GATA4 sequence variants in patients with congenital heart disease

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ORIGINAL ARTICLE

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Background: Recent reports have identified mutations in the transcription factor *GATA4* in familial cases of cardiac septal defects. The prevalence of *GATA4* mutations in the population of patients with septal defects is unknown. Given that patients with septal and conotruncal defect can share a common genetic basis, it is unclear whether patients with additional types of CHD might also have *GATA4* mutations.
Aims: To explore these questions by investigating a large population of 628 patients with either septal or conotruncal defects for *GATA4* sequence variants.
Methods: The *GATA4* coding region and exon–intron boundaries were investigated for sequence variants using denaturing high-performance liquid chromatography or conformation-sensitive gel electrophoresis. Samples showing peak or band shifts were reamplified from genomic DNA and sequenced.
Results: Four missense sequence variants (Gly93Ala, Gln316Glu, Ala411Val, Asp425Asn) were identified in five patients (two with atrial septal defect, two with ventricular septal defect and one with tetralogy of Fallot), which were not seen in a control population. All four affected amino acid residues are conserved across species, and two of the sequence variants lead to changes in polarity. Ten synonymous sequence variants were also identified in 18 patients, which were not seen in the control population.
Conclusions: These data suggest that non-synonymous *GATA4* sequence variants are found in a small percentage of patients with septal defects and are very uncommonly found in patients with conotruncal defects.

Congenital heart disease (CHD) is one of the most common major birth defects, occurring in 4–8 per 1000 live births.[1 2] Despite its prevalence and clinical significance, the aetiology of CHD remains largely unknown. The available data suggest that the disease aetiology is complex, involving multiple genetic and environmental factors.[3 4] Fetal heart development is regulated by a group of highly conserved transcription factors, including *NKX2.5, TBX5* and *GATA4*.[5] Mutations in *NKX2.5* have been identified in familial and sporadic non-syndromic congenital heart disease (with or without atrioventricular block), including septal and conotruncal defects.[6–9] Recent reports have identified *GATA4* mutations in patients with predominantly familial cardiac septal defects.[10–14] *GATA4* maps to a region of chromosome 8, of which deletions have variable association with CHD.[15–17] Given that patients with septal and conotruncal defects can share a common genetic basis, it is possible that patients with other types of CHD also have *GATA4* mutations. To explore these questions, and to correlate genotype with phenotype, a large cohort of 628 subjects with septal and conotruncal defects was investigated for *GATA4* sequence variants.

METHODS

Study cohort

Subjects were drawn from a cohort of patients recruited prospectively into genetic studies of congenital heart disease (table 1). Informed consent was obtained from study participants in accordance with protocols approved by the Institutional Review Board for Human Research at The Children's Hospital of Philadelphia or at the Oregon Health and Science University (Oregon Registry of Congenital Heart Defects). Patients were invited to enrol in the study regardless of gender, race or ethnicity with no selection for or against familial CHD. Subjects with either atrial or ventricular septal defects (ASD or VSD respectively) or conotruncal anomalies were included. Specifically, patients with either a secundum or sinus venosus type ASD were included, whereas those with a primum ASD (endocardial cushion defect) were excluded. Patients with a perimembranous, posterior malalignment or conoseptal hypoplasia type VSD were included, whereas those with only a muscular or endocardial cushion type VSD were excluded. Patients with conotruncal defects, or anomalies of the right or left sided outflow tract of the heart, were recruited including those with: tetralogy of Fallot (TOF), truncus

Table 1 Characteristics of study cohort

Cardiac defect (n)	Patients (n)
Atrial septal defect*	122
Ventricular septal defect†	137
Tetralogy of Fallot	201
D-transposition of the great arteries	76
Double-outlet right ventricle	45
Truncus arteriosus	20
Interrupted aortic arch	11
L-transposition of the great arteries	10
Other	6
Total patients	628

*Secundum or sinus venosus atrial septal defect; †perimembranous, posterior malalignment, or conoseptal hypoplasia types of ventricular septal defect.

