Identification of two novel *GATA6* mutations in patients with nonsyndromic conotruncal heart defects

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Abstract. GATA binding protein 6 (*GATA6*) encodes a zinc-finger transcription factor that is essential for normal heart development. Mutations in this gene lead to conotruncal heart defects associated with cyanotic congenital heart disease; however, it remains unclear whether the mutations in *GATA6* are also responsible for the development of the nonsyndromic conotruncal heart defects. The coding region exons and flanking intron sequences of *GATA6* were screened in 157 patients with nonsyndromic conotruncal heart defects and 300 control subjects. Three heterozygous missense mutations, c.151G>A (E51K), c.551G>A (S184N) and c.733G>C (G245R), were identified in patients with tetralogy of Fallot or persistent truncus arteriosus. The two novel mutations (E51K and G245R) identified in the current study are located in evolutionarily conserved residues of the *GATA6* protein. It was demonstrated that these two mutations lead to a significant reduction in the transactivation capacity of downstream genes. The current study presents two novel *GATA6* mutations in patients with nonsyndromic conotruncal heart defects and provides novel insights into the pathogenesis of this disease.

Introduction

Cardiac conotruncal defects (CTDs) are cardiac outflow tract defects that occur during the embryonic development of complex congenital heart malformations. Examples of CTDs include: Tetralogy of Fallot (TOF); pulmonary atresia with ventricular septal defect (PA/VSD); double outlet of right ventricle (DORV); transposition of the great arteries (TGA); persistent truncus arteriosus (PTA); and interrupted aortic arch (IAA). CTDs accounted for 25-33% of all congenital heart defects and 70% of cyanotic congenital heart disease in 1996 (2). CTDs are the main cause of complex cardiac malformations, mortality and overall serious harm to health in infants, creating a heavy financial burden for families and society (1,2).

Studies have shown that genetic factors are pivotal in the pathogenesis of cardiac CTDs, but the mode of inheritance, the penetrance, and the identities of susceptibility genes are not yet clear (3,4). Previous studies have shown that the 22q11.2 microdeletion is associated with 75-85% of cardiac CTDs, but only 6.1-17.9% of patients with nonsyndromic CTD have 22q11.2 microdeletions (5,6). Therefore, the genetic mechanisms of >80% of nonsyndromic cardiac conotruncal malformations are unknown. As a result of this, the screening of disease genes and candidate genes in patients with CTD is a focus of current cardiovascular research. Studies in animal models have demonstrated that the GATA binding protein 6 (*GATA6*) regulates differentiation and affects the development of CTDs. Homozygous *GATA6*-knockout mice exhibit developmental endoderm defects that lead to embryonic death (7,8). Studies have demonstrated that conditional inactivation of *GATA6* in vascular smooth muscle cells (VSMCs) in mice results in perinatal mortality from a spectrum of cardiovascular defects, including IAA and PTA. Inactivation of *GATA6* in the neural crest recapitulates these abnormalities, demonstrating a cell-autonomous requirement for *GATA6* in neural crest-derived smooth muscle cells (9). It is therefore necessary to screen for *GATA6* mutations in patients with nonsyndromic conotruncal heart defects to enable early disease intervention and genetic counseling.

Materials and methods

Subjects. The present study was approved by the Medical Ethics Committee of Xinhua Hospital (Shanghai, China). Once informed consent was obtained from the parents of all patients and control subjects, venous blood samples from all participants

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Table I. Primers used to amplify the *GATA6* gene.

Fragment	Forward primer (5'-3')	Reverse primer (5'-3')	Fragment size (bp)
1	ccgtcccctccccaccctcttt	gagatcgcgcgcgaggaggaagca	361
2-1	tggaggcgaggtagcgtgcag	ctcgggtgcgaaggggctcag	544
2-2	cccgctcgctgctgctcagtt	ccatgggcgggctgggagagt	591
2-3	cacctgcaggggtcgggcagt	aaacagggcccgagtggagca	616
3	ctactggggcgctccgggtgt	agcgggtgggcgttggaacag	583
4	tggagaagaaaccagggatga	tgcattcaaatttttcacttgag	590
5-6	cggcggccaaattctttta	aaccataaaaaaatgataccgatct	619
7	tggccagggtcaggtcagtgg	gagtggcccaagcgcccagtt	610

GATA6, GATA binding protein 6.

