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The Nkx2-5 Gene Mutations Related to Congenital Heart Diseases in Iranian Patients Population

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ABSTRACT

Background: Despite the clear role of the Nkx2-5 gene mutations as the trigger for Congenital Heart Disease (CHD) in different populations, the condition of these mutations in our population remains obscure.

Objectives: The present study aimed to assess different Nkx2-5 gene mutations in a sample of Iranian patients with CHD.

Patients and Methods: This cross-sectional study was conducted on 79 consecutive suspected non-syndromic CHD patients at Rajaie Cardiovascular Medical and Research Center between 2016 and 2017. Detailed clinical evaluations were performed and CHD was confirmed by echocardiography. The exons of the Nkx2-5 gene were sequenced. In silico analysis was done using Mutation taster, SNP nexus, and Vienna RNA package. In addition, statistical analysis was performed using the SPSS statistical software, version 16.0. $P \leq 0.05$ was considered to be statistically significant.

Results: The study results revealed four synonymous polymorphisms; i.e., rs2277923, rs703752, rs3729753, and c.217C > T, the last of which was novel. Regarding the frequency of different Single Nucleotide Polymorphism (SNP) genotypes, the overall frequency of wild, heterozygous, and mutant genotypes was respectively 65.8%, 31.6%, and 2.5% for rs2277923, 54.4%, 0.0%, and 45.6% for rs703752, 96.2%, 3.8%, and 0.0% for rs3729753, and 93.7%, 6.3%, and 0.0% for c.217 C > T. Bioinformatics analysis demonstrated that the detected novel variants were not pathogens. Moreover, the genotypic variants of all SNPs were independent of gender, type of heart defect, and hereditary form of the disease.

Conclusions: The results could not show any major roles for different exon-related SNPs on the Nkx2-5 gene as the candidate risk profile for CHD. The results also demonstrated no significant associations between such mutations and increased likelihood of specific heart defects.

1. Background

Congenital Heart Diseases (CHD) are the most frequent heart defects discoverable within childhood sourced from any abnormality in developmental embryonic processes. These abnormalities can include both intra-cardiac structures and extra-cardiac components, such as the great vessels (1, 2). The overall prevalence of CHD has been estimated to be 8 - 10 per thousand live births in the world, which leads to life-threatening morbidities, such as prematurity, abortion, and even early death (3). The exact etiology of CHD remains unclear, but it is originally the result of the interaction between genetic and environmental factors (4). Regarding the genetic sources, the chromosomal and genomic predispositions have been recently identified to explain the occurrence of CHD. In this regard, some chromosomal abnormalities, such as different types of trisomy, have been revealed to expose the affected child to heart defects (5). Moreover, recent advances in genetic techniques, such as whole body sequencing, have paved the way for discovering genetic changes associated with

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increased likelihood of CHD (6). Employment of whole genome or whole exome sequencing techniques has provided new opportunities for discovering new point mutations and gene variants associated with CHD (7, 8). Using these techniques led to identification of new de novo mutations related to CHD (9). Besides, Genome-Wide Association Studies (GWAS) showed various polymorphisms and allelic deviations related to the increased risk of CHD (10). One of the recent interesting gene polymorphisms related to CHD risk belong to the Nkx2-5 gene (one of the main families of homeobox genes) that is located on the long arm of chromosome 5 at position 35.1. The critical role of this gene in cardiac morphogenesis has been recently discovered (11). Both animal and human studies have indicated over-expression of the Nkx2-5 gene in myocardium as well as its key function in congenital heart development. However, knockout of the gene resulted in embryonic lethality and, consequently, normal expression of the gene had a protective role (12). Some recent studies have determined a close link between mutations in the Nkx2-5 gene and increased risk of some cardiac defects, such as Atrioventricular Arrhythmias (AVSD), Tetralogy Of Fallot (TOF), Atrial Septal Defect (ASD), and Hypoplastic Left Heart Syndrome (HLHS) (13, 14). Despite the clear role of the Nkx2-5 gene mutations in CHD in different populations, the condition of these mutations in Iranian population remains obscure.

2. Objectives

The present study aims to assess different Nkx2-5 gene mutations in a sample of Iranian patients with CHD.

