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Identification of *GATA6* Sequence Variants in Patients With Congenital Heart Defects

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ABSTRACT: Although the etiology for the majority of congenital heart disease (CHD) remains poorly understood, the known genetic causes are often the result of mutations in cardiac developmental genes. *GATA6* encodes for a cardiac transcription factor, which is broadly expressed in the developing heart and is critical for normal cardiac morphogenesis, making it a candidate gene for congenital heart defects in humans. The objective of this study was to determine the frequency of *GATA6* sequence variants in a population of individuals with a spectrum of cardiac malformations. The coding regions of *GATA6* were sequenced in 310 individuals with CHD. We identified two novel sequence variations in *GATA6* that altered highly conserved amino acid residues (A178V and L198V) and were not found in a control population. These variants were identified in two individuals (one with tetralogy of Fallot and the other with an atrioventricular septal defect in the setting of complex CHD). Biochemical studies demonstrate that the GATA6 A178V mutant protein results in increased transactivation ability when compared with wild-type GATA6. These data suggest that nonsynonymous *GATA6* sequence variants are infrequently found in individuals with CHD. **(*Pediatr Res* 68: 281–285, 2010)**

Congenital heart defects (CHDs) are the most common developmental anomaly with an incidence of ~1% and are the leading noninfectious cause of infant mortality (1). Advances in developmental biology have led to the identification of numerous transcriptional regulators, signaling molecules, and structural genes that are critical for normal cardiac morphogenesis. This molecular understanding of cardiac embryogenesis has assisted in the discovery of CHD-causing genes, which have been identified using positional cloning or candidate gene screening approaches (2,3). Despite these significant advances, the etiology of most CHD remains unknown.

Mutations in the GATA family of zinc finger transcription factors have been linked to human disease, and specifically, *GATA4* mutations were found to be associated with CHDs (4–6). Similar to GATA4, GATA6 is expressed in the developing heart but also has additional areas of expression in the developing vascular smooth muscle (7,8). Both are highly conserved genes and bind identical nucleotide sequences in genomic DNA and regulate similar target genes (9–11). GATA4 null mice exhibit early defects in heart formation and ventral foregut closure (12,13), whereas *GATA6*-null mice die after implantation because of defects in the visceral endoderm and extraembryonic development. More recent studies have demonstrated that conditional deletion of *GATA6* in neural crest-derived smooth muscle leads to malformations of the cardiac outflow tract and the aortic arch arteries (14,15). Consistent with this role in outflow tract development, two loss-of-function *GATA6* mutations were recently reported to cause persistent truncus arteriosus in humans (16). In addition to its role in outflow tract defect development, *GATA6* has also been shown to genetically interact with the cardiac transcription factors, *GATA4* and *TBX5*, which cause cardiac septation defects in humans, suggesting that mutations in *GATA6* may be associated with other forms of CHD (11,17,18).

To determine the frequency of *GATA6* sequence variants and define the phenotypic subset of CHDs that is associated with genetic variations in *GATA6*, we screened the *GATA6* gene in 310 children with a spectrum of CHDs. We hypothesized that mutations in *GATA6* are found in individuals with a spectrum of CHD as reported for *GATA4* (5,6). Here, we have identified two novel *GATA6* sequence variants that altered highly conserved amino acid residues and that were not found in control individuals. Biochemical assays suggested gain-of-function effects for the *GATA6* A178V variant, which was identified in an individual with tetralogy of Fallot. Our data suggest that sequence variants in *GATA6* contribute to a subset of human CHD.

METHODS

Radioactive section* in situ *hybridization. C57Bl6 mice were maintained on a 0600 and 1800 h light-dark cycle with noon on the day of observation of a vaginal plug defined as embryonic day (E) 0.5. Mothers were killed and the embryos harvested at E13.5. *In situ* hybridization was performed using ^{35}S-labeled antisense probes for GATA6 as previously described (18).

