GATA5 loss-of-function mutations associated with congenital bicuspid aortic valve

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Abstract. Bicuspid aortic valve (BAV) is the most common form of congenital cardiovascular defect in humans worldwide and is responsible for substantial morbidity and mortality. Accumulating evidence has demonstated that genetic risk factors are involved in the pathogenesis of BAV. However, BAV is genetically heterogeneous and the genetic basis underlying BAV in a large number of patients remains unknown. In the present study, the coding regions and splice junction sites of the *GATA5* gene, which codes for a zinc-finger transcription factor crucial for the normal development of the aortic valve, was sequenced initially in 110 unrelated patients with BAV. The available relatives of the mutation carriers and 200 unrelated healthy individuals used as controls were subsequently genotyped for *GATA5*. The functional effect of the mutations was characterized by using a luciferase reporter assay system. As a result, two novel heterozygous GATA5 mutations, p.Y16D and p.T252P, were identified in two families with autosomal dominant inheritance of BAV, respectively. The variations were absent in 400 control chromosomes and the altered amino acids were completely conserved evolutionarily. Functional assays revealed that the two GATA5 mutants were associated with significantly reduced transcriptional activity compared with their wild-type counterpart. To the best of our knowledge, this is the first study on the association of GATA5 loss-of-function mutations with enhanced susceptibility to BAV, providing novel insight into the molecular mechanism involved in human BAV and suggesting a potential role for the early prophylaxis and personalized treatment of this common congenital heart disease.

Introduction

Bicuspid aortic valve (BAV) is the most common form of congenital heart disease in humans, with an estimated prevalence of 0.5-2% in the general population as well as a pronounced male predominance of ~3:1 (1-6). While BAV can occur in isolation, it is frequently associated with other congenital cardiovascular malformations, such as coarctation of the aorta, interruption of the aorta, ventricular septal defect, atrial septal defect, patent ductus arteriosus and hypoplastic left heart syndrome, leading to a wide spectrum of clinical presentations ranging from severe disorder detected *in utero* to asymptomatic condition in old age (7). Patients with BAV are at high risk for the development of severe complications, including aortic valve regurgitation, aortic valvular stenosis, aortic dilation or even aneurysm, aortic dissection, thrombus formation, and infective endocarditis (8-11). BAV accounts for 70-85% of aortic stenosis in pediatric patients and at least 50% of stenotic aortic valve in adults (12,13). Moreover, individuals with BAV have an 8-fold increased risk of aortic dissection and 25-year risk of valve replacement of 53%, aneurysm formation of 26% and aortic surgery of 25% (14). Therefore, BAV confers a heavier burden of disease than all other congenital cardiac lesions combined (3,7). Despite its high prevalence and significant clinical importance, the underlying pathogenic basis of BAV remains largely unclear.

Cardiac valve morphogenesis occurring early in fetal development is a complex and dynamic process that requires the temporal and spatial cooperation of cardiac cell

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commitment, differentiation, proliferation, and migration, and both environmental and genetic risk factors may interrupt this biological process, resulting in abnormal valvulogenesis and the formation of BAV (15,16). Mounting evidence suggests that genetic defects play an important role in the pathogenesis of BAV (7,13). Previous studies have established the substantial familial clustering of BAV, with a prevalence ranging from 9 to 24% in the first-degree relatives of BAV patients and a heritability as high as 89%, suggesting a Mendelian pattern of inheritance (7,13). By genome-wide scan of the available family members with polymorphic microsatellite markers and linkage analysis, BAV-susceptibility loci have been mapped on chromosomes 9q34-35, 18q, 5q15-21 and 13q33-qter, and *NOTCH1* has been identified as the first culprit gene accountable for BAV in the genomic region of 9q34 (17,18). Moreover, candidate gene strategy has led to the identification of various novel mutations in the *NOTCH1* gene that are associated with BAV (19-21). Additionally, mutations in other genes, including *KCNJ2*, *TGFBR2* and *NKX2-5*, were detected in patients with BAV (22-24). Nevertheless, BAV is of pronounced genetic heterogeneity and the genetic determinants underpinning BAV in a large number of patients remain to be determined.

