

# Somatic *GATA5* mutations in sporadic tetralogy of Fallot

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**Abstract.** Tetralogy of Fallot (TOF) is the most common form of cyanotic congenital heart disease, with high morbidity and mortality rates. Accumulating evidence has demonstrated that genetic defects play an important role in the pathogenesis of TOF. However, the molecular basis of TOF in the majority of patients remains to be determined. In the present study, sequence analysis of the coding exons and exon-intron boundaries of *GATA5*, a gene encoding a zinc finger-containing transcriptional factor crucial for cardiogenesis, was performed on genomic DNA isolated from resected cardiac tissue and matched blood samples of 85 unrelated patients who underwent surgical repair of TOF. Genotyping was performed on the cardiac tissue and matched blood samples from 63 unrelated patients who underwent cardiac valve replacement due to rheumatic heart disease as well as the blood samples obtained from 200 unrelated healthy individuals. The functional effect of the mutations was evaluated by using a luciferase reporter assay system. As a result, the novel heterozygous *GATA5* mutations, p.D203E and p.Y208X, were found in the cardiac tissues of two TOF patients, respectively. There were no mutations in the cardiac tissues obtained from 63 patients with rheumatic heart disease nor in the blood samples obtained from the 348 subjects. Functional analysis revealed that the *GATA5* mutants were consistently associated with significantly decreased transcriptional activity compared with their wild-type counterpart. Thus, results of this study showed an association of somatic *GATA5* mutations with TOF, providing further insight into the underlying molecular mechanism of TOF.

## Introduction

Congenital heart disease (CHD) is the most common type of birth defect in newborns, affecting ~1% of all live births, and is the most frequent non-infectious cause of infant death, responsible for at least 10% of early miscarriages (1). Based on specific anatomic or hemodynamic lesions, CHD is clinically classified into >21 distinct categories, of which tetralogy of Fallot (TOF), a tetrad of ventricular septal defect, right ventricular outflow tract obstruction, overriding aorta, and right ventricular hypertrophy, is the most common form of cyanotic CHD. TOF occurs in ~3/10,000 neonates alive, accounting for ~7-10% of all congenital cardiac malformations. Surgical treatment is crucial, otherwise 25% of TOF patients with severe obstruction would succumb to CHD in the first year of life, 40% by the age of 3 years, 70% by 10 years and 95% by 40 years (1-3). Various cardiovascular developmental abnormalities, such as ventricular septal defect, atrial septal defect, tetralogy of Fallot, patent ductus arteriosus, atrioventricular septal defect, double outlet right ventricle, pulmonary stenosis, and transposition of great arteries, may occur individually or in combination. These anomalies may result in degraded quality of life, delayed fetal brain development, cardiac enlargement or hypertrophy, pulmonary hypertension, infective endocarditis, thromboembolism, Eisenmenger's syndrome, congestive heart failure, arrhythmias, as well as sudden cardiac death in the absence of surgical or catheter-based repairs (4-10). Despite the high prevalence and significant clinical importance, the molecular mechanism of CHD remains poorly understood (11).

In vertebrate embryo, the heart is the first functional organ to form and embryonic heart development involves five sequential stages, including heart tube formation, cardiac looping, septal formation, outflow tract septation and heart valve formation. Thus it is obvious that cardiogenesis is a complex and dynamic process, which requires the harmonious orchestration of cardiac cell commitment, differentiation, proliferation and migration, and both environmental and genetic risk factors may disrupt the finely regulated biological process, resulting in CHD (12-14). Mounting evidence has demonstrated that an evolutionarily conserved network of transcription factors connecting signaling pathways with genes related to muscle growth, patterning, and contractility, including the most extensively investigated *GATA* and *NK* families, are crucial in cardiovascular morphogenesis (15-17), and germline mutations in *NKX2-5* (18-28), *GATA4* (29-41), *GATA5* (42-46), and *GATA6* (47-55) have been

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associated with CHD. Nevertheless, the genetic basis underlying CHD remains to be clarified. Patients may have mutations in other genes: mutations in *TBX5* and *FOXC1* have been found to be involved in CHD, although these CHD-associated mutations seem to be rare and in most cases are associated with extra-cardiac anomalies, a syndromic phenotype (56,57). Another possible explanation for the incomplete identification of mutation-positive CHD may be somatic mosaicism.