Study population. The subjects comprised 310 unrelated individuals (174 males and 136 females) who received their cardiovascular care at Children's Medical Center of Dallas. The individuals were of varied ethnicity (153 European Americans, 123 Hispanics, and 34 African Americans) and had a variety of CHDs (Table 1). Between January 2002 and December 2008, subjects were prospectively recruited for genetic testing and informed consent

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Abbreviations: α-MHC, alpha-myosin heavy chain; **ANF,** atrial natriuretic factor; **CHD,** congenital heart disease

Table 1. *Diagnoses of screened study population with congenital heart defects*

Cardiac diagnosis	Number of patients
Septation defects	110
Ostium secundum ASD	38
Sinus venosus ASD	8
Perimembranous VSD	51
Muscular VSD	13
Left-sided defects	58
AS or subAS	8
CoA	24
HLHS	26
Right-sided defects	20
PA (without VSD)	13
PS	7
Conotruncal defects	45
TOF	33
Truncus arteriosus	12
Complex	13
DILV, DORV, or single ventricle	13
Endocardial cushion defects	26
AVSD	17
Ostium primum ASD	9
Other	38
TGA	17
TAPVR	10
PDA	9
CA (anomalous)	2

ASD, atrial septal defect; VSD, ventricular septal defect; AS, aortic stenosis; CoA, coarctation; HLHS, hypoplastic left heart syndrome; PA, pulmonary valve atresia; PS, pulmonary valve stenosis; TOF, tetralogy of Fallot; DILV, double inlet left ventricle; DORV, double outlet right ventricle; AVSD, atrioventricular septal defect; TGA, d-transposition of the great arteries; TAPVR, total anomalous pulmonary venous return; PDA, patent ductus arteriosus; CA, coronary.

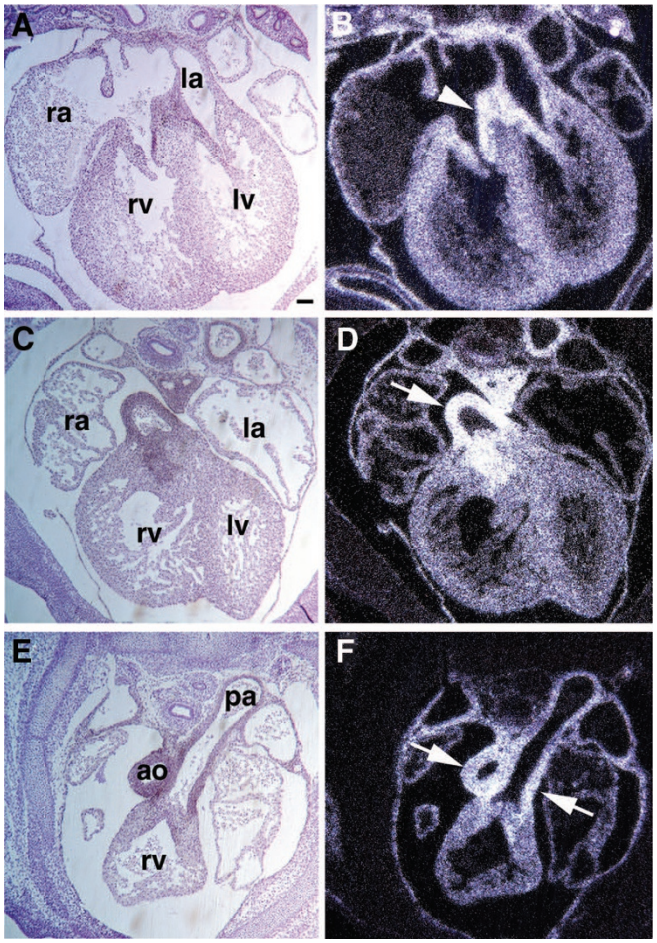

Figure 1. Expression of GATA6 in embryonic heart by radioactive section *in situ* hybridization. (*A–F*) Coronal sections of E13.5 mouse hearts. (*B*) GATA6 transcripts are highly expressed in the developing atrioventricular valve leaflets (*arrowhead*) along with lower levels of expression in the atrial and ventricular myocardium. (*D* and *F*) Sections through the cardiac outflow tract demonstrate the strongest expression in the smooth muscle surrounding the aorta and pulmonary artery (*arrows*) in addition to expression in the chamber myocardium. Corresponding bright field images for *B*, *D*, and *F* are shown in *A*, *C*, and *E*. Right atrium, *ra*; right ventricle, *rv*; left atrium, *la*; left ventricle, *lv*; aorta, *ao*; and pulmonary artery, *pa*.

obtained according to protocol as approved by the Institutional Review Board at the University of Texas Southwestern Medical Center. The cohort was randomly selected from this population, and patients with known chromosomal abnormalities were excluded from the study. Patients underwent complete cardiac evaluation at Children's Medical Center of Dallas, and echocardiogram, cardiac catheterization, and operative reports were reviewed, when available. Venous blood samples were collected and genomic DNA isolated using the PUREGENE kit (Gentra Systems, Minneapolis, MN) from affected subjects. Genomic DNA was obtained from a control population consisting of 288 individuals of variable ethnicity (96 European Americans, 96 Hispanics, and 96 African Americans). The control population did not have known congenital heart defects, but subclinical cardiac malformations such as bicuspid aortic valve or patent foramen ovale were not excluded.

