identification

We gained the skin tissue after obtaining the permission of the parents and extracted the DNA using special kits, then the DNA sample was sent to a biotechnology company for whole-exome sequence. The quality of the raw data was screened and the selection conditions of candidate genes were limited. We ruled out synonymous SNVs and max population frequency of sites higher than 0.001 and mutations located in exonic regions, and gave priority to those in protein coding regions. After screening the quality and pathogenicity of date, we searched for the function and related diseases of the candidate genes by GeneCards and PubMed and aimed to pick out those involved in embryonic development, especially cardiac or valvular development and led to congenital heart defects. We finally found one mutation of NFATC1 gene in our data (Sanger confirmation is showed in Figure 1B). This mutant represents that 964 guanine (G) of NFATC1(NM_172390) coding sequence changed to adenine (A), correspondingly 322 aspartic acid (D) turned into asparagine (N). A traceback of the family suggested that...
this mutation of the proband may inherit from its mother, because our detection of the mother’s peripheral blood also found this mutation but the father did not. This mutation was not found in 1000G database and ExAC Asian population; two heterozygous were detected in ExAC European population, while the minor allele frequency of total global population was 0.00001745, far below the incidence of congenital heart disease (0.007), suggesting it a rare mutation and may has the potential to cause disease. Homology analysis suggested that amino acid 322 of NFATC1 protein was highly conserved among species (Figure S1). What’s more, PROVEAN, PolyPhen, and MutationTaster consistently predicted that the mutation site was intolerable and could lead to disease.

Our study also screened three myocardial development-related genes NDUFV2 (OMIM 600532), TTN (OMIM 188840), and TCAP (OMIM 604488) and three left-right patterning-related genes DNAH5 (OMIM 603335), SHROOM2 (OMIM 300103), and C2CD3 (OMIM 615944), mutations of which could lead to cardiomyopathy or heterotaxia. Despite the significant role in heart development or heart disease, correlation between tricuspid atresia and these six genes was weaker than that of NFATC1 according to recent studies. Detailed information of candidate genes and mutants was listed in Table 1.

3.3 | Functional analyses of NFATC1 c.964G>A mutation

MutationTaster provided the information of conservation of specific site and showed that NFATC1 p.D322 was highly conserved among species, indicating that mutation occurred in the loci had higher danger to alter protein function. HOPE showed a difference in charge between the wild type and mutant amino acid, charge loss of wild-type residue could disturb interactions with other molecules or residues.

Western blotting was applied to research on protein expression alteration caused by mutation. 293T cells and C2C12 cells were used to express wild-type NFATC1 protein and mutant p.D322N protein. By anti-flag antibody hybridization and gray value analysis, we found that expression of mutant p.D322N protein was less than that of wild type in both 293T and C2C12 cells (Figure 2, only showed result in C2C12 cells). We used cycloheximide chase assay to verify the stability of the mutant protein, the result showed both NFATC1 and mutant p.D322N were stable in vitro in our study (Figure S2).

We performed a cellular immunofluorescence experiment to further verify whether the mutation has an effect on protein localization. In our study, wild-type NFATC1 protein was expressed in a large amount in the cytoplasm, also seen in the nucleus. Mutant p.D322N was also expressed in the cytoplasm and nucleus, and the expression trend was not significantly different from that in the wild type (Figure S3).

Next, we used dual-luciferase reporter assay to assess the transcriptional impact which might be arisen from mutation. C2C12 cells were co-transfected with 25 ng wild-type NFATC1 or mutant plasmid and 100 ng DEGS1 (OMIM 615843) promoter reporter plasmid. As shown in Figure 2, wild-type NFATC1 moderately activated the DEGS1 promoter which had harbored a consensus NFAT-binding site, while this transcriptional activity of mutant p.D322N was decreased compared to wild type ($p = 0.0074$), indicating that mutation p.D322N could impact the transcriptional activity of NFATC1 protein. The results represent the means of three independent experiments performed in triplicate and $p < 0.05$ was considered to be significant.

