Functional analysis of the congenital heart disease-associated *GATA4* H436Y mutation *in vitro*

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Abstract. Congenital heart disease (CHD) is the most common type of developmental defect, with high rates of morbidity in infants. The transcription factor GATA-binding factor 4 (*GATA4*) has been reported to serve a critical role in embryogenesis and cardiac development. Our previous study reported a heterozygous *GATA4* c.1306C>T (p.H436Y) mutation in four Chinese infants with congenital heart defects. In the present study, functional analysis of the *GATA4* H436Y mutation was performed *in vitro*. The functional effect of *GATA4* mutation was compared with *GATA4* wild-type using a dual-luciferase reporter assay system and immunofluorescence. Electrophoretic mobility-shift assays were performed to explore the binding affinity of the mutated *GATA4* to the heart and neural crest derivatives expressed 2 (*HAND2*) gene. The results revealed that the mutation had no effect on normal nuclear localization, but resulted in diminished GATA-binding affinity to *HAND2* and significantly decreased gene transcriptional activation. These results indicated that this *GATA4* mutation may not influence cellular localization in transfected cells, but may affect the affinity of the GATA-binding site on *HAND2* and decrease transcriptional activity, thus suggesting that the *GATA4* mutation may be associated with the pathogenesis of CHD.

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Introduction

Congenital heart disease (CHD) is the most common type of developmental abnormality at birth, with an incidence of ~1% of live births worldwide (1,2). Furthermore, it is the leading non-infectious cause of mortality in newborns (3). The most common types of congenital heart defects, including ventricular septal defect (VSD) and atrial septal defect (ASD), account for a high proportion of total congenital heart disease (4). Therefore, it is urgent to determine the pathogenesis of these congenital cardiovascular diseases. VSD and ASD are anatomically characterized by an interatrial and ventricular septum that is defective or absent, causing the blood to flow directly between the atria and ventricles of the heart (5).

Cardiogenesis from the early embryo to formation of the fully functional four-chambered heart is a highly dynamic and complex process that requires numerous factors (6); cardiac transcription factors are considered to be the leading contributors to the normal development of the embryonic heart and include the GATA family (7). In addition, an increasing number of studies have reported that genetic risk factors may disrupt the biological process of heart development and subsequently lead to CHD (8,9). Several genes that are essential for heart development have been identified in the occurrence of CHD, including GATA-binding protein 4 (*GATA4*), T-box transcription factor 5 (*TBX5*) and NK2 homeobox 5 (7,10,11).

GATA4 belongs to a family of DNA-binding proteins with conserved zinc finger domains that can specifically bind the consensus DNA sequence GATA motif present in the promoter of several target genes involved in cardiogenesis, such as atrial natriuretic factor (*ANF*) (12,13), and can interact with other transcriptional factors. *GATA4* serves an important role in heart development, including in the proliferation of cardiomyocytes, endocardial cushion formation, development of the right ventricle and septation of the outflow tract. A previous study reported that the *GATA4* transcription factor is required for ventral morphogenesis and heart tube formation

in mice via knockout of the *GATA4* gene (14). Furthermore, mice homozygous for the *GATA4* G295S mutant allele exhibit normal ventral body patterning and heart looping, but have a thin ventricular myocardium, single ventricular chamber and lethality at embryonic day 11.5 (15). The importance of *GATA4* in cardiac development in other organisms, such as chicks, flies and fish, has also been demonstrated (16). These findings indicate that the GATA4 transcription factor may be closely associated with cardiac development in humans and other animals. Based on our previous study, we have established a mouse model of the *GATA4* p.H435Y mutation and propagated it successfully for further research (17). To date, numerous mutations in the *GATA4* gene have been reported in patients with CHD.

Our previous study reported a heterozygous *GATA4* c.1306C>T (pH436Y) mutation in four Chinese infants with congenital heart defects (18). In the present study, further functional analysis of the *GATA4* H436Y mutation was performed *in vitro*, and the molecular mechanism underlying the effect of this mutation on gene function was explored.

Materials and methods

GATA4 amino acid sequence conservation and mutation prediction. In our previous study (18), a heterozygous *GATA4* c.1306C>T (p.H436Y) mutation was detected in four infants with sporadic cardiac septal defects via sequencing of all exons and flanking intron sequences. Conservation of the amino acids was estimated by aligning genes from various species using National Center for Biotechnology Information Blast (blast.ncbi.nlm.nih.gov/Blast.cgi).