Sequencing of GATA6. All seven exons of *GATA6* were sequenced in the patient population and all sequence variations identified. Only sequence variations that predicted a nonsynonymous amino acid substitution were screened in the control population by direct sequencing. The sequencing primers are available on request. PCR amplification was performed using the Advantage GC Genomic PCR kit following the manufacturer's instructions, with an annealing temperature of 60°C (BD Biosciences, Palo Alto, CA).

Plasmid construction and site-directed mutagenesis. The human GATA6 expression vector was generously provided by W.L. Miller (19). Point mutations were introduced into this plasmid containing the human GATA6 cDNA to generate the GATA6 A178V and GATA6 L198V mutant expression vectors, which were verified by direct sequencing.

Transactivation studies. HeLa cells were transfected using Fugene 6 (Roche) with 100 ng of either alpha myosin heavy chain (αMHC) or atrial natriuretic factor (ANF) luciferase reporter, 100 ng of cytomegalovirus (CMV)-LacZ plasmid, and 300 ng of wild-type GATA6, GATA6 A178V, or GATA6 L198V plasmids. Immunoblots were used to verify appropriate protein expression. Transactivation assays were performed, and luciferase activity was measured 48 h after transient transfection as previously described (20). Three independent experiments were performed in triplicate with the αMHC and ANF luciferase reporters. Luciferase data are shown as fold activation as they are normalized for transfection efficiency using beta-galactosidase. Statistical comparisons were performed using *t* test, and $p < 0.05$ was considered significant.

RESULTS

Cardiovascular expression of GATA6. To determine expression of GATA6 in the later stages of heart development, *in situ* hybridization was performed in wild-type E13.5 mouse embryos. GATA6 transcripts were expressed in the atrial and ventricular myocardium with higher levels of expression seen in the atrioventricular valve leaflets (Fig. 1*A* and *B*) similar to previous reports in E9.5 to E12.5 embryos (15,18). The endocardial cushions, the precursors of the mature atrioventricular valve leaflets, also express GATA6 mRNA in E11.5 murine hearts (18). The highest levels of GATA6 expression were seen in the smooth muscle cells of the aorta and pulmonary artery, as previously described, whereas lower levels were detectable in the pulmonary valve leaflets (Fig. 1*C–F*) (15).

Table 2. *Unique nonsynonymous sequence variations identified in children with CHD*

Nucleotide change	Amino acid change	Cardiac phenotype	Allele frequency (%) in patients	Allele frequency (%) in control population
C740T	A178V	Unbalanced AVSD	0.2 (1/620)	0 (0/576)
C799G	L198V	TOF	0.2 (1/620)	0 (0/576)

Identification of GATA6 sequence variations. We screened for mutations by direct DNA sequencing of the coding regions of *GATA6* in 310 individuals with diverse forms of nonsyndromic CHD (Table 1). Analysis of the sequencing data resulted in the identification of two novel sequence variations that predicted nonsynonymous amino acid substitutions at codons 178 and 198, A178V and L198V, respectively (Table 2 and Fig. 2). The L198V variant was identified in a patient with isolated tetralogy of Fallot (single malalignment ventricular septal defect with subvalvar/valvar pulmonary stenosis and a normal aortic arch), whereas the A178V variant was found in a patient with an unbalanced atrioventricular septal defect, hypoplastic left ventricle, and two muscular ventricular septal defects with no additional evidence of heterotaxy syndrome (Table 2). Both of these changes were identified in individuals of Hispanic ethnicity; however, neither of these nucleotide changes was observed in the control population of 288 individuals (576 alleles), of which 96 individuals (192 alleles) were of Hispanic ethnicity. The A178V variation was identified in an unaffected parent, whereas the inheritance of the L198V variation was unable to be tested. Both of these nucleotide changes altered highly conserved amino acid residues in the GATA6 protein (Fig. 2*C*). Although the substitutions did not lie in known functional domains, the altered alanine residue was located in a conserved polyalanine tract. In addition, we identified a sequence variation that predicted a nonsynonymous amino acid substitution (G15R) with a minor allele frequency of 2.6% (16/620 alleles) in the affected population. This sequence variation was also found in the control population (6.9% = 40/576 alleles) suggesting that it likely represents a single nucleotide polymorphism. We identified several other novel sequence variations that predicted synonymous amino acid changes in individuals with CHD (Table 3).

