Novel and functional DNA sequence variants within the *GATA5* gene promoter in ventricular septal defects

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Original article

Background: **Congenital heart disease (CHD) is the most common human birth defect. Genetic causes for CHD remain largely unknown. GATA transcription factor 5 (GATA 5) is an essential regulator for the heart development. Mutations in the *GATA5* gene have been reported in patients with a variety of CHD. Since misregulation of gene expression have been associated with human diseases, we speculated that changed levels of cardiac transcription factors, GATA5, may mediate the development of CHD.**

Methods: **In this study, *GATA5* gene promoter was genetically and functionally analyzed in large cohorts of patients with ventricular septal defect (VSD) (*n*=343) and ethnic-matched healthy controls (*n*=348).**

Results: **Two novel and heterozygous DNA sequence variants (DSVs), g.61051165A>G and g.61051463delC, were identified in three VSD patients, but not in the controls. In cultured cardiomyocytes, *GATA5* gene promoter activities were significantly decreased by DSV g.61051165A>G and increased by DSV g.61051463delC. Moreover, fathers of the VSD patients carrying the same DSVs had reduced diastolic function of left ventricles. Three SNPs, g.61051279C>T (rs77067995), g.61051327A>C (rs145936691) and g.61051373G>A (rs80197101), and one novel heterozygous DSV, g.61051227C>T, were found in both VSD patients and controls with similar frequencies.**

Conclusion: **Our data suggested that the DSVs in the *GATA5* gene promoter may increase the susceptibility to the development of VSD as a risk factor.**

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Key words: **congenital heart disease; *GATA5* promoter; ventricular septal defect**

Introduction

Congenital heart disease (CHD) is the most common birth defect in humans, affecting about 1%-2% of live births.[1] Although a huge amount of genetic studies on humans and animals have been reported, only a few genes, such as GATA factor 4 (*GATA4*), T-box transcription factor 5 (*TBX5*) and NK2 transcription factor related, locus 5 (*NKX2-5*), have been implicated in a small portion of familial and sporadic CHD patients.[2,3] Recent studies[4,5] have demonstrated that morbidity and mortality are significantly higher in adult CHD patients than in general populations even after successful correction surgery. Late cardiac complications, such as heart failure, arrhythmia and sudden death, are main causes, which are likely due to genetic defects.[4,5] Therefore, genetic studies of CHD are of basic and clinical importance.

The GATA transcription factor family consists of six members, GATA1-6, each of which contains a highly conserved DNA-binding domain that recognizes the sequence element (A/T)GATA(A/G).[6] GATA factors regulate differentiation, proliferation and survival of a variety of cell types. *GATA1/2/3* genes are expressed in hematopoietic stem cells and related derivatives. *GATA4/5/6* genes are expressed in various mesoderm and endoderm-derived tissues, including the heart.[7,8] In the developing heart, *GATA4*, *GATA5* and *GATA6* genes are expressed in a partial overlapping but distinct spatial and temporal pattern.[7]

The *GATA5* gene is first expressed in the precardiac mesoderm, then in the atrial and ventricular chambers and finally restricted to the atrial endocardium

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during the mouse embryonic development. *GATA5* gene expression is also detected in the pulmonary mesenchyme and diverse smooth muscle cells.[9] Mice null for *GATA5* are viable and lack of cardiac defects. Target deletion of the mouse *GATA5* gene causes only female urogenital development.[10] Mice with compound heterozygous mutations for both *GATA4* and *GATA5* or both *GATA5* and *GATA6* died before birth or at perinatal stage with severe cardiac defects, including double outlet right ventricle and ventricular septal defect (VSD).[11,12] Endocardial cell-specific inactivation of *GATA5* in mice leads to hypoplastic hearts and partial formation of penetrant bicuspid aortic valve.[13] A recent study[14] indicates that *GATA5* efficiently promotes the development of mouse embryonic stem cells into cardiomyocytes expressing cardiac troponin T gene. Therefore, GATA5 plays an essential role in the cardiac morphogenesis.