Abbreviations: ASD, atrial septal defect; CHD, congenital heart disease; DHPLC, denaturing high-performance liquid chromatography; CSGE, conformation-sensitive gel electrophoresis; DORV, double-outlet right ventricle; D-TGA, D-transposition of the great arteries; ESE, exonic splice enhancer; IAA, interrupted aortic arch; L-TGA, L-transposition of the great arteries; TA, truncus arteriosus; TOF, tetralogy of Fallot; VSD, ventricular septal defect

Table 2 Primers for *GATA4*

Reaction	Exon	Forward (5′ to 3′)	Reverse (5′ to 3′)
Fragment 1	5′ exon 1	TGTTGCCGTCGTTTTCTCTC	GTCCCCGGGAAGGAGAAG
Fragment 2	3′ exon 1	CGACGGAGCCGCTTACAC	GCGTTGGTGGAAAAACAAGA
Fragment 3	Exon 2	TGAGAGCTGGGCATAAACAA	AGAGGATGTCCCACCAAGG
Fragment 4	Exon 3	GGGCAGTGCACACCTTTTAC	GAGAGATGGGCATCAGAAGG
Fragment 5	Exon 4	TGTCTTTCAATGCTGTAGCAGA	TGCCTAACCCGGAAGATATG
Fragment 6	Exon 5	CATTAGCTTGCACCCATCC	AGTACTAGGCTGGCCTCTGG
Fragment 7	Exon 6	CCTAGACCTCCCAAGC	GGCCTCCTTCTTTGCTATCC

arteriosus (TA), interrupted aortic arch (IAA), double-outlet right ventricle (DORV) or D-transposition of the great arteries (D-TGA). A small group of patients with L-transposition of the great arteries (L-TGA) or rare outflow tract defects was also investigated. Except for patients with ASD, all patients were tested for chromosome 22q11 deletion. Patients with chromosome 22q11 deletion, trisomy 21 or other identified chromosomal anomaly were excluded. Cardiovascular diagnoses were confirmed by attending paediatric cardiologists who reviewed echocardiograms and/or echocardiogram reports, cardiac catheterisation reports, and surgical notes if applicable. Family history of CHD was ascertained by a genetic counsellor by oral history, and confirmed by medical records when available. An effort was made to obtain parental DNA for sequence analysis in all patients found to have a non-synonymous *GATA4* sequence variant. Medical records of patients with a non-synonymous *GATA4* sequence variant were reviewed to determine whether any non-cardiac congenital malformations or recognised genetic syndrome was present.

Control subjects

In total, 159 control subjects (48 African American, 78 European American, and 33 Hispanic American) with no reported cardiac phenotype were sequenced for variants in *GATA4*, and have been described previously.[18] The DNA samples were obtained from the Dallas Heart Study repository at University of Texas Southwestern Medical Center. A low high-density lipoprotein phenotype was found in 64 of the controls. An additional 105 control subjects of mixed but unspecified ethnicities were tested for sequence variants (Ala411Val, Asp425Asn) in exon 6 by conformation-sensitive gel electrophoresis (CSGE).

Testing for sequence variants

The *GATA4* gene is located on chromosome 8p23.1-p22 and consists of six coding exons (NM_002052). We segmented the coding region, exon–intron junctions and part of the 3′ untranslated sequence of the gene (a scanned region of 1706 bp in total) into seven target sequences (table 2) suitable for denaturing high-performance liquid chromatography (DHPLC) (WAVE, Transgenomics Inc, Omaha, Nebraska, USA)[19] or CSGE analysis. Each target sequence was amplified from genomic DNA by PCR. The PCR contained 20 ng genomic DNA amplified in a 30 µl reaction containing 1.5 U HotStar Taq polymerase and 1× HotStar Taq PCR Buffer (Qiagen Inc., Valencia, California, USA), 1.5 mmol/l $MgCl_2$, 125 µmol/l of each dNTP (Roche, Germany) and 1 µmol/l of each PCR primer. All reactions started with 2 minutes at 95℃ followed by 38–40 cycles of 45 seconds at 95℃, 30 seconds at 61℃ or 63℃ and 45 seconds at 72℃, and finished with a 10-minute extension period at 72℃. PCR products were then examined for sequence alterations by DHPLC (n = 504) or CSGE (n = 124) analysis. Samples that showed peak or band shifts were reamplified from genomic DNA and then sequenced with an automated cycle sequencer in both directions (ABI BigDye Taq FS Terminator V.3.1; ABI Prism 3100sequencer, Applied Biosystems, Foster City, California, USA).

RESULTS

Non-synonymous sequence variants

Four sequence variants conferring a change in the encoded amino acid (Gly93Ala, Gln316Glu, Ala411Val, Asp425Asn) were identified in 5 patients (table 3), and were not seen in 159 control subjects. One of these variants, Ala411Val, was previously reported in a patient with cardiac hypertrophy.[20] However, neither this variant nor that seen in two of the current study subjects (Asp425Asn) were seen in an additional 105 control subjects. All four sequence variants change an amino acid conserved in mouse and rat. Using two programs (SIFT (Sorting Intolerant From Tolerant)[21 22] and PolyPhen[23 24]), which predict whether an amino acid substitution affects protein function, two of the variants (Gln316Glu and Asp425Asn) are expected to affect protein function. One of the variants, Gln316Glu, maps to the nuclear localisation signal region of *GATA4* (fig 1).