GATA5 was reported to have a crucial role in cardiovascular development and valvular morphogenesis (25-31), and the targeted deletion of *GATA5* in mice resulted in partially penetrant BAV (31). Furthermore, the endocardial cell-specific inactivation of *GATA5* led to BAV, similar to that observed in *GATA5*-null mice (31). GATA5 is a zinc finger-containing transcription factor that belongs to a subgroup of the GATA family of DNA binding proteins, which, together with GATA4 and GATA6, is abundantly expressed in various mesoderm- and endoderm-derived tissues, predominantly in embryonic heart (32,33). In humans, mutations in *GATA5* have been found to be associated with a wide variety of congenital cardiovascular anomalies, including ventricular and atrial septal defect, tetralogy of Fallot, double outlet right ventricle, aortic stenosis, and BAV (21,34-39). Taken together, these findings suggested screening *GATA5* for mutations in another cohort of patients with BAV.

Materials and methods

Study population. A cohort of 110 unrelated patients with BAV was recruited from the Chinese Han population for this study. The available relatives of the index patients carrying the identified *GATA5* mutations were also enlisted. Patients underwent clinical evaluation that included individual and familial histories, medical records, complete physical examination, 12-lead electrocardiogram, and two-dimensional transthoracic echocardiography with color flow Doppler. BAV was confirmed by imaging and/or direct view during aortic valve replacement surgery. Familial BAV was defined if two or more affected relatives had proven BAV. The patients with known chromosomal abnormalities or syndromic cardiovascular defects, such as Marfan syndrome, Turner syndrome, and Di George syndrome, were excluded from the study.

A total of 200 unrelated, ethnically matched healthy individuals randomly selected from the subjects undergoing routine physical examinations were used as controls. In terms of medical histories and echocardiographic records, the control individuals had no congenital cardiovascular deformities. The ethnic origin of a participant was determined by a combination of self-reported ethnicity and a personal questionnaire regarding birthplace, language, religion, and ancestry.

Peripheral venous blood samples were obtained from BAV cases and control individuals. The study protocol conforms to the principles outlined in the Declaration of Helsinki and was approved by the local Institutional Ethics Committee. Written informed consent was obtained from all participants or their guardians prior to study.

Mutational screening of GATA5. Genomic DNA from each participant was extracted from blood lymphocytes with a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The coding regions and flanking introns of the *GATA5* gene were sequenced initially in 110 unrelated patients with BAV, and subsequently in the available relatives of the index patients carrying identified mutations and the 200 unrelated, ethnically-matched healthy individuals. The primer pairs used to amplify the coding exons and exon/intron boundaries of *GATA5* by polymerase chain reaction (PCR) were described previously (39). PCR was performed using HotStar Taq DNA Polymerase (Qiagen GmbH, Hilden, Germany) on a Veriti Thermal Cycler (Applied Biosystems, Foster, CA, USA), with standard conditions and concentrations of reagents. The amplified products were analyzed on 1% agarose gels stained with ethidium bromide and purified with QIAquick Gel Extraction kit (Qiagen GmbH). 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 amplifications. The DNA sequences were viewed and analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The variant was validated by resequencing an independent PCR-generated amplicon from the subject and met our quality control thresholds with a call rate of >99%. Additionally, an identified sequence variation was searched in the single-nucleotide polymorphism (SNP) database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), the human gene mutation (HGM) database (http://www.hgmd.org/), and the 1000 genome database (http://www.1000genomes.org/) to confirm its novelty.

Alignment of multiple GATA5 protein sequences among species. Multiple GATA5 protein sequences across various species were aligned using the online program of MUSCLE, version 3.6 (http://www.ncbi.nlm.nih.gov/).

Prediction of the pathogenic potential of a GATA5 sequence variation. The disease-causing potential of a *GATA5* sequence variation was predicted by MutationTaster (an online program at http://www.mutationtaster.org), which automatically gave a probability for the variation to be either a pathogenic mutation or a benign polymorphism. Notably, the P-value used here is the probability of the correct 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). Additionally, the

online program PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) was used to predict the possible impact of an amino acid substitution on the structure and function of GATA5 protein.

Plasmids and site-directed mutagenesis. The recombinant expression plasmid pcDNA3.1-hGATA5 was constructed as described previously (39). The atrial natriuretic factor (ANF)-luciferase reporter gene, which contains the 2600-bp 5'-flanking region of the *ANF* gene, i.e., ANF(-2600)-Luc, was kindly provided by Dr Ichiro Shiojima, from the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chuo-ku, Chiba, Japan. The identified mutation was introduced into the wild-type GATA5 using a QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) with a complementary pair of primers. The mutant was sequenced to confirm the appropriate mutation and to exclude any other sequence variations.