PolyPhen-2 (genetics.bwh.harvard.edu/pph2), SIFT (sift.jcvi.org) and Mutation Taster (www.mutationtaster.org) programs were used to predict the disease-causing potential of the mutation.

Plasmid construction and site-directed mutagenesis. A wild-type *GATA4* expression plasmid was constructed by cloning the entire human *GATA4* cDNA (accession no: NM_002052) into pcDNA3.1 (+) expression vector with a C-terminal flag-tag (Youbio). A point mutation was introduced into the wild-type *GATA4*-pcDNA3.1 plasmid using the KOD-plus-mutagenesis kit (cat. no. SMK-101; Toyobo Life Science), according to manufacturer's protocol, and confirmed by Sanger sequencing (19). The reporter plasmid, ANF-luciferase (ANF-luc), was constructed as previously described (20,21).

Cell culture, transfection and luciferase reporter assay. HeLa cells, originally purchased from the Cell Bank of type culture collection of the Chinese Academy of Sciences, were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2. Cells were seeded in 12-well plates at a density of 1-4x10^5 cells/well at 24 h prior to transient transfection using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). For co-transfection luciferase assays, 2.5 μg pcDNA3.1, 2.5 μg wild-type *GATA4*-pcDNA3.1 or 2.5 μg mutant *GATA4*-pcDNA3.1 were co-transfected with 2.5 μg ANF reporter plasmid. The pRL-TK plasmid (Promega Corporation) was co-transfected with the plasmids mentioned previously to normalize the luciferase activity. Luciferase activity was measured at 48 h after transient transfection; three independent experiments were performed in duplicate with the ANF-luc reporters. The firefly luciferase activity was normalized to *Renilla* luciferase activity, and fold activation of wild-type *GATA4* and mutant *GATA4* luciferase activities were calculated with respect to the pcDNA3.1 value.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from HeLa cells transfected with wild-type *GATA4*-pcDNA3.1 and mutant *GATA4*-pcDNA3.1 expression plasmids using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) 24 h after transfection. RNA samples were reverse transcribed into cDNA using a PrimeScript RT kit (cat. no. #RR036A; Takara Biotechnology Co., Ltd.), according to manufacturer's protocol. Subsequently, relative quantification was performed using the $2^{-\Delta\Delta Cq}$ method (22) and the TB Green system kit (Toyob Life Science), according to manufacturer's protocol. The primer sequences were synthesized by Generay Biotech Co., Ltd. as follows: *GATA4*, forward, 5'-GTCACACATGCTTCCAGGTAATG-3' and reverse, 5'-GGGAACGGTAAATGGCTCTCTA-3'; *GAPDH* forward, 5'-GTCACACATGCTTCCAGGTAATG-3' and reverse, 5'-GGGAACGGTAAATGGCTCTCTA-3'. The PCR thermal cycling conditions were as follows: 95˚C for 60 sec followed by 40 cycles of amplification at 95˚C for 30 sec, and annealing and extension at 60˚C for 30 sec; extension at 60˚C for 5 min. All reactions were performed in triplicate, and *GAPDH* was used as an internal control to normalize expression levels.

Western blot analysis. HeLa cells were transfected with wild-type *GATA4*-pcDNA3.1 or mutant *GATA4*-pcDNA3.1 expression plasmids, and whole cell extracts were obtained using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) containing 1X protease inhibitor cocktail. The protein concentrations were detected using a bicinchoninic acid (BCA) protein assay kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Subsequently, ~10 μg protein extracts were separated using 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked with PBS with 1% Tween-20 (PBST) containing 5% BSA for 2 h at room temperature and then probed with primary antibodies against *GATA4* (dilution 1:10,000; cat. no. ab124265; Abcam) and *GAPDH* (dilution 1:3,000; cat. no. ab9482; Abcam) at 4˚C overnight. The antigen-antibody complex was then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (dilution 1:3,000; cat. no. M21002; Abmart) or anti-mouse secondary antibody (dilution 1:3,000; cat. no. M21001; Abmart) for 1 h at room temperature. Blots were visualized using an ImageQuant LAS 4000 (GE Healthcare). GAPDH served as a loading control.