3. Patients and Methods

3.1. Study Population

This cross-sectional study was performed on 79 consecutive patients as the known cases with CHD admitted to Rajaie Cardiovascular Medical and Research Center from 2016 to 2017. Baseline characteristics of the participants, including demographics, medical history, and familial tendencies to diseases, were collected by interviewing the families. After receiving written informed consent forms, 5 ml venous blood samples were taken from the antecubital veins of the patients and their parents and were kept in EDTA tubes. The study protocol was approved by the Ethics Committee of Rajaie Cardiovascular Medical and Research Center.

3.2. Genomic Assessment

Genomic DNA was extracted from peripheral blood samples using salting out method. To assess the purity of the isolated DNA samples, NanoDrop (Thermo Fisher Scientific, USA) was used. Additionally, the quality of the samples was examined using agarose gel analysis. Two gene-related exons were considered in amplification of the Nkx-2.5 gene with amplicons lengths of 763bp and 1360bp. To amplify of the exons, Polymerase Chain Reaction (PCR) technique was performed in PeqSTAR 96 x Universal/ Gradient (PEQLAB, Germany) considering a 10X concentrated solution of the primers (for exon 1: the forward primer of 5-GAGACCCTTCCAAATGCGTC-3 and the reverse primer of 5-CTCCTGGCCCTGAGTTTCTT-3, for exon 2: the forward primer of 5-CTTACCATTACTGTGCGGCC-3 and the reverse primer of 5-ATCTCAGAAAGTGCCCGACA, 10 pM/µl of each primer), 1.5 mM MgCl2, 200 µM dNTP, 1 U/µl Taq polymerase (Amplicon, UK), and 100 ng/µl DNA. PCR was ordered as follows: an initial-denaturation step at 94 °C for 5 minutes, 35 cycles including a denaturation step at 94 °C for 40 seconds, an annealing step at 60 °C for exon 1 and 64 °C for exon 2 for 30 seconds, and an extension step at 72 °C for 45 seconds, and one final elongation cycle at 72 °C for 10 minutes. PCR products were run on gel electrophoresis on a 2% agarose gel after staining by Fluoro Dye Green, 6x (SMOBIO, Taiwan) and were observed by gel documentation (Vilber, France) under UV light. The confirmed PCR products were directly sequenced through Big Dye termination method using sequencer analyzer ABI Sequencer 3130XL PE (Applied Bio Systems, US). For SNP evaluation, mutation taster with accuracy of 91.1 ± 0.1% (15), SNP nexus (non-synonymous substitution effect prediction based on the UCSC, Ensemble, PolyPhen-2, and SIFT) (16) and Vienna RNA Package version 2.3.1 (RNA secondary structure prediction) (17) were used to evaluate the effect of novel variation on protein structure, protein function, and RNA structure stability. In addition, STRING database version 10.5 (18) was used to find other genes that might have interactions with Nkx2-5 in its performance pathway.

3.3. Statistical Analysis

Chi-square, Fisher's exact test, independent t-test, or ANOVA were used to assess the link between the polymorphisms-related genotypes and alleles and baseline variables, including gender, type of heart defect, and genetics tendency (sporadic or familial). All statistical analyses were done using the SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL), and $P < 0.05$ was considered to be statistically significant.

4. Results

4.1. Baseline Information

This study was performed on 79 patients with definite diagnosis of CHD. The mean age of the participants was 8.89 (SD = 11.39) years ranged from 2 months to 50 years. In addition, 55.7% of the participants were male and 44.3% were female. Regarding the type of CHD, various types of single and complex defects were detected by echocardiography the commonest of which being isolated Ventricular Septal Defect (VSD) (21.5%) followed by TOF (16.5%). Regardless of simultaneous defects and complexity, VSD was found in 49.4%, ASD in 25.3%, Patent Ductus Arteriosus (PDA) in 20.2%, Pulmonary Hypertension (PH) in 11.4%, Pulmonary Stenosis (PS) in 10.1%, and Coarctation of the Aorta (COA) in 6.3% of the patients. Besides, CHD was appeared familiarly in 82.3% and sporadically in 17.7% of the participants.