were collected in an anticoagulant tube with sodium citrate. Karyotype analysis and multiplex ligation-dependent probe amplification were performed in samples from all patients with CTDs. Fluorescence *in situ* hybridization (FISH) was performed in all patients to identify and exclude patients with genetic deletions such as trisomy 18, trisomy 21 and 22q11.2 deletion. Nonsyndromic patients with CTD were diagnosed by transthoracic echocardiography, computed tomography, cardiac catheterization, and/or surgical inspection. A total of 157 unrelated Chinese patients with nonsyndromic CTD were enrolled in the study from January 2009 to January 2011. Patients included 105 males and 52 females, aged between 1 month and 17 years old with a median age of 3.64 years. CTDs in these patients included: TOF (73 cases); PA/VSD (27); DORV (28); TGA (9); PTA(10); and IAA (10). The present study enrolled 300 healthy unrelated children as healthy controls. All participants were of Han ethnicity. Genomic DNA was isolated from 200 μl blood using a standard phenol-chloroform extraction protocol. The families of the probands (parents and siblings) also underwent physical examination and transthoracic echocardiography, and venous blood samples were analyzed for *GATA6* mutations.

Screening for mutations. Seven whole exons and exon-intron boundaries of human *GATA6* were amplified. Oligonucleotide primers were designed based on genomic sequences (GenBank accession number NC_000018) using Primer3 software (http://frodo.wi.mit.edu/primer3/) and were synthesized by Shanghai Genesky Bio-Tech (Shanghai, China). Primers were designed so that each exon was flanked by part of the corresponding intron (Table I). For exons 1, 2, 3 and 7, the polymerase chain reaction (PCR) reaction mixture (total, 10 μl) contained 1.0 μl genomic DNA, 1.0 μl each primer, 0.2 μl dNTP mixture, 5.0 μl 2X GC buffer I, 2.74 μl ddH_20, and 0.06 μl HotTaq DNA polymerase (Takara Biotechnology, Dalian, China). PCR was performed using a GeneAmp 9600 Thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: Predenaturation at 95˚C for 2 min, followed by 35 cycles at 96˚C for 10 sec, and annealing and extension at 72˚C for 4 min. For exons 4 and 5-6, the PCR reaction mixture (total, 10 μl) contained 1.0 μl genomic DNA, 1.0 μl each primer, 0.2 μl dNTP mixture, 1.0 μl 2X GC buffer I, 0.2 μl $MgC1_2$, 6.54 μl ddH_20, and 0.06 μl of HotTaq DNA polymerase. PCR cycling conditions were as follows: 11 cycles of predenaturation at 95˚C for 2 min, denaturation at 96˚C for 20 sec, denaturation at 62˚C for 40 sec, and extension at 72˚C for 2 min (annealing temperature was decreased by 0.5˚C/cycle); 24 cycles of denaturation at 94˚C for 20 sec, annealing at 56˚C for 30 sec and extension at 72˚C for 2 min. All PCR products were gel purified using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and then sequenced using the dideoxy chain termination method on an ABI3130XL sequencer (Applied Biosystems). Sequencing results were aligned with the reference sequence using the GenBank BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The corresponding sequences of healthy controls were amplified and sequenced as above in order to exclude polymorphisms. *GATA6* protein sequences from various species were aligned using ClustalW software (www.clustal.org).

Plasmid construction and cells. The wild-type *GATA6* expression plasmid, pcDNA3.1(+)-Homo *GATA6* was supplied by Professor Hiroyuki Yamagishi (10). To generate the *GATA6* E51K and G245R mutant constructs, mutations were introduced into pcDNA3.1(+)-Homo *GATA6* by site-directed mutagenesis-PCR individually. The vector with a luciferase reporter driven by the ANF promoter was a kind gift from Professor Vidu Garg (11). HEK293T cells (Cell Bank of the Chinese Academy of Science, Shanghai, China) were split and seeded into 96-well plates with 10,000 cells/well in preparation for the following assay.

