

# Involvement of a novel *GATA4* mutation in atrial septal defects

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**Abstract.** Atrial septal defect (ASD) is one of the most common types of congenital heart disease and is associated with a significant increase in the morbidity and mortality of affected individuals. Accumulating evidence indicates that genetic defects play important roles in the pathogenesis of congenital ASD. However, ASD is genetically heterogeneous and the genetic determinants for ASD in the majority of the patients remain to be identified. In this study, the entire coding region of *GATA4*, a gene encoding a zinc-finger transcription factor crucial to embryogenesis, was initially sequenced in 120 unrelated patients with ASD. The available relatives of patients carrying the identified mutation and 200 ethnicity-matched unrelated control individuals were genotyped. The functional characteristics of the *GATA4* mutant were compared to its wild-type counterpart using a luciferase reporter assay system. A novel heterozygous missense *GATA4* mutation, p.G21V, was identified in 2 unrelated families with ASD, which was not detected in the control population nor reported in the human gene mutation database. Alignment of multiple *GATA4* proteins displayed that the affected amino acid residue was highly conserved across species. Functional analysis showed that the p.G21V *GATA4* mutation was associated with a decreased transcriptional activity. The find-

ings underscore the pathogenic link between compromised *GATA4* function and congenital ASD, providing new insight into the molecular mechanism involved in this common form of congenital cardiovascular anomalies.

## Introduction

Congenital heart disease is the most common form of birth defect with a prevalence of approximately 1% in neonates, and is the leading non-infectious cause of mortality in newborns, with more than 29% of infants who die of embryonic developmental abnormalities having cardiovascular malformations (1). Congenital heart disease, an abnormally developed cardiac structure, is recognized with at least 18 distinct types including atrial septal defect (ASD), ventricular septal defect, transposition of the great artery, atrioventricular septal defect, coarctation of the aorta, and hypoplastic left heart syndrome, of which ASD is the second commonest cardiovascular dysmorphism only next to ventricular septal defect (1,2). ASD accounts for about 33% of all congenital cardiovascular deformities, affecting over 3 out of 1,000 live births (2). ASDs are defined by an anatomically deficient interatrial septum allowing blood to flow directly between the left and right atria. ASDs are clinically classified into 5 types by whether they involve other structures of the heart and how they are formed during the cardiac developmental process. These types include the ostium secundum ASD, patent foramen ovale, ostium primum ASD, sinus venosus ASD and common or single atrium, of which the ostium secundum ASD is the most frequent defect, representing 85% of all ASDs (3). Congenital ASD may occur separately or in combination with other cardiac anomalies, such as ventricular septal defect, pulmonary valve stenosis, or conduction defects. Regardless of other anomalies that may accompany ASD, persistent severe blood shunt between two atria may give rise to cardiac enlargement, congestive heart failure, pulmonary hypertension, Eisenmenger's syndrome, arrhythmias, and even sudden cardiac death in the absence

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Table I. The intronic primers used to amplify the coding exons and exon-intron boundaries of *GATA4*.

Exon	Forward primer	Reverse primer	Amplicon (bp)
2-a	5'-GAT CTT CGC GAC AGT TCC TC-3'	5'-GTC CCC GGG AAG GAG AAG-3'	458
2-b	5'-GCT GGG CCT GTC CTA CCT-3'	5'-AAA AAC AAG AGG CCC TCG AC-3'	554
3	5'-GGG CTG AAG TCA GAG TGA GG-3'	5'-GAT GCA CAC CCT CAA GTT CC-3'	437
4	5'-GAG ATC TCA TGC AGG GTC GT-3'	5'-GCC CCT TCC AAA TCT AAG TC-3'	390
5	5'-TCT TTC TCG CTG AGT TCC AG-3'	5'-GGG ATG TCC GAT GCT GTC-3'	379
6	5'-GCC ATC CCT GTG AGA ACT GT-3'	5'-GAG GGT AGC TCA CTG CTT GC-3'	444
7	5'-AAG TGC TCC TTG GTC CCT TC-3'	5'-TTC CCC TAA CCA GAT TGT CG-3'	479

of surgical or catheter based repair (4). Hence ASD is an important contributor to the significantly increased morbidity and mortality in infancy.