4.2. Nkx2-5 Variations

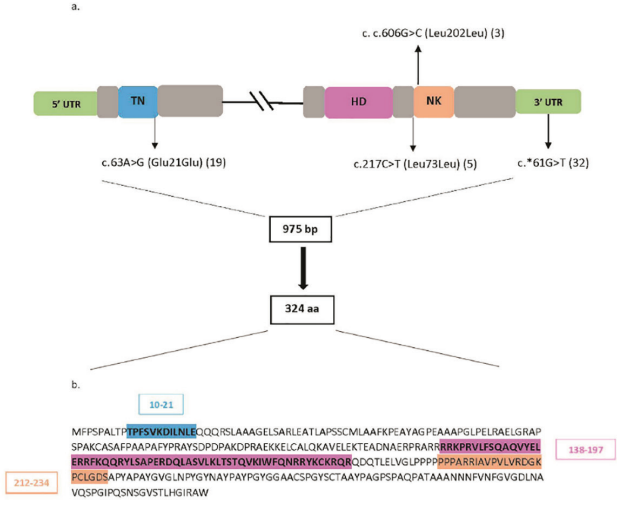
Direct sequencing of Nkx2-5 gene exons showed four variations in different regions of this gene (Figure 1): c.*61G > T in exon 2 that was present in most of the patients

(rs703752), A > G substitution at nucleotide c.63 in exon 1 (rs2277923) (E21E), novel C > T substitution at nucleotide c.217 in exon 1 (L73L), and G > C transversion at nucleotide c.606 in exon 2 (rs3729753) (L164L).

4.3. Frequency of Genotypes

Regarding the frequency of different SNP genotypes in different regions of the Nkx2-5 gene, the overall frequency of wild, heterozygous, and mutant genotypes was respectively 65.8%, 31.6%, and 2.5% for rs2277923, 54.4%, 0.0%, and 45.6% for rs703752, 96.2%, 3.8%, and 0.0% for rs3729753, and 93.7%, 6.3%, and 0.0% for c.217 C > T. As shown in Table 1, the genotyping pattern of all SNPs was independent of patients' gender, age, type of defect, and hereditary form of the disease.

Figure 1. Locations of the Mutations Identified in This Study



a: The number of mutations expressed in parentheses. The broken line shows the single intron. UTR, untranslated region; TN, conserved domain; HD, home domain; NK, conserved domain. Gray boxes are out site of the conserved domain in the coding region. b: The amino acid sequence. The blue sequence is TN, the purple sequence is HD, and the orange sequence is NK.

4.4. Bioinformatic Analysis of c.217C > T (chr5:172661870)

According to mutation taster prediction, c.217C > T is a disease causing variation. SNP nexus gene/protein consensuses (Ensemble: ENSG00000183072 UCSC: uc003mcm.2/uc010jjt.2/ uc011dfe.2) evaluation revealed that synonymous variation in the coding sequence of the gene did not have any effects on protein structure and function. Due to the high accuracy of the mutation taster and its disease causing prediction, the next surveying by Vienna RNA package was done. Comparing the results of normal and mutant mRNA sequences revealed that structural stability of Nkx2-5 mRNA did not change significantly by c.217C > T variation. The Minimum Free Energy (MFE) structure of RNA sequence prediction that was achieved (defaults to 37 °C) by using the dynamic algorithm and loop-based energy model was -752.70 kcal/mol in the normal state and -751.80 kcal/mol in the mutant state (Figure 2). Also, the entropy of nucleotide position in RNA was similar in C and T (U) (Figure 3). The STRING database output showed Nkx2-5 interactions with GATA binding protein 4 (GATA4), Serum Response Factor (SRF), T-Box 5 (TBX5), Heart And Neural Crest Derivatives Expressed 1 (HAND1), Bone Morphogenetic Protein 4 (BMP4), Myocyte Enhancer Factor 2C (MEF2C), Heart And Neural Crest Derivatives Expressed 2 (HAND2), SMAD family member 4 (SMAD4), Noggin (NOG), and Myocyte Enhancer Factor 2A (MEF2A) genes (Figure 4).

