

Somatic *GATA4* mutation contributes to tetralogy of Fallot

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Abstract. Tetralogy of Fallot (TOF) is the most prevalent cyanotic congenital heart pathology and causes infant morbidity and mortality worldwide. GATA-binding protein 4 (*GATA4*) serves as a pivotal transcriptional factor for embryonic cardiogenesis and germline *GATA4* mutations are causally linked to TOF. However, the effects of somatic *GATA4* mutations on the pathogenesis of TOF remain to be ascertained. In the present study, sequencing assay of *GATA4* was performed utilizing genomic DNA derived from resected heart tissue specimens as well as matched peripheral blood specimens of 62 patients with non-familial TOF who underwent surgical treatment for TOF. Sequencing of *GATA4* was also performed using the heart tissue specimens as well as matched peripheral venous blood samples of 68 sporadic cases who underwent heart valve displacement because of rheumatic heart disorder and the peripheral venous whole blood samples of 216 healthy subjects. The function of the mutant was explored by dual-luciferase activity analysis. Consequently, a new *GATA4* mutation, NM_002052.5:c.708T>G;p.(Tyr236*), was found in the heart tissue of one patient with TOF. No mutation was detected in the heart tissue of the 68 cases suffering from rheumatic heart disorder or in the venous blood samples of all 346 individuals. *GATA4* mutant failed to transactivate its target gene, myosin heavy chain 6. Additionally, this mutation

nullified the synergistic transactivation between *GATA4* and T-box transcription factor 5 or NK2 homeobox 5, two genes causative for TOF. Somatic *GATA4* mutation predisposes TOF, highlighting the significant contribution of somatic variations to the molecular pathogenesis underpinning TOF.

Introduction

Congenital heart disease is hypothesized to be the most prevalent type of birth anomaly in humans, occurring in ~1/100 live newborns and 10/100 early miscarriages worldwide (1,2). If minor cardiovascular developmental abnormalities are included, such as aortic bicuspid valve, which represents the most frequent congenital heart deformity with a prevalence of ~1% in the general pediatric population (3), the total prevalence of congenital heart defects is up to 5% in live newborns (4). As a global pediatric concern, congenital heart defects comprise a wide spectrum of cardiovascular developmental defects, which are categorized into >25 distinct clinical subtypes, including tetralogy of Fallot (TOF) (1). Although certain minor congenital heart defects spontaneously resolve, severe congenital heart disease may lead to poor health and quality of life (5-8), diminished physical exercise capacity (9-13), impaired neurodevelopment (the most prevalent extracardiac manifestation in patients with a congenital heart defect) and brain damage (14-18), thromboembolic complications (19-21), acute renal injury and chronic kidney disease (22-24), hepatic dysfunction (25), pulmonary arterial hypertension (26-28), infective endocarditis (29-31), congestive cardiac failure (32-34), miscellaneous cardiac dysrhythmia (35-37) and cardiovascular demise (38-40). Improvement has been made in cardiovascular surgery and transcatheter interventional treatment, which has allowed >90% of children with congenital heart defects to survive to adulthood; adults living with various congenital heart defects outnumber children affected by congenital heart defects (41-43). However, despite the lifespan of these survivors being markedly prolonged, the long-term prognostic effects are suboptimal because of complications, including cerebrovascular infarction, chronic renal dysfunction, hypertension, myocardial fibrosis, congestive cardiac

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failure, cardiac arrhythmias and death (44,45). Therefore, congenital heart disease has resulted in strikingly increased morbidity, mortality and socioeconomic burden, which underscores the need for defining the causes of congenital heart disease (1).

In vertebrates, embryonic cardiac organogenesis arises from complicated biological processes that involve cellular commitment, differentiation, proliferation, apoptosis and migration (46); both non-inheritable/environmental predisposing factors and heritable abnormal components may interrupt the finely controlled process, leading to congenital heart disease (2,47-51). Environmental precipitating factors may contribute to ~10% of congenital heart disease cases, although their underlying mechanisms are largely unclear (2). Non-inheritable factors predisposing congenital heart disease encompass maternal viral infection, folate deficiency, early-onset pre-eclampsia, obesity, diabetes mellitus, autoimmune imbalance and maternal consumption of alcohol, tobacco and medications as well as exposure to toxicants and air pollutants during gestation (47,52,53). However, ever-mounting evidence demonstrates that heritable pathogenic determinants are the leading cause of congenital heart disease (2,51). At present, in addition to copy number variations (loss or gain) and aneuploidies, mutations in >100 genes have been identified as responsible for congenital heart disease (2,51,54-75). Nevertheless, the definitive genetic components for congenital heart disease are identified in only a minority of patients (2,51,54-75), which highlights the genetic heterogeneity of congenital heart disease and makes it essential that new congenital heart disease-causing mutations or genes are investigated.

Recent aggregating evidence has underscored the key roles of some nuclear transcriptional factors in regulating proper cardiovascular morphogenesis, including the guanine-adenine-thymine-adenine (GATA) family of transcriptional factors (2,51,76). At present, six members of the GATA family have been categorized fundamentally into a cardiac subfamily (GATA4/5/6) and a hematopoietic subfamily (GATA1/2/3) (76). *GATA4* and *GATA6*, as well as *GATA5*, are among the first genes expressed abundantly in the embryonic heart with a partially overlapping mode of expression spectrum, and these three cardiogenic GATA factors regulate cardiac organogenesis (76). In addition, germline mutations in all three cardiogenic GATA genes (*GATA4/5/6*) are associated with various forms of congenital heart disease, including TOF (77-81), the most prevalent type of cyanotic birth defect with an estimated prevalence of 3/10,000 in live newborns (46). Furthermore, somatic mutations in both *GATA6* and *GATA5* are causally related to TOF (46,82), which implies that somatic mutations in *GATA4* may also play a role in TOF.