Mutations in the *GATA5* gene have been reported in patients with various types of CHD, including VSD, tetralogy of Fallot and bicuspid aortic valve.[15-17] In familial cases, *GATA5* mutations cause atrial septal defect, VSD and double outlet right ventricle.[18] *GATA5* gene mutations have also been found in Down syndrome-associated atrioventricular septal defects.[19] In addition, *GATA5* gene mutations cause familial atrial fibrillation.[20,21] In different types of human cancer cells, such as gastrointestinal, lung and colorectal cancer, *GATA5* gene promoter hypermethylation has been observed with reduced *GATA5* levels.[22-26] Thus, we speculated that altered *GATA5* gene expression levels, caused by DNA sequence variants (DSVs) within its promoter region, may mediate the development of CHD. In the present study, the promoter region of the human *GATA5* gene was genetically and functionally analyzed in large groups of VSD patients and healthy controls.

Methods

Patients

VSD patients (n=343, male 163, female 180, mean age 8.42 years), who were unrelated, were recruited from the Division of Cardiac Surgery, Jining Medical University Affiliated Hospital, Jining Medical University, China. All VSD patients had no family history of CHD. All VSD patients were diagnosed and confirmed by the following interventional procedures or open heart surgeries. Ethnic-matched healthy controls (n=348, male 283, female 65, mean age 5.25 years) were recruited from the same hospital. Controls with a family history of CHD were excluded. The procedures were in accordance with the ethical standards of the responsible committee on human experimentation of Jining Medical University Affiliated Hospital and with the *Helsinki Declaration* of 1964, as revised in 2000. Informed consents were obtained from participants or their guardians.

Sequence analysis

Peripheral leukocytes were isolated from vein blood and genomic DNAs were extracted. The *GATA5* gene promoter of 836 bp (from -785 bp to +51 bp to the transcription start site at 61051026 of the human *GATA5* genomic sequence) was generated with PCR with the following primers: *GATA5*-forward, 5'-AGTGCGAGCGGGACACGGTT-3', and *GATA5*-reverse, 5'-GAGCACTCACCAGCGGGCAG-3'. PCR primers were designed based on genomic sequence of the human *GATA5* gene (NCBI: NC_000020.10). The PCR products were bi-directionally sequenced with BigDye® Terminator v3.0 reagents and a 3730 DNA analyzer (Applied Biosystems, Foster city, CA, USA) and aligned with the wild type sequence of the *GATA5* gene promoter.

Functional analysis

The DNA fragments of wild type and variant *GATA5* gene promoters (836 bp, from -785 to +51 bp) were generated by PCR with the same set of PCR primers. A KpnI site was added to the *GATA5* forward primer and a HindIII site to the *GATA5* reverse primer. Expression constructs were generated by subcloning PCR products into KpnI and Hind III sites of a reporter vector (pGL3-basic) that express the luciferase gene. Designated expression constructs were transiently transfected into rat cardiomyocyte cells (H9c2), which were cultured with Dulbecco's modified Eagles medium (high glucose). Forty-eight hours post-transfection, the cells were collected and the luciferases activities were measured using dual-luciferase reporter assay system on a Glomax 20/20 luminometer (Promega, Madison, WI, USA). Expression construct expressing renilla luciferase gene (pRL-TK) was used as an internal control. Empty vector pGL3-basic was used as a negative control. The transcriptional activities of the *GATA5* gene promoter were represented as ratios of luciferase activities over renilla luciferase activities. All the experiments were repeated three times independently.

Statistical analysis

The quantitative data were represented as mean±SE and compared by Student's t test. Frequencies of the DSVs within the *GATA5* gene promoter in the VSD patients and controls were compared with SPSS v13.0. P<0.05 was considered statistically significant.