5. Discussion

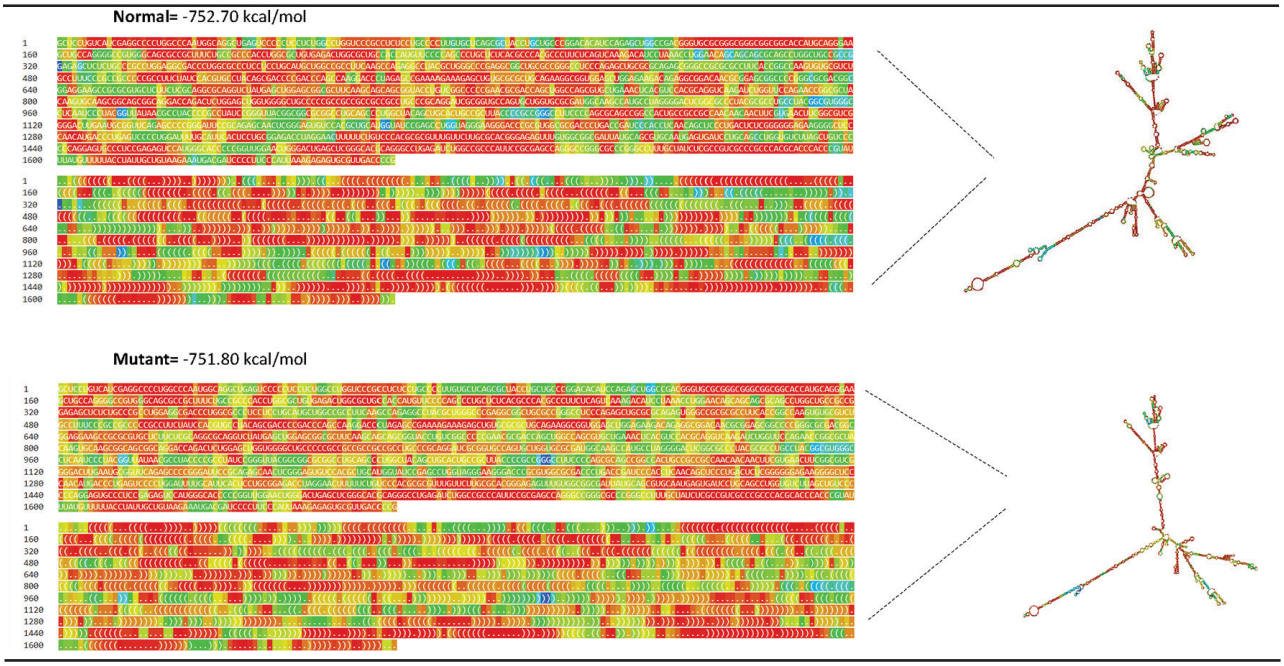
Mutations in the Nkx-2.5 genes have been reported as a cause of CHD in different populations (19-22). In the present study, Nkx-2.5 gene exons were examined in 79 CHD patients, but no pathogenic mutation was found. The results revealed c.63A > G (rs2277923) and c.*61G > T (rs703752) mutations in different types of CHD; both sporadic and familial patients. Yu Cao et al. found rs2277923 mutation in ASD patients and rs703752 mutation in VSD patients, which is nearly inconsistent with our results. They also detected rs3729753 polymorphism in their

Table 1. The Association between the Genotypic Variants on the Nkx-2.5 Gene and Baseline Characteristics

Item	rs2277923			rs703752		rs3729753		c.217 C > T	
	AA	AG	GG	GG	TT	GC	GG	CC	CT
Gender									
Male	61.4%	36.4%	2.3%	43.2%	56.8%	6.8%	93.2%	95.5%	4.5%
Female	71.4%	25.7%	2.9%	48.6%	51.4%	0.0%	100%	91.4%	8.6%
P value	0.599			0.633		0.115		0.650	
Familial/Sporadic									
Familial	64.6%	32.3%	3.1%	46.2%	53.8%	95.4%	4.6%	92.3%	7.7%
Sporadic	71.4%	28.6%	0.0%	42.9%	57.1%	100%	0.0%	100%	0.0%
P value	0.755			0.822		0.999		0.579	
CHD Type									
VSD	68.4%	28.9%	2.6%	52.6%	47.4%	94.7%	5.3%	97.4%	2.6%
ASD	75.0%	18.8%	6.2%	56.2%	43.8%	100%	0.0%	87.5%	12.5%
PDA	70.6%	29.4%	0.0%	29.4%	70.6%	100%	0.0%	100%	0.0%
TOF	58.3%	41.7%	0.0%	41.7%	58.3%	91.7%	8.3%	100%	0.0%
P value	0.456			0.124		0.859		0.889	

