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ORIGINAL ARTICLE

WILEY Congenital Heart Disease

Novel mutation of *GATA4* gene in Kurdish population of Iran with nonsyndromic congenital heart septals defects

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Abstract

Background: The mutations in *GATA4* gene induce inherited atrial and ventricular septation defects, which is the most frequent forms of congenital heart defects (CHDs) constituting about half of all cases.

Method: We have performed High resolution melting (HRM) mutation scanning of *GATA4* coding exons of nonsyndrome 100 patients as a case group including 39 atrial septal defects (ASD), 57 ventricular septal defects (VSD) and four patients with both above defects and 50 healthy individuals as a control group. Our samples are categorized according to their HRM graph. The genome sequencing has been done for 15 control samples and 25 samples of patients whose HRM analysis were similar to healthy subjects for each exon. The PolyPhen-2 and MUpro have been used to determine the causative possibility and structural stability prediction of *GATA4* sequence variation.

Results: The HRM curve analysis exhibit that 21 patients and 3 normal samples have deviated curves for *GATA4* coding exons. Sequencing analysis has revealed 12 nonsynonymous mutations while all of them resulted in stability structure of protein 10 of them are pathogenic and 2 of them are benign. Also we found two nucleotide deletions which one of them was novel and one new indel mutation resulting in frame shift mutation, and 4 synonymous variations or polymorphism in 6 of patients and 3 of normal individuals. Six or about 50% of these nonsynonymous mutations have not been previously reported.

Conclusion: Our results show that there is a spectrum of *GATA4* mutations resulting in septal defects.

KEYWORDS
GATA4, high resolution melt, nonsyndromic ASD and VSD

1 | INTRODUCTION

Congenital heart defects (CHDs) with prevalence of 0.7% in Europe and 0.93% in Asia in newborns disturb most parts of the heart.[1–3] The clinical features of CHDs can be divided into three broad categories: cyanotic heart diseases, left-sided obstruction defects, and septation defects.[2] The septation defects, involves atrial and ventricular septal defects (ASD and VSD) or formation of structures in the central part of the heart (atrioventricular septal defects, AVSDs), may occur as an isolated defect or in combination with other cardiac malformations, such as ASD and VSD are the most common types of CHDs and account about 50% of all cases of CHDs.[4,5]

VSDs, which are classified according their location are foramens in the ventricular septum.These defects can be divided into four anatomical components including membranous VSD, muscular VSD, atrioventricular canal type VSD, and conical septal VSD.[6] There are three major types of ASDs or interatrial communications including ostium primum, sinus venosus defects, and ostium secundum which is the most common type of atrial septal defect.[7]

The atrial and ventricular septal defects are multifactorial especially genetic and nongenetic factors play important roles.[4,8–10] It has been suggested that periconceptional multivitamin or folic acid intake may reduce the risk of cardiac disease in the fetus; however, maternal illnesses, maternal therapeutic, nontherapeutic drug exposures, and environmental exposures are some factors that can increase the risk.[11] Etiologies of single-gene mutations for ASD and VSD have been principally discovered by studying large number of families with autosomal dominant patterns of septal defects using traditional linkage analysis methods.[12,13] The first genetic etiology for ASD discovered by finding mutations in *TBX5* transcription factor that is a leading causes of septation defects in the setting of Holt-Oram syndrome, which is characterized by cardiac and upper limb malformations.[14] The *GATA4* gene was first discovered as a genetic cause of cardiac septal defect in 2003.[15] The GATA family of zinc finger transcription factors plays a central role in vertebrate heart formation.[12,16,17] The *GATA4*, 5 and 6 genes are being expressed in development of cardiomyocytes and their mutations are associated with congenital heart disease.[18–21] The GATA transcription factors bind to upstream region of heart gene promoters to directly regulate them.[22] Congenital mutations in *GATA4* are associated with human atrial and ventricular septal defects.[19,23–28]

High resolution melting (HRM) is a post PCR method that is used to detect any small variation in sequence of genes. The HRM stem from PCR melting dissociation curve techniques. This method can discriminate the sequence of genes in varying ways, such as GC content, length or complementarity of strands.[29] This method is an in vitro technique and requires the addition of a saturating intercalating dye in the PCR mix, in addition, a HRM step after PCR. The technique has already been employed to scan for somatic mutations in the *KIT*, *BRAF*, *EGFR*, *ERBB2*, *TP53* and *KRAS* genes.[30–35] The causes of CHDs in the most cases are unknown, but the molecular evolution and gene analysis of heart have led to identification of some mutations associated with the CHDs. In our previous study in Kurdish patients with atrial and ventricular wall defects referred to Sanandaj and Kermanshah Hospitals, was detected no mutation in *NKX2.5* gene and 3 mutations in *TBX5* gene.[36]

The purpose of this study is to determine the role of *GATA4* mutations in Kurdish patients with nonsyndrome atrial and ventricular wall defects as well as investigation of their genotype-phenotype correlation by HRM referred to Sanandaj and Kermanshah Hospitals.