Transfection and transcriptional assay. Transfection using FuGene HD Transfection reagent (Roche Diagnostics, Mannheim, Germany) was conducted in triplicate as previously described (12), and then the activity of firefly luciferase and *LacZ* in cell lysates was measured by the Dual-Glo Luciferase Assay system (Promega Corporation, Madison, WI, USA) 24 h after transfection. HEK239T cells were transfected with 20 ng of wild-type or mutant *GATA6*, 100 ng reporter construct ANF-luciferase, and 20 ng cytomegalovirus (CMV)-*LacZ* in each well for correcting the transfection efficiency. Results are presented as the relative luciferase activity, normalized to the co-transfected CMV-*LacZ* group.

Statistical analysis. Data are presented as the mean ± standard deviation. The two groups were compared using the χ^2 test for

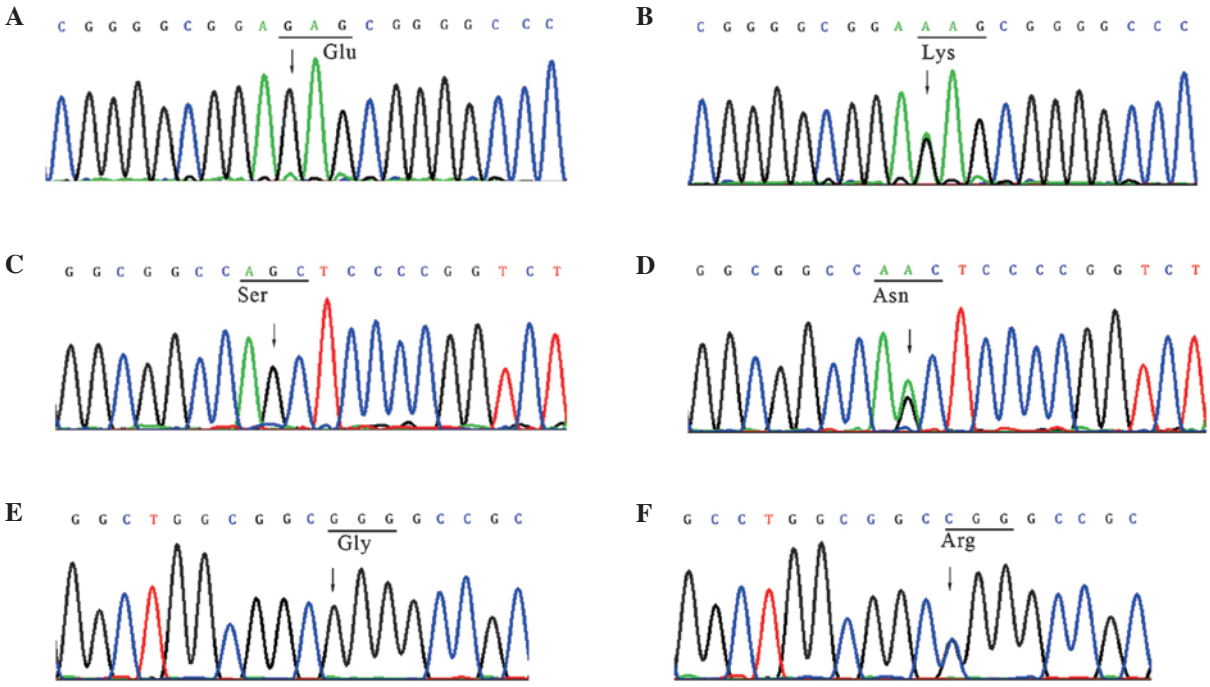

Figure 1. Three heterozygous missense *GATA6* mutations were identified in three nonsyndromic conotruncal heart defect patients. (A,C and E) Wild type; (B,D and F) mutant. p.Glu51Lys (E51K), p.Ser184Asn (S184N), p.Gly245Arg (G245R), and their wild-type counterparts are shown. *GATA6*, GATA binding protein 6.

Figure 2. Location of E51K, S184N and G245R in the structural domains of the *GATA6* protein. Colored lines represent exons 2-7 encoding amino acids 1-595 of the *GATA6* protein. Arrow, location of the mutation; TAD, transcriptional activation domain; ZF, zinc finger domain; NLS, nuclear localization signal; *GATA6*, GATA binding protein 6.

