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Genetic Analysis of Congenital Heart Disease in Iranian Pediatric Patients

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ABSTRACT

Background: Congenital Heart Disease (CHD) occurs in nearly 1% of newborns due to genetic and environmental factors. There are many genes involved in CHD. Variants of Gap Junction Protein Alpha 1 (GJA1), Zic Family Member 3 (ZIC3), Nodal Growth Differentiation Factor (NODAL), and Forkhead Box H1 (FOXH1) genes are common to develop CHD.

Objectives: To date, no study has been published about CHD patients in Iran. Therefore, the present study aimed to evaluate the sequence variants of these genes in Iranian patients.

Methods: This study was conducted on 73 patients with familial CHD and their family members. Genetic investigations were performed using Polymerase Chain Reaction (PCR) and direct DNA sequencing of the exons and flanking regions of the genes. The variants were evaluated using available online software tools. Mutation taster, PROVEAN, SIFT, PolyPhen-2, and CADD were used to predict the effects of the variants and I-TASSER was applied to evaluate the possible structural effects of the genetic variations.

Results: c.612G > A, c.717G > A, and c.895C > T in GJA1 were found in the study participants. c.1248T > G in the ZIC3 was observed in a twin with CHD. Besides, c.193 + 12C > T, c.-109T > C, c.494A > G, c.417C > T, and c.357C > T variants were detected in the NODAL gene. Additionally, c.-314T > G, c.175-30C > T, and c.373A > T sequence changes were determined in the FOXH1 gene. Two novel heterozygous variants, namely c.1061C > G and c.-465C > A, were also found in the FOXH1 gene. Bioinformatics analysis indicated that the detected reported/novel variants might not have a damaging effect among Iranian CHD patients.

Conclusion: The study results indicated the first variation screening of GJA1, ZIC3, NODAL, and FOXH1 genes in Iranian familial CHD patients. The results also suggested a minor role for GJA1, ZIC3, NODAL, and FOXH1 genes in familial CHD pathogenesis. However, their exact role in CHD causation entails further research.

1. Background

Congenital Heart Diseases (CHDs), defined as defects in heart structure and vessels (1), are present in about 9 per 1000 Asian newborns (2). Heart malformations vary broadly and consist of venous defects (both pulmonary and systemic), septal anomalies, Transposition of the Great Arteries (TGA), occlusion of left and right outflow tracts, and Double Outlet Right Ventricle (DORV). CHD is usually diagnosed during childhood (0 - 15 years) and is sometimes found in adults (3). Delay in detection and referral for the needed treatments increases mortality in the affected individuals (4). Thus, early diagnosis of CHDs is critical for improving the population's healthcare (2).

CHDs are highly heterogeneous. Although the exact CHD pathogenesis is mostly unknown, genetic agents appear to play a major role in non-syndromic and syndromic forms of CHDs (5). Moreover, recent evidences have led to the

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analysis of candidate genes variations as CHD risk factors (2). Some genes, namely Gap Junction Protein Alpha 1 (GJA1) (6), Zic Family Member 3 (ZIC3) (7), Nodal Growth Differentiation Factor (NODAL) (8), and Forkhead Box H1 (FOXH1), have been reported to be related to the CHD pathogenesis (9). GJA1 gene encoding connexin 43 protein (a molecular weight mass of 43 KD) assists to progress cell to cell communication (10). This gene was mapped to 6q22 (11) and the study by Lo et al. showed its important role in CHD (12). Similarly, the results of a clinical study on GJA1 mutations in patients with hypoplastic heart syndrome (6) and visceroatrial heterotaxia (13) suggested the significant role of GJA1 in CHD.

The mutations of ZIC3 gene (Xq26) encoding a transcription factor lead to complex cardiac defects and lung, spleen, renal, and anal abnormalities (14). Recognition of loss-of-function mutations in X-linked heterotaxy proved that ZIC3 is critical for the development of left–right axis in humans (15).

NODAL is a member of the NODAL conduction pathway (10). Roessler et al. showed that defects in NODAL pathway played roles in Tetralogy Of Fallot (TOF) and, to a minor extent, DORV and TGA (16). The FOXH1 encoding a DNA binding protein conducts NODAL pathway by binding to the NODAL enhancer sequence. Mutation in FOXH1 displayed defects in axis evolution in some animal models, such as mouse, zebra fish, and schmalspur (8). In another study, some patients with CHD, mostly TOF, carried a mutation in FOXH1 (16).