2 | MATERIALS AND METHODS

2.1 | Study subjects

The target Kurdish population consisted of 50 healthy controls and 100 cases (62 females and 38 male) of nonsyndromic patients including 57 patients with VSD (42 membranous, 14 muscular, 1 Conal septal), 39 patients with ASD (36 ostium secundum, 2 sinus venosus, 1 without information) and four patients possessing AVSD (atrioventricular) both defects from Kermanshah Imam Ali Cardiovascular Hospital and Sanandaj Tohid Hospital. Regarding parental relationship of patients, 38 of them have parents who are relative. However, the rest of patients have parents who are not relative (Table 4).

2.2 | DNA extraction

Genomic DNA was extracted from whole blood by *AccuPrep* Genomic DNA Extraction Kit (Bioneer Corporation, Seoul, South Korea).

2.3 | Real time PCR and HRM assay

The human *GATA4* gene (NM_002052) consists of 7 exon, 6 coding exons and 6 introns introns that encode a 442 amino acid protein. All coding exons and the intron-exon flanking regions of The *GATA4* were amplified by real-time polymerase chain reaction (RT-PCR) for a mutation screening. The primers were designed by CLC main workbench 5 for the coding exons of *GATA4* gene (Table 1) and were synthesized by TIB MOLBIOL Berlin, Germany). The final primer sequences are listed in Table 1. PCR cycling and HRM analysis were performed on the Rotor-Gene 6000 (Corbett Research, Sydney, Australia). PCR was performed in 20 μL reaction tube containing 20 ng of template DNA, 1.5 mM MgCl2, 250 μM dNTP, 10 μM of forward and reverse primers, 1X LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA), 2 U/μL of pfu Taq Polymerase, and 1× PCR buffer supplied by the manufacturer Promega (Madison, Wisconsin, USA). The amplification was initiated at 95°C for 5 min then followed by 45 cycles of 3 steps consisting of 95°C for 10 s, 60°C for 30 s and 72° for 15 s, and a HRM step from 72 to 95°C rising at 0.1°C per second.

TABLE 1 *GATA4* sequencing primers and product size

Fragments of exon		*Sequence*		
Fragment	**Coding exon**	**PCR product**	**Forward**	**Reverse**
Fragment 1	EXON 2A	382bp	TGTTGCCGTCGTTTTCTCTC	GTGTAAGCGGCTCCGTCG
Fragment 2	EXON 2B	431bp	CGACGGAGCCGCTTACAC	CGAAGGCGTTGGTGGAAAAA
Fragment 3	EXON3	316bp	ATCCTCTGTGTCTTTTCTTGTC	TCCCCGATGCACATCCCTCA
Fragment 4	EXON4	257bp	GGGCAGTGCACACCTTTTA	ACAAAGGAAGAAGACAAGGG
Fragment 5	EXON5	216bp	GACTACGCAGAAATGGAA	CTAACCCGGAAGATATGA
Fragment 6	EXON 6	352bp	TGTTCGTTTGTCCCTGCCG	TCAATGGCTGGGTCTTCCTA
Fragment 7	EXON[a] 7	270bp	TCATCGTGTGCTTTCTGCT	TCCTTCTTTGCTATCCTCC

[a]Coding region.

2.4 | Sequencing analysis

The PCR products of patient samples at this stage were sequenced by ABI 3730XL DNA Analyzers in Bioneer Corporation. The results were analyzed using CLC main workbench 5.

2.5 | Structural stability prediction for sequence variation

Prediction of protein stability changes resulting from single amino acid mutations was performed online in MUpro site http://www.ics.uci.edu/~baldig/mutation.html using support vector machines (SVM) and neural network methods.[37,38]

2.6 | Prediction of the causative possibility of *GATA4* sequence variation

The pathogenic potential of a *GATA4* sequence variation was anticipated by the online program PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), automatically giving a probability scores for each modification to be either pathogenic or benign (Table 2).

2.7 | Statistical analysis

Comparison of the categorical variables was made using Pearson's chi-square test or Fisher's exact test when appropriate. A two-tailed *P* value $< .05$ indicated significant differences.

