

A novel GATA4 mutation responsible for congenital ventricular septal defects

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Abstract. Ventricular septal defect (VSD) is the most common type of cardiovascular developmental anomaly and is an important risk factor for the substantially increased morbidity and mortality in newborns. Aggregating evidence implicates genetic defects in the pathogenesis of congenital VSD. However, VSD is genetically heterogeneous and the genetic determinants for VSD in most patients remain to be identified. In this study, the whole coding region of the *GATA4* gene, which encodes a zinc-finger transcription factor pivotal to cardiogenesis, was initially sequenced in 210 unrelated patients with VSD. The relatives of the index patient carrying the identified mutation and 200 unrelated ethnically-matched healthy individuals used as controls were subsequently genotyped. The functional effect of the mutant *GATA4* was characterized in contrast to its wild-type counterpart using a luciferase reporter assay system. A novel heterozygous *GATA4* mutation, p.G296R, was identified in a family with VSD inherited as an autosomal dominant trait. Absent in 200 control individuals, the mutation co-segregated with VSD in the family with 100% penetrance and was completely conserved evolutionarily across species. Functional analysis displayed that the p.G296R mutation of *GATA4* was associated with a decreased transcriptional activity. The findings expand the spectrum of mutations in *GATA4* linked to VSD and provide more insight into the molecular mechanism involved in VSD. The results of the present study imply the potential implications in the genetic

diagnosis and gene-specific therapy of this common malformation in infancy.

Introduction

Congenital heart defects are the most common developmental abnormality with a prevalence of approximately 1% in neonates, and are the leading non-infectious cause of infant mortality, accounting for more than 29% of infants who die from a birth defect (1). Congenital cardiovascular anomalies are clinically classified into at least 18 distinct categories with many additional anatomical variations, of which ventricular septal defects (VSDs) are the most common form. VSDs occur in nearly 50% of all babies with congenital cardiovascular deformations, and account for 14-16% of cardiac defects that require an invasive treatment within the first year of life (1,2). Congenital VSDs may occur alone or in combination with other cardiac malformations, such as atrial septal defects, pulmonary artery stenosis or tetralogy of Fallot. Despite other deformities that may accompany VSD, larger VSDs may give rise to cardiac enlargement, congestive heart failure, pulmonary hypertension, Eisenmenger's syndrome, delayed fetal brain development, arrhythmias, and even sudden cardiac death in the absence of surgical or catheter based repair (3-7). Nevertheless, the etiology underlying VSDs in the majority of the patients remains largely unclear (8,9).

Congenital VSDs arise from perturbations of cardiac development during embryogenesis and both environmental and genetic risk factors have been implicated in VSDs (8-10). Growing evidence highlights the key role of several transcription factors, including *GATA4*, in septogenesis (9). The human *GATA4* gene maps to chromosome 8p23.1-p22 and consists of 7 exons, encoding a protein of 442 amino acids (11). It is expressed throughout cardiac morphogenesis and is essential for normal cardiac development (12-17). Hence, *GATA4* has been one of the prime candidate genes in identifying the inheritable components for structural congenital cardiovascular defects. Presently, more than 40 mutations of the *GATA4* gene have been identified in patients with a variety of congenital heart aberrations including VSDs, atrial septal defects,

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tetralogy of Fallot, endocardial cushion defects, patent ductus arteriosus, pulmonary stenosis, and hypoplastic right ventricle (18–33). Nevertheless, the molecular mechanisms responsible for VSD in most patients remain to be elucidated (18–33).

In this study, the coding exons and exon/intron boundaries of *GATA4* were sequenced initially in a cohort of 210 unrelated patients with congenital VSDs and a novel heterozygous *GATA4* mutation, p.G296R, was identified in a VSD patient with positive family history. Subsequently, genetic analysis of the family members showed that the mutation co-segregated with VSDs inherited in an autosomal dominant fashion. Functional analysis demonstrated that the p.G296R mutation of *GATA4* was associated with a decreased transcriptional activity. These findings expand the spectrum of mutations in *GATA4* linked to VSDs and provide more insight into the genetic mechanisms involved in human heart development.