In genetics, somatic mosaicism refers to the condition in which multiple cell clones with distinct genotypes exist in the same individual that has developed from a single fertilized zygote. Somatic mutation leading to mosaicism is prevalent in cancer and is responsible for most leukemia, lymphomas and solid tumors (58). Somatic mutations have been identified in *GATA4* and *GATA6* as well as their transcriptionally cooperative partners, *NXK2-5* and *TBX5*, in the cardiac tissue derived from a collection of hearts with CHD (59-68). The expression and function of *GATA5*, a member of the *GATA* family, overlap at least partially with those of *GATA4*, *GATA6*, *NXK2-5* and *TBX5* during embryogenesis (12-17). Thus, it may be hypothesized that somatic *GATA5* mutations potentially underlie TOF in a subset of patients.

In the present study, in order to determine the prevalence and spectrum of somatic *GATA5* mutations in patients with sporadic TOF and explore the mechanism by which mutated *GATA5* confers susceptibility to TOF, the coding regions and splice junction sites of *GATA5* were analyzed in patients as compared to control individuals, and the functional effect of the mutant *GATA5* was characterized as compared to its wild-type counterpart using a luciferase reporter assay system.

## Materials and methods

**Study participants.** The study included a cohort of 85 unrelated patients with sporadic TOF, who underwent heart surgery at the Shanghai Renji Hospital in China during the period January 2009 to June 2013. The age range was 5 months to 8 years, with an average of 1.46 years at the time of surgical repair. Skillful cardiologists assessed the patients and a diagnosis of TOF was made using echocardiography and confirmed by direct view during surgery. The patients with known chromosomal abnormalities or syndromic cardiac deformities, such as Holt-Oram, Noonan, Alagille, DiGeorge, Marfan and Char syndrome, were excluded from the present study. According to the report by Draus *et al* (66), the sample size was sufficient to conclude that the absence of somatic mutations was significant.

Controls included 63 unrelated patients (35 males and 28 females) with rheumatic heart disease undergoing cardiac valve replacement, and 200 unrelated healthy individuals (110 males and 90 females) randomly selected from those undergoing routine physical examinations. Based on the individual medical history and echocardiographic record, the control individuals had no overt congenital cardiovascular deformations, apart from subclinical cardiac aberrations such as bicuspid aortic valve or patent foramen ovale.

All the participants were Chinese Han. The ethnic origin of a subject was ascertained by a combination of self-reported ethnicity and a personal questionnaire with regards to the birthplace, language, religion and ancestry. The study protocol conformed to the ethical guidelines of the 2008 Declaration

of Helsinki and was approved by the local Institutional Ethics Committee. Written informed consent from each participant's guardian was obtained prior to investigation.

**Sample preparation.** The heart tissues from the right ventricular outflow tracts of TOF patients were resected during the routine cardiac surgery procedures. Following resection, the discarded cardiac tissue samples were collected after cleaning the blood stain by sterile normal saline. The samples were then stored at -80°C. Discarded peripheral venous blood samples with sodium citrate were collected from the patients (the blood samples were mostly used for activated partial thromboplastin time and prothrombin time tests prior to surgery). The discarded cardiac samples from the cardiac valves and matched blood samples of the patients undergoing cardiac valve replacement because of rheumatic valvular disease, and the peripheral venous blood samples of healthy individuals were prepared as controls.