Four of the five patients with non-synonymous sequence variants had septal defects, and the fifth had tetralogy of Fallot (table 3). Only one subject (Gln316Glu) had additional cardiac defects including small muscular VSDs and mild pulmonary valve stenosis. Review of medical records found that this subject (Gln316Glu) also had additional non-cardiac anomalies, including hydrocephalus and developmental delay. The other four subjects had no other reported malformations or medical issues. None of the five subjects was reported to have cardiac conduction abnormalities or arrhythmias.

The patient carrying the Gly93Ala alteration was identified as having an ethnic ancestry of >1 race. The patient with the Gln316Glu alteration is Native American/Hispanic. The other three patients are of European American descent (Caucasian).

Characterisation of relatives

There was no family history of CHD in three patients carrying an alteration (Gly93Ala, Ala411Val). Another patient (Asp425Asn) had a vague history of CHDs: one sibling with heart and lung anomalies was miscarried, one paternal cousin had an abnormal valve and another had tachycardia. A distant family member died early in life with a ''blue spell'', which may have resulted from unrepaired tetralogy of Fallot. The family history was unavailable for one subject (Gln316Glu).

If available, parental genomic DNA was tested for the sequence variant identified in the corresponding offspring. In two patients, reportedly unaffected mothers were carriers (table 3).

Synonymous sequence variants

Ten synonymous sequence variants were observed in 18 subjects, which were not seen in 159 control subjects (table 4). Two of these sequence variants have been previously reported in two patients with cardiac hypertrophy.[20] Nine of these alterations occurred within a conserved amino acid.

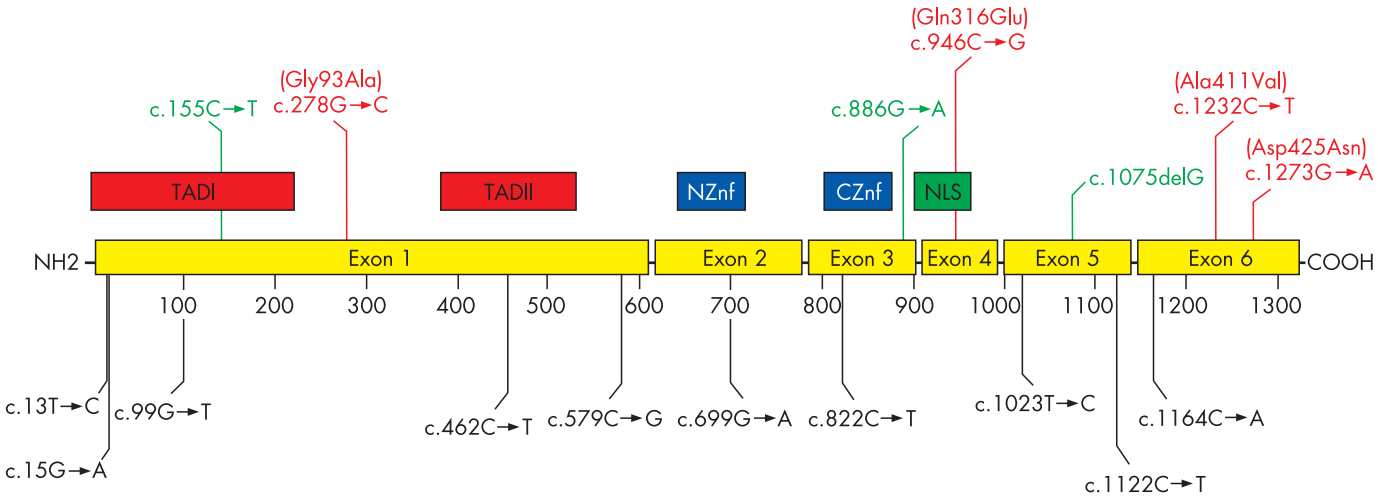

Figure 1 Location of GATA4 sequence variants identified in study cohort. Schema of GATA4 with exons (n = 6) and functional domains. Red, non-synonymous sequence variants; black, synonymous sequence variants; green, previously published sequence variants that associate with familial congenital heart disease (CHD).[10–13] Sequence variants are listed by nucleotide changes (amino acid change in parentheses). CZnf, C-terminal zinc finger; NLS, nuclear localisation signal; NZnf, N-terminal zinc finger; TADI, transcriptional activation domain 1; TADII, transcriptional activation domain 2.