Materials and methods

Human research individuals. The present human case-control study adhered to ethical standards outlined in the Declaration of Helsinki (2013). The protocol was approved by The Medical Ethics Committee of Tongji Hospital [approval no. LL(H)-09-07, Shanghai, China]. Informed consent was signed by each individual's legal guardian prior to recruitment. A total of 62 patients with sporadic TOF (33 male cases and 29 female

cases) who underwent cardiac surgery were recruited from the Tongji Hospital (Shanghai, China) between March 2009 and October 2022. The age range of patients was 6-12 months, with a mean age of 0.91 years (~11 months) at the time of surgical treatment. TOF was diagnosed by echocardiographic images and validated by cardiologist direct view during surgery. The inclusion criteria for the patients included a diagnosis of sporadic TOF, available heart tissue and peripheral blood samples as well as clinical data, and informed consent. The exclusion criteria included a positive familial history of congenital heart disease, a known monogenic mutation or pathogenic copy number variation responsible for TOF, and presence of acquired risk factors predisposing to congenital heart disease. Cases with definite anomalous chromosomes or syndromic cardiac deformations, such as Marfan, Char, DiGeorge, Alagille, Noonan, Holt-Oram and Turner's syndrome, were also excluded. Controls comprised 68 patients with rheumatic heart disorder who underwent cardiac valve displacement (36 male and 32 female cases) and 216 healthy subjects (115 male and 101 female subjects). The age range and location and date range of recruitment for the control subjects were the same as those for the patients with TOF. In terms of echocardiograms, no control patients presented with cardiovascular developmental deformation. All the study subjects were unrelated and enrolled from the Chinese population of the Han race.

Sample preparation and DNA extraction. A section of heart tissue was routinely resected from the right ventricular outflow tract of patients with TOF during cardiac surgery. The right outflow tract tissue from TOF repair was collected and cleared of blood contaminants with sterile normal saline, then stored in a -80°C refrigerator. The peripheral blood samples from the patients with TOF were collected (2 ml for each patient). The cardiac tissue from the heart valves and venous blood specimens of cases who underwent cardiac valve displacement because of rheumatic heart disorder, as well as venous blood specimens of healthy subjects, were collected as control specimens. Somatic genomic DNA was isolated from cardiac tissue samples using the DNeasy Blood & Tissue Kit (cat. no. 69504; Qiagen, Inc.) following the manufacturer's instructions. Purification of genomic DNA from blood leucocytes was performed using the Wizard® Genomic DNA Purification Kit (cat. no. A1125; Promega Corporation) according to the manufacturer's instructions.

Genetic investigation. The oligonucleotide primers applied to amplify coding exons and splicing donors/acceptors of the *GATA4* gene via PCR, as well as the reaction mixtures and conditions for the PCR, were as previously described (83). Briefly, the HotStar Taq DNA Polymerase (cat. no. 203205; Qiagen, Inc.) was used according to the manufacturer's instructions. The primers to amplify the whole coding regions of *GATA4* by PCR were as follows: Exon 2 (part a) forward, 5'-GATCTTCGCGACAGTTCCTC-3' and reverse, 5'-GTC CCCGGAAGGAGAAG-3' (amplicon size, 458 bp); exon 2 (part b) forward, 5'-GCTGGGCCTGTCTACCT-3' and reverse, 5'-AAAACAAGAGGCCCTCGAC-3' (amplicon size, 554 bp); exon 3 forward, 5'-GGGCTGAAGTCAGAG TGAGG-3' and reverse, 5'-GATGCACACCCTCAAGTTCC-3'

(amplicon size, 437 bp); exon 4 forward, 5'-GAGATCTCATGCAGGGTTCG-3' and reverse, 5'-GCCCTTCCAAATCTAAGTC-3' (amplicon size, 390 bp); exon 5 forward, 5'-TCTTTC TCGCTGAGTTCAG-3' and reverse, 5'-GGGATGTCCGATGCTGTC-3' (amplicon size, 379 bp); exon 6 forward 5'-GCCATCCCTGTGAGAACTGT-3' and reverse, 5'-GAGGGTAGCTCACTGCTTGC-3' (amplicon size, 444 bp) and exon 7 forward, 5'-AAGTGCTCCTTGGTCCCTTC-3' and reverse, 5'-TTCCCCTAACCGATTGTCG-3' (amplicon size, 479 bp). The PCR-amplified products were fragmented by electrophoresis on 1.3% agarose gel and isolated with the QIAquick Gel Extraction Kit (cat. no. 28704; Qiagen, Inc.). The amplicons were sequenced and analyzed as previously described (83). For each *GATA4* variation detected, databases such as gnomAD (gnomad-sg.org/) and SNP (ncbi.nlm.nih.gov/SNP) were consulted to evaluate its novelty. Additionally, once a *GATA4* mutation was identified, it would be deposited in a genetics database (<https://databases.lovd.nl/shared/genes/GATA4>).