**DNA isolation.** Somatic DNA was isolated from freshly frozen tissues using QIAamp DNA FFPE Tissue kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's protocol. Genomic DNA was extracted from peripheral blood lymphocytes using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA).

**Genetic analysis.** The primer pairs used to amplify the coding exons and exon-intron boundaries of the *GATA5* gene were described in a previous study (46). Polymerase chain reaction (PCR) was carried out in Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The reaction mixture for PCR included 2 µl of genomic DNA (50-100 ng/µl), 2.5 µl of 10X Taq buffer, 5 µl of 5X Q Solution, 2 µl of dNTP mixture (2.5 mM each), 0.5 µl of each primer (20 mM each), 0.25 µl (1.25 units) of HotStar TaqDNA polymerase (Qiagen), and 12.25 µl of deionized H<sub>2</sub>O, with a final volume of 25 µl. PCR was performed under the following conditions: Pre-denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 30 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified products were analyzed on 1% agarose gels stained with ethidium bromide and purified using QIAquick Gel Extraction kit (Qiagen). The two strands of each PCR product were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing kit under an ABI PRISM 3130XL DNA analyzer (both from Applied Biosystems). The sequencing primers were the same as previously designed for specific region amplification. The DNA sequences were analyzed using the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The sequence variation was validated by re-sequencing an independent PCR-generated amplicon from the same subject. For each *GATA5* sequence variation identified in this study, the single-nucleotide polymorphism (SNP) database of the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/SNP>), the 1000 Genome Project (1000GP) database (<http://www.1000genomes.org/>), and the Human Gene Mutation (HGM) database (<http://www.hgmd.cf.ac.uk/>) were searched to confirm its novelty.

**Multiple alignments of *GATA5* amino acid sequences among species.** Alignment of a number of *GATA5* amino acid

sequences across various species was carried out using the online program of Muscle, version 3.6 (<http://www.ncbi.nlm.nih.gov/>).

**Prediction of the disease-causing potential of a *GATA5* sequence variation.** The causative potential of a *GATA5* sequence variation was predicted by the MutationTaster (an online program at <http://www.mutationtaster.org/>), which automatically yielded a probability for the variation to be a disease-causing mutation or a benign polymorphism. In addition, another online program PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2>) was also used to evaluate the pathogenicity of an amino acid substitution in *GATA5*.

**Expression plasmids and site-directed mutagenesis.** The recombinant expression plasmid pcDNA3.1-h*GATA5* was constructed as described previously (46). The atrial natriuretic factor (ANF)-luciferase reporter gene, containing 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 mutation identified was introduced into the wild-type *GATA5* by using a QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) with a complementary pair of primers. The mutant was sequenced to confirm the required mutation and to exclude any other sequence variations.

**Reporter gene assay.** HEK-293 cells were seeded in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The ANF(-2600)-Luc reporter vector and an internal control reporter plasmid pGL4.75 (hRluc/CMV; Promega) were employed in transient transfection assays to examine the transcriptional activation function of the *GATA5* mutants. The HEK-293 cells were transfected with 0.4 µg of wild-type or mutant pcDNA3.1-h*GATA5* expression vector (D203E or Y208X), 0.4 µg of ANF(-2600)-Luc reporter construct, and 0.04 µg of pGL4.75 control reporter vector using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). For co-transfection experiments, 0.2 µg of wild-type pcDNA3.1-h*GATA5*, 0.2 µg of empty pcDNA3.1 plasmid or 0.2 µg of mutant pcDNA3.1-h*GATA5* (D203E or Y208X), 0.4 µg of ANF(-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. The activity of the *ANF* promoter was presented as fold activation of Firefly luciferase relative to *Renilla* luciferase. Three independent experiments were performed at minimum for wild-type and mutant *GATA5*.

**Statistical analysis.** Data were presented as means ± standard deviation. Continuous variables were tested for normality of distribution. The Student's unpaired t-test was utilized to compare numeric variables between 2 groups. A two-tailed P-value <0.05 indicated a significant difference.