Materials and methods

Study participants. A cohort of 210 unrelated patients with VSD was identified among the Chinese population. Subjects were evaluated by individual and familial history, review of the medical records, complete physical examination, 12-lead electrocardiogram (ECG) and two-dimensional transthoracic echocardiography with color flow Doppler. All patients had a classic form of VSD, with a defect diameter of >3 mm and nearly all patients underwent cardiac catheterization and, if required, cardiac surgery. A total of 200 ethnically matched unrelated healthy individuals, which were derived from the general population, were recruited as controls to screen for the identified mutation in *GATA4*. Peripheral venous blood specimens from VSD cases and control individuals were prepared. The study protocol was reviewed and approved by the local institutional ethics committee and written informed consent was obtained from all participants or their guardians prior to investigation.

Genetic studies. Genomic DNA from all participants was extracted from blood lymphocytes with the Wizard Genomic DNA Purification kit (Promega). The candidate gene *GATA4* was screened initially in 210 unrelated patients with VSD and subsequently genotyping *GATA4* in the available relatives of the index patient harboring an identified mutation and the 200 control individuals was conducted. The genomic DNA reference sequence of *GATA4* was derived from GenBank (accession no. NC_000008). The primer pairs used to amplify the complete coding region of *GATA4* by polymerase chain reaction (PCR) were designed as previously described (33). Polymerase chain reaction (PCR) was carried out using HotStarTaq DNA polymerase (Qiagen) on a PE9700 thermal cycler (Applied Biosystems), with standard conditions and concentrations of reagents. Amplified products were analyzed on 1% agarose gels stained with ethidium bromide and purified with the QIAquick Gel Extraction kit (Qiagen). Both strands of each PCR product were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) under an ABI PRISM 3130XL DNA Analyzer (Applied Biosystems). The sequencing primers used were the same as previously designed for specific region amplification. The DNA sequences were viewed and analyzed with the DNA

Sequencing Analysis software v5.1 (Applied Biosystems). The variant was validated by re-sequencing an independent PCR-generated amplicon from the subject and met our quality control thresholds with a call rate >99%.

Multiple sequence alignments. The multiple *GATA4* protein sequences across species were aligned using the program MUSCLE (version 3.6, an online program at <http://www.ncbi.nlm.nih.gov>).

Prediction of the disease-causing potential of a *GATA4* sequence variation. The disease-causing potential of a *GATA4* sequence variation was predicted automatically by MutationTaster (an online program at <http://www.mutationtaster.org>), giving a probability for the alteration to be either a causative mutation or a harmless polymorphism. Notably, the P-value used here is the probability of the prediction rather than the probability of error as used in t-test statistics, i.e. a value close to 1 indicates a high ‘security’ of the prediction.

Plasmids and site-directed mutagenesis. The recombinant expression plasmids pSSRa-*GATA4* and atrial natriuretic peptide-luciferase reporter gene, which contains the 2600-bp 5'-flanking region of the atrial natriuretic peptide gene, namely atrial natriuretic peptide (ANP)(-2600)-Luc, were kindly provided by Ichiro Shiojima, Chiba University School of Medicine. The identified mutation was introduced into the wild-type *GATA4* using a QuickChange II XL Site-Directed Mutagenesis kit (Stratagene) with a complementary pair of primers. The mutant was sequenced to confirm the desired mutation and to exclude any other sequence variations.

Reporter gene assays. COS-7 cells were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% fetal calf serum. The ANP(-2600)-Luc reporter construct and an internal control reporter plasmid pGL4.75 (hRluc/CMV, Promega) were used in transient transfection assays to examine the transcriptional activation function of the *GATA4* mutant. COS-7 cells were transfected with 0.2 µg of wild-type or mutant pSSRa-*GATA4* expression vector, 0.2 µg of ANP(-2600)-Luc reporter construct, and 0.04 µg of pGL4.75 control reporter vector using the PolyFect Transfection reagent (Qiagen). For co-transfection experiments, 0.1 µg of wild-type pSSRa-*GATA4*, 0.1 µg of mutant pSSRa-*GATA4*, 0.2 µg of ANP(-2600)-Luc and 0.04 µg of pGL4.75 were used. Firefly luciferase and *Renilla* luciferase activities were measured with the Dual-Glo luciferase assay system (Promega) 48 h after transfection. Three independent experiments were performed at minimum for wild-type and mutant *GATA4*.

Statistics. Data are denoted as the mean ± SD. Differences between the two groups were compared with the Student's t-test for continuous variables and a 2-tailed P<0.05 was considered to be statistically significant.

Results

Characteristics of the study subjects. A cohort of 210 unrelated patients with VSD was registered and clinically evaluated in contrast to a cohort of 200 ethnically matched unrelated

Table I. Clinical characteristics of the 210 unrelated patients with ventricular septal defects.