Construction of expression vectors. The expression vectors of GATA-binding protein 4 (GATA4)-pSSRa, T-box transcription factor 5 (TBX5)-pcDNA3.1 and K2 homeobox 5 (NKX2.5)-pEFSA, which express human GATA4, TBX5 and NKX2.5, respectively, reporter vector of atrial natriuretic peptide (ANP)-luciferase (Luc), where the *ANP* promoter drives the expression of firefly luciferase, and the reporter plasmid of myosin heavy chain 6 (*MYH6*)-luciferase (Luc), where the promoter of *MYH6* (expressing myosin heavy chain 6) drives the expression of firefly luciferase, were generated as previously described (84). Expression vectors of GATA4-pSSRa and NKX2.5-pEFSA as well as the reporter vector ANP-Luc were provided by Dr Ichiro Shiojima at The Department of Cardiovascular Science and Medicine of Chiba University (Chiba, Japan). The mutant-type GATA4-pSSRa plasmid harboring the c.708T>G (p.Tyr236*) mutation was created via site-directed mutagenesis using the GeneArt Site-Directed Mutagenesis System (Invitrogen; Thermo Fisher Scientific, Inc.) and an overlapping pair of primers containing the target mutation (forward, 5'-TGGGACGGGTCCTACTA GCTGTGCAACGCCTGC-3' and reverse, 5'-GCAGGCGTTGCACAGCTAGTGACCCGTCCTCA-3') and was validated via PCR-sequencing assay performed as aforementioned. The primers used for site-directed mutagenesis are located in the cDNA of human *GATA4* (Fig. S1).

Cellular transient transfection with vectors and reporter activity assay. COS-7 cells (an African green monkey kidney fibroblast-like cell line) from the Cell Bank of Chinese Academy of Sciences were maintained as previously described (84). COS-7 cells plated onto a 24-well plate at an initial density of 1×10^5 cells/well were grown in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. COS-7 cells at ~80% confluency were transiently transfected with the aforementioned expression vectors using Lipofectamine[®] 3000 (Thermo Fisher Scientific, Inc.), as described previously (84). As an internal control, the vector pGL4.75 (Promega Corporation), which expresses *Renilla*

luciferase, was used for normalized transfection efficiency. A total of 1.0 µg wild-type GATA4-pSSRa was used to mimic the human physiological status, 1.0 µg Tyr236*-mutant GATA4-pSSRa was used to mimic pathogenic status of patients harboring the homozygous mutation and 0.5 µg wild-type GATA4-pSSRa + Tyr236*-mutant GATA4-pSSRa was used to mimic the pathogenic status of patients harboring the heterozygous mutation. Additionally, 0.5 µg wild-type GATA4-pSSRa + empty pcDNA3.1 was compared with 0.5 µg wild-type GATA4-pSSRa + Tyr236*-mutant GATA4-pSSRa to determine whether the Tyr236*-mutant GATA4 exerted a dominant-negative effect on the wild-type GATA4. For each transfection, three independent replicates were performed. Cells were collected 48 h after transfection and lysed. The lysate was used to assess dual-luciferase activity under a microplate luminometer (Promega Corporation) with the Dual-Luciferase[®] Reporter Assay System (cat. no. E1910; Promega Corporation) according to the manufacturer's instructions. The activity of the *MYH6* or *ANP* promoter was expressed as a relative value of firefly luciferase activity divided by *Renilla* luciferase activity. The results were representative of three independent experiments in triplicate.

Statistical analysis. Analyses of categorical data (such as demographic data, including ethnicity, sex and family history) between two groups were performed by χ^2 or Fisher's exact test. For the quantitative parameters given as mean \pm standard deviation (such as age and the *MYH6* or *ANP* promoter activity), Student's unpaired t-test was applied to perform comparisons between two groups. For comparisons between ≥ 3 groups, one-way ANOVA followed by Tukey's post hoc test was applied. Statistical analysis was performed employing SPSS version 16.0 (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data of patients. The present research included 62 non-familial cases affected with TOF who underwent cardiac surgery, 68 sporadic cases who underwent cardiac valve displacement because of rheumatic heart disorder and 216 healthy patients as controls. All research subjects, who were of Han race, had no known family history of congenital heart defect and had no identified environmental factors contributing to congenital heart disease, such as maternal disease, medication and exposure to ionizing radiation, chemicals and toxins during pregnancy. There was no significant difference in the ages ($t = -0.104976$, $P = 0.9165$) between the case group of 62 patients with TOF (with an average of 0.91 ± 0.59) and the control group of 216 healthy individuals (with an average of 0.92 ± 0.68). The baseline phenotypical data of the 62 non-familial cases with sporadic TOF are summarized in Table I.

Discovery of a somatic GATA4 mutation causative for TOF. Sequencing analysis of the *GATA4* gene was performed with the genomic DNA isolated from the diseased cardiac tissue (the resected right ventricular outflow tract muscle to release right ventricular outflow tract obstruction) of 62 non-familial patients with TOF and the heart valve tissues of 68 patients

Table I. Baseline phenotypical data of 62 unrelated patients with sporadic TOF.