3 | RESULTS

3.1 | HRM assay

The coding sequences of *GATA4* gene for all samples were initially screened using HRM analysis to find out if there is any difference with normal status. The results of HRM analyses of *GATA4* exons are illustrated in Figures 1–4 illustrated both normalized and difference plots of the results of PCR for exons 4 to 7 compared to that of the wild-type. The sensitivity, which is defined as increased degree at each step of proliferation and specificity, which is the confidence percentage of HRM for sequence variant were 0.1 and 0.78, respectively. The *GATA4* HRM curve assays yielded 24 HRM-positive samples including 21 of patients and 3 of normal samples with deviated curves for *GATA4* coding exons that displayed aberrant HRM curves.

3.2 | Sequencing analysis

Sequencing analysis of samples with deviated HRM curves have revealed that all of patients had heterozygous changes. The detected mutations included 15 modification consisted of 12 nonsynonymous variations, a nucleotide deletion and an indel mutation, both resulted in frame shifts (Table 2). Four synonymous mutations were also detected in 6 of patient and 3 of normal samples (Table 3). 6 of these nonsynonymous mutations have not been previously reported, sequencing result of exon 6 was shown in Figures 5–8 as sample.

3.3 | Nonsynonymous sequence variants

15 sequence variants conferring a change in the encoded amino acids were identified in 15 patients (Table 2), which were not seen in 50 control subjects.

Ten of the sixteen patients with nonsynonymous sequence variants had ventricular septal defects (VSD); two of them had AVSD and three had ASD (Table 3). Four of these variants, was in exon 3 include 196G > A, 526G > C, 350T > G, 278G > C that resulted in amino acid change at Ala 66 Thr, Ala176 Pro in patients with VSD- Patent Ducts Arteriosus and VSD and Leu 117Arg, Gly93Ala in patients ASD.

Three of these variants, were in exon 4 include 766A > G, 674C > G, that resulted in amino acid changes at Lys 256 Glu, Thr 225 Ser in patients with VSD and 717 del C, that result in frame shift and termination [FSM &T] after 8 amino acids at position 246 (nonsense mutation)

Two of these variants, were in exon 5 include 818A > G, 881C > T, that resulted in amino acid change at Asn273Ser, Ala294Val in patients with AVSD.

Three of these variants, were in exon 6 include 958C > T, 983C > G that resulted in amino acid changes at Arg320Trp, Ser328 Cys, respectively, in patients with VSD and an indel mutation AAC > TTTTT that resulted in frame shift and termination after 8 amino acids (nonsense mutation) at position 327 in patient with small muscular VSDs and mild pulmonary valve stenosis (VSD, PS)

Two of these variants, were in exon7 include 1079A > G that resulted in amino acid change at Glu 360 Gly in patients with VSD and deletion mutation 1074 delC that result in frame shift and termination after 45 amino acid (nonsense mutation) at position 403 in patient with ASD

One of these variants, were in exon7 include 1325C > T that resulted in amino acid change at Ala 442 Val in patients with VSD.

Two subjects had additional cardiac defects including small muscular VSDs and mild pulmonary valve stenosis (AAAC > TTTTT) and second including medium VSD and patent ductus arteriosus (Ala66Thr). Two subjects had AVSD. The other subjects had no other reported malformations or medical issues. All of these alterations occurred within a conserved amino acid.

3.4 | Synonymous sequence variants

Four synonymous sequence variants were observed in 4 subjects, which were seen in 3 of 50 control subjects. One of these sequence variants (1221A > G) has been previously reported in patients with VSD but 3 sequence variants have not been previously reported in patients with ASD and VSD (Table 3).

3.5 | Prediction of change amino acid on protein

Accurate prediction of protein stability changes resulting from single amino acid mutations is important for understanding protein Function and structures in healthy and patient's subjects. The bioinformatics investigation showed decrease of stability in 8 samples by VSM method and neural network, decrease of stability by VSM and increase

TABLE 2 GATA4 nonsynonymous variations identified in 57 patients with VSD, 39 patients with ASD and 50 healthy control