Characteristics	Number or mean value	Percentage or range
Gender (Male, female)	82, 128	64, 100
Age at the diagnosis of VSD (years)	2.05	0.17-14
Age at the present study (years)	5.61	0.17-18
Positive family history	18	8.57
Distribution of different types of VSDs		
Subpulmonary	17	8.10
Perimembranous	146	69.52
Atrioventricular canal	22	10.48
Muscular	25	11.90
Prevalence of VSDs with other defects		
Isolated VSD	165	78.57
VSD and ASD	30	14.29
VSD, ASD and PDA	6	2.86
VSD, ASD and DORV	4	1.90
VSD, PDA	3	1.43
VSD and PS	2	0.95
Incidence of arrhythmias		
Atrioventricular block	8	3.81
Atrial fibrillation	3	1.43
Treatment		
Surgical repair	168	80.00
Percutaneous closure	18	8.57
Follow-up	24	11.43

VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; DORV, double outlet right ventricle; PS, pulmonary stenosis.

healthy individuals used as controls. None of them had overt traditional risk factors for VSD. The baseline clinical characteristics of the 210 unrelated patients with VSD are summarized in Table I.

GATA4 mutations. Direct sequencing of the coding regions of the *GATA4* gene was performed after PCR amplification of genomic DNA from the 210 unrelated VSD patients. A heterozygous substitution of cytosine (C) for guanine (G) in the first nucleotide of codon 296 of the *GATA4* gene (c.886G>C), predicting the transition of glycine to arginine at amino acid position 296 (p.G296R), was identified in 1 out of 210 patients. The total population prevalence of *GATA4* mutations based on the cohort patients was approximately 0.48%. The sequence chromatogram showing the detected heterozygous *GATA4* variation of c.886G>C in comparison to the control sequence is displayed in Fig. 1. The variant was neither detected in the control population nor reported in the SNP database at the website <http://www.ncbi.nlm.nih.gov/SNP>. Genetic scanning of the relatives of the mutation carrier displayed that the gene variant was present in all affected family members alive, but absent in unaffected family members examined. Analysis of the pedigree demonstrated that the mutation co-segregated

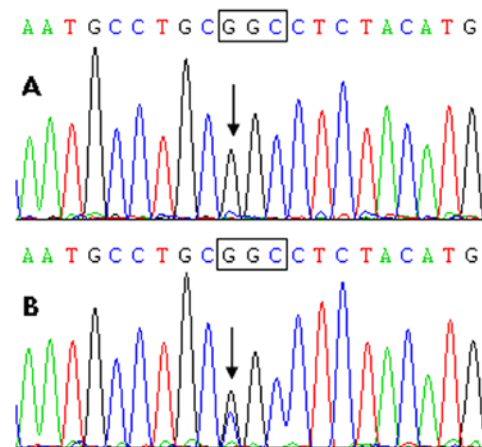


Figure 1. Sequence chromatogram showing the *GATA4* mutation identified in family 1. The arrow indicates the heterozygous nucleotides of C/G in the proband from family 1 (B) or the homozygous nucleotides of G/G in a control individual (A). The square denotes the nucleotides comprising a codon of *GATA4*.

with VSD transmitted as an autosomal dominant trait in the family with complete penetrance. The pedigree structure of the family is illustrated in Fig. 2. The phenotypic characteristics

Table II. Phenotypic characteristics and status of the *GATA4* mutation in the affected pedigree members.

Subject information				Phenotype			Genotype
Identity	Gender	Age at time of study (years)	Age at diagnosis of VSD (years)	VSD (mm)	Other structural defects	AVB	G296R
I-1	M	56 ^a	16	20	ASD, PS	+	N/A
II-1	M	34	9	14	ASD	-	+/-
II-6	F	29	4	11	-	-	+/-
III-2	F	5	2	12	-	-	+/-

^aAge at death. F, female; M, male; VSD, ventricular septal defect; N/A, not available or not applicable; AVB, atrioventricular block; ASD, atrial septal defect; PS, pulmonary stenosis. +, indicates present and -, denotes absent.