4 | DISCUSSION

NFATC1 belongs to the nuclear factor in activated T cells (NFAT) family which has been proved to be critical for
proliferation, differentiation, and homeostasis in several cells during embryonic development and postnatal life (Crabtree & Olson, 2002). Functional NFATC1 locates in nuclear and promotes numerous biological activities such as neuronal guidance, immune cell function, skeletal and cardiac muscle hypertrophy, osteoclast differentiation, and cardiac valve development (Hogan et al., 2003; Li et al., 2013; Yehya et al., 2006). NFATC1 is specifically expressed in the AVC and OFT endothelial cells at the early stages of endocardial cushion formation and promotes endocardial cell proliferation of these regions during cardiac valve formation. Studies similarly found that NFATC1 knockout mice died due to defect in cardiac cushion formation (Pompa et al., 1998; Ranger et al., 1998). Mutation screening carried out by Georges and his colleagues among tricuspid atresia patients indicated the pathogenic role of NFATC1 mutations in tricuspid atresia (Abdul-Sater et al., 2012).

Here, we found a healthy couple who consecutively had two fetuses aborted due to tricuspid atresia. Fetal echocardiography indicated the proband of the study, the second fetus of the couple, carried right ventricular dysplasia, perimembranous ventricular septal defect, and tricuspid atresia. Considering severe intrauterine growth retardation and cardiac dysfunction, the proband was treated with induction of labor just as the fetus of the previous pregnancy. We obtained the skin tissue of the proband and extracted the DNA after gaining the permission of the parents. Whole-exome sequencing was applied to explore the genetic etiology of the proband’s disease. Data output of whole-exome sequencing technique was huge and complicated due to its capacity of sensitively capturing alterations of nucleotide acids in the sample. Therefore, effective, correct, and reasonable analyses of the data were one of the most critical parts of our research. We strictly screened the raw data from the aspects of sequencing quality, mutation site attributes, and gene functions. Finally, we found one mutation of NFATC1, which had been reported as tricuspid atresia-related gene among seven candidate genes.

After finding a gene associated with heart valve development, we analyzed the pathogenicity of the mutation site of NFATC1 gene. This mutant showed an alteration from 964 guanine (G) of NFATC1 (NM_172390) coding sequence to adenine (A), correspondingly 322 aspartic acid (D) to asparagine (N). NFATC1 p.D322 was highly conserved among species, mutation in the loci was possible to alter protein function. This mutation was not newly found because two heterozygotes were detected in ExAC European population. The minor allele frequency of total global population was 0.00001745, according to ExAC database, far below the incidence of congenital heart disease (0.007), suggesting it a rare mutation and may also has the potential to cause disease. The possible pathogenic significance of this site was confirmed by other online software. PROVEAN, PolyPhen, and

| Gene   | mRNA RefSeq | mRefSeq | Probe change | Protein change | Difference | PolyPhen (score) | PROVEAN (score) | MutationTaster | Software prediction |
|--------|-------------|---------|--------------|----------------|------------|-----------------|-----------------|-----------------|-------------------|
| DNAH5  | NM_172390   | NM_0012866577 | c.A376G | p.I126V | Neutral (-0.15) | Benign (0.001) | Delerious (−2.631) | Disease causing |
| C2CD3  | NM_001649   | NM_001649 | c.C3588G | p.D322N | Deleterious (-6.55) | Benign (0.175) | Delerious (−2.631) | Disease causing |
| SHROOM2 | NM_001649 | NM_001649 | c.C588G | p.S196R | Deleterious (-4.614) | Benign (0.175) | Delerious (−2.631) | Disease causing |
| DNAH5  | NM_0012866577 | NM_0012866577 | c.A376G | p.I126V | Neutral (-0.15) | Benign (0.001) | Delerious (−2.631) | Disease causing |
| C2CD3  | NM_172390   | NM_172390 | c.G964A | p.D322N | Deleterious (-2.631) | Benign (0.001) | Delerious (−2.631) | Disease causing |
| TTN    | NM_003673   | NM_003673 | c.T338C | p.V112A | Deleterious (-3.200) | Benign (0.175) | Delerious (−2.631) | Disease causing |
| TCAP   | NM_003673   | NM_003673 | c.T338C | p.V112A | Deleterious (-3.200) | Benign (0.175) | Delerious (−2.631) | Disease causing |
| NDUFV2 | NM_001074   | NM_001074 | c.T338C | p.V112A | Deleterious (-3.200) | Benign (0.175) | Delerious (−2.631) | Disease causing |
| DNAH5  | NM_172390   | NM_172390 | c.G964A | p.D322N | Deleterious (-2.631) | Benign (0.001) | Delerious (−2.631) | Disease causing |
| Table 1 Detailed information of candidate gene mutations | | | | | | | |
MutationTaster consistently predicted that the mutation site was intolerable and could lead to disease. HOPE indicated a difference in charge between the wild type and mutant amino acid, which might disturb interactions with other molecules or residues.