Number Location	1 Exon[a]2	2 Exon 2	3 Exon 2	4 Exon 2	5 Exon 3	6 Exon 3	7 Exon 3	8 Exon 4	9 Exon 4	10 Exon 5	11 Exon 5	12 Exon 5	13 Exon 6	14 Exon 6	15 Exon 7
Nucleotide change in CDS	196G>A	350T>G	278G>C	526G>C	766A>G	717 delC	674C>G	881C>T	818A>G	983C>G	958C>T	AAAC> TTTTT	1079A>G	1074delC	1325C>T
Amino acid change	Ala66Thr	Leu117Arg	Gly93Ala	Ala176Pro	Lys256Glu	FSM & T	Thr225Ser	Ala294Val	Asn273Ser	Ser328Cys	Arg320Trp	FSM &T 955–958, 959+T	Glu360Gly	FSM &T	Ala442 Val
Type of cardiac defects	VSD^{M} PDA	ASD^{O}	ASD^{O}	VSD^{M}	ASD^{O}	VSD^{M}	VSD^{M}	AVSD^{A}	AVSD^{A}	VSD^{M}	VSD^{M}	VSD^{Mu}, PS	VSD^{Mu}	ASD^{o}	VSDM
Parents carriers	NO	Mat	Mat	Pat	Pat	De novo	No	Mat	NO	Mat	Mat	denov	Pat	Mat	no
Mutation novel/ report	[Zhang et al. 2008]	Novel	[Tomita-Mitchell et al. 2007]	Novel	Novel	Novel	Novel	[Reamon-Buettner and Borlak 2005]	[Reamon-Buettner and Borlak 2005]	Novel	Novel	Novel	[Wang et al. 2013]	[Chen et al. 2010]	[Zhang et al. 2008]
Stability by SVM	Decrease	Decrease	Decrease	Decrease	Increase	Truncated protein	Decrease	Decrease	Decrease	Decrease	Decrease	Truncated protein	Decrease	Truncated protein	Increase
Neural network methods	Decrease	Increase	Decrease	Increase	Decrease		Decrease	Decrease	Decrease	Decrease	Decrease		Decrease		Increase
Polyphen-2	Benign: score: 0.000 (Sen: 1.00; Spe: 0.00)	Possibly damaging score 0.586 (Sen: 0.88; Spe: 0.91)	Possibly damaging score: 0.943 (Sen: 0.80; Spe.: 0.95)	Benign score: 0.026 (Sen: 0.95; Spe: 0.81)	Possibly damaging score: 0.894 (Sen: 0.82; Spe: 0.94)		Probably damaging score: 1.00 (Sen: 0.00; Spe: 1.00)	Probably damaging score:1.00 (Sen: .00; Spe: 1.00)	Probably damaging score 1.000 (Sen: 0.00; Spe: 1.00)	Possibly damaging: score: 0.915 (Sen: 0.81; Spe: 0.94)	Probably damaging score: 1.000 (Sen: 0.00; Spe: 1.00)		Probably damaging score:0.985 (Sen: 0.74; Sep: 0.96)		Probably damaging: score 0.99 (Sen: 0.14 Spe: 0.99)

[a]Frame shift mutation & termination.

Abbreviations: FSM & T, frame shift mutation and termination; M, membranous; Mat, maternal; Mu, muscular; O, ostium secundum; Pat, paternal; Sen, sensitivity; Spe, specificity.

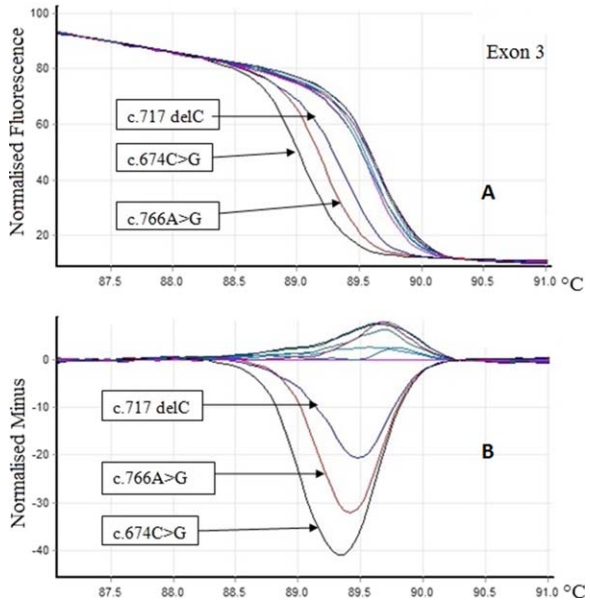

FIGURE 1 (A) Normalized plots for exon 3. (B) Difference plots for variation of exon 3. The three below curves in (B) show the differences between patients and normal cases