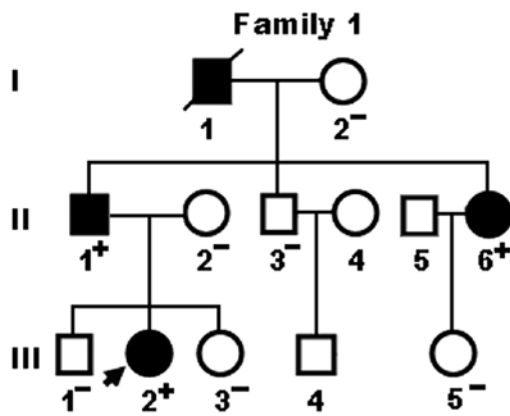


Figure 2. Pedigree structure of the family with a ventricular septal defect. Family members are identified by generations and numbers. Squares indicate male family members; circles, female members; symbols with a slash, the deceased members; closed symbols, affected members; open symbols, unaffected members; arrow, proband; +, carriers of the heterozygous mutations; -, non-carriers.

and results of genetic screening of the affected pedigree members are listed in Table II. All the 3 living patients underwent surgical repair of the VSDs.

Multiple alignments of the *GATA4* protein sequences across species. A cross-species alignment of *GATA4* protein sequences demonstrated that the altered amino acid G296 was completely conserved evolutionarily as shown in Fig. 3, suggesting that the amino acid is functionally important.

Disease-causing potential of a sequence variation. The sequence variation of c.886G>C detected in *GATA4* was automatically predicted to be disease-causing with a P-value of 0.99999963, providing evidence for the alteration to be a malicious disease mutation rather than a benign polymorphism.

Transcriptional activity of the *GATA4* mutant. The transcriptional activation function of *GATA4* in COS-7 cells was examined using one of its direct cardiac downstream target genes, *ANP*, as a luciferase reporter, and the activity of the *ANP* promoter was presented as fold-activation of the firefly

luciferase relative to the *Renilla* luciferase. The same amounts of wild-type (0.2 μ g) and mutant *GATA4* (0.2 μ g) activated the *ANP* promoter by ~5.5- and ~2.1-fold, respectively. When the same amount of wild-type *GATA4* (0.1 μ g) was cotransfected with mutant *GATA4* (0.1 μ g), the induced activation of the *ANP* promoter was ~3.6-fold. These results suggest that mutant *GATA4* has significantly reduced activation activity compared with wild-type *GATA4* (as shown in Fig. 4).

Discussion

In the present study, we report a previously unrecognized missense mutation of *GATA4* identified in a family with congenital VSD. The novel heterozygous mutation was present in all the affected family members alive but absent in unaffected relatives examined and in the 400 normal chromosomes from a matched control population. A cross-species alignment of *GATA4* protein sequences showed that the altered amino acid was completely conserved evolutionarily. Functional analysis displayed that the p.G296R mutation of *GATA4* was associated with a significantly decreased transcriptional activity. Therefore, it is very likely that mutated *GATA4* is responsible for the VSD in this family.

GATA transcription factors are a family of transcription factors characterized by their ability to bind to the consensus DNA sequence 'GATA'. In vertebrates, six members of the *GATA* family have been identified, of which *GATA4*, *GATA5* and *GATA6* are expressed mainly in the developing heart (34). *GATA4* contains 2 transcriptional activation domains (TAD1, amino acids 1-74; TAD2, amino acids 130-177), 2 zinc finger domains (ZF1, amino acids 215-240; ZF2, amino acids 270-294), and 1 nuclear localization signal (NLS, amino acids 295-324) (31). The two TADs are essential for the transcriptional activity of *GATA4*. The C-terminal ZF1 is required for DNA sequence recognition and binding to the consensus motif, while the N-terminal ZF2 is responsible for sequence specificity and stability of protein-DNA binding. The NLS sequence is associated with the subcellular trafficking and distribution of *GATA4* (31,34). The *GATA4* G296R mutation identified in this study is located in the NLS, and it thus may influence the transcriptional activity of *GATA4* via a failure to target the protein to the cell nucleus through the nuclear pore complex.

		280		G296R		312
Human (NP_002043.2)	---	TLWRRNAEGEPVCNAC	G	↓	LYMKLHGVPRLAMRK	---
Chimpanzee (XP_528070.2)	---	TLWRRNAEGEPVCNAC	G		LYMKLHGVPRLAMRK	---
Dog (XP_543205.2)	---	TLWRRNAEGEPVCNAC	G		LYMKLHGVPRLAMRK	---
Cattle (XP_616466.2)	---	TLWRRNAEGEPVCNAC	G		LYMKLHGVPRLAMRK	---
Mouse (NP_032118.2)	---	TLWRRNAEGEPVCNAC	G		LYMKLHGVPRLAMRK	---
Rat (NP_653331.1)	---	TLWRRNAEGEPVCNAC	G		LYMKLHGVPRLAMRK	---
Fowl (XP_420041.1)	---	TLWRRNAEGEPVCNAC	G		LYMKLHGVPRLAMRK	---
Zebrafish (NP_571311.1)	---	TLWRRNAEGEPVCNAC	G		LYMKLHGVPRLAMRK	---
Fruit fly (NP_732098.1)	---	SLWRRNPAGEPVCNAC	G		LYYKLH SVPRPLTMKK	---

Figure 3. Alignment of multiple NKX2-5 protein sequences across species. The altered amino acid of p.G296 is completely conserved evolutionarily among the examined species.