Results

GATA5 gene promoters were bi-directionally sequenced in the VSD patients (n=343) and healthy controls (n=348). Distributions of the identified DSVs are summarized in Table. The DSVs' locations were indicated in Fig.1A. Two novel heterozygous DSVs (g.61051165A>G and g.61051463delC) were identified in three VSD patients, but not in the controls. DSV g.61051165A>G was found in an 11-year-old girl and a 31-year-old man, both with membranous VSD. DSV g.61051463delC was found in a 14-year-boy with a membranous VSD. In addition, a novel heterozygous DSV, g.61051227C>T,

Table. DSVs within the *GATA5* gene promoter in VSD patients and controls

DSVs	Genotype	Location*	Controls (n=348)	VSD (n=343)	P value
g.61051379-80GG>AA	GG/AA	-353 bp	1	0	-
g.61051373G>A (rs80197101)	GA	-347 bp	43	27	0.051
g.61051363G>C	GC	-337 bp	1	0	-
g.61051463delC	C/-	-437 bp	0	1	-
g.61051327A>C (rs145936691)	AC	-301 bp	10	9	0.841
g.61051279C>T (rs77067995)	CT	-253 bp	43	27	0.051
g.61051227C>T	CT	-201 bp	2	1	1.000
g.61051165A>G	AG	-139 bp	0	2	-

*: locations of the DSVs upstream to the transcription start site at 61051026 (NC_000020.10). DSVs: DNA sequence variants; VSD: ventricular septal defect.