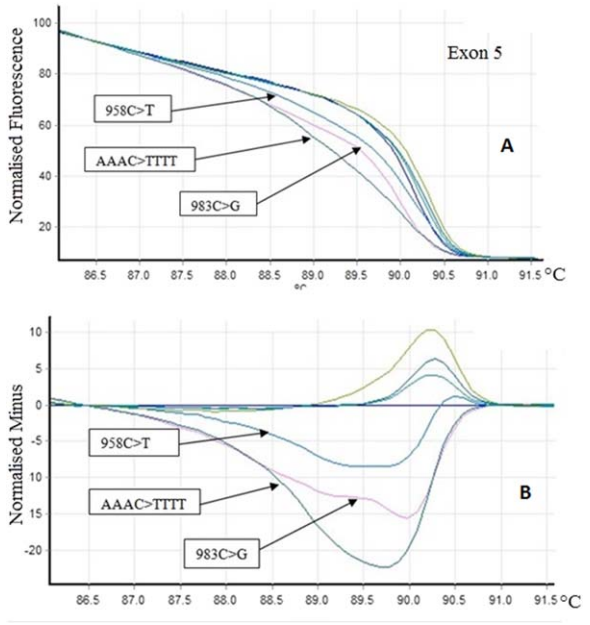

FIGURE 3 (A) Normalized plots for exon 5. (B) Difference plots for variation of exon 5. The three below curves in (B) show the differences between patients and normal cases

by neural network in 2 samples, increase by VSM and decrease by neural network and increase of stability one sample with both of these methods (Table 2).

3.6 | Pathogenic potential of *GATA4* sequence variation

The GATA4 amino acid substitutions were predicted to be possibly damaging by Poly-Phen-2, with a score including sensitivity; specificity

suggesting that mutated GATA4 might contribute to the development of congenital septal heart defects in this patients (Table 2).

3.7 | Statistical analysis result

We analyzed statistically a sample of 100 patients with nonsyndrome congenital heart septal defect to compare the clinical characteristics and dependence of some factors, although there was no statistically

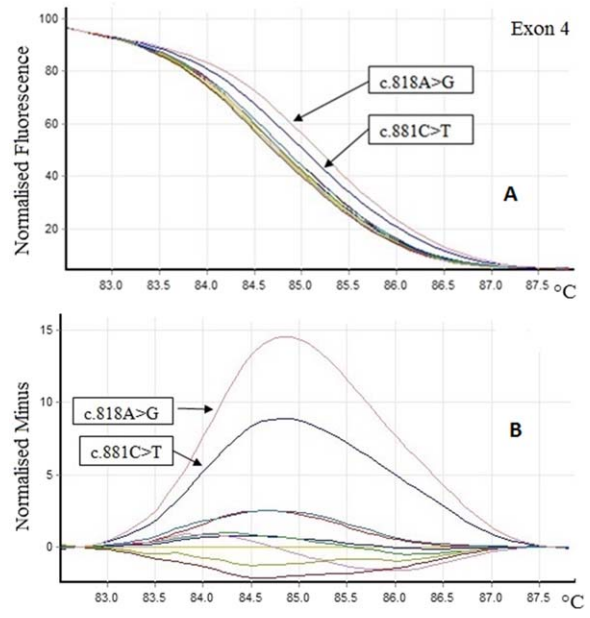

FIGURE 2 (A) Normalized plots for exon 4. (B) Difference plots for variation of exon 4. The two up curves in (B) show the differences between patients and normal cases

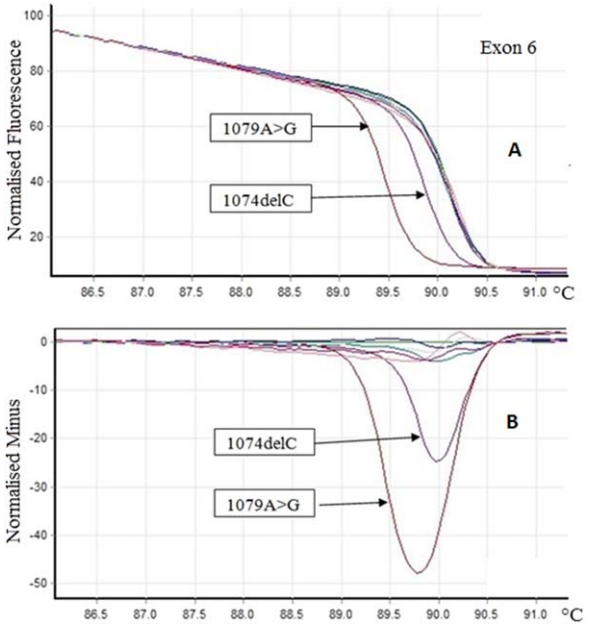

FIGURE 4 (A) Normalized plots for exon 6. (B) Difference plots for variation of exon 6. The two below curves in (B) show the differences between patients and normal cases

TABLE 3 GATA4 synonymous variations identified in 57 patients with VSD, 39 patients with ASD and 50 healthy control