Table 3. *Single nucleotide polymorphisms and rare synonymous sequence variations in children with CHD and in control population*

Nucleotide change	Amino acid change	Allele frequency (%) in patients	Allele frequency (%) in control population
G43C	G15R	2.6 (16/620)	6.9 (40/576)
G222A	P74P	0.2 (1/620)	NT
G768T	A256A	0.2 (1/620)	NT
G855T	A285A	0.2 (1/620)	NT

NT, not tested.

***GATA6 A178V has increased transcriptional activity* in vitro.** To determine whether the amino acid substitutions in GATA6 resulted in functional abnormalities, we tested them using *in vitro* transfection assays. We generated mutant expression constructs for the GATA6 A178V and GATA6 L198V nucleotide variants. Luciferase reporter assays were performed using the GATA-dependent cardiac enhancers, αMHC and ANF, upstream of a luciferase reporter. The GATA6 A178V and L198V proteins were equally expressed in HeLa cells when compared with wild-type GATA6 (data not shown). Transfection of GATA6 A178V expression plasmid demonstrated a 2-fold increase in transactivation ability on αMHC-luciferase reporter compared with transfection with equal amounts of wild-type GATA6 plasmid ($p = 0.013$; Fig. 3). A similar pattern of activation increase was found with ANF-luciferase reporter ($p = 0.001$; Fig. 3). The GATA6 L198V variant did not show any difference in transactivation ability compared with wild-type GATA6.

DISCUSSION

Mutations in cardiac transcription factors have been implicated as genetic etiologies of human CHD (21). We have identified two rare *GATA6* sequence variants, in a subset of individuals with CHD, which resulted in nonsynonymous amino acid substitutions in two subjects after screening a population of 310 affected individuals. *In vitro* transactivation studies demonstrated that the GATA6 A178V variant is a gain-of-function mutation with increased transactivation ability when compared with wild-type GATA6. These findings highlight the importance of the *GATA6* as an etiologic gene

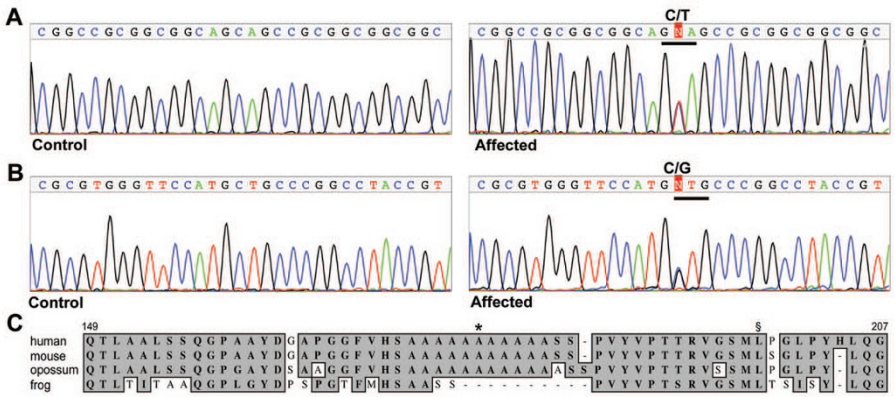

Figure 2. Novel GATA6 sequence variations alter highly conserved amino acids. (*A*) Sequence chromatogram showing heterozygous C to T transition in affected subject compared with control individual. The nucleotide variation predicts a nonsynonymous amino acid substitution at codon 178 (A178V). (*B*) Sequence chromatogram showing heterozygous C to G transversion that predicts a valine at codon 198 in affected subject compared with control. (*C*) Alignment of human GATA6 protein sequence with orthologues from multiple species. The alanine and leucine at codons 178 and 198, respectively, are highly conserved. Location of A178V and L198V is indicated by (*) and (§), respectively.