Reporter gene assay. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and seeded in 12-well plates prior to transfection. The ANF(-2600)-Luc reporter construct and an internal control reporter plasmid pGL4.75 (hRluc/CMV, Promega) were used in the transient transfection assay to evaluate the transcriptional activity of the *GATA5* mutants. HEK-293 cells were transfected with 0.4 μg of wild-type or mutant pcDNA3.1-hGATA5 expression vector, 0.4 μg of ANF(-2600)-Luc reporter construct, and 0.04 μg of pGL4.75 control reporter vector using a PolyFect Transfection Reagent (Qiagen). For cotransfection experiments, 0.2 μg of wild-type pcDNA3.1-hGATA5 together with 0.2 μg of mutant pcDNA3.1-hGATA5 or 0.2 μg of empty pcDNA3.1 vector were used in the presence of 0.4 μg of ANF(-2600)-Luc and 0.04 μg of pGL4.75. Firefly and *Renilla* luciferase activities were measured with the Dual-Glo® luciferase assay system (Promega) 48 h after transfection. The activity of the ANF promoter was presented as fold activation of Firefly luciferase relative to *Renilla* luciferase. A minimum of three independent experiments were performed for wild-type and mutant *GATA5*.

Statistical analysis. Data are expressed as means ± standard deviation. Continuous variables were tested for normality of distribution, and the Student's unpaired t-test was used to compare the numeric variables between the two groups. Comparison of the categorical variables between the two groups was completed by using Pearson's χ^2 test or Fisher's exact test when appropriate. A two-tailed P-value <0.05 was considered to indicate statistical difference.

Results

Clinical characteristics of the study subjects. A cohort of 110 unrelated patients with BAV was enrolled and clinically evaluated as well as 200 unrelated control individuals. All the participants had no established environmental risk factors for congenital heart disease, such as maternal illness and drug use in the first trimester of pregnancy, parental smoking, and long-term exposure to toxicants and ionizing radiation. The baseline clinical characteristics of the 110 unrelated BAV cases are shown in Table I.

Table I. Clinical characteristics of the 110 unrelated patients with bicuspid aortic valve (BAV).

Parameter	Statistic
Age (years)	45.4±11.8
Male gender (%)	72 (65.5)
Positive family history (%)	36 (32.7)
Abnormal valve function[a] (%)	73 (66.4)
Concomitant aortopathy[b] (%)	67 (60.9)
Concomitant other cardiac structural defects[c] (%)	20 (18.2)
Atrial fibrillation (%)	5 (4.5)
Surgical repair (%)	96 (87.3)

[a]Aortic regurgitation and/or aortic stenosis with at least moderate severity; [b]Aortic dilation ≥40 mm, affecting any part of the aorta from sinus of Valsalva to proximal descending aorta; [c]Seven patients with coarctation of the aorta (CoA); three with ventricular septal defect (VSD); three with patent ductus arteriosus (PDA); two with atrial septal defect (ASD); one with patent formamen ovale; one with anomalous origin of the left coronary artery; one with CoA and VSD; one with CoA and ASD; one with CoA, VSD and PDA.

GATA5 sequence variation. Two heterozygous *GATA5* sequence variations were identified in 2 of 110 unrelated BAV patients, respectively, with a mutational prevalence of ~1.82%. Specifically, a change of thymine into guanine at the first nucleotide of codon 16 of the *GATA5* gene (c.46T>G), predicting the transition of tyrosine into aspartic acid at amino acid position 16 (p.Y16D), was identified in the index patient from family 1. A substitution of cytosine for adenine in the first nucleotide of codon 252 (c.754A>C), equivalent to the replacement of threonine by proline at amino acid 252 (p.T252P), was identified in the proband from family 2. The sequence electropherograms showing the identified heterozygous *GATA5* variations in contrast to corresponding control sequences are shown in Fig. 1. A schematic diagram of GATA5 protein showing the structural domains and the locations of the detected mutations is shown in Fig. 2. The variations were not observed in the 400 control chromosomes or found in the SNP, HGM and 1,000 genome databases, which were consulted again on November 10, 2013.