Location	Polymorphism	Amino acid	Cardiac defects	Number of patient	Parents	Normal people[a]	Mutation novel/report
E3	657C>T	Asn219Asn	VSD^{Mu}	1	Normal	1	Novel
E2	93C>G	Gly31Gly	ASD^{O}	2	Mother	0	Novel
E4	807 C>T	Leu 269 Leu	VSD^{Mu}	2	Both parents	1	Novel
E7	1221A>G	Pro407pro	VSD^{M}	1	No information	1	[Tomita-Mitchell et al. 2007]

Abbreviations: M, membranous; Mu, muscular; O, ostium secundum.
[a]All of parents were normal.

significant difference in any of the comparisons. Clinical characteristics of patients are summarized in Table 4.

4 | DISCUSSION

The *GATA4* gene is located on chromosome 8p23.1-p22, and consists of six coding exons and codes for a zinc finger transcription factor (NM_002052). It is an essential transcription factor for mammalian cardiac development and crucial for survival of the embryo. It acts in combination with other cardiac transcription factors such as Nkx2–5 and Tbx5, as well It is expressed in both fetus and mature cardiomyocytes where it acts as a transcriptional regulator for various cardiac genes, and also adjusts development of the heart.[39] Variety of mutations in this gene have been discovered in many cardiac defects including abnormal ventral folding, and defects in the cardiac septum separating the atria and ventricles, and hypoplasia of the ventricular myocardium.[40,41] We have found 15 heterozygous *GATA4* mutations in a nonesyndrome ASD and VSD of 100 Kurdish patients and 50 healthy controls.

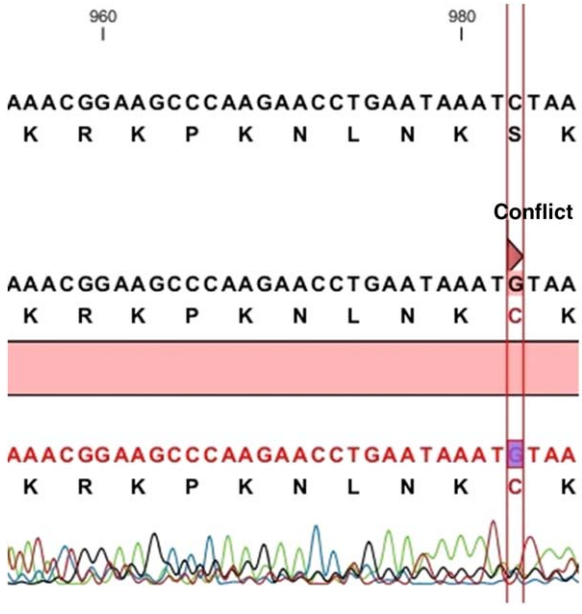

FIGURE 5 Sequencing show 983C > G mutation of exon 5 in patient with VSD that result in Ser328Cys

From these 15 mutations, 6 of them have not been previously reported and for the first time are reported in this study. The prevalent frequency of *GATA4* nonsynonymous mutations specifically identified in ASD and VSD patients in our study was approximately 15% (15/100). This prevalence was including 4% in ASD and 9%VSD and 2% AVSD. This was the first mutation scanning of *GATA4* gene in nonsyndromic atrial septal defect and ventricular septal defect in Iran and among Kurdish population.

These results are consistent with the *GATA4* mutation prevalence found in an earlier study on Germany among 68 patients with ASD, VSD and AVSD subtype of CHD that showed 23 mutation type, and studies of mix population of America by Schluterman 21 patients (13.3%) in 157.[42,43] The prevalence of *GATA4* mutation in Kurdish patients is noticeably different from studies in China, and American patients. For the 628 American patients 0.8% *GATA4* mutation prevalence was discovered and 2.1% in 384 and 2.5% on 486 Chinese CHD subjects' studies with different types of CHD and No pathogenic mutations were found among Danish patients. These mutations occurred in subgroup of CHD including VSD and ASD in mentioned studies.[24,25,44–46] This study limited CHD to nonsyndrome ASD and VSD in Kurdish population with nonrelative marriage to identify mutation spectrum of *GATA4* gene in Kurdish population, but due to Ancestral relative marriage costume between this population, the frequency of mutation of this gene may have been increased. These results suggest that *GATA4* nonsynonymous mutations contribute to ASD and VSD differently between various ethnic populations as we did not detect any mutation in *NKX2.5* genes in these patients in our previous study and 3 mutations in *TBX5*.[36] Therefore, it can be estimated that relative and ethnic marriages play a role in frequency of *GATA4* mutations.