The aberrant development of the atrial septum is implicated in a heterogeneous, complex biological process associated with environmental and genetic risk factors (5,6). Growing evidence highlights the crucial role of several transcription factors, including *GATA4*, in septogenesis (6). The human *GATA4* gene maps to chromosome 8p23.1-p22, consists of 7 exons, and encodes a zinc-finger transcription factor, a protein of 442 amino acids (7). The zinc finger-containing transcription factor *GATA4* is expressed throughout cardiac morphogenesis and is essential for normal cardiac development (8-12). Therefore, *GATA4* has been a prime candidate gene in studies to identify the genetic determinants for congenital cardiovascular defects, and to date, more than 40 mutations within the *GATA4* gene have been identified in patients with a wide variety of congenital heart malformations including ASD, ventricular septal defect, endocardial cushion defect, tetralogy of Fallot, patent ductus arteriosus, pulmonary stenosis, and hypoplastic right ventricle (13-26). Nevertheless, the molecular etiology responsible for ASD in most patients remains to be identified (5,6).

In this study, the coding exons and exon/intron boundaries of *GATA4* were initially sequenced in a cohort of 120 unrelated patients with ASD and a novel heterozygous *GATA4* mutation, p.G21V, was identified in 2 patients. Subsequently, genetic analysis of the available relatives of the patients harboring the mutation demonstrated that in each family, the mutation co-segregated with autosomal dominantly inherited ASD. The mutation was absent in 400 control chromosomes and the altered amino acid was highly conserved evolutionarily. Functional analysis demonstrated that the p.G21V mutation of *GATA4* was associated with a significantly decreased transcriptional activity. These findings expand the spectrum of mutations in *GATA4* linked to ASD and provide new insight into the molecular mechanism involved in the pathogenesis of ASD.

## Materials and methods

**Study participants.** A cohort of 120 unrelated patients with ASD was recruited from the Chinese population. Subjects were evaluated by individual and familial history, review of the medical records, complete physical examination, 12-lead electrocardiogram (ECG) and a two-dimensional

transthoracic echocardiography with a color flow Doppler. All patients had a classic form of ASD, with a defect diameter of >5 mm and nearly all patients underwent cardiac catheterization and, if required, cardiac surgery. A group of 200 ethnically matched unrelated healthy individuals, which were derived from the general population, were used as controls to screen for the identified *GATA4* mutation. Peripheral venous blood specimens from subjects and control individuals were collected. 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). Initially, the candidate gene *GATA4* was screened in 120 unrelated patients with ASD and subsequently, genotyping of *GATA4* in the available relatives of the index patients carrying an identified mutation and the 200 control individuals was performed. The genomic DNA reference sequence of *GATA4* was derived from GenBank (accession no. NC\_000008). The primers used to amplify the coding exons (exons 2-7) and intron-exon boundaries of *GATA4* by polymerase chain reaction (PCR) were designed with the on-line Primer 3 software (<http://frodo.wi.mit.edu>) and are shown in Table I. PCR was carried out using the HotStarTaqDNA polymerase (Qiagen) on a PE 9700 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 3130 XL DNA Analyzer (Applied Biosystems). The sequencing primers 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 of >99%.

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

Table II. Clinical characteristics of 120 unrelated patients with atrial septal defects.

	Number or mean value	Percentage or range
Male: female	52:68	43.33
Age at the diagnosis of ASD (year)	3.20	0.67-9.50
Age at the present study (year)	4.15	0.83-10.25
Positive family history	14	11.67
Distribution of different types of ASDs		
Ostium secundum	111	92.50
Patent foramen ovale	4	3.33
Ostium primum	3	2.50
Sinus venosus	1	0.83
Common or single atrium	1	0.83
Prevalence of ASDs with other defects		
Isolated ASD	102	85.00
ASD and VSD	10	8.33
ASD, VSD and PDA	4	3.33
ASD, VSD and DORV	2	1.67
ASD and PDA	1	0.83
ASD and PS	1	0.83
Incidence of arrhythmias		
Atrioventricular block	5	4.17
Atrial fibrillation	2	1.67
Treatment		
Surgical repair	87	72.50
Percutaneous closure	24	20.00
Follow-up	9	7.50

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

**Prediction of the disease-causing potential of a *GATA4* sequence variation.** The disease-causing potential of a *GATA4* sequence variation was predicted automatically by the online program MutationTaster (<http://www.mutationtaster.org>), giving the 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 the error as used in t-test statistics, with a value close to 1 indicating 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 ANP(-2600)-Luc, were kindly provided by Dr Ichiro Shiojima, Chiba University School of Medicine, Japan. 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  $\mu$ g wild-type or mutant pSSRa-GATA4 expression vector, 0.2  $\mu$ g ANP(-2600)-Luc reporter construct, and 0.04  $\mu$ g pGL4.75 control reporter vector using the PolyFect Transfection Reagent (Qiagen). For co-transfection experiments, 0.1  $\mu$ g wild-type pSSRa-GATA4, 0.1  $\mu$ g mutant pSSRa-GATA4, 0.2  $\mu$ g ANP(-2600)-Luc, and 0.04  $\mu$ g pGL4.75 were used. Firefly and *Renilla* luciferase activities were measured with the Dual-Glo luciferase assay system (Promega) 48 h after transfection. At least three independent experiments were performed for wild-type and mutant *GATA4*.