Table 3 Non-synonymous sequence variants

Nucleotide	Amino acid	Obs (n)	Patient	Family history of CHD	Mother	Father	Conserved in mouse and rat	SIFT	Poly-Phen	Other congenital anomalies
c.278G→C	Gly93Ala	1	2° ASD	No	Carrier	NA	Yes	T	B	–
c.946C→G	Gln316Glu	1	2° ASD	Unknown	NA	NA	Yes	NT	PD	+
c.1232C→T*	Ala411Val	1	VSD	No	NA	NA	Yes	T	B	–
c.1273G→A	Asp425Asn	2	2° ASD	No	Carrier	No	Yes	NT	PD	–
			TOF	Possible†	NA	NA				–

2° ASD, secundum atreial septal defect; AA, amino acid; B, benign; NA, not available; NT, not tolerated; Obs, observations; PD, possibly damaging; PolyPhen, prediction of functional effect of human non-synonymous single nucleotide polymorphisms; SIFT, Sorting Intolerant From Tolerant; T, tolerated; TOF, tetralogy of Fallot with pulmonary valve stenosis; VSD, perimembranous ventral septal defect.
*Previously observed in a patient with cardiac hypertrophy.[20]
†See Results section for details.

Table 4 Synonymous sequence variants

Nucleotide	Amino acid	Obs (n)	Patient	Conserved in mouse and rat	ESE motif
c.13T→C	Leu5Leu	1.6	TOF/PA	Yes	Yes
c.15G→A	Leu5Leu	1	TOF/PA	Yes	No
c.99G→T	Ala33Ala	1	TA	Yes	Yes
c.462C→T	Phe154Phe	2	2° ASD TOF	Yes	Yes
c.579C→G	Pro193Pro	1	SV ASD	Yes	Yes
c.699G→A*	Thr233Thr	4	TOF/PA VSD TOF VSD	Yes	Yes
c.822C→T*	Cys274Cys	3	TOF/PA DORV VSD	Yes	Yes
c.1023T→C	Pro341Pro	3	TOF DORV TOF	Yes	Yes
c.1122C→T	Tyr374Tyr	1	2° ASD	Yes	Yes
c.1164G→A	Ala388Ala	1	VSD	No	No

2° ASD, secundum atrial septal defect; DORV, double-outlet right ventricle; ESE, exonic splice enhancer; Obs, observations; SV ASD, sinus venosus atrial septal defect; TOF/PA, tetralogy of Fallot with pulmonary valve atresia.
*Previously observed in patients with cardiac hypertrophy.[20]

Translationally silent alterations that change exonic splice enhancer (ESE) motifs have recently come under scrutiny because these sequence alterations can cause exon skipping. The list of "silent mutations" with confirmed association to genetic diseases is growing.[25] Eight of the synonymous *GATA4* sequence variants map into ESE motifs.[26]

Control subjects

Five sequence variants were observed both in controls and subjects (Asn352Asn (c.1056C→T), Ser371Ser (c.1113A→G), Ser377Gly (c.1129A→G), Val380Met (c.1138G→A), Pro407Pro (c.1221A→C). Three of these five sequence variants are listed in database SNP lists (Asn352Asn as rs3729855, Ser377Gly as rs3729856, Pro407Pro as rs7830178).

DISCUSSION

This study sought to investigate the frequency with which *GATA4* sequence variants occur in a large population with a variety of predominantly sporadic congenital heart disease. Despite reports of *GATA4* mutations in some familial cases of septal defects, our data indicate that non-synonymous sequence variants of *GATA4* occur in only a small percentage of overall cases with septal anomalies and rarely in patients with conotruncal defects (table 5). Two of three parents available for testing were found to carry the same sequence variant as the proband even though the same changes were not seen in control subjects. These individuals were reportedly unaffected, although this was not confirmed by echocardiogram. This finding is consistent with incomplete penetrance, a phenomenon commonly observed in complex traits and other genetic disorders characterised in part by CHD. Whether these particular missense sequence variants are disease-causing, or increase the susceptibility to disease, or are even unrelated to the risk of disease is debatable in the absence of supportive functional data.