Immunofluorescence and subcellular localization. HeLa cells were seeded onto 20 mm glass-bottom cell culture dishes (NEST Scientific) at a density of 0.6x10^5 cells/ml 24 h before transfection with wild-type *GATA4*-pcDNA3.1 or mutant *GATA4*-pcDNA3.1 expression plasmids using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific,

Inc.). HeLa cells were fixed with 3.7% formaldehyde/PBS for 20 min at room temperature and permeabilized with 0.1% Triton X-100/PBS for 1 h at room temperature 48 h after transfection. Subsequently, cells were incubated with a primary antibody against *GATA4* (dilution 1:800; cat. no. ab124265; Abcam) overnight at 4˚C and then detected using anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (dilution: 1:500, cat. no. ab150080; Abcam) at room temperature for 2 h. Nuclear staining was performed with a 1:1,000 dilution of DAPI at room temperature for 20 min. The cells were observed under a Leica TCS SP8 Laser Scanning Confocal microscope (Leica Microsystems GmbH).

Electrophoretic mobility shift assay (EMSA). The enhancer region of the heart and neural crest derivatives expressed 2 (*HAND2*) gene contains two conserved consensus binding sites for GATA factors (23). The biotin-labeled oligonucleotide corresponding to the conserved GATA-binding sites at -3,039 and -3,140 (i.e. G1: 5'-TGATAA-3') of the *HAND2* gene was synthesized by Generay Biotech Co., Ltd., as follows: Forward 5'-GCA GTTAACTGATAATGACACTGTG-3' and reverse 5'-CAC AGTGTCATTATCAGTTAACTGC-3'. An unlabeled oligonucleotide with the same sequence was used as the competitor. Oligonucleotide pairs were annealed into double strands; the DNA-binding ability was detected by EMSA using a scientific light-shift EMSA kit (cat. no. 20148; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. HeLa cells were harvested 48 h after transfection with wild-type *GATA4*-pcDNA3.1 or mutant *GATA4*-pcDNA3.1 expression plasmids Whole cell extracts were prepared using RIPA lysis buffer (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a BCA protein assay kit. Whole cell extracts (10 μg) were incubated with 20 fmol of biotin-labelled probe in binding buffer containing poly (dI-dC), 50% glycerol and 1% NP-40 (included with EMSA kit) for 30 min at room temperature. A 200-fold excess of unlabeled probe was added to the reaction for competition experiments to confirm the specificity of the binding. Supershift analysis was performed by adding 1 μl neat *GATA4* antibody (cat. no. ab124265; Abcam) to the whole cell extracts for 20 min prior to the addition of the labelled probe. Protein-DNA complexes were separated from the free probe by 6% polyacrylamide gel electrophoresis. The DNA-protein complexes were analyzed using GE ImageQuant LAS4000 mini (GE Healthcare).

Statistical analysis. All data are presented as the mean ± standard deviation of three independent experiments. Differences between multiple groups were analyzed using one-way ANOVA and the least significant difference post hoc test. Statistical analysis was performed using SPSS software v20.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of the GATA4 mutation on gene transcription activity. Our previous study (18) reported a heterozygous *GATA4* c.1306C>T (p.H436Y) mutation in four Chinese children with congenital heart defects, which was located on exon 7. This mutation exhibits conserved evolution, and was predicted to be deleterious and disease causing, as determined using SIFT, Polyphen-2 and Mutation Taster. In order to confirm whether the *GATA4* H436Y mutation affects the functional activity of *GATA4,* the present study used ANF-luc in HeLa cells, as described previously (24). As shown in Fig. 1, the *GATA4* mutation significantly reduced reporter gene transcription activity compared with the wild-type controls (P<0.01), thus suggesting that the mutant *GATA4* significantly diminished the transcriptional activity of *GATA4*.