A genetic scan of the family members of the mutation carriers showed that in each family, the variation was present in all the affected family members available, but absent in the unaffected family members examined. Analysis of the pedigrees revealed that the variations cosegregated with BAV with complete penetrance. The pedigree structures of the two families are shown in Fig. 3. In addition, in family 1, the proband's father (I-1) and brother (II-3) had ventricular septal defect, aortic stenosis and the electrocardiogram documented atrial fibrillation. In family 2, the proband's father (I-1) also had aortic stenosis. The phenotypic characteristics and results of genetic screening of the affected pedigree members are listed in Table II.

Table II. Phenotypic characteristics and status of the GATA5 mutations of the affected pedigree members.

Subject information			Phenotypes	Genotypes
Identity	Gender	Age (years)	Congenital cardiac structural defects	GATA5 mutations
Family 1				Y16D
I-1	M	61[a]	BAV, VSD, AS	NA
II-3	M	48	BAV, VSD, AS	+/−
II-6	F	45	BAV	+/−
III-3	M	19	BAV	+/−
Family 2				T252P
I-1	M	55[a]	BAV, AS	NA
II-1	M	42	BAV	+/−
III-1	F	16	BAV	+/−

[a]Age at death. M, male; F, female; BAV, bicuspid aortic valve; VSD, ventricular septal defect; NA, not available or not applicable; AS, aortic stenosis; +, presence of mutation; −, absence of mutation.
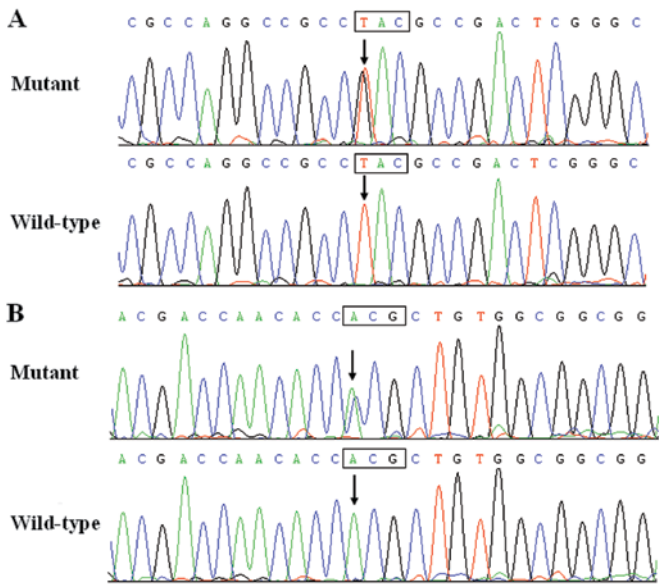

Figure 1. Sequence electropherograms showing the GATA5 mutations in contrast to the corresponding controls. The arrow indicates the heterozygous nucleotides of A/G in the proband from family 1 or A/C in the proband from family 2 (mutant); or the homozygous nucleotides of A/A in the corresponding control individuals (wild-type). The rectangle represents the nucleotides constituting a codon of GATA5.
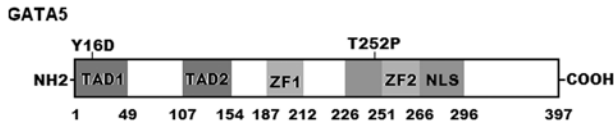

Figure 2. Schematic diagram of GATA5 protein structure with the mutations responsible for congenital bicuspid aortic valve. The mutations associated with congenital bicuspid aortic valve are shown above the structural domains. NH2, amino-terminus; TAD, transcriptional activation domain; ZF, zinc finger; NLS, nuclear localization signal; COOH, carboxyl-terminus.
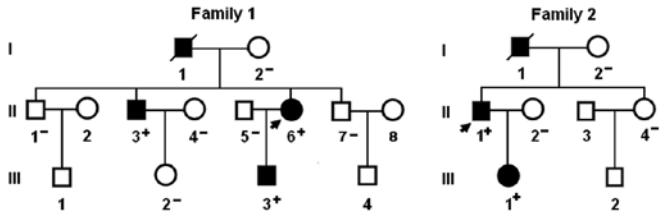

Figure 3. Pedigree structures of the families with congenital bicuspid aortic valve. Families are designated as families 1 and 2, respectively. Family members are identified by generations and numbers. Squares indicate male family members; circles, female members; closed symbols, affected members; open symbols, unaffected members; symbols with a slash, the deceased members; arrows, probands; '+', carriers of the heterozygous mutations; and '-', non-carriers.