Variable	Value
Male, n (%)	33 (53.23)
Age at time of surgery, years	0.91±0.59
Age at time of recruitment, years	0.87±0.62
Family history of TOF, n (%)	0 (0.00)
Form of TOF, n (%)	
Isolated	30 (48.39)
Bicuspid pulmonary valve	8 (12.90)
Patent ductus arteriosus	6 (9.68)
Atrial septal defect	5 (8.06)
Persistent left superior vena cava	4 (6.45)
Anomalous pulmonary venous connection	2 (3.23)
Partial common atrioventricular canal	2 (3.23)
≥2 other cardiovascular defects	5 (8.06)
Dysrhythmia, n (%)	
Atrioventricular block	4 (6.45)
Supraventricular tachycardia	2 (3.23)
Atrial fibrillation	1 (1.61)
Surgical repair, n (%)	100 (100.00)
TOF, tetralogy of Fallot.	

with rheumatic heart disorder, as well as the blood leucocytes of all the 346 research participants. A heterozygous *GATA4* mutation, NM_002052.5: c.708T>G; p.(Tyr236*), was discovered in the pathological myocardial tissue from an 11-month-old male patient with TOF. The sequencing chromatograms illustrating the detected *GATA4* mutation (G/T) as well as its corresponding control counterpart (T/T) are exhibited in Fig. 1A. The schematic diagrams delineating the key structural domains of wild-type *GATA4* and Tyr236*-mutant *GATA4* are presented in Fig. 1B. The discovered heterozygous *GATA4* mutation was not detected in the heart valve tissue samples from 68 cases with rheumatic heart disorder or blood cells of all 346 patients and was not released in the SNP and gnomAD databases (accessed August 2023).

Functional insufficiency of Tyr236*-mutant *GATA4*. In the cultured COS-7 cells transiently transfected with various expression vectors, wild-type *GATA4* (*GATA4*) and Tyr236*-mutant *GATA4* (Tyr236*) transcriptionally activated *MYH6* by ~13-fold and ~1-fold, respectively (t=14.6834; P=0.00013; Fig. 2). When Tyr236* and *GATA4* were co-expressed, transactivation on *MYH6* was ~6-fold (t=7.69231; P=0.00154). Wild-type *GATA4* retained its activity in the presence of Tyr236*-mutant *GATA4*, indicating no significant dominant-negative effect for this *GATA4* mutation. Similar results were obtained when the comparison of multiple groups (among all the control and experimental groups) was performed (P=6.555x10⁻⁸; F=94.859). Specifically, multiple comparisons were conducted between pcDNA3.1 and *GATA4* (t=11.6767, P<0.00001), pcDNA3.1 and Tyr236* (t=0.03, P=1.0), pcDNA3.1 and pcDNA3.1 + *GATA4* (t=5.3767, P=0.00013),

pcDNA3.1 and pcDNA3.1 + Tyr236* (t=5.01, P=0.00023), *GATA4* and Tyr236* (t=11.6467, P<0.00001), *GATA4* and pcDNA3.1 + *GATA4* (t=6.3, P=0.00003), *GATA4* and *GATA4* + Tyr236* (t=6.6667, P=0.00002), Tyr236* and *GATA4* + pcDNA3.1 (t=5.3467, P=0.00013), Tyr236* and *GATA4* + Tyr236* (t=4.98, P=0.00024) and *GATA4* + pcDNA3.1 and *GATA4* + Tyr236* (t=0.3667, P=0.98237).

Synergistic transactivation dysfunction of Tyr236*-mutant *GATA4* with *NKX2.5* or *TBX5*. Cultivated COS-7 cells transiently transfected with multiple expression vectors, *GATA4* and Tyr236* transcriptionally activated *ANP* by ~7-fold and ~2-fold, respectively (t=9.7248, P=0.00063; Fig. 3). In the presence of *NKX2.5*, *GATA4* and Tyr236* transactivated *ANP* by ~32- and ~11-fold, respectively (t=13.4306, P=0.00018); while in the presence of *TBX5*, *GATA4* and Tyr236* transactivated *ANP* by ~38- and ~15-fold, respectively (t=12.4266, P=0.00024). Additionally, similar results were obtained when the comparisons of multiple groups were conducted [P=2.249x10⁻¹¹ (F=220.56) for the synergy of *GATA4* with *NKX2.5* and P=2.852x10⁻¹¹ (F=211.89) for the synergy of *GATA4* with *TBX5*]. Specifically, multiple comparisons were conducted between pcDNA3.1 (-) and *GATA4* (t=5.92, P=0.00123), pcDNA3.1 and Tyr236* (t=0.04, P=1.0), pcDNA3.1 and *NKX2.5* (t=9.92, P=0.00001), pcDNA3.1 and *GATA4* + *NKX2.5* (t=30.02, P<0.00001), pcDNA3.1 and Tyr236* + *NKX2.5* (t=9.5533, P=0.00001), *GATA4* and Tyr236* (t=5.88, P=0.00130), *GATA4* and *NKX2.5* (t=4.0, P=0.02408), *GATA4* and *GATA4* + *NKX2.5* (t=24.1, P<0.00001), *GATA4* and Tyr236* + *NKX2.5* (t=3.6333, P=0.04329), Tyr236* and *NKX2.5* (t=9.88, P=0.00001), Tyr236* and *GATA4* + *NKX2.5* (t=29.98, P<0.00001), Tyr236* and Tyr236* + *NKX2.5* (t=9.5133, P=0.00001), *NKX2.5* and *GATA4* + *NKX2.5* (t=20.1, P<0.00001), *NKX2.5* and Tyr236* + *NKX2.5* (t=0.3667, P=0.99914), *GATA4* + *NKX2.5* and Tyr236* + *NKX2.5* (t=20.4667, P<0.00001); pcDNA3.1 and *TBX5* (t=14.7533, P<0.00001), pcDNA3.1 and *GATA4* + *TBX5* (t=36.72, P<0.00001), pcDNA3.1 and Tyr236* + *TBX5* (t=13.52, P<0.00001), *GATA4* and *TBX5* (t=8.8333, P=0.00028), *GATA4* and *GATA4* + *TBX5* (t=30.8, P<0.00001), *GATA4* and Tyr236* + *TBX5* (t=7.6, P=0.00109), Tyr236* and *TBX5* (t=14.7133, P<0.00001), Tyr236* and *GATA4* + *TBX5* (t=36.68, P<0.00001), Tyr236* and Tyr236* + *TBX5* (t=13.48, P<0.00001), *TBX5* and *GATA4* + *TBX5* (t=21.9667, P<0.00001), *TBX5* and Tyr236* + *TBX5* (t=1.2333, P=0.93282) and *GATA4* + *TBX5* and Tyr236* + *TBX5* (t=23.2, P<0.00001).