GATA4 is a transcription factor which is conserved evolutionary and plays a vital role in normal heart development, in this study the various mutations were detected to show that *GATA4* is a hyper mutable protein in ASD, VSD, and AVSD patients. These findings seem to be in contrast in comparison with previously published data suggesting that *GATA4* is a hypo mutable protein, but consistent with them that indicated mutation spectrum is dispersive.[25,44–46]

Until now, reported nonsynonymous mutations in the GATA4 protein are more than hundreds, which one third of them are located in highly evolutionarily conserved regions and some mutation are found in well conserved zinc finger domains.[45] Mutation detection rate of

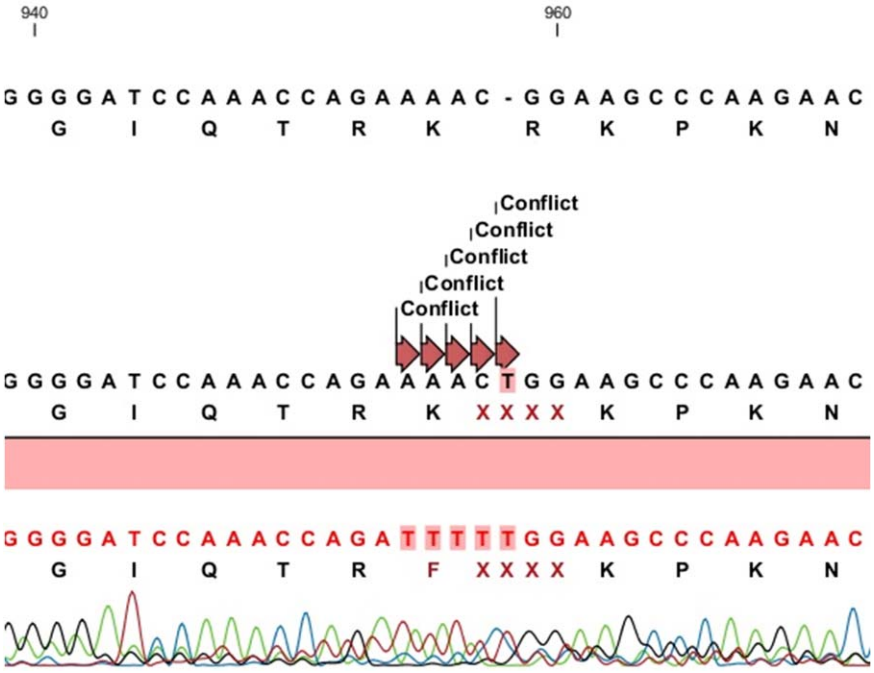

FIGURE 6 Sequencing show indel mutation (AAAC > TTTTT) of exon 5 in patient with VSD and PS that result in nonsense mutation

GATA4 was various from range 0–12.5 percent depending on ethnic population[28,45,47] and our detection rate 15% (15/100) was more than this range because we limited our study in Kurdish population and subset group of congenital heart disease including VSD and ASD of those results. All of these findings suggest that *GATA4* mutations are frequently detected in ASD and VSD. In this study, we detect mutations which decrease stability of protein and some of them increase stability of protein while both type of mutation result in defecting in development of heart. Although according to bioinformatics assays(Stability by SVM and neural network methods) of this study, for example the A442V mutation increase protein stability of GATA4, polyphen-2 program predicted this mutation as a destructive mutations and also by immunostaining(real functional study) results by Wang study showed that wild-type GATA4 localized to the nucleus, while the GATA4 mutants A442V were partially detected in the cytoplasm, which suggests that these mutants do not properly localize to the nucleus.[45] The different form or misfolding of these mutants may limit GATA4 protein transportation. These mutations are not situated in the previously known NLS region,[24,48] but they influence the dispersion of the protein. Likewise, mutations which affect outside of the recognized NLS of TBX5 also impede nuclear localization.[49,50] Not only GATA4 mutant E360G show decrease of stability but according to Wang study it also localized in cytoplasm similarity to GATA4 mutant A442V.These outcomes recommend that the nuclear localization of these transcription elements is impacted by an extensive region of the protein that is not restricted to the traditional NLS region.[45] Also in this study The