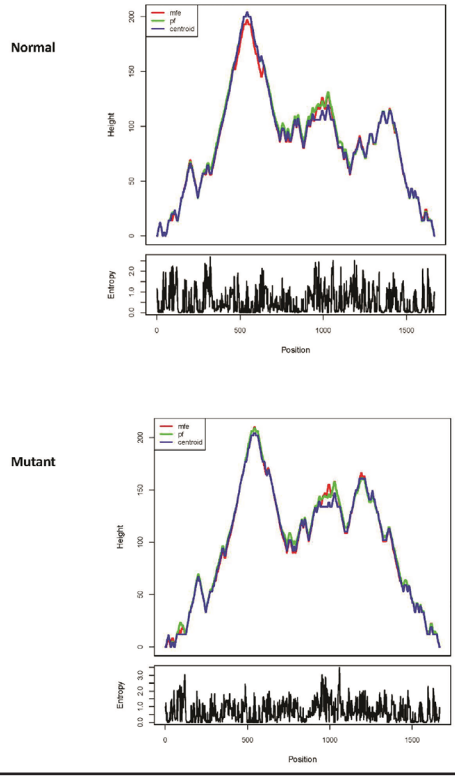
Abbreviations: VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; TOF, tetralogy of fallot

Figure 2. Minimum Free Energy Calculation



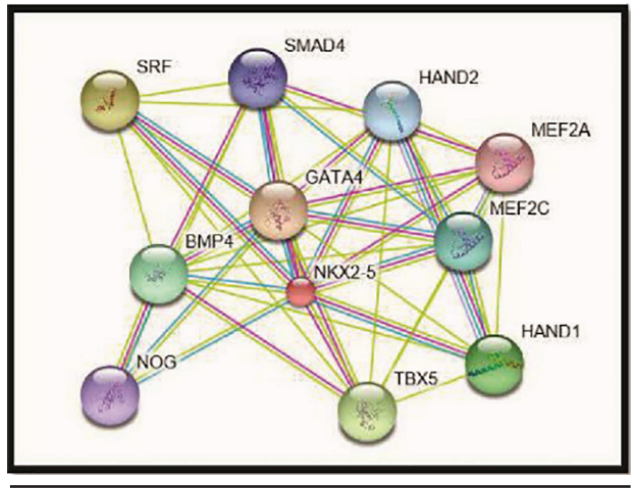
Left drawing is the optimal secondary structure in dot-bracket notation with a minimum free energy that is colored by positional entropy. Right drawing is interactive drawing of the minimum free energy structure that is colored by base-pairing probabilities (:::::: free extremes; ((())) stem; <<<< >>> internal stem; ----- loop; ,,,,,, internal loop). This figure was obtained from Vienna RNA Package site (V2.3.1).

Figure 3. Mountain Plot Representation of the Thermodynamic Ensemble of RNA Structures, the Minimum Free Energy Structure, and the Centroid Structure



The height (m) is given by the number of base pairs enclosing the base at position k. Loops correspond to plateaus and stems correspond to slopes. The closer the two curves, the better the structure could be defined. MFE, minimal free energy; pf, folding probability. This figure was obtained from Vienna RNA Package site (V2.3.1).

Figure 4. Protein-Protein Interaction Network of Nkx2-5



The following proteins have a main role in heart development: GATA binding protein 4 (GATA4), Serum Response Factor (SRF), T-Box 5 (TBX5), Heart And Neural Crest Derivatives Expressed 1 (HAND1), Bone Morphogenetic Protein 4 (BMP4), Myocyte Enhancer Factor 2C (MEF2C), Heart And Neural Crest Derivatives Expressed 2 (HAND2), SMAD family member 4 (SMAD4), Noggin (NOG), and Myocyte Enhancer Factor 2A (MEF2A). This figure was obtained from STRING site (V10.5).