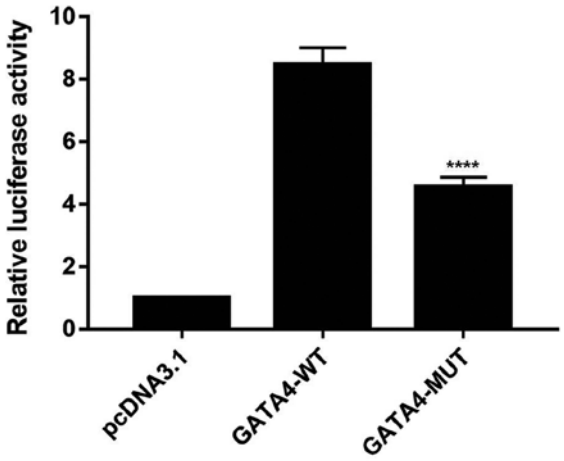

Figure 1. Atrial natriuretic factor-luciferase reporter assay in HeLa cells. The results revealed that the *GATA4* mutation significantly reduced the gene transcriptional activity of *GATA4*. All experiments were repeated three times and each was performed using duplicate samples. Data are presented as the mean ± standard deviation. ****P<0.0001 vs. *GATA4*-WT. *GATA4*, GATA-binding factor 4; MUT, mutant; WT, wild-type.

Expression of wild-type and mutant GATA4 in HeLa cells. RT-qPCR was performed to measure the mRNA expression levels of wild-type and mutant *GATA4* following extraction of total RNA from HeLa cells. The results of RT-qPCR revealed that the mRNA expression levels of *GATA4* were significantly lower in the mutant group compared with in the wild-type group (Fig. 2A; P<0.01). The present study also performed western blot analysis to detect the protein expression levels of *GATA4* in the wild-type and mutant groups; the results demonstrated that the *GATA4* mutation significantly reduced *GATA4* protein expression (Fig. 2B). These results are consistent with those of RT-qPCR, in which the *GATA4* mutation reduced transcriptional activity of the gene.

Subcellular localization of the wild-type and mutant GATA4 proteins. GATA4 is a nuclear transcription factor that is localized in the nucleus. To determine whether the *GATA4* c.1306C>T (p.H436Y) mutation altered distribution of the *GATA4* protein in cells, the present study performed immunofluorescence analysis in HeLa cells, in order to detect its cellular localization. As shown in Fig. 3, although the protein expression of mutant *GATA4* was reduced compared with the wild-type, the protein localization of mutant *GATA4* was similar to wild-type *GATA4*, with both localized in the nucleus, thus suggesting that the *GATA4* mutation did not influence the protein subcellular localization.

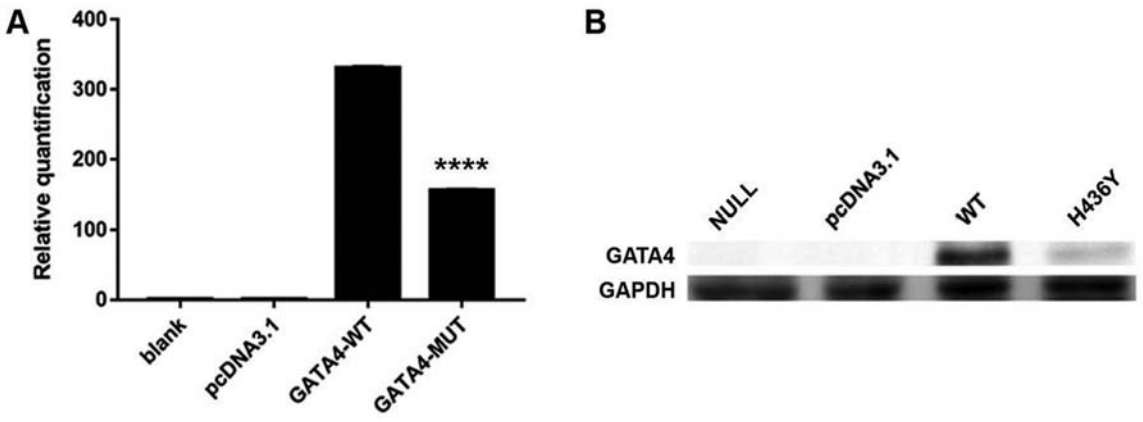

Figure 2. Expression of WT and MUT *GATA4* in HeLa cells. (A) Reverse transcription-quantitative PCR results revealed that the mRNA expression levels of *GATA4* were decreased in the mutant group when compared with the wild-type group. ****P<0.0001 vs. *GATA4*-WT. (B) Western blot analysis detected the protein expression levels of *GATA4* in the MUT and WT groups. Anti-GATA4 antibody was used as the primary antibody and anti-GAPDH was used as the internal control. The c.1306C>T mutation significantly reduced GATA4 protein expression. *GATA4*, GATA-binding factor 4; MUT, mutant; WT, wild-type.