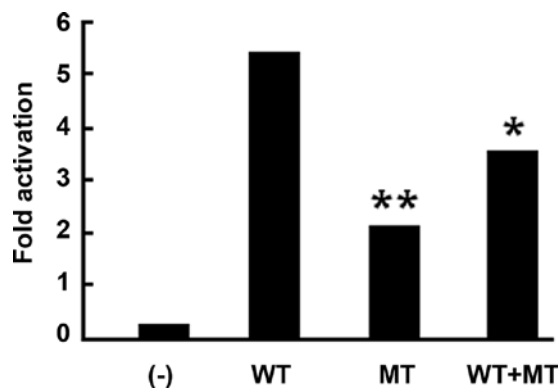


Figure 4. Transcriptional activity of the mutant *GATA4* on the *ANP* promoter. COS-7 cells were transfected with 0.2 μ g of wild-type or mutant pSSRa-*GATA4* expression vector, 0.2 μ g of the *ANP*(-2600)-Luc reporter construct, and 0.04 μ g of pGL4.75 control reporter vector. For co-transfection experiments, 0.1 μ g of wild-type pSSRa-*GATA4*, 0.1 μ g of mutant pSSRa-*GATA4*, 0.2 μ g of *ANP*(-2600)-Luc, and 0.04 μ g of pGL4.75 were used. The activity of the *ANP* promoter was presented as fold-activation of the firefly luciferase relative to the *Renilla* luciferase. Values are the mean \pm SD of data from three independent experiments performed in triplicate. ***Both represent $P < 0.0001$, when compared with the wild-type *GATA4*.

The association of *GATA4* variations with VSD in humans was first described in 1999. Pehlivan and colleagues (35) investigated patients with the 8p23 syndrome and found that *GATA4* haploinsufficiency was responsible for congenital heart disease including VSD. Garg *et al* (18) identified the first two unrelated families with congenital heart defects caused by two *GATA4* mutations, respectively, of which in family A, the pG296S mutation was detected in three patients with VSD in combination with atrial septal defects and pulmonary stenosis or patent ductus arteriosus. Functional assays of the pG296S mutation revealed a significantly impaired affinity to the promoter DNA and a failure of *GATA4* to interact with *TBX5* resulting in a significant decrease of target gene expression. Subsequently, the pG296S mutation of *GATA4* was observed in another family with atrial septal defects, VSD and pulmonary valve stenosis, and in a third family with atrial septal defects, and pulmonary valve stenosis (36). Interestingly, the pG296C mutation of *GATA4* was observed in a family with atrial septal defects and pulmonary stenosis (24). In the present study, the pG296R mutation of *GATA4* was observed in a family with VSD, atrial septal defects and pulmonary stenosis. These results demonstrated that the glycine at amino

acid position 296 of *GATA4* is functionally important and a mutation hotspot for VSD, although a long list of *GATA4* mutations linked to isolated or syndromic VSD have been reported, such as p.A6V (25), p.H28Y (27), p.S46del (25), p.A66T (27), p.A118_A119insA (25), p.A125_A126insAA (25), p.P163S (24), p.E216D (22), p.Q316E (23), p.E359K (25), p.P407Q (32), p.A411V (23), p.D425N (23), p.S429T (25), p.H436Y (27) and p.A442V (25).