2. Objectives

Due to lack of surveys on the above-mentioned candidate genes for CHD patients in Iranian families, the present study aims to investigate the mutations on GJA1, ZIC3, NODAL, and FOXH1 genes in familial CHD patients.

3. Patients and Methods

3.1. Patients

CHD patients were selected from the individuals referred to Rajaei Cardiovascular, Medical, and Research Center. According to clinical and echocardiographic evaluations by the related specialists, a total of 73 patients with familial CHD were recruited to genetic investigations during 2014-2016. This study was reviewed by the Ethics Committee of Rajaei Cardiovascular, Medical, and Research Center. Once written informed consents were received from all participants, the patients' and their parents' peripheral blood samples were collected in EDTA tubes.

3.2. DNA Extraction and Molecular Analysis

Genomic DNA was extract from the blood using the standard salt extraction method (17). The primers were designed for the coding regions and splice junctions of GJA1 (2 exons), ZIC3 (3 exons), NODAL (3 exons), and FOXH1 (3 exons) genes.

The SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, USA) was used for 25-µL PCR reactions, including 10X buffer, 1.5 mM/L MgCl2, 200 mM/L dNTP, 10 pM/L primers, 100 ng of DNA, and 1 U of Taq DNA polymerase (Amplicon, UK). The PCR procedures were as follows: 35 cycles at 95 °C for 5 minutes: denaturation at 95 °C for 40 seconds, annealing for 30 seconds, and extension at 72 °C for 30 seconds. The PCR products were evaluated by 2% agarose gel electrophoresis and Fluoro Dye Green, 6x (SMOBIO, Taiwan) staining. The primers sets were applied for sequencing on the ABI Sequencer 3500XL PE (Applied Bio Systems, US). The sequences were aligned using FinchTV 1.4.0 (www.geospiza.com/finchTV) and were visually evaluated for polymorphisms. Variants were named based on the Human Genome Variation Society (HGVS) nomenclature (18).

3.3. Bioinformatics

Mutation taster (http://www.mutationtaster.org/) (19), Protein Variation Effect Analyzer (PROVEAN) (http://provean.jcvi.org/index.php) (20), Sorting Intolerant From Tolerant (SIFT) (http://sift.bii.a-star.edu.sg/) (21), Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/) (22), and Combined Annotation Dependent Depletion (CADD) (http://cadd.gs.washington.edu/home) (23) were used to evaluate the possible effects of the missense variants on protein function and structure. Furthermore, Iterative Threading Assembly Refinement (I-TASSER) (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to evaluate the secondary structure resulting from missense sequence variants (24). Any missense variant was considered to be possibly disease-causing if even three out of the five independent prediction programs represented a high score of damaging effect. In cases with suspected variants, parents of the probands were invited to participate in the segregation study regardless of their situation as healthy or affected (Table 1).

3.4. Statistics

This study was an observational research on a highly-selected group. The numerical data have been presented as median and range.

4. Result

The 73 familial CHD patients included 53 families with multiple affected individuals in pedigree and various cardiac defects (Table 2). The patients underwent the evaluation of the coding region and exon–intron boundaries; i.e., about 100 bp upstream and downstream from the exons, of GJA1, ZIC3, NODAL, and FOXH1 genes by direct sequencing of PCR. Based on the results, three nucleotide sequence variants were identified in the GJA1 gene, one in the ZIC3 gene, five in the NODAL gene, and five in the FOXH1 gene in Iranian familial CHD patients, with the most common variant being related to GJA1 (4%) followed by ZIC3 (2.7%), NODAL (63%), and FOXH1 (57.5%). Two out of the five variants in FOXH1 had not been reported previously (Table 3).

4.1. GJA1 Variants

The sequence variants c.612G > A, c.717G > A, and c.895C > T were found in the second exon of the GJA1 gene. c.612G > A variant with no changes in amino acids (p.Thr204Thr) was detected in heterozygous form in a four-month-old boy with TOF. c.717G > A was diagnosed in homozygous form in two patients and in heterozygous

Table 1. In Silico Analysis of Missense Variants

Variant	CADD Phred *	Mutation taster	PROVEAN (Score) **	SIFT (Score) ***	Polyphen-2 (Score) ****
c.895C > T	23.4	Diseases causing	Neutral (-1.06)	Tolerated (0.06)	Probably damaging (0.99)
c.1248T > G	2.6	Diseases causing	Neutral (-0.12)	Tolerated (1.0)	Benign (0.004)
c.494A > G	0.0	Polymorphism	Neutral (-0.38)	Tolerated (0.46)	Benign (0.00)
c.373A > T	23.2	Polymorphism	Neutral (-0.79)	Deleterious (0.1)	Probably damaging (0.99)
c.1061C > G	22.9	Polymorphism	Neutral (-1.59)	Deleterious (0.01)	Benign (0.14)