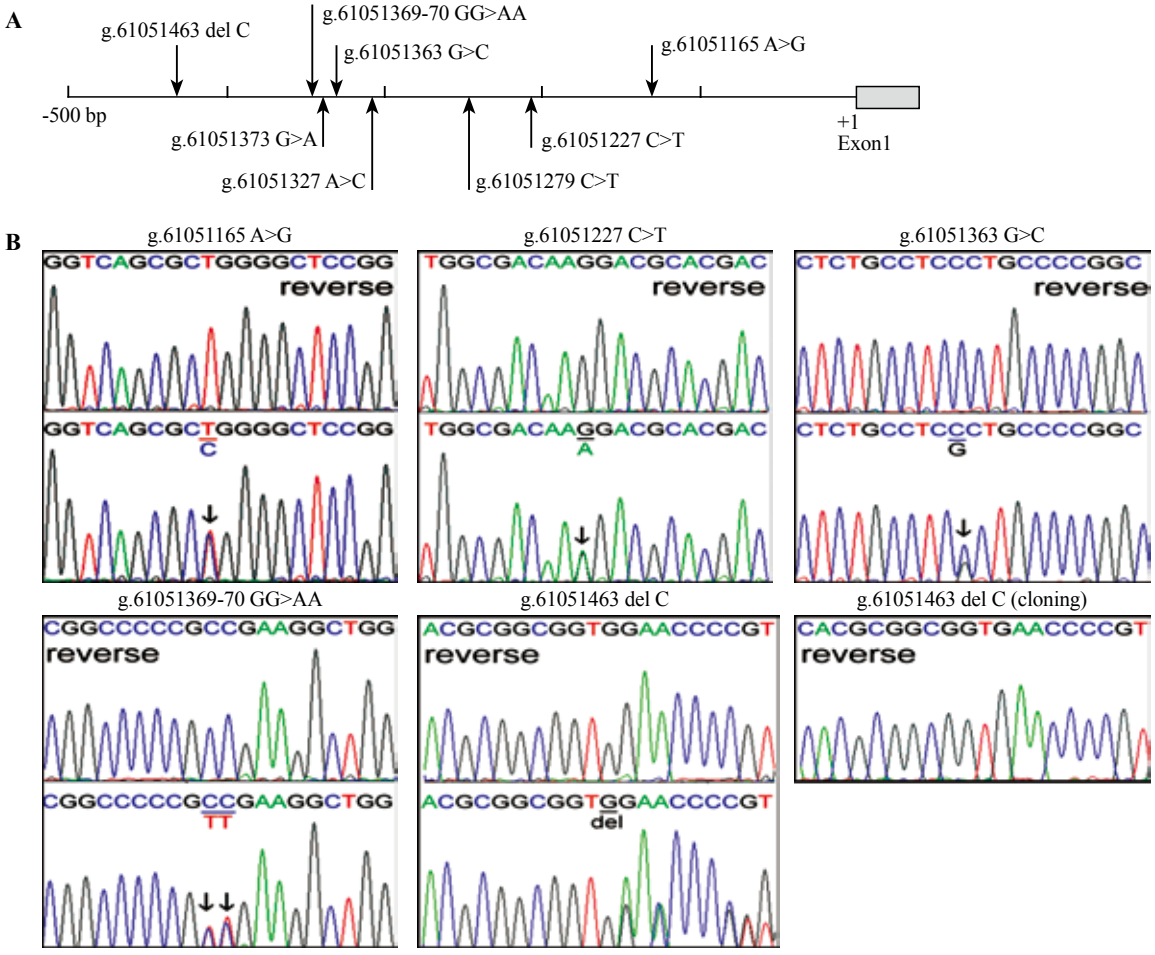

Fig. 1. DSVs within the *GATA5* gene promoter in VSD patients and controls. **A:** Schematic representation of the identified DSVs. The DSVs were named according to their locations in *GATA5* genomic sequences (NCBI: NC_000020.10). The transcription starts at 61051026 in the first exon, which is not translated; **B:** Chromatograms of the novel and heterozygous DSVs. The orientations are indicated. Top panels show wild type and bottom panels heterozygous DSVs. Deletions are underlined and DSVs are marked with arrows. The heterozygous deletion DSV, g.61051463delC, was confirmed by subcloning into expression vector pGL3-basic and directly sequenced, which is shown and marked. DSVs: DNA sequence variants; VSD: ventricular septal defect.

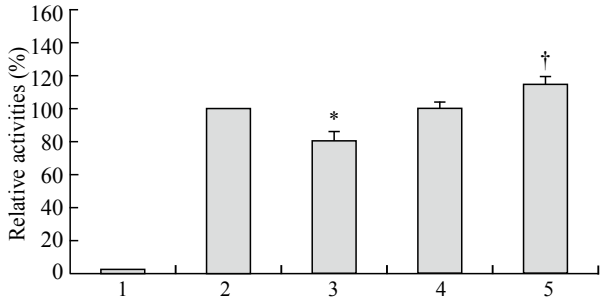

Fig. 2. Transcriptional activities of the wild type and variant *GATA5* gene promoters. *GATA5* gene expression constructs were transfected into H9c2 cells and dual-luciferase activities were measured. The transcriptional activity of wild type *GATA5* gene promoter was designated as 100%. The data were represented as mean±SE from three independent transfection experiments, in triplicate. Lane 1, pGL3-basic, a negative control; 2, WT, wild type; 3, pGL3- 61051165G; 4, pGL3-61051227T, which was used as an internal negative control; 5, pGL3-61051463delC. *: $P<0.05$, compared to pGL3-WT; †: $P<0.01$, compared to pGL3-WT.

and three single-nucleotide polymorphisms (SNPs), g.61051279C>T (rs77067995), g.61051327A>C (rs145936691) and g.61051373G>A (rs80197101), were found in both VSD patients and controls with similar frequencies. In this population, SNPs, g.61051279C>T (rs77067995) and g.61051373G>A (rs80197101), were closely linked. Chromatograms of the novel DSVs were shown in Fig. 1B. The deletion DSV, g.61051463delC, was confirmed by subcloning the DNA fragments into expression vector and direct sequencing.

Analysis of the *GATA5* gene promoter region with TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) suggested that the two novel DSVs (g.61051165A>G and g.61051463delC), which were only identified in VSD patients, did not alter binding sites of known transcription factors. To examine their transcriptional activities, expression constructs for wild type (pGL3-WT) and variant *GATA5* gene promoters (pGL3-61051165G, pGL3-61051227T and pGL3-61051463delC) were generated. The constructs were transfected into H9c2 cells and dual-luciferase activities were measured. The results showed that the DSV, g.61051165A>G, significantly decreased the transcriptional activities of the *GATA5* gene promoter ($P<0.05$). The DSV, g.61051463delC, significantly increased the transcriptional activities of the *GATA5* gene promoter ($P<0.01$) (Fig. 2). The DSV, g.61051227C>T, which was found in both VSD patients and controls, did not affect the *GATA5* gene promoter activity ($P>0.05$).