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

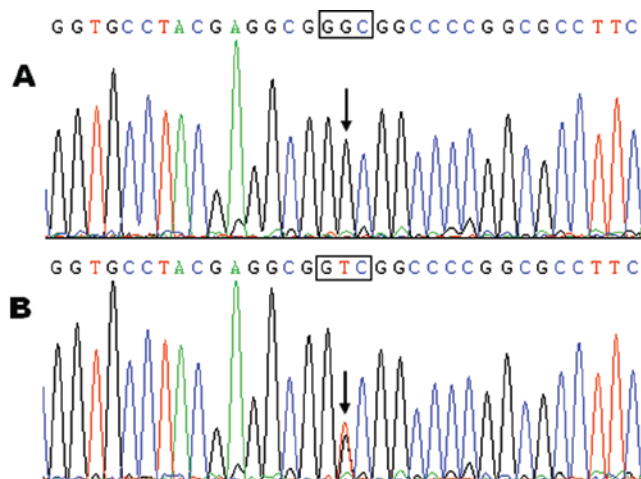


Figure 1. Representative sequence chromatogram showing the *GATA4* mutation identified in 2 families. The arrow indicates the heterozygous nucleotides of T/G in a proband (B) or the homozygous nucleotides of G/G in a control individual (A). The square denotes the nucleotides comprising a codon of *GATA4*.

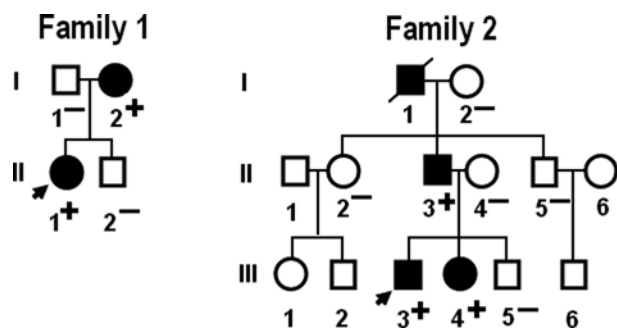


Figure 2. Pedigree structures of families with the atrial septal defect. Families are designated as family 1 and family 2, respectively. Family members are identified by generations and numbers. Squares indicate male family members; circles, female members; the symbol with a slash indicates the deceased member; closed symbols, the affected members; open symbols, unaffected members; arrow, proband; +, carriers of the heterozygous mutations; and -, non-carriers.

## Results

**Characteristics of the study subjects.** A cohort of 120 unrelated patients with ASD was recruited and clinically evaluated in contrast to a group of 200 ethnically matched unrelated healthy individuals as controls. None of them had traditional risk factors for ASD. The clinical characteristics of the 120 unrelated patients with ASD are summarized in Table II.

***GATA4* mutation.** Direct sequencing of the coding regions of the *GATA4* gene was performed after PCR amplification of genomic DNA from the 120 unrelated ASD patients. A heterozygous missense mutation in *GATA4* was identified in 2 out of 120 patients. The total population prevalence of the *GATA4* mutation based on the patient cohort was ~1.67%. A substitution of thymine (T) for guanine (G) in the second nucleotide of codon 21 of the *GATA4* gene (c.62G>T), predicting the transition of glycine into valine at amino acid 21 (p.G21V), was identified in 2 unrelated patients. The sequence chromatogram showing the detected heterozygous *GATA4* variation of c.62G>T in comparison to the control sequence is shown in Fig. 1. The variant was not detected in 200 unrelated control individuals nor described in the human gene mutation database (HGMD, <http://www.hgmd.cf.ac.uk/ac/index.php>). Genetic scanning of the family members available displayed that the gene variant was present in all the affected family members alive, but absent in unaffected family members tested in each family. Analysis of the pedigrees demonstrated that the mutation co-segregated with ASD transmitted as an autosomal dominant trait in the families with complete penetrance. The pedigree structures of the 2 families are illustrated in Fig. 2. The phenotypic characteristics and results of genetic screening of the affected pedigree members are listed in Table III.