studied population (23). c.606G > C(rs3729753) was only recognized in two families; one family with two sons both of whom were suspected to have VSD and another family with one son suffering from TOF. c.217C > T was seen in ASD, VSD, COA, Transposition of the Great Arteries (TGA), PDA, and AVSD types of CHD. These observations are corresponding with the results of a recent study performed by Reamon-Buettner SM and Borlak J. They

stated that although Nkx2-5 had many reported variations, there was no association between the genotype and specific phenotype of CHD (24). There was no pathogenic mutation in our population and our detected variations were similar to those of other studies. Wang et al. conducted a meta-analysis on Chinese population and found rs2277923 SNP in 7 studies on 1243 CHD patients. Therefore, they reported that rs2277923 might be associated with CHD risk in their population (25). Similarly, Ketharnathan et al. (26) found rs2277923 mutation in their studied Indian population and reported the same conclusion. We analyzed the novel c.217C > T in RNA level, but the stability of RNA structure in wild and mutant types was almost the same. This result indicated that although codon changed with the same amino acid and might not influence the protein/mRNA structure, the tRNA responsible for carrying a specific codon to ribosome for the changed codon may be less in the heart. Thus, this possibility can be surveyed at expression level. Not detecting pathogenic variations in the present study might be attributed to other reasons of CHD; i.e., epigenetic or genetic. In the same line, Winston J. et al. (27) stated that Nkx2-5 variations led to CHD with incomplete penetrance and the modifier gene could affect Nkx2-5 mutations pathogenicity in CHD.

Since CHD is a heterogeneous disorder and many genes can induce CHD, Nkx-2.5 may have no major roles and other genes may cause CHD in the Iranian population. In the present study, STRING database, version 10.0 was used to identify the genes that have interactions with the Nkx-2.5 gene, including GATA4, TBX5, BMP4, HAND1, HAND2, MEF2C, MEF2A, NOG, SMAD4, and SRF (Figure 4). Among these genes, GATA4 and TBX5 are essential transcription factors in heart evolution and have the most interaction with Nkx2.5 (28). Sequence analysis of GATA4 and TBX5 in the Iranian CHD population with normal Nkx2.5 sequence will be useful to identify Iranian specific genes and polymorphisms. CHD is a multifactorial disease; therefore, existence of rs2277923 in an individual and its interaction with environmental risk factors may lead to CHD. Yet, this claim needs to be confirmed by increasing the number of evaluated patients and comparing them to normal control participants. Another polymorphism (rs703752) was also found in the current study, which was not in the coding sequence. Nevertheless, its frequency in some patients indicates that this SNP might have a role in the regulation of Nkx-2.5 function. This hypothesis should be confirmed in future studies.

Reviewing the recent literature revealed two important points. First, the Nkx2-5 gene variants are associated with various types of CHD with no specification to an especial type. More importantly, recent studies focused on the promoter regions as the candidate sequences for CHD (29). The critical role of non-homeodomain regions of the Nkx2-5 gene in the pathogenesis of CHD has been highlighted, as well. In this regard, it has been demonstrated that although none of the mutations revealed by direct sequencing are located in the homeodomain region, some important mutations as even deletion sites have been shown in non-homeodomain regions of the gene (30, 31). It should also be expressed that along with SNPs, some specific haplotypes comprising multiple mutations have been discovered in relation to the occurrence of CHD (32). Considering Iranian population, limited studies have been done on the genetic basis of CHD. A systematic review on similar studies among Iranians showed only two similar studies. In a study by Kheirollahi et al. (33), patients were assessed with respect to mutations on homeodomain-encoding region of the Nkx2-5 gene that led to discovering only one SNP (c.543G > A) associated with the risk for TOF. However, this mutation was observed only in one patients and, consequently, could not be generalized to all TOF patients. In another study by Soheili et al., 30 patients with ASD and 57 ones with VSD were scheduled for high resolution melt scanning for Nkx2-5 exons. They indicated no significant associations between the polymorphisms identified in the exons of the gene and increased risk of both anomalies.

Although the insignificant association between the revealed mutations on the Nkx2-5 exons and CHD may be a true characteristic in the present study population, the results might have been influenced by some errors and biases, such as small sample size and consequently low study power, ignoring baseline cardiovascular determinants and risk factors especially during pregnancy, and the cross-sectional design of the study leading to inability to assess causality. Future studies comparing different populations with a specific type of CHD regarding the frequency of the Nkx2-5 genotype might provide an explanation for the high frequency of some variants in our population.