To date, at least 20 germline mutations in the coding region of *GATA4* have been identified in VSD patients with or without other concurrent cardiovascular defects, showing that although *GATA4* mutations are involved in a great variety of congenital cardiac malformations, one of the most frequent phenotypes due to a mutation in *GATA4* is VSD (18,22-25,27,32,36,37). Nevertheless, the substantially varied prevalence of *GATA4* mutations associated with congenital cardiovascular diseases was reported, which was roughly 0.0 (0/99), 0.5 (1/205), 0.8 (5/628), 0.8 (1/120), 1.0 (1/104), 1.4% (5/357), 1.7 (2/120), 2.2 (3/135), 2.5 (12/486), 3.7 (4/107), 6.9 (2/29), 10.0 (5/50) or 12.5% (2/16), with a total prevalence of 1.8% (43/2456) based on these reports (21-27,32,33,36-39). Similarly, a *GATA4* mutation prevalence of nearly 0.5% (1/210) was observed in our VSD population, implying that the *GATA4* mutations could be a minor cause of congenital VSD. It was noteworthy that remarkable genetic heterogeneity of VSD was substantiated by an inability to find mutations in about 99.5% of our cohort patients, despite somatic *GATA4* mutations as a potential mechanism of VSD in some patients (20,40,41). Therefore, the contribution of genes other than *GATA4* to VSD pathogenesis seems likely.

Mutations in other transcription factors associated with cardiogenesis, such as *NKX2-5*, *GATA6* and *TBX5*, have also been identified in patients with VSD (42-47) and also mutations in cardiac structural proteins such as troponin I type 3 (*TNNI3*) and α myosin heavy chain (*MYH6*) were identified in VSD patients (48,49). However, in the current study population, genetic analysis of *NKX2-5* and *GATA6* was performed as previously described (43,45) and no novel mutations were observed, implying the importance of other genes in the pathogenesis of VSD.

Association of impaired *GATA4* with increased susceptibility to VSD and other cardiac dysmorphias have been documented in animals. In the embryonic hearts of knock-down chicks generated by using small interfering RNAs targeted to *GATA4*, the bilateral myocardial rudiments failed

to travel to the midline, resulting in the formation of two separate hearts in lateral positions, an anomaly of cardia bifida (50). In other studies, mice lacking both GATA4 alleles died between Days 7.0 to 9.5 of embryonic development because of abnormal embryogenesis and heart tube formation, including septal defects, right ventricular hypoplasia, endocardial cushion defect, tetralogy of Fallot, double outlets of the right ventricle, and cardiomyopathy (51-53). In a study of transgenic mice expressing a missense mutation in a highly conserved zinc-finger domain of GATA4 (p.V217G), the embryos died after embryonic Day 12.5 exhibiting features in common with congenital cardiovascular defects in humans (54). Additionally, GATA4 was also found to be indispensable for cardiogenesis in the fly, fish and *Xenopus* (55). Taken together, these experimental results from animals have clarified that GATA4 plays an important role in the morphogenesis and differentiation of the heart and compromised GATA4 may underlie a variety of congenital cardiac abnormalities including VSD in humans.

Previous studies have revealed that GATA4 is an upstream regulator or transcriptional activator of several genes expressed during cardiac development including the *ANP* gene, the brain natriuretic peptide gene, and the α -cardiac actin gene (34). GATA4 binding to target DNA may occur in conjunction with other partners including the transcription factors TBX5 and NKX2-5, and the synergistic transcriptional activation mediated by TBX5 and NKX2-5 has been previously reported (18,56,57). Therefore, the functional characteristics of the GATA4 mutations can be explored by analysis of the transcriptional activity of the *ANP* promoter in cells transfected with GATA4 mutants in contrast to wild-type. In this study, the functional role of the novel p.G296R mutation of GATA4 identified in our familial VSD patients was characterized by transcriptional activity assays and showed a significantly decreased transcriptional activity on a downstream gene, consistent with the loss-of function effects of other GATA4 mutations associated with cardiac septal defects on the transcriptional activity of the *ANP* promoter (18,22,33). These findings indicate that haploinsufficiency or the dominant negative effects of GATA4 mutations are the major pathophysiological mechanisms involved in congenital VSD.

The finding of a novel GATA4 mutation in patients with congenital VSD contributes to making an early and precise diagnosis, which is clinically important, because leaving it uncorrected until adulthood may result in congestive heart failure and pulmonary hypertension, although some patients with minor VSD can often lead an otherwise healthy life. As the mortality rate of VSD operations is very low in patients without cardiac failure at an early age, timely surgical repair or catheter based closure of VSD is feasible. This information will be especially useful for genetic counseling of the family, because testing for p.G296R in new family members can be followed by careful medical examination and prophylactic intervention if VSD is found.

In conclusion, this study links a novel mutation in the cardiac transcription factor GATA4 to VSD and provides new insight into the molecular mechanism implicated in the pathogenesis of VSD, suggesting the potential implications for the genetic diagnosis and gene-specific therapy for this common disease in children.

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