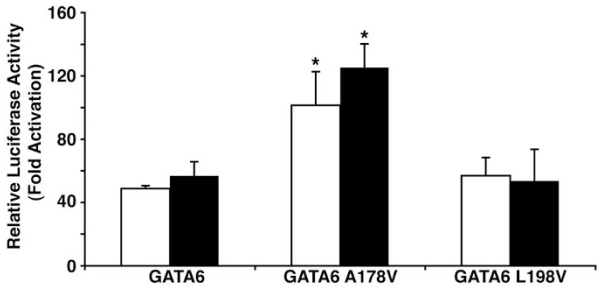

Figure 3. *In vitro* functional analysis of GATA6 sequence variations. Transactivation assays in HeLa cells transfected with 300 ng of GATA6 A178V or GATA6 L198V along with cotransfection of either αMHC-luciferase (□) or ANF-luciferase reporter (■). More than 2-fold increased luciferase activation was found with the GATA6 A178V mutant on both luciferase reporters. No significant difference was demonstrated with transfection of the GATA6 L198V mutant plasmid. Experiments were performed in triplicate and mean and standard deviations are shown. $*p < 0.05$.

for a subset of CHD and suggest that appropriate levels of GATA6 are critical for normal heart development in humans.

The prevalence of *GATA6* sequence variants in our study was 0.6%, consistent with similar large population-based studies of nonfamilial CHD where the mutation prevalence of a single CHD-causing gene is <2% (5,6,22). The report by Kodo *et al.* (16) identified two *GATA6* mutations in a population of 21 individuals with truncus arteriosus. Interestingly, we did not identify any *GATA6* mutations in 12 subjects with truncus arteriosus. However, we did identify a gain-of-function mutation in an individual with tetralogy of Fallot, which is embryologically related to truncus arteriosus. Therefore, it is possible that the prevalence of *GATA6* mutations is higher in a population of subjects with conotruncal abnormalities. In our study, this would represent an incidence of 2% (1/45). Of note, the gain-of-function *GATA6* A178V mutation was inherited from an unaffected parent. The finding of incomplete penetrance has been most recently shown with *NOTCH1* variants, which exhibit *in vitro* functional deficits, and were identified in individuals with left-sided cardiac malformations and their unaffected parents (23). For the L198V variant, we did not identify any *in vitro* functional abnormalities in the assays that we used. The nucleotide variant is rare and alters a highly conserved leucine residue, and ultimately, biochemical deficits may exist in transactivation assays with other luciferase reporters or protein-protein interactions because of alterations in structure.

Normal cardiac development requires adequate gene dosage of cardiac transcription factors. This has been shown in both murine and human studies and classic examples include *NKX2-5*, *TBX5*, *GATA4*, and *TBX1* where loss-of-function mutations disrupt cardiac morphogenesis in humans and mice (24). Previous studies suggest that increased dosage of *TBX5* or *TBX1* is associated with phenotypes consistent with Holt-Oram syndrome and Shprintzen syndrome, respectively, whereas a gain-of-function mutation in *TBX20* was recently reported to be associated with atrial septal defects and cardiac valve defects (25–27). In addition, gain-of-function mutations in *PTPN11* have been well described to be associated with the cardiac defects found in Noonan syndrome (28). Our *in vitro* studies suggest that the *GATA6* A178V sequence variant represents a gain-of-function mutation with its increased transactivation ability. Consistent with this, the alanine is located within a polyalanine tract, which has been proposed to function as a transcriptional repression domain (29). This finding is particularly interesting in light of studies in *Xenopus*, which have demonstrated that overexpression of GATA-6 disrupts cardiac development by preventing differentiation and blocking expression of GATA target genes, cardiac actin and XMLC2, a heart-specific myosin light chain (30). Consistent with this, mutations in the GATA target αMHC (*MYH6*) are linked to atrial septal defects in humans (31). Further experimentation is necessary to understand the effect of this substitution on GATA6 protein structure and function during cardiac development.

In conclusion, genetic abnormalities involving cardiac development genes are increasingly being discovered to be associated with CHD in humans. In this study, we have identified two novel sequence variations in the cardiac transcription factor, *GATA6*, to be associated with CHD, and similar to other investigations, genetic variants of single gene are associated with only a small subset of CHD. Studies of larger well-phenotyped populations will need to be performed for improved genotype-phenotype correlations and to determine whether *GATA6* mutations are found in a higher frequency with cardiac septal defects and conotruncal abnormalities. Ultimately, further studies elucidating the role of GATA6 in the developing cardiovascular system will be required to increase our knowledge of the genetic basis of CHD and to provide more personalized genetic counseling and develop novel preventive therapies.

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