* CADD, Phred ≤ 20: damaging; Phred > 20: natural

** PROVEAN, score ≤ -2.5: deleterious; score > -2.5: natural

*** SIFT, score ≤ 0.05: deleterious; score > 0.05: tolerable

**** Polyphen-2, score = 0 - 0.15: benign; score = 0.15-0.85: possibly damaging; score = 0.85 - 1: probably damaging

Table 2. The Number of Familial CHD Patients with Molecular and Clinical Futures. Some Patients Showed Concurrent Cardiac Defects or Variants

Variant	ASD	VSD	TOF	COA	PDA	TGA	PS	AVSD	Complex
GJA1	1	1	1	-	-	-	-	-	2
ZIC3	-	-	-	-	-	-	-	-	2
NODAL	3	18	9	-	-	1	1	2	16
FOXH1	5	17	9	-	2	1	-	2	17
Total patients	5	20	16	-	2	3	1	3	23

Abbreviations: ASD, atrial septal defect; VSD, ventricular septal defect; TOF, tetralogy of Fallot; CoA, Co-arctation of the aorta; PDA, patent ductus arteriosus; TGA, transposition of the great arteries; PS, pulmonary tenosis; AVSD, atrioventricular septal defect; DORV, double outlet right ventricle.

Table 3. The GJA1, ZIC3, NODAL, and FOXH1 Variants in This Study

Gene	dbSNP	Variant	Exon/ Intron	Mutation Type	Amino Acid Change	Frequency of Variant (%)				
						The present study	Iranome	ExAC	1000Genome	TOPMED
GJA1	rs766082259	c.612G > A	Exon2	Synonymous	p.Thr204 =	1.4	-	0.00	-	0.00
	rs57946868	c.717G > A	Exon2	Synonymous	p.Arg239 =	4.0	0.0175	0.0199	0.0343	0.0439
	rs748954821	c.895C> T	Exon2	Missense	p.Arg299Cys	1.4	0.0006	0.00	-	0.00
ZIC3	rs755343529	c.1248T > G	Exon3	Missense	p.Asp416Glu	2.7	0.0012	0.0001	-	-
NODAL	rs10999338	c.193 + 12C > T	Inron2	Intronic	-	49.3	0.335	0.4705	0.3397	0.3044
	rs74139636	c.-109T > C	Intron1	Intronic	-	8.2	0.1007	-	0.1538	0.0759
	rs1904589	c.494A > G	Exon2	Missense	p.His165Arg	63	0.5437	0.3829	0.3343	0.4617
	rs764201898	c.417C > T	Exon2	Synonymous	p.Val139 =	1.4	-	0.00	-	-
	rs77151171	c.357C > T	Exon2	Synonymous	p.Pro119 =	1.4	0.005	0.0014	0.0026	0.0004
FOXH1	rs750472	c.-314T > G	Exon1	5'UTR	-	57.5	-	-	0.4443	0.4766
	-	c.-465C > A *	Exon1	5'UTR	-	23.3	-	-	-	-
	rs1871545	c.175-30C > T	Intron1	Intronic	-	12.3	0.1239	0.0811	0.0427	0.0689
	rs112028242	c.373A > T	Exon3	Missense	p.Thr125Ser	1.4	0.0031	0.0081	0.0266	0.0354
	-	c.1061C > G *	Exon3	Missense	p.Pro354Arg	1.4	-	-	-	-

* Novel variant

form in one patient. This variant had no effects on amino acids, as well (p.Arg239Arg). A heterozygous c.895C > T variant was present in a six-month-old boy with Ventricular Septal Defect (VSD), leading to a substitution of arginine to cysteine at codon 299 (p.Arg299Cys). The patient's father also had VSD (Figure 1A).