To further confirm the pathogenicity of the mutant, we reconstructed the NFATC1 expression vector containing this mutation site and detect the functional difference between the mutant plasmid and wild-type plasmid. We failed to gain embryonic cardiac cells for experiments, so we chose 293T cell and C2C12 cell which are relatively well cultured and have higher transfection efficiency for our research. Western blotting indicated that the expression of p.D322N was lower than the wild-type NFATC1. One possible reason might be due to the alteration of amino acid properties caused by mutation decreased the stability of NFATC1 protein. We, therefore, used cycloheximide chase assay to verify the stability of the mutant protein and found there was no significant difference between half-life of mutant protein than that of wild-type protein. Therefore, the decrease in protein expression may not be due to the decline in the stability of the synthesized protein, but due to other causes that needed to be further explored. NFATC1 is widely expressed in cells. One research from Rao pointed out that NFATC1 can be stimulated by calcineurin to migrate from the cytoplasm into the nucleus, and play the biological role of transcription factors (Rao et al., 1997). Several researches showed NFATC1 mutations can lead to changes in the location of NFATC1 protein (Abdul-Sater et al., 2012; Ferese et al., 2018). While, we find no significant difference in protein localization between wild-type and mutant NFATC1 in our study. We then examined the effect of mutation on the transcriptional activity of transcription factor NFATC1 by dual-luciferase reporter assay. DEGS1, which was involved in the development and formation of cardiac valves through mediating cell apoptosis, was chosen as reporter gene. Results indicated that mutant p.D322N could affect the transcriptional activity of wild-type NFATC1, leading to aberrant expression of downstream DEGS1. Decrease of transcriptional activity might be caused by decrease of binding ability of protein to DNA, or abnormal expression of transcriptional factor. In our study, we believed that the anomalous transcriptional activity of p.D322N might be associated with the decrease in protein expression. Although specific mechanism has not yet been clarified, our experimental results are consistent with those of bioinformatic analyses, that is mutant p.D322N might alter the protein function of NFATC1 and lead to disease.

There is an atypical genotype–phenotype inconsistency in our case; DNA sequencing indicated that the normal mother of the proband also carried the pathogenic c.964G>A mutation, which means mutation contributed to disease in the proband while it had no effect on the mother. Similar phenomenon was also found in other studies (Faria et al., 2008; Guo et al., 2015), while the underlying mechanism has not yet been fully elucidated. Traditional genetic central dogma could not precisely explain the relationship between genotype and phenotype because studies confirmed that genetic modification or environmental factors can influence the final product of gene expression. We speculated whether there were different environmental stimulations among individuals, or altered gene modifications caused by molecular interactions that cooperatively promote the genotypic–phenotypic heterogeneity. It was also a pity that we failed to gain any sample of the first fetus, which was of great significance for genetic etiological diagnosis.
Though some problems still remain unsolved and discussed, our study screened out one **NFATC1** mutation and proved its potential pathogenic significance by bioinformatic analyses and functional experiments. We aimed to deepen the understanding of etiology of tricuspid atresia and gene function of **NFATC1**, and provide some references or suggestions for genetic diagnosis of tricuspid atresia in the future.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

Bojian Li: complete experiment design, experiment operation and article writing; Tingting Li: participate in experiment design, operation and writing; Tian Pu and Chunjie Liu: data analysis and completion of part of the experimental operation; Sun Chen and Kun Sun: consultants of clinical part and participate in article review; Rang Xu: design of experiments and revision of article.

**DATA AVAILABILITY STATEMENT**

Additional supporting information may be found online in the Supporting Information section.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.