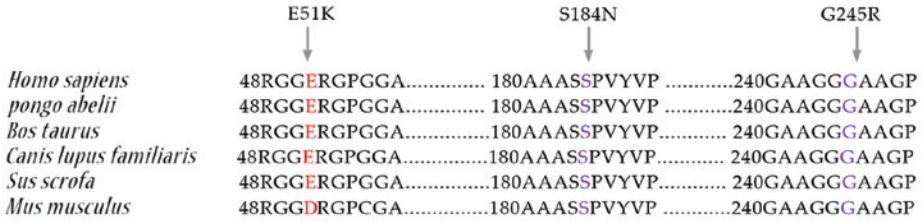

Figure 3. Multiple alignment of the sequences of the *GATA6* protein from various species, indicating that the three heterozygous missense mutations are located at conserved sites. Arrow indicates the location of the mutations in conserved residues. *GATA6*, GATA binding protein 6.

continuous variables and P<0.05 was considered to indicate a statistically significant difference.

Results

GATA6 mutations in patients with nonsyndromic CTD. Three heterozygous missense mutations (E51K, S184N and G245R) were identified in the *GATA6* gene in 3/157 unrelated cases of nonsyndromic CTD. These sequence variants, which are located in coding exons and generate the amino acid alterations of E51K, S184N and G245R, were absent in the chromosomes of all healthy controls (Fig. 1). Overall, the mutation frequency of the *GATA6* gene in nonsyndromic CTD was 1.9% (3/157). To the best of our knowledge, the current study is the first to report the E51K and G245R heterozygous missense mutations. The c.151G>A in exon 2, identified in a patient with TOF, changes a relatively conserved glutamic acid residue to lysine at position 51 (E51K). This change was expected to have a significant impact on the structure and function of the *GATA6* protein since glutamic acid is acidic, while lysine is basic. The

Table II. Identification of *GATA6* sequence variants in nonsyndromic CTD.

Location	Nucleotide change	dbSNP database	Amino acid change	Allele frequency	
				Patients	Controls
Exon 2	c.43G>C	rs116262672	Gly15Arg	8/157 (0.051)[a]	21/300 (0.070)
Exon 2	c.151G>A		Glu51Lys	1/157 (0.006)[b]	0/300 (0.000)
Exon 2	c.551G>A		Ser184Asn	1/157 (0.006)[b]	0/300 (0.000)
Exon 2	c.733G>C		Gly245Arg	1/157 (0.006)[b]	0/300 (0.000)
Intron 2	c.5825+19C>G	rs76308670		1/157 (0.006)[a]	2/300 (0.007)
Intron 2	c.10501-60C>T	rs3764504		4/156 (0.026)[a]	8/300 (0.027)
Intron 2	c.10501-85T>C			1/157 (0.006)[a]	2/300 (0.006)
Intron 6	c.16589+6T>C			1/157 (0.003)[a]	2/300 (0.007)
Intron 6	c.16589+7A>G	rs3764962		9/157 (0.057)[a]	22/300 (0.073)
3'-UTR	c.+72G>A	rs1941084		67/157 (0.427)[a]	156/300 (0.520)
3'-UTR	c.+77G>A	rs1941083		26/157 (0.166)[a]	56/300 (0.187)

[a]P>0.05; [b]P<0.05 compared with the control group. *GATA6*, GATA binding protein 6; CTD, conotruncal defect.

c.733G>C missense mutation in exon 2, identified in a patient with PTA, leads to the substitution of a highly conserved glycine residue with arginine at position 245 (G245R). This mutation may also have a significant impact on the structure of a salt bond and thus the structure of the *GATA6* protein since glycine is a non-polar hydrophobic amino acid, while arginine is basic. In addition, the c.551G>A missense mutation in exon 2 was identified in a patient with TOF, and substitutes a serine with an asparagine at position 184 (S184N). However, this mutation has been reported previously (13). As the two novel mutations are likely to alter the structure of the *GATA6* protein, they may be pathogenic. The location and sequence alignment data for these mutations are shown in Figs. 2 and 3. Nonsyndromic CTD pathogenesis is not only related with the 22q11.2 microdeletion and *TBX1* gene mutations, but may also be involved in pathogenesis mediated by the *GATA6* zinc-finger transcription factor.

Seven sequence variants were identified in introns and the 3'-UTR of *GATA6* in patients with nonsyndromic CTD and control subjects. A previously reported sequence variant in exon 2 (c.43G>C) predicted to lead to an amino acid change (p.Gly15Arg) was also identified in the present study. This nucleotide substitution was identified in 8/157 nonsyndromic CTD patients (5.1%) and in 21/300 control subjects (7.0%) (P>0.05). Six of these sequence variants were already present in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). The other two intron sequence variants (c.10501-85T>C, intron 2; c.16589+6T>C, intron 6) were novel single nucleotide polymorphisms, but displayed no significant difference in their allele frequencies between patients with nonsyndromic CTD and control subjects. All sequence variants and their allele frequencies are summarized in Table II.