## Results

**Clinical characteristics of the study subjects.** This study included a cohort of 85 unrelated patients with sporadic

TOF who underwent cardiac surgery, 63 unrelated patients undergoing cardiac valve replacement due to rheumatic heart disease and 200 healthy individuals used as controls. All the subjects were Chinese Han and did not have a positive family history of CHD or established environmental risk factors for CHD, such as maternal illness and drug use during the first trimester of pregnancy, parental smoking, or chronic exposure to toxicants and ionizing radiation. The baseline clinical characteristics of the 348 participants are provided in Table I.

**Sample sources.** Peripheral venous blood samples were obtained from the 348 participants. Malformed myocardial tissue samples were collected from 85 unrelated patients with sporadic TOF who underwent surgical resection of the right ventricular outflow musculature to relieve stenosis. The cardiac muscle fragment collected was ~5x5 mm in size. The cardiac valvular tissue samples used as controls were obtained from surgical discards of the 63 unrelated patients undergoing cardiac valve replacement.

***GATA5* mutations.** Genomic DNA from the malformed cardiac tissues of 85 patients with sporadic TOF, the cardiac valvular tissues of 63 cases with rheumatic heart disease, and the peripheral blood lymphocytes of all the 348 participants, were genetically scanned for *GATA5* mutations. Two heterozygous *GATA5* mutations were identified in the fresh pathological myocardial tissues of two unrelated TOF patients, respectively, with a mutational prevalence of ~2.35%. Specifically, a substitution of guanine for cytosine in the third nucleotide of codon 203 of the *GATA5* gene (c.609C>G), predicting the transition of aspartic to glutamic acid at amino acid position 203 (p.D203E), was identified in the cardiac tissue of a 2-year-old male TOF patient. A change of cytosine into adenine at coding nucleotide 624 (c.624C>A), resulting in a truncated protein with only N-terminal 207 amino acids left (p.Y208X), was identified in the cardiac tissue of a 1-year-old male TOF patient. The sequence chromatograms showing the detected heterozygous *GATA5* mutations as compared to their corresponding control sequences are shown in Fig. 1. A schematic diagram of *GATA5* showing the structural domains and locations of the identified mutations is provided in Fig. 2. The two mutations were not observed in the cardiac valvular tissues of 63 patients with rheumatic heart disease or identified in the peripheral blood samples of the 348 participants. Neither mutation was reported in the SNP, 1000 GP and HGM databases, which was consulted again on October 26, 2013. Furthermore, no atrial fibrillation was observed in the two mutation carriers during a 24-h period of ambulatory electrocardiographic monitoring.

**Alignment of multiple *GATA5* protein sequences across species.** Alignment of multiple *GATA5* protein sequences across species revealed that the affected amino acids p.D203 and p.Y208 were completely conserved evolutionarily (Fig. 3). Furthermore, the p.Y208X mutation deleted a number of functionally important structural domains, including the nuclear localization signal, C-terminal zinc finger and part of the N-terminal zinc finger (Fig. 2).

Table I. Baseline clinical characteristics of the 85 unrelated patients with sporadic tetralogy of Fallot (TOF).

Variable	Statistic
Male gender, n (%)	47 (55.29)
Age at the initial diagnosis of TOF (year)	1.02±0.85
Age at the time of surgery (year)	1.46±1.19
Positive family history of TOF, n (%)	0 (0)
Distribution of various types of TOF, n (%)	
Isolated TOF	42 (49.41)
TOF and bicuspid pulmonary valve	14 (16.47)
TOF and pulmonary stenosis	10 (11.76)
TOF and right-sided aortic arch	8 (9.41)
TOF and atrial septal defect	5 (5.88)
TOF and atrioventricular septal defect	1 (1.18)
TOF and anomalous pulmonary venous return	1 (1.18)
TOF and at least two other anatomical defects	4 (4.71)
Incidence of arrhythmias, n (%)	
Atrioventricular block	5 (5.88)
Atrial fibrillation	4 (4.71)
Treatment, n (%)	
Surgical repair	85 (100)