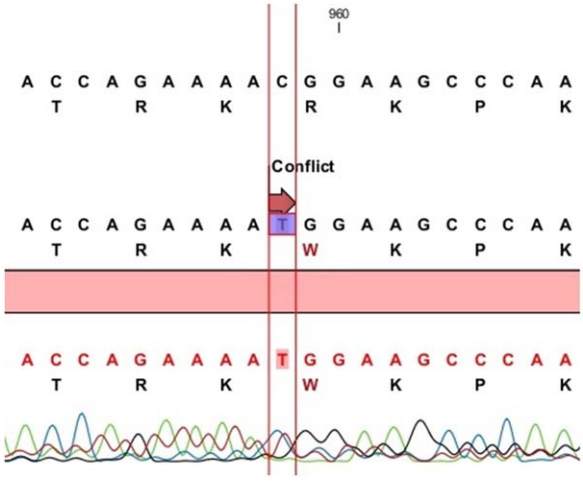

FIGURE 7 Sequencing shows 958C > T mutation of exon 5 that result in Arg320Trp in patients with VSD

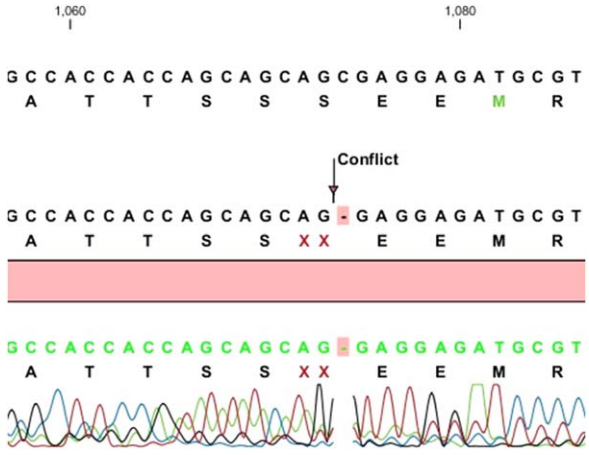

FIGURE 8 Sequencing shows del 1074 C mutation of exon 6 that result in nonsense mutation

TABLE 4 Clinical characteristics of the patients with congenital heart septal defect

	ASD N (%)	VSD N (%)	VASD N (%)	*P* value
Total	39(39)	57(57)	4(4)	
Gender				*F*: 0.391, P:.391
Male	12(12)	23(23)	2 (2)	
Female	28(27)	34(34)	2 (2)	
Family history				*F*: 0.362, *P*:.237
YES	29	43	3	
NO	2	8	1	
No information	8	6	0	
Parent consanguinity				*F*: 0.761, *P*:.810
Relative	14	22	2	
Nonrelative	25	35	2	
Parent consanguinity				*F*: 0.802, *P*:.715
	Mutation	Nonmutation		
Relative	7	31		
Nonrelative	14	48		
Size of heart septal defect				*F*: 0.689, *P*: .721
	Small	Medium	Large	
GATA 4 mutation[a]	7	6	6	
Nonmutation	16	14	25	
Subtype of heart septal defect				*F*: 0.383, *P*: .366
Total:97	AO	AS	VC	VM VU B
Female	24	2	1	19 9 2
Male	12	0	0	21 5 2
Subtype of heart septal defect				*F*: 0.566, *P*:.599
	AO	AS	VC	VM VU B
GATA 4 Mutation[a]	6	0	0	9 4 2
Nonmutation	30	2	1	31 10 2

Abbreviations: AO, ASD ostium secundum; AS, ASD sinus venosus; VC, VSD conal septal; VM, VSD memebranous; VU, VSD muscular; B, both of VSD and ASD.
[a]Synonymous and nonsynonymous mutation.

bioinformatics investigation by both method VSM and neural network, showed decrease of stability in A66T mutation while the polyohen-2 predicted it as benign and on the another hand. In study that carried out by Wang the in vitro luciferase assay revealed that this mutant increase transcriptional activity, but its ability could not be explained by DNA binding affinity.

The bioinformatics investigation by both method VSM and neural network, showed decrease of stability in Gly93Ala, Asn273Ser, Ala294Val, mutants and new GATA4 mutants Gly93Ala, Thr225Ser, Ser328Cys,Arg320Trp that were detected in this study and the polyohen-2 predicted it as Pathogenic potential mutants (Table 2)

VSM show decrease of stability new GATA4 mutants Leu117Arg, Ala176Pro while neural network shows increase of stability in these mutants and polyphen-2 program project them as ruin and benign mutation, respectively. Also VSM show increase stability in new GATA4 mutants Lys256Glu and neural network show decrease of stability while it was predicted as pathogenic mutation by polyphen-2.

These findings suggest that heterogeneity of *GATA4* mutation, and although these mutations can increase or decrease stability of protein and predict the pathogenic potential by bioinformatics software, real functional study each mutation is necessary because they can affect localization and conformation or misfolding them that can result in disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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