Characteristics and mutation screening of family members. The c.151G>A proband was a male with TOF, and family members included two parents and siblings. A young brother of the proband was confirmed to be suffering from trisomy 21 associated with an atrioventricular septal defect (AVSD), but his parents and sister had normal cardiac morphology. The c.151G>A missense mutation was identified in his father and brother. The c.551G>A and the c.733G>C probands were males with TOF and PTA, respectively, and family members included parents but no siblings in both cases. These parents all had normal cardiac morphology and there were no *GATA6* sequence variants identified (Fig. 4).

E51K and G245R cause a significant reduction in the capacity of GATA6 to transactivate downstream genes. Among the three mutations (E51K, S184N and G245R), S184N has been reported in a previous study by Lin *et al* (13). In the current study, the

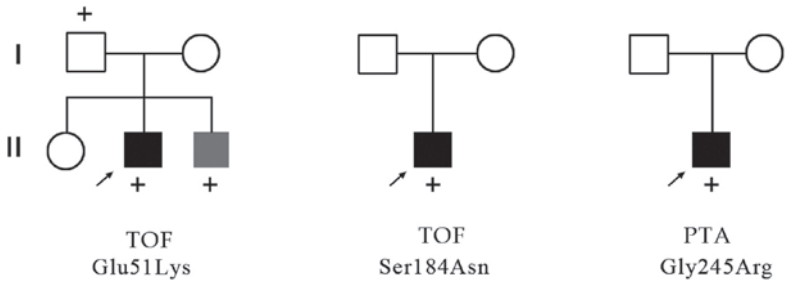

Figure 4. Characteristics and presence of mutations in the three families. Arrow, proband; +, carriers of the mutations Glu51Lys, Ser184Asn and Gly245Arg; black box, nonsyndromic conotruncal heart defects patient; grey box, trisomy 21 patient; TOF, tetralogy of Fallot, PTA, persistent truncus arteriosus.

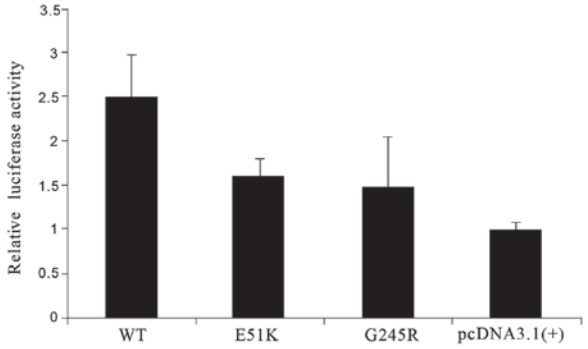

Figure 5. The transactivation function of mutant *GATA6* was disrupted. The data are expressed as the mean ± standard deviation of two independent experiments performed in triplicate, and the relative luciferase value of control, pcDNA3.1 (+) was set at 1. WT, wild type; *GATA6*, GATA binding protein 6.

transcription capacities of the E51K and G245R proteins in cultured cells were determined. The ANF-luciferase construct in which the reporter gene expression was driven by the ANF promoter was co-transfected into HEK293T cells with wild-type or mutant *GATA6*. The expressed *GATA6* molecules bound to the ANF sequence and then transactivated luciferase expression downstream. The luciferase activity normalized to the *LacZ* value was regarded as the relative transactivation function of *GATA6*. E51K diminished the transactivation activity by almost 40% and G245R interrupted it more markedly (>40%) compared with that of wild-type *GATA6* (Fig. 5).