Discussion

In the present study, through sequencing analysis a new *GATA4* mutation in a heterozygous status, NM_002052.5:c.708T>G;p.(Tyr236*), was found in diseased heart tissue derived from one male patient out of 62 non-familial patients with sporadic TOF. The mutant allele was not detected in the diseased heart tissues of 68 cases with rheumatic heart disorder or in the blood cells of all the 346 research subjects, encompassing 216 healthy participants matched for ethnicity and sex, suggesting the identified mutation was somatic in origin. This mutation in *GATA4* was absent from gnomAD and SNP databases. Quantitative

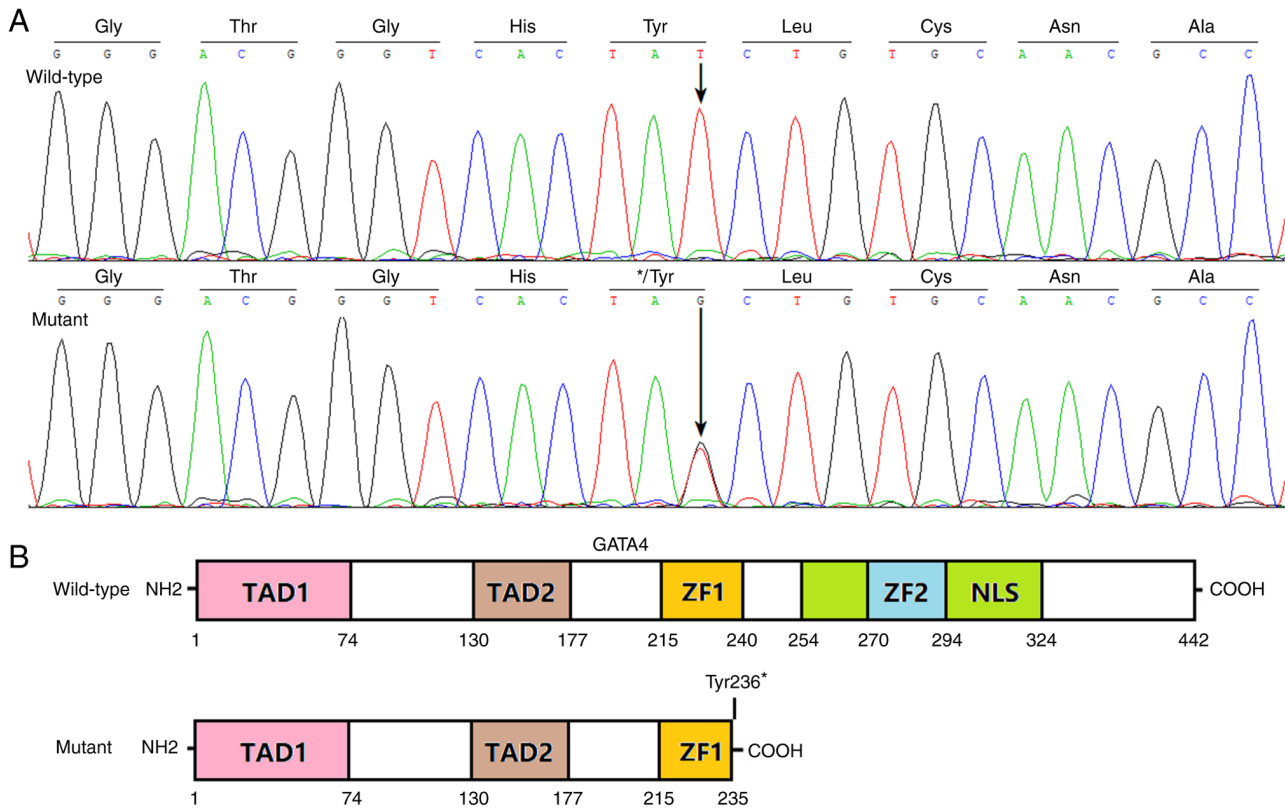


Figure 1. Somatic GATA4 mutation accountable for TOF. (A) Sequence chromatograms illustrating GATA4 mutation identified in a case with TOF (mutant) compared with a healthy subject (wild-type). Arrow sign points to the mutation site. (B) Schematics displaying the critical functional domains of GATA4 with the Tyr236* mutation shown. NLS, nuclear localization signal; ZF, zinc finger; TAD, trans-activation domain; TOF, tetralogy of Fallot.