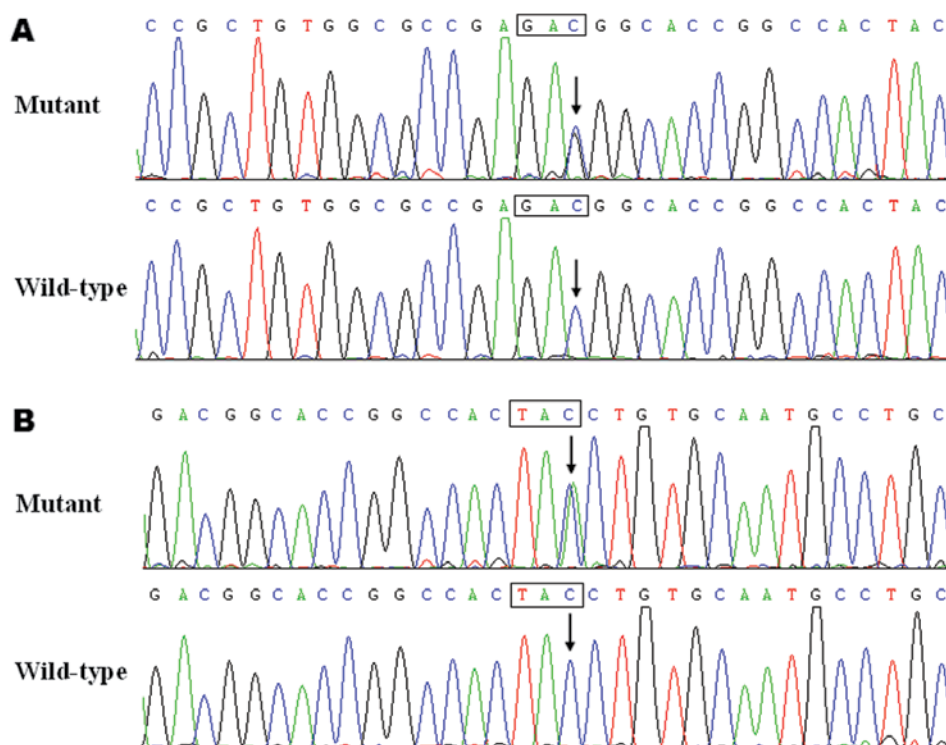


Figure 1. Sequence electropherograms showing the *GATA5* mutations as well as their corresponding controls. The arrows indicate the heterozygous nucleotides of (A) C/G and (B) C/A in the 2 unrelated patients, respectively (mutant) or the homozygous nucleotides of C/C in the corresponding control individuals (wild-type). The rectangle denotes the nucleotides comprising a codon of *GATA5*.

**Pathogenic potential of *GATA5* variations.** The nucleotide sequence variations of c.609C>G and c.624C>A in *GATA5* were predicted to be causative by the MutationTaster, with P-values of 0.9995 and 1.0000, respectively. No SNPs were

detected in the altered regions in the MutationTaster database. The amino acid substitution of p.D203E in *GATA5* was also predicted to be damaging by PolyPhen-2, with a score of 1.000 (sensitivity 0.00; specificity 1.00).