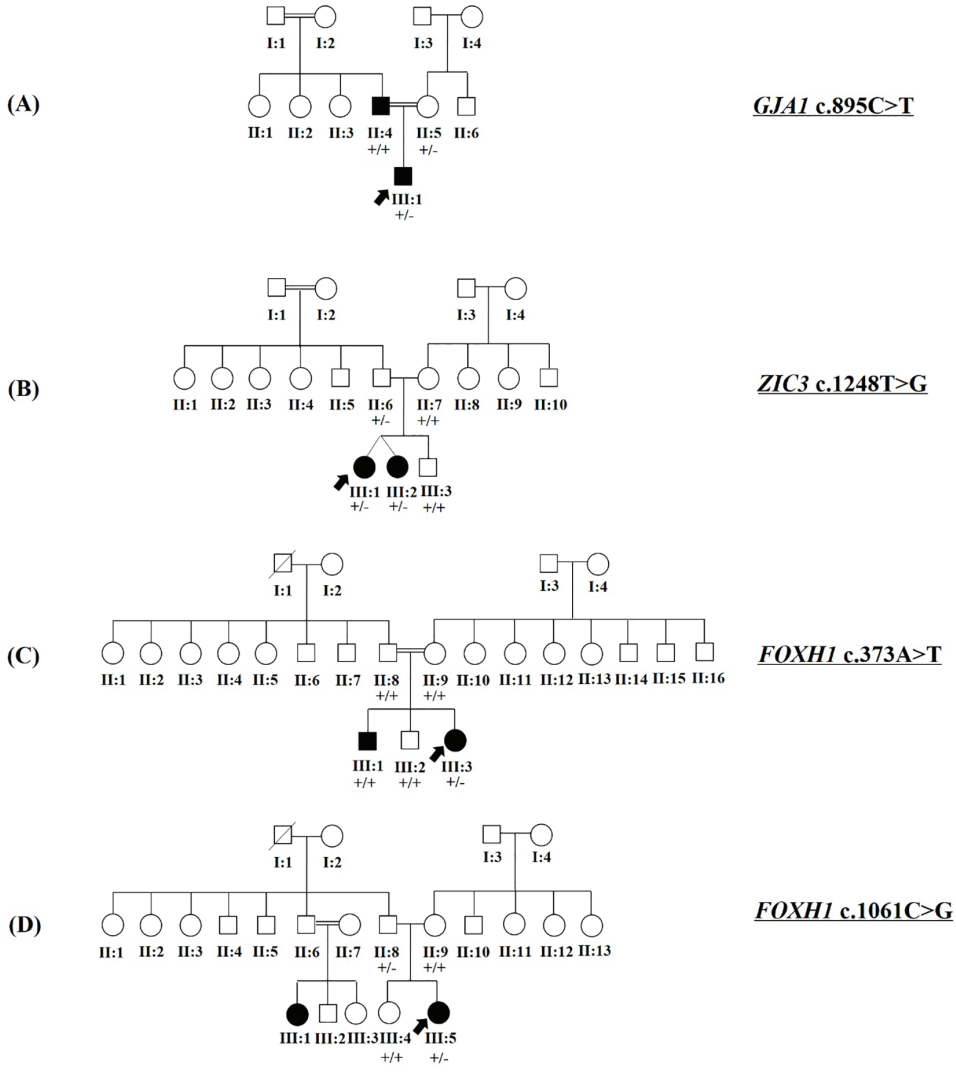
4.2. ZIC3 Variants

A single heterozygous c.1248T > G variant was detected in the third exon of the ZIC3 gene. This variant was observed only in twin three-year-old sisters suffering from Atrial Septal Defect (ASD) and Patent Ductus Arteriosus (PDA). This variant represented a codon change from GAT to GAG, which was translated to glutamate (p.Asp416Glu). Although in the mutation taster tool, p.Asp416Glu was defined as a disease causing variant, it was a benign one in other prediction tools (CADD, PROVEAN, Polyphen2, and SIFT) (Table 1). Further sequence analysis of the patients' healthy father displayed this variant, as well (Figure 1B).

4.3. NODAL Variants

c.193 + 12C >T and c.-109T > C were found in the intronic regions of the NODAL gene. c.193+12C > T was present in many patients. c.494A > G variant, the most frequent variant in the population, was identified in the second exon of the NODAL gene. This polymorphism was observed in heterozygous/homozygous form and caused the substitution of the amino acid at codon 165 (p.His165Arg), which was predicted to be not deleterious by means of the computational tools (Table 1). Moreover, c.417C > T and

Figure 1. Pedigrees of Patients with *GJA1* (A), *ZIC3* (B), and *FOXH1* (C and D) Genes Variants.