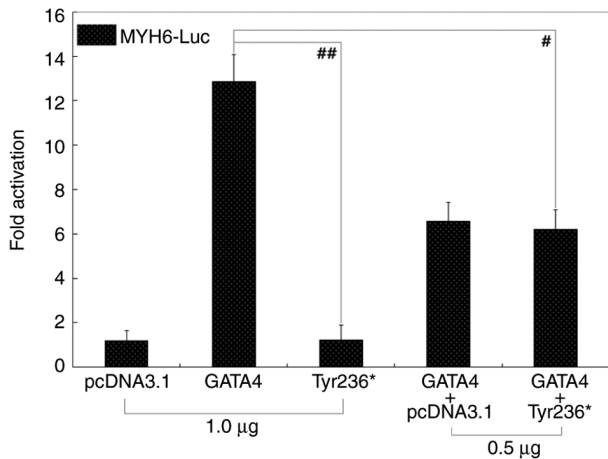


Figure 2. Functional loss of Tyr236*-mutant GATA4. In routinely cultivated COS-7 cells overexpressing various interest proteins (Tyr236*-mutant GATA4, wild-type GATA4, firefly luciferase and *Renilla* luciferase), dual-luciferase reporter gene assay of the transactivation of the *MYH6* promoter-driven firefly luciferase by Tyr236*-mutant or wild type GATA4, singly or in combination, unveiled that the Tyr236* mutant lost transactivation function. ## $P < 0.001$ and # $P < 0.005$ vs. GATA4 (1.0 µg). Luc, luciferase; GATA4, GATA-binding protein 4; MYH6, myosin heavy chain 6.

reporter gene measurements unveiled that Tyr236*-mutant GATA4 was unable to trans-activate the key target genes of *MYH6* and *ANP*, singly or in synergy with *NKX2.5* or *TBX5*, two other TOF-causative genes (85-88). *ANP* and *MYH6* are well-characterized downstream target genes of GATA4 and

GATA4 loss-of-function mutations decrease the transcription of *ANP* or *MYH6* (89-91). Additionally, GATA4, alone or in synergy with transcriptionally cooperative partners such as *NKX2.5* and *TBX5*, has been shown to activate transcription of target genes such as *ANP* and *MYH6*, highlighting the important role of physical and functional interactions between GATA4 and *NKX2.5* as well as *TBX5* in proper heart development (89,92,93). Furthermore, multiple germline deleterious mutations in *GATA4* cause cardiac developmental deformations, including bicuspid aortic valve, atrial septal defect, double-outlet right ventricle, Ebstein's anomaly, ventricular septal defect and TOF (77,78,94). The present results strongly support that somatic *GATA4* mutation is responsible for the molecular pathogenesis underpinning TOF in the mutation carrier, although the mechanism by which the somatic *GATA4* mutation causes TOF remains to be elucidated.

Although progress has been made in the discovery of germline mutations contributing to occurrence of congenital heart defects (2,51,54-75) and the significant effects of somatic mutations on genesis and progression of cancer and aging are well defined (95-97), the roles of somatic mutations in the development of congenital heart disease are unclear. Furthermore, depending on the type of disease and class of mutation (insertion/deletion, single nucleotide substitution, copy number variation, chromosomal aberration and transposon-mediated mutation), somatic mutations may be causative in 6-20% of patients and the frequency of gene mutation in embryonic cells is not significantly different from that in germline cells (98). Given the intensive oxidative metabolism of cardiomyocytes,