Two of the four missense alterations (Gln316Glu and Asp425Asn) are predicted by SIFT[21 22] and PolyPhen[23 24] to affect protein function and hence, potentially alter phenotype. Gln316Glu maps to the nuclear localisation signal region of *GATA4*, which is important for DNA binding affinity and the transactivation of downstream targets. It is possible that all four missense alterations may be disease-related, but the functional significance is difficult to demonstrate. Functional studies can help define the significance of these variants, but are themselves limited by the assay used, given the variable location of the changes in the coding region of the gene.

Table 5 Frequency of *GATA4* sequence variants in 628 patients with CHD

Cardiac defect	Non-synonymous variants		Synonymous variants	
	Patients (n)	%	Patients (n)	%
ASD	2/122	1.6	3/122	2.5
VSD	2/137	1.5	4/137	2.9
TOF	1/201	0.5	8/201	4
D-TGA	0/76	0	0/76	0
DORV	0/45	0	2/45	4.4
TA	0/20	0	1/20	5
IAA	0/11	0	0/11	0
L-TGA	0/10	0	0/10	0
Other	0/6	0	0/6	0
Total	5/628	0.8	18/628	2.9

ASD, atrial septal defects; DORV, double-outlet right ventricle; D-TGA, D-transposition of the great arteries; IAA, interrupted aortic arch; L-TGA, L-transposition of the great arteries; TA, truncus arteriosus; TOF, tetralogy of Fallot; VSD, ventricular septal defects.

A surprising number of non-synonymous sequence variants was found in our study cohort, but it is unclear whether these sequence variants are disease-related. It was previously assumed that translationally silent sequence alterations would not have a phenotypic effect; however, there is increasing evidence that many human disease genes harbour exonic mutations, including silent mutations, that affect pre-mRNA splicing.[25–27] It is possible that silent *GATA4* sequence variants alter gene function by modifying ESE or exonic splicing silencing sites. A recent query on AceView (National Center for Biotechnology Information) found that numerous isoforms of *GATA4* are expressed in humans, although at present it appears that there is only one primary isoform in the heart. The production of several isoforms from the same transcriptional unit by various types of alternative splicing is common, occurring in 40–60% of all genes,[28 29] and balancing these heterogeneous isoforms appears to have an important role in normal cell regulation. Other genes involved during fetal development appear to be very sensitive to changes in isoform balance. For instance, the alternative splicing of the *WT1* gene, a transcription factor involved in growth and cell differentiation, generates four different isoforms.[30 31] It was recently shown that environmental activation of the aryl hydrocarbon receptor pathway disrupts the heterogeneous balance of *WT1* isoforms, and that this balance is extremely sensitive and necessary for correct nephrogenesis.[32] It is possible that *GATA4* is similarly sensitive to changes in isoform expression. In fact, animal data indicate that multiple aspects of cardiac morphogenesis and function are exquisitely sensitive to very small changes in *GATA4* expression levels, and it has been hypothesised that altered *GATA4* activity may contribute to CHD through both genetic and environmental factors.[33 34] Another possibility is that synonymous sequence variants may alter the translational kinetics of mRNA, affecting final protein conformation, as was recently shown to occur with a translationally silent mutation in the multidrug resistance 1 (*MDR1*) gene.[35 36] If some of the synonymous *GATA4* sequence variants found in patients with conotruncal defects are functionally significant, then *GATA4* alterations may make a greater contribution to the risk of conotruncal defects than is otherwise suggested by the notable rarity of non-synonymous variants in this subset of patients.

Sequence variants of *GATA4*, synonymous or otherwise, were not identified in subjects with D-TGA. Although the number of subjects is relatively small, this study supports previous observations that D-TGA does not necessarily share a common genetic aetiology with other conotruncal defects or isolated perimembranous VSDs. For example, a 22q11 deletion is commonly identified in patients with tetralogy of Fallot, truncus arteriosus, interrupted aortic arch and perimembranous ventricular septal defects, but rarely identified in patients with D-TGA.[37] Likewise, mutations of *NKX2.5*, a molecular partner of *GATA4*, were found in a subset of patients with conotruncal or ventricular septal defects but not in the cohort with D-TGA.[8]

In conclusion, four potentially disease-related, missense *GATA4* sequence variants were found in five patients with ASD, VSD, and TOF, which were not found in control subjects. Additional synonymous sequence variants were found in subjects with both septal and conotruncal defects (excluding those with D-TGA). These data suggest that *GATA4* sequence variants may be aetiologically important in a small subset of patients with septal defects and an even smaller subset of patients with conotruncal defects. These findings further underline the marked heterogeneity and aetiological complexity of congenital heart disease.

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