The variants detected in each family have been stated on the right sides of the pedigrees. Arrows represent probands. (A) The proband (III:1) manifested Ventricular Septal Defect (VSD) at the age of six months. His father (III:6) was also affected with VSD. (B) The proband (III:1) and her sister (III:1); the three-year-old girls had VSD. (C) The proband (III:3); a six-month-old girl and her brother (III:1) showed VSD. (D) The proband (III:5) was diagnosed with Atrial Septal Defect (ASD) at the age of three years. III:1 showed Tetralogy Of Fallot (TOF) and was not available for molecular testing.

c.357C > T heterozygous transitions each coding the same amino acid (p.Val139Val and p.Pro119Pro) were recognized in the second exon of the NODAL gene. c.417C > T was present just in one out of the two affected children, a six month-old girl from a kindred with VSD. c.357C > T variant was also observed just in one out of the two children, a one-year-old boy with PDA and ASD. These two patients also harbored c.193 + 12C > T and c.494A > G variants in homozygous form.

4.4. FOXH1 Variants

A c.-314T > G transversion was detected within 5'UTR of the FOXH1 gene in 42 patients; 12 cases with the homozygous form and the others with the heterozygous form. In the first intron of this gene, a c.175-30C > T transition was identified in two homozygotes and seven heterozygotes. In the third exon of the FOXH1 gene, the heterozygous c.373A > T variant resulting in a missense change (p.Thr125Ser) was found just in a six-month-old girl with VSD (Figure 1C). This patient also harbored c.417C > T variant described in the previous section. Indeed, two novel variants were recognized in the FOXH1 gene; a heterozygous c.-465C > A variant found in 17 patients and a heterozygous c.1061C > G variant leading to amino acid change (p.Pro354Arg) in a three-year-old girl with ASD (Figure 1D). This patient also had c.-314T > G variant in the homozygous form.

4.5. In Silico Analysis of c.895C>T, c.373A>T and c.1061C > G

Segregation analysis was performed for c.895C > T, c.373A > T, and c.1061C > G variants, because they had probably adverse effects by at least three of the prediction tools or they had not been reported previously. In additions,

in silico analysis was performed for the mutated proteins (p.Arg299Cys, p.Thr125Ser, and p.Pro354Arg) using the I-TASSER software. It predicted the proteins' secondary structures and 3D models.

CADD (Phred of 23.4), Polyphen-2 (Score 0.99), and Mutation taster tools predicted c.895C > T variant in the GJA1 gene as potentially pathogenic. However, the segregation analysis showed that this heterozygous variant was present in a healthy mother, but not a father with VSD. Therefore, it could not have pathogenic effects. The I-TASSER indicated the same secondary structure and 3D model for the wild type and mutated protein. c.373A>T in the FOXH1 gene was found in a six-month-old girl with VSD. Although this variant was predicted to be pathogenic, deleterious, and probably damaging with the scores of 23.2 (CADD), 0.1 (SIFT), and 0.99 (Polyphen-2), it was not found to be pathogenic by Mutation taster and PROVEAN tools (Table 1). Moreover, it was not present in her brother with the same CHD. Indeed, the secondary structure was not changed by this variant. c.1061C > G in the FOXH1 gene was predicted as a deleterious variant with the SIFT score of 0.01 and CADD Phred of 22.9. Nonetheless, three prediction tools (Mutation taster, PROVEAN, and Polyphen-2) regarded it as a polymorphism. Furthermore, this variant was transmitted by a healthy father. Moreover, I-TASSER indicated that c.1061C > G (p.Pro354Arg) variant was located in an adopting coil segment of the protein's secondary structure, which was not changed with the mentioned variant.

5. Discussion

CHD is a complex disorder with a heterogeneous etiology (25). Previous studies have indicated the roles of different genes, such as NKX2-5, ZIC3, CITED2, FOXH1, NOTCH1, NODAL, MYH6, TBX5, GJA1, and GATA4, in CHD development (3, 10). This study aimed to present the genetic analysis of GJA1, ZIC3, NODAL, and FOXH1 genes in Iranian families with familial non-syndromic CHD. The results of genetic analysis revealed no bona fide pathogenic mutations in the patients. However, some sequence variants were identified in these genes; i.e., two novel variants in the FOXH1 gene (c.-465C > A and c.1061C > G) and known variants including rs766082259, rs57946868, rs748954821, rs755343529, rs10999338, rs74139636, rs1904589, rs764201898, rs77151171, rs750472, rs1871545, and rs112028242.