**Multiple alignments of the *GATA4* protein sequences across species.** Alignment of *GATA4* amino acid sequences from human, chimpanzee, dog, cattle, mouse, and rat revealed that

Table III. 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 ASD (years)	ASD (mm)	Other structural defects	AVB	G21V
Family 1							
I-2	F	30	24	14	-	-	+/-
II-1	F	4	4	12	-	-	+/-
Family 2							
I-1	M	68 <sup>a</sup>	N/A	18	VSD	+	N/A
II-3	M	34	12	15	VSD	-	+/-
III-3	M	7	7	21	-	-	+/-
III-4	F	5	5	9	-	-	+/-

F, female; M, male; ASD, atrial septal defect; N/A, not available or not applicable; AVB, atrioventricular block; VSD, ventricular septal defect; +, present; -, absent. <sup>a</sup>Age at death.

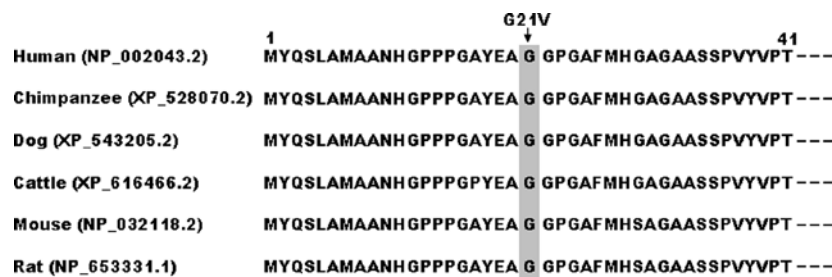


Figure 3. Alignment of multiple GATA4 protein sequences across species. The G21 affected amino acid is completely conserved evolutionarily across mammals.

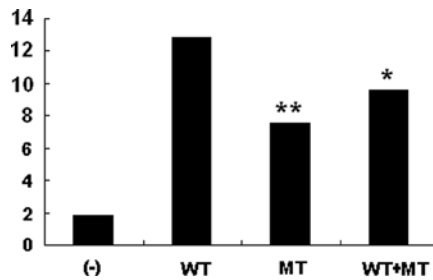


Figure 4. Transcriptional activity of the mutant *GATA4* on the *ANP* promoter. COS-7 cells were transfected with 0.2  $\mu$ g of wild-type or mutant pSSRa-*GATA4* expression vector, 0.2  $\mu$ g of ANP(-2600)-Luc reporter construct, and 0.04  $\mu$ g of pGL4.75 control reporter vector. For co-transfection experiments, 0.1  $\mu$ g of wild-type pSSRa-*GATA4*, 0.1  $\mu$ g of mutant pSSRa-*GATA4*, 0.2  $\mu$ g of ANP(-2600)-Luc and 0.04  $\mu$ g of pGL4.75 were used. The activity of the *ANP* promoter was presented as fold-activation of firefly luciferase relative to the *Renilla* luciferase. Values are the mean  $\pm$  SD of data from three independent experiments performed in triplicate. \* $P < 0.01$  and \*\* $P < 0.001$ , respectively, when compared with the wild-type *GATA4*.

the affected amino acid 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.62G>T detected in *GATA4* was automatically predicted to be disease-causing with a P-value of 0.887668, providing evidence for the variation 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 (0.2  $\mu$ g) of wild-type and mutant *GATA4* activated the *ANP* promoter by ~7-fold and ~4-fold, respectively. When the same amount of wild-type *GATA4* (0.1  $\mu$ g) was co-transfected with mutant *GATA4* (0.1  $\mu$ g), the induced activation of the *ANP* promoter was ~5-fold. These results suggest that mutant *GATA4* has a significantly reduced transcriptional activity compared to its wild-type counterpart (Fig. 4).

## Discussion

In the present study, we report the previously unrecognized p.G21V *GATA4* mutation identified in 2 unrelated families

with ASD. The novel heterozygous missense mutation was present in all the affected family members alive but absent in unaffected relatives and 400 normal chromosomes from a matched control population. A cross-species alignment of *GATA4* protein sequences showed that the affected amino acid was highly conserved evolutionarily. Prediction of a causative potential of a sequence alteration demonstrated that the p.G21V mutation is disease-causing with a probability value as high as ~1. Functional analysis displayed that the p.G21V *GATA4* mutation is associated with a significantly decreased transcriptional activity. Therefore, it is likely that the mutated *GATA4* is responsible for the ASD in these families.

*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 *GATA1*, *GATA2* and *GATA3* are expressed predominantly in haematopoietic cells and in some ectodermal derivatives, whereas *GATA4*, *GATA5* and *GATA6* are expressed mainly in the developing heart and in several endodermal lineages (27). *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) (26). The two TADs are both 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* (26,27). The *GATA4* mutation, p.G21V, identified in this study is located in TAD1, thus may directly influence the transcriptional activity of *GATA4*, which has been confirmed by reporter gene assay experiments.