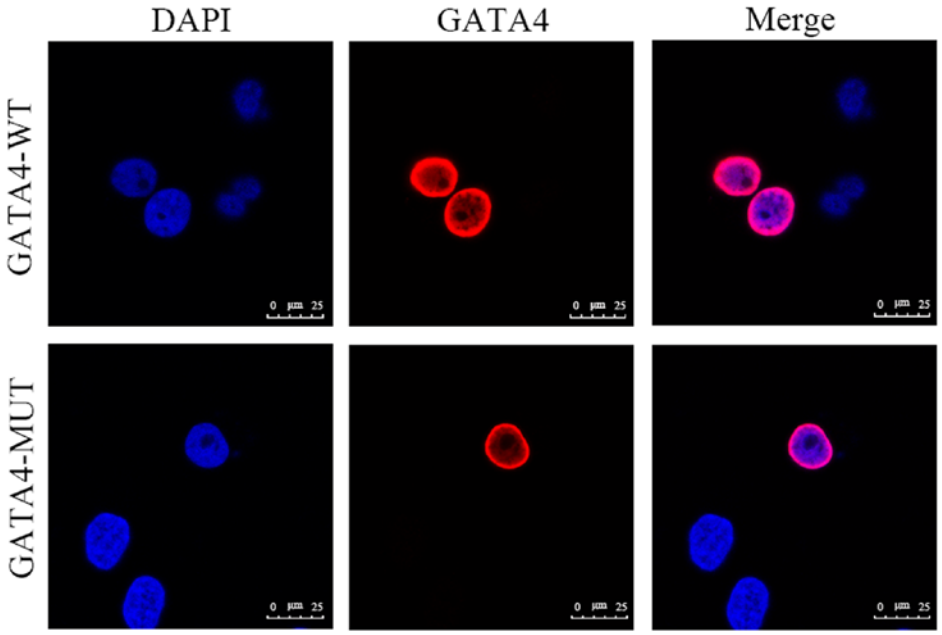

Figure 3. Subcellular localization of *GATA4*, as determined by immunofluorescence in HeLa cells. For each construct, anti-FLAG (red) and DAPI (blue) staining was presented individually and merged. WT and MUT *GATA4* were localized exclusively to the nuclei with normal nuclear distribution. *GATA4*, GATA-binding factor 4; MUT, mutant; WT, wild-type.

Effects of the GATA4 mutation on DNA-binding affinity. A previous study (25) demonstrated that *GATA4* could interact with the cardiac-expressed basic helix-loop-helix transcription factor *HAND2* gene to regulate transcription of the downstream gene by binding to the conserved GATA-binding sites on the *HAND2* gene (G1: 5'-TGATAA-3'; G2: 5'-CTATCT-3'; Fig. 4A). To determine whether the *GATA4* mutant (c.1306C>T; p.H436Y) affects the binding ability of the *GATA4* protein to the conserved GATA-binding site in the promoter of the *HAND2* gene, the present study performed EMSA using wild-type and mutant *GATA4* proteins from transfected HeLa cells, with a biotin-labeled probe. As shown in Fig. 4B (lane 2), the wild-type *GATA4* protein could bind to the conserved GATA-binding site. In order to confirm the binding specificity of the *GATA4* protein to the conserved GATA-binding site on the *HAND2* gene, an unlabeled probe at 100X was used to compete with the biotin-labeled probe at 1X bound to the wild-type *GATA4* protein. As shown in Fig. 4B (lane 3), the protein/DNA complex could compete with an excessive amount of unlabeled probe. However, when the equivalent amount of mutant *GATA4* protein was added, the DNA/protein band showed a lighter band than the *GATA4*-WT group but a stronger band than the competitor group (Fig. 4B, lane 4), thus suggesting that the *GATA4* mutation reduces DNA-binding affinity. In addition, supershift analysis was conducted to prove that binding was caused by the *GATA4* protein (Fig. 4B, lane 5). These results indicated that the mutant *GATA4* protein decreased the ability to bind the conserved GATA-binding site on the *HAND2* gene, which may contribute to abnormal expression of the *HAND2* gene.