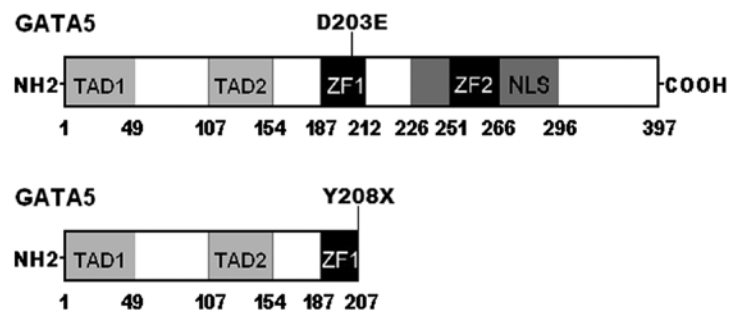


Figure 2. Schematic representation of GATA5 protein structure with the tetralogy of Fallot related mutations indicated. The mutations identified in patients with tetralogy of Fallot are shown above the structural domains. The p.Y208X mutation produces a truncated protein that lacks multiple important structural domains including a small part of ZF1, ZF2, nuclear localization signal, and C-terminus. NH2, amino-terminus; TAD, transcriptional activation domain; ZF, zinc finger; NLS, nuclear localization signal; and COOH, carboxyl-terminus.

	188	D203E	Y208X	223
NP_536721.1 (Human)	--- ECVNCGALSTPLWRR	D GTGH	Y LCNACGLYHKMNGVN	---
XP_514767.3 (Chimpanzee)	--- ECVNCGALSTPLWRR	D GTGH	Y LCNACGLYHKMNGVN	---
XP_001115055.2 (Monkey)	--- ECVNCGALSTPLWRR	D GTGH	Y LCNACGLYHKMNGVN	---
NP_001029393.1 (Cow)	--- ECVNCGALSTPLWRR	D GTGH	Y LCNACGLYHKMNGVN	---
XP_543086.2 (Dog)	--- ECVNCGALSTPLWRR	D GTGH	Y LCNACGLYHKMNGVN	---
NP_032119.2 (Mouse)	--- ECVNCGALSTPLWRR	D GTGH	Y LCNACGLYHKMNGVN	---
NP_001019487.1 (Rat)	--- ECVNCGALSTPLWRR	D GTGH	Y LCNACGLYHKMNGVN	---
NP_990752.1 (Fowl)	--- ECVNCGAMSTPLWRK	D GTGH	Y LCNACGLYHKMNGIN	---
NP_571310.2 (Zebrafish)	--- ECVNCGSISTPLWRR	D GTGH	Y LCNACGLYHKMNGIN	---

Figure 3. Alignment of multiple GATA5 protein sequences across species. The altered amino acids p.D203 and p.Y208 are completely conserved evolutionarily among a great variety of species.

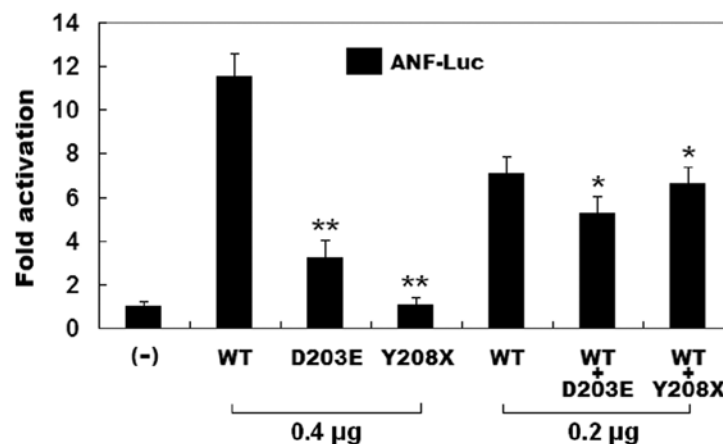


Figure 4. Functional defects resulted from GATA5 mutations. Activation of ANF-luciferase reporter in HEK-293 cells by wild-type (WT) or mutant (D203E or Y208X) GATA5, alone or in combination, showed significantly decreased transcriptional activity by mutant proteins. Experiments were performed in triplicate and mean and standard deviations are shown. \*\*P<0.001 and \*P<0.005, compared with WT GATA5.