Furthermore, the parents of the boy carrying g.61051463delC variant and the girl carrying g.61051165A>G variant were screened. The parents of the man carrying g.61051165A>G variant were not available for screening. Both 52-year-old father of the boy and 39-year-old father of the girl had the same *GATA5* variants. Echocardiographic examination revealed that both fathers had a significantly reduced diastolic function of left ventricles, though no VSD or other cardiac defects were found. These results suggested that the DSVs in *GATA5* gene promoter may affect biological function of cardiomyocytes in adults. Taken together, these *GATA5* variants may not play a causal role, but act as a risk factor for the development of VSD.

Discussion

Growing evidence has suggested that rare monogenic mutations and alleles play a major role in the etiology of common complex disorders.[27,28] In the present study, we genetically and functionally analyzed the promoter region of the *GATA5* gene in large groups of VSD patients and controls. Two novel heterozygous DSVs were found within the *GATA5* gene promoter in three VSD patients, but not in the controls. Functionally, these DSVs significantly altered the transcriptional activities of the *GATA5* gene promoter in cultured cardiomyocytes. The fathers of the VSD patients carried the same *GATA5* variants and had a significantly reduced diastolic function of left ventricles. Therefore, these *GATA5* gene promoter DSVs may increase the susceptibility to VSD development as a risk factor, probably by changing GATA5 levels.

The human *GATA5* gene has been mapped to chromosome 20q13.2-q13.3.[29] The promoter region of the human *GATA5* gene has been partially characterized, which is lack of TATA elements. An E-box within the proximal region of the *GATA5* gene promoter (-164 to -159 bp upstream to the transcription start site) has been identified, through which upstream stimulatory factor 1 activates *GATA5* gene expression.[30] In mice, the DNA fragment (from -150 bp to +311 bp to the transcription start site) containing a conserved E-box exhibits the greatest promoter activity.[30] In the mouse *GATA5* gene, an alternate promoter within its first intron has been reported, suggesting the complexity of the *GATA5* gene expression and regulation.[31] In differentiating human colon cancer cells, the *GATA5* gene is upregulated, suggesting that *GATA5* gene expression could be induced.[32] In this study, we identified the DSVs within the *GATA5* gene promoter, through which *GATA5* gene expression may be manipulated with genetic or pharmaceutical approaches.

Misregulation of gene expression programs has been implicated in a broad range of human diseases, including cancer, inflammation, diabetes and cardiovascular diseases.[33] Heart development is strictly controlled by a conserved network of cardiac transcription factors,

cofactors and chromatin regulators. Balanced dosages of cardiac transcription factors are required for the cardiac morphogenesis.[34] For example, *NKX2-5* and cardiac-myosin heavy chain genes have been demonstrated to be directly regulated by *GATA5*.[35-38] *GATA5* interacts with *GATA4* and *GATA6* in the outflow tract formation.[11] *GATA5* cooperates with *GATA4* in regulating the cardiomyocyte proliferation.[12] In the developing heart, *GATA5* directly interacts with *TBX20* and *P300* cofactor in regulation of gene expression.[39,40] In the differentiation of cardiogenic precursors into endothelial endocardial cells, *GATA5* and nuclear factor of activated T cells c (NF-ATc) synergistically activate cardiac gene expression.[41] NF-ATc has been shown to be essential for endocardial development.[42,43] As a critical factor for heart development, decreased or increased *GATA5* levels may interfere with cardiac gene regulatory network, leading to the development of CHD.

In conclusion, two novel and heterozygous DSVs were identified in VSD patients, which significantly altered transcriptional activities of the *GATA5* gene promoter. Our findings suggested that these DSVs may increase the susceptibility to the development of VSD as a risk factor. Genetic and pharmaceutical manipulation of *GATA5* gene expression may provide some insight into designing novel and personalized therapies for adult patients with CHD.

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Ethical approval: Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the editor of this journal.

Competing interest: None declared.

Contributors: SJP, WXL, QYG and WYHX collected clinical samples and information. HWH and PSC performed the experiments and analyzed the results. YB designed the study and wrote the paper. All authors contributed to the content and approved the final version.

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