5.1. Conclusion

The findings of the current study could not show any major roles for different exon-related SNPs on the Nkx2-5 gene as the candidate risk profile for CHD. The results also demonstrated no significant associations between such mutations and increased likelihood of specific heart defects. The small sample size of the study may be the main reason for this insignificancy. Thus, further assessments by employing huge samples from different regions of the country are warranted.

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Authors' Contribution

Samira Kalayinia: Performance of the project, data analysis/interpretation, drafting article; Alireza Biglari: Scientific Advisor (Genetic); Hassan Roknizaheh: Scientific Advisor (Genetic); Mohammad Mahdavi: Scientific Advisor (Cardiology); Bahareh Rabbani: Scientific Advisor (Genetic); Majid Maleki: Scientific Advisor (Cardiology); Nejat Mahdieh: Concept/Design, data analysis/interpretation, drafting article.

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References

1. Hoffman JI. Incidence of congenital heart disease: I. Postnatal incidence. *Pediatric cardiology*. 1995;**16**(3):103-13.
2. Mitchell SC, Korones SB, Berendes HW. Congenital heart disease in 56,109 births. Incidence and natural history. *Circulation*. 1971;**43**(3):323-32.
3. Fahed AC, Gelb BD, Seidman JG, Seidman CE. Genetics of congenital heart disease: the glass half empty. *Circulation research*. 2013;**112**(4):707-20.
4. Warkany J. Etiology and Morphogenesis of Congenital Heart Disease. *Archives of Pediatrics & Adolescent Medicine*. 1981;**135**(4):389.
5. Wessels M, Willems P. Genetic factors in non-syndromic congenital heart malformations. *Clinical genetics*. 2010;**78**(2):103-23.
6. Jia Y, Louw JJ, Breckpot J, Callewaert B, Barrea C, Sznajer Y*, et al.* The diagnostic value of next generation sequencing in familial nonsyndromic congenital heart defects. *American journal of medical genetics Part A*. 2015;**167A**(8):1822-9.
7. Arrington CB, Bleyl SB, Matsunami N, Bonnell GD, Otterud BEM, Nielsen DC*, et al.* Exome Analysis of a Family With Pleiotropic Congenital Heart Disease. *Circulation: Cardiovascular Genetics*. 2012;**5**(2):175-82.
8. Choi M, Scholl UI, Ji W, Liu T, Tikhonova IR, Zumbo P*, et al.* Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;**106**(45):19096-101.
9. de Ligt J, Veltman JA, Vissers LE. Point mutations as a source of de novo genetic disease. *Current opinion in genetics & development*. 2013;**23**(3):257-63.
10. Zaidi S, Choi M, Wakimoto H, Ma L, Jiang J, Overton JD*, et al.* De novo mutations in histone-modifying genes in congenital heart disease. *Nature*. 2013;**498**(7453):220-3.
11. Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP*, et al.* Congenital heart disease caused by mutations in the transcription factor NKX2-5. *Science*. 1998;**281**(5373):108-11.
12. Yang JH, Xu XY, Mi HY, Jiang Y, Ma XM, Li L. [NKX2.5 and TBX5 gene mutations in in vitro fertilization children with congenital heart disease]. *Zhongguo dang dai er ke za zhi = Chinese journal of contemporary pediatrics*. 2017;**19**(6):652-7.
13. Tong YF. Mutations of NKX2.5 and GATA4 genes in the development of congenital heart disease. *Gene*. 2016;**588**(1):86-94.
14. Xu YJ, Qiu XB, Yuan F, Shi HY, Xu L, Hou XM*, et al.* Prevalence and spectrum of NKX2.5 mutations in patients with congenital atrial septal defect and atrioventricular block. *Molecular medicine reports*. 2017;**15**(4):2247-54.
15. Schwarz JM, Rodelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. *Nature methods*. 2010;**7**(8):575-6.
16. Dayem Ullah AZ, Lemoine NR, Chelala C. A practical guide for the functional annotation of genetic variations using SNPnexus. *Briefings in bioinformatics*. 2013;**14**(4):437-47.