Alignment of multiple GATA5 protein sequence. As shown in Fig. 4, a cross-species alignment of multiple GATA5 protein sequences demonstrated that the affected amino acids of p.Y16 and p.T252 were completely conserved evolutionarily, suggesting that the amino acids are functionally important.

Causative potential of GATA5 sequence variations. The *GATA5* sequence variations of c.46T>G and c.754A>C were automatically predicted to be disease-causing, with P-values of 0.82270 and 1.00000, respectively. No SNPs in the altered regions were identified in the MutationTaster database. The two variations were predicted to be probably damaging by the

software PolyPhen-2, with the same score of 1.000 (sensitivity: 0.00; specificity: 1.00)

Reduced transcriptional activity of the GATA5 mutants. As shown in Fig. 5, the wild-type GATA5, the Y16D-mutant, and the T252P-mutant GATA5 activated the ANF promoter by ~12-, ~6- and ~3-fold, respectively. When wild-type GATA5 was coexpressed with the same amount of Y16D- or T252P-mutant GATA5, the induced activation of the ANF promoter was ~9- or ~5-fold. These results suggest that the two GATA5 mutants are associated with significantly reduced transactivational activity compared with their wild-type counterpart.

Figure 4. Multiple alignments of GATA5 protein sequences among species. The altered amino acids of p.Q113 and p.T252 are highly conserved evolutionarily across various species.
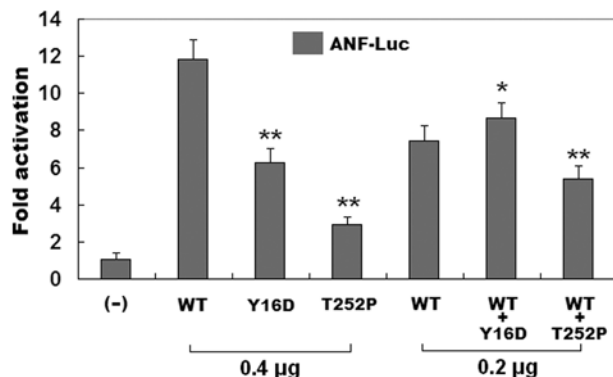

Figure 5. Functional defects of GATA5 resulted from mutations. Activation of the ANF-luciferase reporter in cultured HEK-293 cells by wild-type GATA5 (WT) or mutant (Q13R or T252P), alone or in combination, showed significantly reduced transcriptional activity by mutant proteins. Experiments were performed in triplicate and means ± standard deviations is shown. **P<0.005 and *P<0.05, when compared with wild-type GATA5.

Discussion

In the current study, the novel heterozygous mutations in GATA5, p.Y16D and p.T252P, were identified in two unrelated families with BAV. In each family, the mutant allele was present in all the affected family members, but absent in the unaffected relatives examined and 400 referral chromosomes from an ethnically-matched control population. A cross-species alignment of GATA5 amino acid sequences demonstrated that the altered amino acids were completely conserved evolutionarily. The p.Y16D and p.T252P variations were predicted to be pathogenic mutations, and the functional analysis revealed that the GATA5 mutant proteins were consistently associated with significantly reduced transcriptional activity. Therefore, it is likely that functionally impaired GATA5 contributes to BAV in these families. To the best of our knowledge, this is the first study to associate GATA5 loss-of-function mutations with enhanced susceptibility to BAV in humans.

At present, six members (GATA1-6) of the GATA transcription factor family have been identified in vertebrate. GATA1-3 are important regulators of hematopoietic stem cells and some ectodermal derivatives, whereas GATA4-6 are associated with cardiogenesis and the formation of a subset of endoderm-derived tissues (32,33). The human *GATA5* gene was mapped on chromosome 20q13.33 by fluorescence *in situ* hybridization, which encodes for a protein of 397 amino acids (40). By alignment of GATA5 with GATA4, the structural domains of GATA5 protein are predicted to encompass two transcriptional activation domains (TAD1, amino acids 1-49 and TAD2, amino acids 107-154), two adjacent zinc fingers (ZF1, amino acids 187-212 and ZF2, amino acids 242-266), which comprise the DNA-binding domain with a Cys-X_2-Cys-X_{17}-Cys-X_2-Cys consensus (where X represents any amino