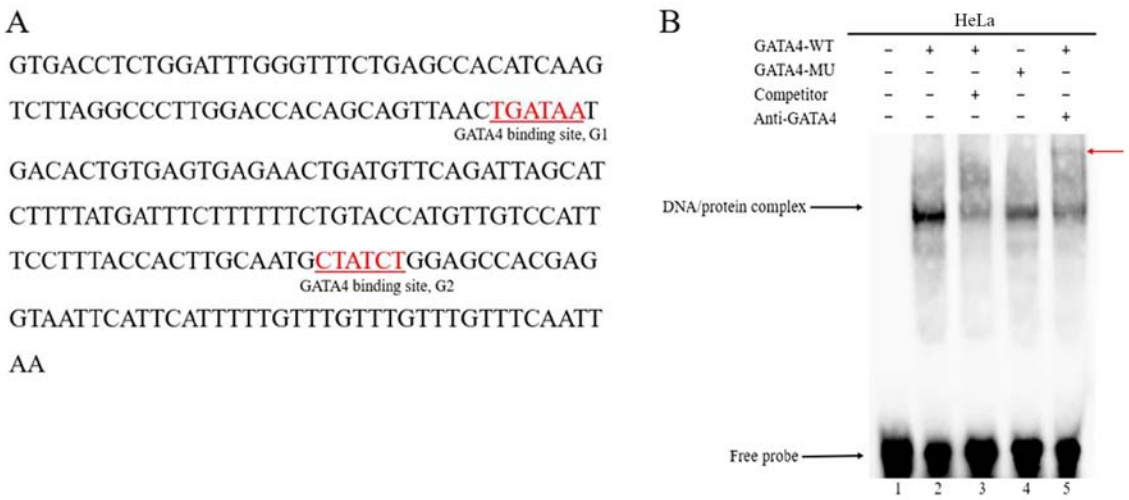

Figure 4. *GATA4* binds to the conserved GATA-binding sites on the *HAND2* gene. (A) Enhancer region of the *HAND2* gene contains two conserved GATA-binding sites (underlined sequence, transcription factor binding sequence). (B) EMSA results revealed that the MU *GATA4* protein exhibited decreased DNA-binding affinity. Lane 1, labeled probe; lane 2, protein from HeLa cells transfected with *GATA4* (WT) + labeled probe; lane 3, protein from HeLa cells transfected with *GATA4* (WT) + unlabeled competitor probe + labeled probe; lane 4, protein from HeLa cells transfected with *GATA4* (MU) + labeled probe; lane 5, protein from HeLa cells transfected with *GATA4* (WT) + labeled probe + anti-*GATA4*. The specific DNA/protein complexes are indicated by arrows. A supershift, indicated by the red arrow, revealed that the *GATA4* antibody could specifically bind with whole cell lysate, which was transfected with the *GATA4* plasmid. EMSA, electrophoretic mobility shift assay; *GATA4*, GATA-binding factor 4; *HAND2*, heart and neural crest derivatives expressed 2; MU, mutant; WT, wild-type.

Discussion

The *GATA4* gene serves an important role in cardiac development, and numerous mutations in this gene have previously been reported in congenital heart defects. For example, a heterozygous G296S missense mutation in *GATA4* results in reduced DNA-binding affinity and transcriptional activity of *GATA4*. Furthermore, the *GATA4* mutation abrogates a physical interaction between *GATA4* and *TBX5* (15). *GATA4* R311W resides in the nuclear localization signal domain (NLS), and the mutant protein does not alter its intracellular distribution; however, the mutation reduces the ability of GATA4 to activate its downstream target gene (26). Furthermore, the *GATA4* K300T mutation may impair cardiogenesis by impeding the GATA4-DNA-binding process and the transcription of GATA4 target genes (27). In our previous study, a heterozygous missense mutation, *GATA4* c.1306C>T (p.H436Y), was identified in four children with sporadic cardiac septal defects, including two VSDs, one VSD associated with ASD, and one VSD associated with an ASD and patent foramen ovale (18).

It has been reported that GATA4 is an upstream transcriptional regulator of *ANF* and *HAND2* (12), and that it regulates their protein expression. *GATA4* can interact with the *HAND2* gene to regulate transcription of the downstream gene by binding to the conserved GATA-binding sites on the promoter region of the *HAND2* gene. In this study, two different methods (*ANF* luciferase assay and *HAND2* EMSA assay) were used to determine the functional consequences of the *GATA4* H436Y mutation, in order to obtain more realistic and reliable results.