Discussion

GATA6 is an early marker of cardiac precursor cells, an important transcription factor for cardiac cell differentiation and development, and one of the major candidate genes in the pathogenesis of congenital heart diseases, particularly conotruncal heart defects (10,14). The human *GATA6* gene is localized to chromosome 18q11.1-11.2, contains seven exons spanning 34,812 bp and encodes a protein comprising 595 amino acids (15). *GATA6* expression in the cardiac neural crest during embryonic development can be visualized with FISH, and data obtained using this technique supports the hypothesis that *GATA6* not only regulates differentiation of smooth muscle cells in the cardiac neural crest but also regulates development of the aorticopulmonary septum through signaling from cells derived from the neural crest (9,16). In the wild-type embryonic heart, *GATA6* mRNA is expressed in the cardiac outflow tract, atrium and ventricles 9.5 days post-conception. *GATA6* is expressed in the ascending aorta, pulmonary artery smooth muscle cells, and the endocardial cushion that differentiates into the cardiac outflow tract conotruncus 11.5 days post-conception. Furthermore, *GATA6* mRNA detected at 12.5 days post-conception is localized in the aorta, pulmonary artery, VSMCs of the ductus arteriosus, cardiomyocytes of the atrium and ventricles, and the endocardial cushion of the conotruncus (17,18). It is reported that of the GATA gene family, only *GATA6* is expressed in VSMCs and it mediates their differentiation through modulating the expression of VSMC-specific genes, including α-MHC, α-actin and *SM22*, in order to maintain various differentiated vascular smooth muscle phenotypes (19-23).

Three *GATA6* missense mutations that were demonstrated to be associated with single conotruncal heart defects were identified in the current study, and two of these were novel (E51K and G245R). The E51K mutation was not demonstrated to be located in the transcriptional activation, zinc finger or nuclear localization signaling domains; however, this mutation results in the replacement of an acidic with a basic amino acid at position 51. Furthermore, the region of the *GATA6* sequence containing this mutation is important in the transactivation of target genes. It is therefore reasonable to suggest that the E51K mutation significantly affects the structure, stability, and hence, the transcriptional activity of the *GATA6* protein. It was also indicated that the G245R mutation results in changes to salt bonds and may alter the structure of the *GATA6* protein.

These assumptions were confirmed by functional analysis in the present study. The E51K and G245R mutations reduced the capacity of *GATA6* to transactivate downstream genes by ~40% compared with that of wild-type *GATA6*. Lin *et al* (13) previously identified S184N missense mutations in one patient with TOF and one with an atrial septal defect, and then demonstrated that the mutation led to reduced transcriptional activity of *GATA6* (13). The mutant *GATA6* appears to attenuate the expression of certain downstream genes which modulate the formation of the cardiac outflow tract, endocardial cushion and the atrioventricular septa, and this may be the genetic mechanism underlying nonsyndromic CTDs.

Thus far, studies have reported *GATA6* mutations in congenital heart disease. Kodo *et al* (10) reported the N466H mutation in the zinc finger domain and the E486del mutation in the nuclear localization signaling domain in patients with PTA. They also demonstrated that the mutant proteins did not

transactivate target genes, thus disrupting normal regulation of the semaphorin-plexin signaling pathway and resulting in cardiac outflow tract malformations. Maitra *et al* (24) identified two missense mutations of the *GATA6* gene in one patient with TOF (A178V) and one with AVSD (L198V). They also demonstrated that the A178V mutation leads to increased *GATA6* transcriptional activity. In the present study, a detailed phenotype and genotype characterization of the family members of all patients with *GATA6* mutations was performed. The results displayed that the father and brother of the proband with the E51K mutation also had the mutation (c.151G>A); however, the father had normal cardiac morphology, suggesting incomplete penetrance of this mutation. The brother was diagnosed with trisomy 21 syndrome with AVSD. It is reported that ~40-60% of patients with trisomy 21 syndrome exhibit some form of congenital heart disease; however, trisomy 21 alone is insufficient to cause congenital heart disease as ~50% of patients with trisomy 21 have a normal heart (25,26). It has been suggested that the etiology for heart defects in trisomy 21 with AVSD involves mutations in the *CRELD1*, *HEY2* and ALK2 genes (26-28). The present study suggests that mutation of the *GATA6* gene may also be involved in the pathogenesis of AVSD.

The results of the present study demonstrated that mutations of the *GATA6* gene are closely related to nonsyndromic CTDs, suggesting an important role for this gene in the development of the human heart conotruncus. However, the development and differentiation of the human heart is a complex process and many factors contribute to the pathogenesis of abnormal heart structures, including genetic mutation, epigenetic modification and abnormal gene-environment interactions. Further studies are therefore required to clarify the role of the *GATA6* mutations in the pathogenesis of heart malformations, particularly in CTD.

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