**Reduced transcriptional activity of the GATA5 mutants.** The wild-type GATA5, the D203E-mutant, and the Y208X-mutant GATA5 activated the *ANF* promoter by ~12-, ~3- and ~1-fold, respectively. When wild-type GATA5 was co-expressed with the same amount of D203E- or Y208X-mutant GATA5, the

induced activation of the *ANF* promoter was ~5- or ~7-fold, respectively. Findings of the present study indicate that the two GATA5 mutants are associated with markedly reduced activation activity compared to their wild-type counterpart (Fig. 4).

## Discussion

In the present study, two novel heterozygous GATA5 mutations, p.D203E and p.Y208X, were identified in the deformed heart tissues from 2 of 85 unrelated patients with TOF. The mutant alleles were absent in the pathogenic cardiac tissues of 63 unrelated patients with rheumatic heart disease and in the peripheral blood samples of all 348 participants, including 200 ethnically matched healthy individuals. A cross-species alignment of multiple GATA5 protein sequences showed that the altered amino acids were completely conserved evolutionarily. The two GATA5 sequence variations were predicted to be pathogenic, and the functional analysis revealed that the mutant GATA5 proteins were associated with significantly decreased or no transcriptional activity. Therefore, it is very likely that somatic GATA5 loss-of-function mutations predispose these two mutation carriers to TOF.

There are two major categories of cells in humans: somatic and germ cells. Somatic cells, also known as body cells, make up the body of an organism, forming all the internal organs, skin, bones, blood, and connective tissue. Numerous endogenous factors such as mobile elements, DNA polymerase slippage, DNA double-strand break, inefficient DNA repair, unbalanced chromosomal segregation and certain exogenous factors such as nicotine and UV exposure can contribute to the generation of somatic mutations, thereby leading to somatic mosaicism (69). Mutations that occur in the genetic material of somatic cells are not transmitted to the next generation, but occur in all cells derived from the mutated cell (70). Therefore, disease-associated somatic mutations can be identified by screening the genetic substance from diseased tissue cells, but cannot be detected by the genetic analysis of DNA from peripheral blood lymphocytes alone, and mosaicism may reduce the likelihood of detection in the affected tissue. Thus, mutations in cardiac tissues may be absent or sporadic in blood lymphocytes of the same person (64). In this study, two novel GATA5 mutations were identified in the anomalous heart tissues, although neither mutation was found in the peripheral blood lymphocytes, suggesting that somatic mutation may be an alternative mechanism of CHD.

GATA transcription factors belong to a group of DNA binding proteins characterized by preferential binding to the consensus DNA sequence (T/A)GATA(A/G) of target gene promoters. At present, six members of the GATA family have been identified in vertebrates, of which GATA1, GATA2 and GATA3 are mainly involved in haematopoiesis and some ectodermal derivatives, whereas GATA4, GATA5 and GATA6 are predominantly associated with cardiogenesis and the formation of certain endoderm-derived tissues in embryonic heart (16). Human GATA5 maps to chromosome 20q13.33 by fluorescence *in situ* hybridization, which codes for a zinc finger-containing protein with 397 amino acids (71). GATA5 is predicted to comprise two transcriptional activation domains (TADs), two adjacent zinc fingers (ZFs) and one nuclear localization signal (NLS). The two TADs are crucial for the transcriptional activity of GATA5. The C-terminal ZF2 is essential for DNA sequence recognition and binding to the consensus motif, while the N-terminal ZF1 is associated with the sequence specificity and stability of protein-DNA binding.

The NLS is responsible for the sub-cellular trafficking and distribution of GATA5 (46). The GATA5 mutation p.Y208X eliminates the functionally important domains of NLS and ZF2 as well as part of ZF1, and may therefore be expected to nullify GATA5. Another GATA5 mutation p.D203E is located at ZF1, which is likely to reduce the transcriptional activity of GATA5 by interfering with the specific binding and nuclear localization of GATA5.