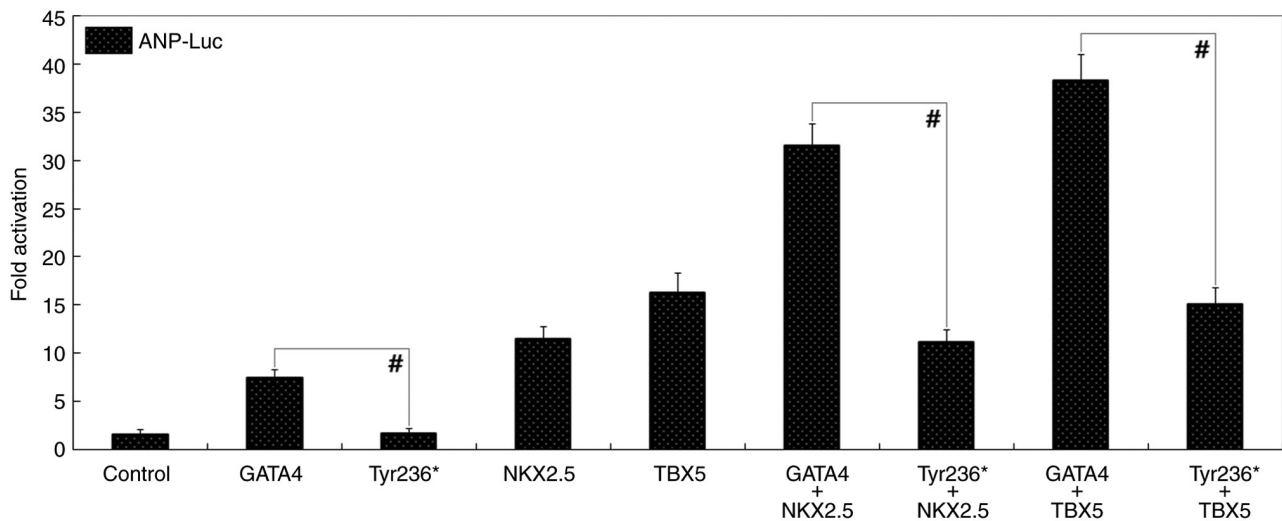


Figure 3. Lost synergistic transactivation between Tyr236*-mutant GATA4 and NKX2.5 or TBX5. In cultured COS-7 cells overexpressing various interest proteins (Tyr236*-mutant GATA4, wild-type GATA4, NKX2.5, TBX5, firefly luciferase and *Renilla* luciferase), dual-luciferase activity measurement of the synergistic activation of ANP by GATA4 in combination with NKX2.5 or TBX5 showed that synergy was disrupted by the Tyr236* mutation. * $P < 0.001$. Luc, luciferase; GATA4, GATA-binding protein 4; NKX2.5, NK2 homeobox 5; ANP, atrial natriuretic peptide; TBX5, T-box transcription factor 5.

increased oxidative DNA damage and/or decreased base excision repair as well as defective mismatch repair of damaged DNA may lead to somatic mutations in cardiomyocytes, and emerging evidence indicates that non-inherited/acquired mutations involving somatic cells are key in cardiovascular disorder (99,100). In agreement with this evidence, the present sequencing analysis of *GATA4* on genomic DNA from resected cardiac tissue along with peripheral blood leucocytes of a patient with TOF identified a somatic mutation responsible for TOF, suggesting that TOF could be partially due to cardiac somatic mutations and somatic mosaicism may be an alternative molecular mechanism of TOF.

The prevalence of somatic *GATA4* variations in patients suffering from congenital heart disease undergoing cardiac surgery has been examined. Salazar *et al* (101) analyzed the *GATA4* gene in fresh-frozen pathological heart tissues as well as corresponding non-diseased tissue obtained from 62 patients with sporadic congenital heart disease (35 cases with cardiac septal defects and 27 cases presented with other heart deformities), and detected six rare variants as well as two frequent polymorphisms in *GATA4* in both the cardiac and the corresponding normal tissues, indicating that they were constitutional variations rather than somatically derived mutations. Wang *et al* (102) performed a sequencing assay of *GATA4* derived from muscle tissue of the right ventricular outflow tract as well as peripheral venous blood leucocytes of 38 patients with isolated TOF undergoing routine cardiac surgery and identified a previously reported *GATA4* mutation (p.Pro407Gln) in an affected child, both in the diseased heart tissue and in blood lymphocytes, implying that a germline *GATA4* mutation contributes to non-syndromic TOF. Cheng *et al* (103) sequenced *GATA4* on DNA samples obtained from cardiac tissue and peripheral blood leucocytes of 20 patients undergoing surgery for ventricular septal defects; seven novel variations in a heterozygous status were observed in the heart tissues but none in the blood leucocytes of patients or in the control samples of 500 healthy individuals, indicating

that they are of somatic origin. Esposito *et al* (104) utilized freshly frozen cardiac tissue samples of right ventricular myocardium and matched blood samples from nine cases undergoing surgical treatment for TOF and 24 patients with left heart hypoplasia to evaluate the incidence of somatic *GATA4* mutations in heart tissue by direct sequencing analysis; no somatic or germline mutations were identified. Yin *et al* (105) performed direct PCR-sequencing analysis of *GATA4* on genomic DNA purified from heart tissue and peripheral blood cells of 98 cases with sporadic congenital heart disease and found two well-known SNPs (rs3729856 and rs56166237) in *GATA4* in both heart tissue and blood samples, indicating a role of germline *GATA4* variations in development of congenital heart disease. Given these conflicting reports on the contribution of somatic mutations to congenital heart disease, the finding of a somatic mutation of *GATA4* in a case of TOF is rare and may depend on various factors such as analytical methods, ethnicity and environmental factors. More in-depth investigations with larger samples sizes from individuals of different ethnicities are required to determine the genetic contribution of somatic mosaicism to pathogenesis of congenital heart defects.

A number of germline *GATA4* mutations have been causally implicated in distinct forms of congenital heart disease, including TOF. Nemer *et al* (94) screened exon 2 of *GATA4* in 26 patients with TOF and 94 cases with other types of congenital heart defect and identified a novel heterozygous *GATA4* mutation, namely NM_002052.5: c.648C>G; p.(Asp216Glu), in two of 26 patients with TOF. Asp216Glu-mutant *GATA4* decreases transactivation of a downstream target gene, ANP, although this mutation has no effect on the binding affinity of *GATA4* to its target gene promoter DNA or the physical and functional interaction of *GATA4* with zinc finger protein FOG family member 2. Yang *et al* (77) sequenced *GATA4* in 52 probands with TOF with a positive family history and found three novel heterozygous mutations, namely p.Ala9Pro, p.Leu51Val and p.Asn285Ser, in three TOF families. Functional analysis