Despite the genetic evidences denoting that sequence changes in GJA1, ZIC3, NODAL, and FOXH1 genes play a significant role in CHD (13-16), there are some studies that, similar to the present work, demonstrated no pathogenic variations of these genes in CHD patients. In a study on 300 patients with CHD, Huang et al. identified only two silent variants in the GJA1 gene in eight patients. They concluded that GJA1 variation was not likely a major participant to CHD (26). ZIC3 variants may show variable expressivity and penetrance (27). Therefore, the ZIC3 variant found in the family in the current study (Figure 1B) might have a reduced penetrance although variable expressivity of its phenotype could not be excluded because the father was not completely examined. The associations between NODAL gene variants and different types of CHD have been surveyed by Roessler et al. They indicated that some NODAL variants acted as modifiers. Although their identified variants of the NODAL gene did not act as CHD risk factors, they suggested that these variants' effects might interact with other genes (16). Furthermore, the same authors reported in another research that more than one variation existed in NODAL pathway genes in CHD patients (9). Some studies have also reported variations in more than one gene in patients with CHD. For example, Bassi et al. described a patient affected by heterotaxy, bearing both a NODAL mutation and a FOXA2 variant (28). Furthermore, Bamford et al. indicated CFC1 and NODAL mutations in an individual with TGA and dextrocardia (29). Altogether, although the multigenic etiology of CHD is more common, the importance of monogenic types of CHD could not be ignored.

In silico analyses were performed to evaluate the most likely functional missense variants of GJA1, ZIC3, NODAL, and FOXH1 genes. However, no single bioinformatics tool is able to get a perfect picture of the functional effects of the variants. Hence, the present investigation was carried out using some prediction tools. Although c.373A > T variant was found in one out of the two affected siblings, it existed in their healthy father. Furthermore, two out of the five prediction tools considered it to be a polymorphism. This variant could not be deleterious because if it was regarded as pathogenic in the patient, another variant was expected to cause the disease in her brother. Another notable variant, the novel c.1061C > G, was predicted as a pathogenic variant by two of the computational tools. Yet, the healthy father had this variant, as well. The absence of a CHD in the transmitting father might reflect the reduced penetrance of the pathogenic variant, and it is likely that he had been undiagnosed. Therefore, the reported/novel missense variants (Table 1) were not considered to have functional significance and pathogenic importance according to the computational prediction tools.

Although all identified variants in the present study were not pathogenic, they could be applied as markers to evaluate the genetic association with other pathogenic loci. Absence of pathogenic mutations in the sample might be attributed to some reasons. First of all, the heterogeneous etiology of CHD is more common and acceptable in our population (10), and genes other than GJA1, ZIC3, NODAL, and FOXH could have a role in Iranian CHD pathogenicity. Second, the sample size should be expanded to detect causal variants. Third, duplications and deletions of GJA1, ZIC3, NODAL, and FOXH1 genes should be checked in the studied group. Fourth, promoter regulatory elements and intronic regions may have a role and should be analyzed.

5.1. Conclusion

The existence of pathogenic mutations in GJA1, ZIC3, NODAL, and FOXH1 genes in Iranian CHD patients is still a matter of debate, and a large Iranian cohort study is required to reveal the role of these genes in Iranian patients. The research projects on families with heart diseases display how much more information is required concerning genetic and environmental effects. Future studies should

be conducted on patients with regard to the CHD specific types in patients or Iranian subpopulations. Furthermore, evaluation of sequence variants in other genes involved in cardiac development may be helpful in identifying the pathology of CHDs. Although many different genes have already been indicated to be involved in familial CHD, many human genes will be recognized within the coming years. The sequencing potency of Next-Generation Sequencing (NGS) can identify additional genes involved in CHDs (30). Reported and novel variants detected in the current study could be useful in the investigation of GJA1-, ZIC3-, NODAL-, and FOXH1-associated diseases.

5.2. Ethical Considerations

Informed consent forms were obtained by the authors.

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This study was performed in Rajaei Cardiovascular, Medical, and Research Center, Tehran, Iran and Zanjan University of Medical Sciences, Zanjan, Iran.

Authors' Contribution

SK, TSH and NM wrote the manuscript. SK carried out the experiments. NM, AB and MM contributed to patient's diagnosis. HRZ performed computational analysis of the data. SK, TSH and NM contributed to project management, genetic analyses, interpretation of data, revision of the initial manuscript, and final approval.

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