GATA5 is considered an upstream transcriptional regulator of multiple genes transcribed during embryogenesis, including the genes that encode atrial natriuretic peptide, brain natriuretic peptide,  $\alpha$ -myosin heavy chain,  $\beta$ -myosin heavy chain, and cardiac troponin C and I (16). Therefore, the functional roles of the GATA5 mutations may be deciphered by examining the transcriptional activity of the *ANF* promoter in cultured cells. In this study, the functional effect of the novel GATA5 mutations identified in TOF patients was characterized by the transcriptional activation assay, and the results demonstrated a significantly decreased transcriptional activity on a downstream gene. These data indicate that compromised GATA5 increases the vulnerability to TOF.

It has been reported previously that germline mutations in the GATA5 gene play a role in CHD, including TOF. Jiang *et al* (42) screened GATA5 in 320 index patients with CHD, and identified four novel heterozygous mutations, including p.R132G, p.V190A, p.A266P and p.H274R, in 4 of 320 unrelated patients with CHD, respectively, with a mutational prevalence of 1.25%. Among these CHD probands harboring GATA5 mutations, one V190A-mutation carrier and one A266P-mutation carrier presented with TOF. However, the functional characteristics of these CHD-related GATA5 mutations remain to be delineated. Wei *et al* (43) genotyped GATA5 in 130 unrelated patients with TOF, and detected the novel heterozygous GATA5 mutations, p.R187G and p.H207R, in two families with autosomal dominantly inherited TOF, respectively, with a mutational prevalence of ~1.54% based on the proband population. Functional analysis revealed that the GATA5 mutants were consistently associated with significantly decreased transcriptional activation. Additionally, Wei *et al* (44) performed sequence analysis of GATA5 in 120 unrelated patients with congenital ventricular septal defect and identified a novel heterozygous mutation of p.L199V in one patient with a positive family history, with a mutational prevalence of ~0.83%. Biochemical assays demonstrated that the mutant GATA5 had significantly decreased transcriptional activity. Moreover, some carriers with GATA5 mutation of p.W200G, p.K218T or p.A266P had congenital cardiac septal defect in addition to atrial fibrillation (45,46). However, in the present study, no germline GATA5 mutation was detected with the exception of two somatic GATA5 mutations, which underscores a genetic mosaic basis for the pathogenesis of TOF in a subset of patients.

The association of genetically impaired GATA5 with enhanced susceptibility to CHD has been demonstrated in animal experiments. In *Xenopus*, GATA5 is required for the early development of the embryonic heart and acts earlier than GATA4 and GATA6 to regulate cardiac morphogenesis. Downregulation of GATA5 in *Xenopus laevis* and *Xenopus tropicalis* by using two non-overlapping translation-blocking

morpholino oligonucleotides and by using a splice-site blocking morpholino oligonucleotide leads to a substantial reduction in the number of heart precursors at the time of or shortly after their specification (72). In zebrafish, targeted disruption of the *GATA5* gene resulted in embryonic lethality due to defects in endocardial and myocardial differentiation and migration, a similar phenotype to cardia bifida of *GATA4*-null zebrafish (73). Although the *GATA5*-null mice were viable and had no apparent cardiac defects, the mice that were compound heterozygous for *GATA5* and *GATA4* or for *GATA5* and *GATA6* knockout died embryonically or perinatally because of severe defects of the outflow tract development, including double outlet right ventricle and ventricular septal defect (74). These experimental findings highlight the exquisite sensitivity of the developing cardiovascular system to the levels of *GATA4*, *GATA5* and *GATA6*, and indicate that these GATA factors act synergistically to regulate target genes.

In conclusion, the present study has demonstrated an association between somatic *GATA5* loss-of-function mutations and TOF, providing insight into the molecular mechanism of CHD, and suggesting the potential implications for the antenatal diagnosis and potentially the personalized strategy of CHD.

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