17. Lorenz R, Bernhart SH, Honer Zu Siederdissen C, Tafer H, Flamm C, Stadler PF*, et al.* ViennaRNA Package 2.0. *Algorithms for molecular biology : AMB*. 2011;**6**:26.
18. Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P*, et al.* The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic acids research*. 2011;**39**(Database issue):D561-8.
19. Bouveret R, Waardenberg AJ, Schonrock N, Ramialison M, Doan T, de Jong D*, et al.* NKX2-5 mutations causative for congenital heart disease retain functionality and are directed to hundreds of targets. *eLife*. 2015;**4**.
20. Cao Y, Wang J, Wei C, Hou Z, Li Y, Zou H*, et al.* Genetic variations of NKX2-5 in sporadic atrial septal defect and ventricular septal defect in Chinese Yunnan population. *Gene*. 2016;**575**(1):29-33.
21. Li J, Cao Y, Wu Y, Chen W, Yuan Y, Ma X*, et al.* The expression profile analysis of NKX2-5 knock-out embryonic mice to explore the pathogenesis of congenital heart disease. *Journal of cardiology*. 2015;**66**(6):527-31.
22. Zakariyah AF, Rajgara RF, Veinot JP, Skerjanc IS, Burgon PG. Congenital heart defect causing mutation in Nkx2.5 displays in vivo functional deficit. *Journal of molecular and cellular cardiology*. 2017;**105**:89-98.
23. Cao Y, Lan W, Li Y, Wei C, Zou H, Jiang L. Single nucleotide polymorphism of NKX2-5 gene with sporadic congenital heart disease in Chinese Bai population. *International journal of clinical and experimental pathology*. 2015;**8**(11):14917-24.
24. Reamon-Buettner SM, Sattlegger E, Ciribilli Y, Inga A, Wessel A, Borlak J. Transcriptional defect of an inherited NKX2-5 haplotype comprising a SNP, a nonsynonymous and a synonymous mutation, associated with human congenital heart disease. *PloS one*. 2013;**8**(12):e83295.
25. Wang Z, Zou L, Zhong R, Zhu B, Chen W, Shen N*, et al.* Associations between two genetic variants in NKX2-5 and risk of congenital heart disease in Chinese population: a meta-analysis. *PloS one*. 2013;**8**(8):e70979.
26. Ketharnathan S, Koshy T, Sethuratnam R, Paul S, Venkatesan V. Investigation of NKX2.5 gene mutations in congenital heart defects in an Indian population. *Genetic testing and molecular biomarkers*. 2015;**19**(10):579-83.
27. Winston JB, Schulkey CE, Chen I-BD, Regmi SD, Efimova M, Erlich JM*, et al.* Complex Trait Analysis of Ventricular Septal Defects Caused by Nkx2-5 MutationClinical Perspective. *Circulation: Genomic and Precision Medicine*. 2012;**5**(3):293-300.
28. Su W, Zhu P, Wang R, Wu Q, Wang M, Zhang X*, et al.* Congenital heart diseases and their association with the variant distribution features on susceptibility genes. *Clin Genet*. 2017;**91**(3):349-54.
29. Benson DW, Silberbach GM, Kavanaugh-McHugh A, Cottrill C, Zhang Y, Riggs S*, et al.* Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. *The Journal of clinical investigation*. 1999;**104**(11):1567-73.
30. Goldmuntz E, Geiger E, Benson DW. NKX2.5 mutations in patients with tetralogy of fallot. *Circulation*. 2001;**104**(21):2565-8.
31. Pabst S, Wollnik B, Rohmann E, Hintz Y, Glanzer K, Vetter H*, et al.* A novel stop mutation truncating critical regions of the cardiac transcription factor NKX2-5 in a large family with autosomal-dominant inherited congenital heart disease. *Clinical research in cardiology : official journal of the German Cardiac Society*. 2008;**97**(1):39-42.
32. Liu XY, Wang J, Yang YQ, Zhang YY, Chen XZ, Zhang W*, et al.* Novel NKX2-5 mutations in patients with familial atrial septal defects. *Pediatric cardiology*. 2011;**32**(2):193-201.
33. Kheirollahi M, Khosravi F, Ashouri S, Ahmadi A. Existence of mutations in the homeodomain-encoding region of NKX2. 5 gene in Iranian patients with tetralogy of Fallot. *Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences*. 2016;**21**.