Our results are supported by the findings of other *GATA4* mutations predisposing to congenital ASD. Garg *et al* (13) identified the heterozygous missense mutation, p.G296S (c.886G>A), and the heterozygous frame-shift mutation, p.E359RfsX44 (c.1075delG), in *GATA4* in 2 large unrelated families with ASD. Luciferase assays demonstrated the decreased trans-activation caused by the mutant proteins. This was the first report linking *GATA4* loss-of-function mutation with an isolated congenital heart defect. Okubo *et al* (14) found the novel *GATA4* mutation, p.S358RfsX45 (c.1074delC), in a large Japanese family with ASD. By PCR and direct sequencing, Hirayama-Yamada *et al* (16) screened *GATA4* in 16 unrelated families with ASD and identified the novel mutation, S52F



(c.155C>T), and the known mutation, E359fsX45 (c.1075delG), in 2 families, with a mutation prevalence of 12.5% in the probands with ASD. Tomita-Mitchell *et al* (18) investigated the *GATA4* coding region and exon-intron boundaries in a large population of 628 unrelated patients with either septal or conotruncal defects and 4 missense sequence variants were identified in 5 patients, of which p.G93A and p.Q316E were found in 2 out of 122 ASD patients, p.A411V and p.D425N in 2 out of 122 patients with ventricular septal defects, and p.D425N in 1 out of 201 patients with tetralogy of Fallot. So far, more than 25 germline mutations in *GATA4* have been implicated in congenital cardiovascular anomalies, of which more than half of the mutations have been linked to ASD with or without other defects. The data suggest that although *GATA4* mutations are involved in a long list of cardiac malformations, the most frequent phenotype resulting from a *GATA4* mutation is ASD (13-26). In most of these patients, the ASD causing mutations are familial, whereas sporadic cases remain relatively infrequent (13-26). Similarly to these findings, the figure of 2/120 mutations (roughly 1.67%) in our patient cohort suggests that the *GATA4* mutations could be an uncommon cause of ASD. Notably, remarkable genetic heterogeneity of ASD was proved by an inability to detect mutations in over 98% of our cohort patients, despite somatic *GATA4* mutations as a likely mechanism of ASD in some patients (15). Hence, the contribution of genes other than *GATA4* to ASD pathogenesis appears likely.

Mutations in other transcription factors associated with cardiogenesis, such as *NKX2-5* (28-31), *TBX5* (32-34), *TBX20* (35,36) and *GATA6* (37), have also been detected in patients with ASD, and also mutations in cardiac structural proteins such as  $\alpha$ -myosin heavy chain (*MYH6*) and  $\alpha$ -cardiac actin (*ACTC1*) were identified in familial ASD (38,39). Therefore, genetic analysis of these candidate genes in our cohort with ASD is warranted. However, these ASD associated genes have also been reported to contribute to other cardiac or even extracardiac defects, which highlights the clinical heterogeneity and the suggestive roles of the established genotype-phenotype relationship of these genes. Specifically, *NKX2-5* mutations were reported to cause ASD and progressive atrioventricular block, while the two most common phenotypes caused by mutated *NKX2-5* were ASD and atrioventricular conduction disturbance, indicating the pivotal role of *NKX2-5* not only in the morphogenesis of the heart, but also in the construction of the cardiac conduction system (40). Moreover, *TBX5* mutations underlay the Holt-Oram syndrome, a rare dominant inherited disease clinically characterized by upper limb and multiple heart defects including ASD, ventricular septal defects, tetralogy of Fallot, hypoplastic left heart and conduction abnormalities. Although the clinical manifestations were variable, upper limb abnormalities were always present (41). These observations underscore the important role of *TBX5* in the development of both the heart and upper limbs.

Association of impaired *GATA4* with increased predisposition to ASD has been observed 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 (42). Homozygous *GATA4*-deficient mice died

between day 7.0 to 9.5 and analysis of the *GATA4*-null embryo substantiated the lethal failure to form a linear heart tube (43,44). Transgenic mice expressing *GATA4* mutants demonstrated a wide variety of cardiac malformations including septal defects, right ventricular hypoplasia, endocardial cushion defect, tetralogy of Fallot, double outlets of the right ventricle, and cardiomyopathy, similar to the anomalies seen in humans (43-47). Taken together, these results from animal experiments define a critical role for *GATA4* in regulating normal cardiac morphogenesis.

In conclusion, the present study links a novel mutation in the cardiac transcription factor *GATA4* to ASD and provides new insight into the molecular mechanism implicated in the pathogenesis of this common congenital cardiovascular disease.

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