A previous study demonstrated that *GATA4* is a transcriptional activator of numerous genes expressed during cardiac development, including the *ANF* gene (23). Therefore, the functional characteristics of this mutation could be analyzed by investigating the transcriptional activity of the *ANF* promoter in HeLa cells expressing *GATA4*. In the present study, the functional effects of the *GATA4* mutation were studied by ANF-luc assays; the results revealed that the *GATA4* c.1306C>T, p.H436Y mutation was associated with decreased transcriptional activity. Furthermore, the present study performed RT-qPCR and western blotting to explore the expression of *GATA4* at the mRNA and protein levels, and revealed that the mutation induced decreases in the protein and mRNA expression levels of *GATA4*. These results indicated that the haploinsufficiency or dominant-negative effect resulting from the *GATA4* mutation may be a pathogenic mechanism underlying congenital heart defects.

As reported previously, the human *GATA4* gene is located on chromosome 8p23.1-p22 and consists of sevens exons, encoding a protein containing 442 amino acids (25). The GATA4 protein is comprised of two transcriptional activation domains [(TAD)-1, amino acids 1-74; TAD2, amino acids 130-177], two highly conserved zinc finger domains [(ZF)-1, amino acids 215-240; ZF2, amino acids 270-294], and one NLS (amino acids 254-32) (28). Additionally, the results revealed that subcellular localization of *GATA4* was not affected by the *GATA4* mutations analyzed in the present study, which may be associated with the fact that the mutation is absent in the NLS region, not affecting the nuclear distribution of *GATA4*.

Notably, the present study demonstrated that the DNA-binding affinity was weakened by the mutation, as determined using EMSA, although the *GATA4* p.H436Y mutation is not located in the ZF2 domain, which is essential for DNA sequence recognition and binding to the consensus motif (29). The *GATA4* C-finger domain interacts with the basic helix-loop-helix domain of *HAND2* to synergistically activate the expression of cardiac-specific genes, including *ANF* and the brain type natriuretic peptide (12). In the mutation investigated in the present study, the amino acid at site 436 of the *GATA4* protein was changed from histidine to tyrosine. The substitution of polar positively charged histidine to neutral

tyrosine may alter the structure and charge of the residue. From the EMSA results, it may be hypothesized that the significantly decreased affinity of *GATA4* to *HAND2* caused by the p.H436Y mutation may be associated with an alteration in the three-dimensional structure of the mutated protein, obstructing its interaction with the DNA, further affecting the subsequent malfunction in transcriptional regulation, which may lead to the occurrence of CHD. This finding is consistent with previous studies (7,30).

A limitation of the study is that the HeLa cell line was used instead of a cardiac cell line for *in vitro* experiments. Although cardiac cell lines could be used to perform the *in vitro* experiments, endogenous *GATA4* gene expression in cardiac cells may interfere with the results of the gene mutation studies. Notably, the HeLa cell line has a strong proliferative ability and is easy to culture for experimental research. The endogenous *GATA4* gene expression was very low and had little effect on the experimental results of transfection with wild-type and mutant *GATA4* plasmids in the HeLa cell line. Considering these advantages, the *in vitro* experiments were performed using the HeLa cell line instead of a cardiac cell line in the present study. In additions, further studies using additional cell lines, including cardiac cell lines, should be conducted in future to further the work presented in the present study.

Similar to previous studies (27,31), congenital heart defects were observed in four patients bearing the same *GATA4* mutation in our previous study (18), indicating that the *GATA4* mutation (c.1306C>T; p.H436Y) may be closely associated with the occurrence of VSD. However, congenital heart defects are multifactorial, and both genetic and environmental factors serve an important role in their occurrence. The same CHD phenotype can be caused by different mutations, and the same mutation may lead to different phenotypes in different patients (32). The occurrence of CHD is a complex process, involving genetic and environmental factors, epigenetic regulation and many other factors (33). In conclusion, the results of the present study may broaden the spectrum of known mutations in the *GATA4* gene associated with congenital heart defects, and could provide novel insights into the mechanism underlying CHD. In addition, these findings may contribute to the future development of genetic diagnostic techniques and therapies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WS and MC were responsible for study design and revision of the manuscript. GH conceived and designed the study. TF and YJZ performed the research and analyzed the data. YLZ wrote the manuscript. AX and QW conducted the statistical analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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