acid), and one nuclear localization signal (NLS, amino acids 226-396). The two TADs are required for the normal transcriptional activity of GATA5. The C-terminal ZF2 is essential for DNA sequence recognition and binding to the consensus motif (T/A)GATA(A/G), within the promoters of target genes; while the N-terminal ZF1 is responsible for sequence specificity and stability of protein-DNA binding, and both ZFs can also interact directly with other regulatory proteins. The NLS is crucial to the sub-cellular trafficking and nuclear distribution of GATA5 (41). The GATA5 mutations of p.Y16D and p.T252P identified in this study are located in TAD1 and ZF2, respectively, and may be expected to exert an effect on the transcriptional activity of GATA5 by direct inhibition or interfering with the nuclear localization and specific binding ability of GATA5.

In a previous study, it was substantiated that GATA5 regulates multiple downstream molecules expressed during embryogenesis and cardiac morphogenesis, including ANF, brain natriuretic peptide, α- and β-myosin heavy chains, and cardiac troponin C and I (32). Thus, the functional characteristics of the *GATA5* mutations may be delineated by analyzing the transcriptional activity of the *ANF* promoter in tool cells. In this study, the functional effect of the novel p.Y16D and p.T252P mutations of GATA5 identified in our familial BAV patients were explored by a transcriptional activity assay and the results revealed a significantly reduced transcriptional activation of the *ANF* promoter. These data suggest that genetically compromised GATA5 is potentially an alternative molecular mechanism of BAV.

The relationship between *GATA5* variants and human BAV was previously investigated. Padang *et al* screened the coding regions and splice signal sequences of the *GATA5* gene in 100 unrelated BAV patients, and found four rare non-synonymous variations within the GATA5 transcriptional activation domains, i.e., p.Q3R, p.S19W, p.Y142H and p.G166S, in 4 of 100 unrelated patients, respectively, with a mutational prevalence of 4% (37). However, the functional roles of these GATA5 variations remain to be determined. Foffa and colleagues screened by direct sequencing all the coding exons including adjacent intronic as well as 5'- and 3'-untranslated of *GATA5* in a cohort of 11 index patients with familial BAV, however, no pathogenetic mutation was identified in GATA (21). The discrepancy in the mutational prevalence of these reports including the present study may be partially explained by different sample size and ethnicity.

It has been validated in animals that genetically defective GATA5 predisposes to congenital cardiovascular defects. In zebrafish, the targeted disruption of *GATA5* led to embryonic lethality due to defects in endocardial and myocardial differentiation and migration, a phenotype similar to cardia bifida of *GATA4*-null zebrafish (43). In mice, *GATA5* knockout led to partially penetrant BAV, with a penetrance of 25%, and endocardial cell-specific inactivation of *GATA5* resulted in BAV, similar to that observed in *GATA5*-null mice (31). Furthermore, the mice that were compound heterozygous for *GATA5* and *GATA4* or for *GATA5* and *GATA6* knockout died embryonically or perinatally due to severe defects of the outflow tract development including double outlet right ventricle and ventricular septal defect (30). These experimental results emphasize the notable sensitivity of the developing cardiovascular system to the levels of GATA4, GATA5 and GATA6,

and indicate that these GATA factors may act cooperatively to regulate some target genes.

Of note, ventricular septal defect and atrial fibrillation were documented in two BAV patients harboring the p.Y16D mutation of GATA5. Similar to our findings, GATA5 has been previously reported to be involved in various congenital heart diseases as well as atrial fibrillation (38,39,42). Additionally, the GATA family members GATA4 and GATA6 have an expression profile and functional characteristics that overlap with those of GATA5, and a long list of mutations in GATA4 and GATA6 have also been connected with a large variety of congenital cardiovascular deformations and atrial fibrillation (44-77). These findings support that the transcription factors of the GATA family are pivotal for the cardiovascular morphogenesis.

In conclusion, to the best of our knowledge, this study provides the first genetic evidence for the association of functionally compromised GATA5 with increased vulnerability to BAV, highlighting the role of a GATA signaling pathway in BAV and other developmental cardiovascular malformations, and suggesting the potential implications for genetic counseling and clinical care of the families presenting with BAV.

Acknowledgements

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