indicated that all three *GATA4* mutants had markedly reduced DNA-binding ability and significantly diminished transcriptional activity. Moreover, Asn285Ser mutation prevented the functional interplay of *GATA4* with *TBX5*. Additionally, Dixit *et al* (78) screened *GATA4* in 285 probands with congenital heart defects and detected nine heterozygous mutations (p.Pro407Gln, p.Trp228Arg, p.Ala8Asp, p.Ala75Ser, p.Glu128Val, p.Thr355Ser, p.Ser358Thr, p.Ser133Cys and p.Ala9Thr) in 22 unrelated patients with congenital heart disease. Notably, *GATA4* mutants were more commonly involved in TOF (45%) and pulmonary stenosis (22.7%) regardless of the profusion of cardiac septal defects in the research cohort. Biochemical measurements showed that three of the nine *GATA4* mutants, p.Trp228Arg, p.Ser133Cys and p.Glu128Val, had impaired combinatorial synergy with *TBX5*, *NKX2.5* or serum response factor (SRF) and diminished DNA-binding affinity. Here, no germline *GATA4* mutations were found except for one somatic *GATA4* mutation, highlighting a somatic mosaic basis of TOF in a minority of patients.

In humans, *GATA4* is located at chromosome 8p23.1 and comprises seven exons, coding for a protein with 442 amino acids (77). *GATA4*, one of the earliest genetic markers expressed in the developing heart, is amply expressed in the embryonic heart; *GATA4* transactivates expression of multiple target genes in the cardiovascular system during embryonic development, including genes that encode *MYH6*, *ANP*, β myosin heavy chain, brain natriuretic factor, vascular endothelial growth factor, cardiac troponin I and cardiac troponin C, alone or synergistically with cofactors such as *TBX5*, *NKX2.5*, *GATA6*, heart and neural crest derivatives expressed 2 and SRF, which indicates the key role of *GATA4* in embryonic cardiac organogenesis (77,106,107). In chick embryos, knockdown of *Gata4* by small interfering RNAs targeting *Gata4* in the cardiac mesodermal cells inhibits ability of bilateral cardiac rudiments to migrate to the midline, resulting in development of two isolated hearts at lateral locations, a deformity of cardia bifida, due to the downregulated expression of N-cadherin (108). In mice, knockout of *Gata4* causes embryonic lethality due to anomalous morphogenesis of the heart tube, including TOF, endocardial cushion defect, cardiac septal defect, right ventricular hypoplasia, double-outlet right ventricle and cardiomyopathy (109-111). In a transgenic murine model overexpressing Val217Gly-mutant *GATA4*, embryonic death occurs, manifesting similar cardiovascular developmental defects with those observed in humans carrying *GATA4* mutations (112). In a knock-in mouse model expressing Gly295Ser-mutant *GATA4*, homozygous mice manifested a single ventricular chamber, thin ventricular myocardium and embryonic lethality while heterozygous mice are viable, with minor structural aberrations of the atrial septum and semilunar valve stenosis (113). Moreover, *Gata4* is required for normal cardiovascular morphogenesis in the xenopus, fly and fish (114). Collectively, these observations from experimental animals highlight the sensitivity of the heart to *GATA4* mutants during cardiac organogenesis, suggesting that *GATA4* exerts a pivotal role in the developing heart and functionally defective *GATA4* predisposes humans to numerous types of congenital heart disease, including TOF.

Notably, in addition to a range of congenital heart defects, germline *GATA4* mutations cause dilated cardiomyopathy

and atrial fibrillation in humans (115,116). As indicated by the present research findings and others (46,82), a higher rate of gene mutations in heart tissue and peripheral blood samples suggests a genetic contribution to dilated cardiomyopathy and atrial fibrillation. Sequencing analysis of *GATA4* from resected cardiac tissue of patients with dilated cardiomyopathy and atrial fibrillation may reveal cardiac somatic mutations contributing to dilated cardiomyopathy and atrial fibrillation.

There are some limitations to this investigation. Firstly, the sample size of the study is relatively small, and larger sample sizes may lead to the discovery of more pathogenic mutations. Secondly, in this study, a pathogenic *GATA4* mutation was identified through candidate gene analysis, hence it cannot be ruled out that other genetic defects may also play a pathogenic role. Whole exome or genome sequencing analysis can help address this problem. Thirdly, the subcellular localization and distribution of the mutated *GATA4* protein, as well as the changes in its ability to bind target gene promoters, remain to be clarified. Finally, the pathogenicity of the *GATA4* mutation is still to be further explored at the level of genetically modified animal models.

In conclusion, the present study identified a somatic *GATA4* loss-of-function mutation predisposing TOF, which indicated that somatic mosaicism plays a prominent role in the molecular pathogenesis of TOF in a minority of cases.

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Availability of data and materials

All data generated or analysed during this study are included in this published article. The *GATA4* mutation, NM_002052.5: c.708T>G; p.(Tyr236*), was deposited in a genetics database (<https://databases.lovd.nl/shared/genes/GATA4>), having an individual ID of 00436129 (phenotype ID: 0000326313; screening ID: 0000437610; variant ID: 0000932923).

Authors' contributions

JW and YQY conceived the study and wrote the manuscript. PA, YJL, RTH, XYL, JNG, CXY, YJX, JW and YQY performed clinical research including collection and analysis of clinical data. PA, YJL, JNG, CXY, JW and YQY performed genetic and biochemical experiments. All authors have read and approved the final manuscript. JW and YQY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by The Medical Ethics Committee of Tongji Hospital [approval no. LL(H)-09-07;

Shanghai, China]. Informed consent was signed by the legal guardians of all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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