ORIGINAL ARTICLE

Existence of mutations in the homeodomain-encoding region of NKX2.5 gene in Iranian patients with tetralogy of Fallot

Majid Kheirollahi[1,2], Fereshteh Khosravi[3], ,Saeideh Ashouri[1], Alireza Ahmadi[1]

[1]Pediatric Inherited Diseases Research Center, Research Institute for Primordial Prevention of Non-Communicable Disease, [2]Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, [3]Department of Biology, Islamic Azad University, Yazd, Iran

Background: Tetralogy of Fallot (TOF), the most common cyanotic heart defect and one of the most common congenital heart diseases, occurs mostly sporadically and nonsyndromically. The underlying molecular genetic mechanism is not known. Therefore, the existence of mutations in the homeodomain-encoding region of NKX2.5 gene in Iranian patients with tetralogy of Fallot is evaluated. **Materials and Methods:** In the present study, we analyzed the peripheral blood samples of27 patients in order to find any mutation in the 180 bp homeodomain-encoding region of NKX2.5 gene, which is known to be involved in heart development and diseases. DNA was extracted and all the samples were amplified by polymerase chain reaction (PCR) and sequenced. **Results:** Twenty-seven patients were included in the study. Twenty-five of them were infants and children (6 days to 11 years of age), one was a teenager (14-years of age), and another was a 33-year-old man [mean ± standard deviation (SD): 5.80 ± 3.90 years]. Thirteen patents were males (mean ± SD: 6.587077 ± 5.02 years) and 14 were females (mean ± SD: 5.0726 ± 2.81 years). One synonymous variant, i.e., c.543G>A was identified in one patient. **Conclusion:** Mutations in the homeodomain-encoding region of NKX2.5 gene may not have an outstanding role in etiology of tetralogy of Fallot patients in Iran.

Key words: Cardiac defect, Iranian patient, NKX2.5 gene, tetralogy of Fallot (TOF)

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INTRODUCTION

Tetralogy of Fallot (TOF) is the most prevalent form of cyanotic congenital cardiac defect, which is repaired by congenital heart defect corrective surgery. This condition occurs because of incorrect development of the right side of the heart.[1,2] In 1671, TOF, the most common conotruncal cardiac defect, was described by Niels Stenson for the first time. Then, in 1784 its detailed anatomical description was provided by William Hunter at St. Georges Hospital Medical School in London, England.[3,4] Around 3.5% of all the newborns affected by congenital heart diseases have TOF and men and women are equally affected. Similar to most of other congenital heart diseases, the exact cause of TOF is not known. It seems that most cases are sporadic but with no other first-degree affected relatives, the risk of recurrence in siblings is around 3%.[5]

A ventricular septal defect between the anterior and posterior limbs of the trabecular septal band, right ventricular outflow tract obstruction, and overriding of the aortic valve due to anterocephalad deviation of the outlet septum are characterizations of TOF. It is considered as the malformation of the cardiac outflow tract. Most patients affected by this disorder have sufficient pulmonary blood flow at birth but there is increasing cyanosis during the first few weeks and months after birth. These days, in countries with

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Address for correspondence: Dr. Majid Kheirollahi, Pediatric Inherited Diseases Research Center, Research Institute for Primordial Prevention of Non-Communicable Disease, Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: mkheirollahi@med.mui.ac.ir

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developed cardiac services for infants, diagnosis of TOF is not postponed and palliative procedures and complete repairs are done; therefore, severe cyanosis and other consequences of severely reduced pulmonary blood flow are uncommon.[1]

The etiology of TOF is not exactly known. Apart from this, numerous genes have been identified to have a role in inherited and sporadic congenital heart diseases. Most of them encoding transcription factors play a significant role in heart development.[6] Some evidence suggests that environmental factors may play a role in its etiology.[7] It has been suggested that almost 10-15% of the patients affected by TOF carry a 22q11 deletion (del22q11) (DiGeorge syndrome)[8,9] and trisomy of chromosome 21 (Down syndrome) has been found in 7% of these patients.[10] Additionally, TOF occurs in patients with Alagille syndrome (with mutations in JAG1),[11,12] VACTERL association, and CHARGE syndrome.[13,14] Previous studies have shown that the occurrence of mutations in NKX2.5 is a nonsyndromic cause of TOF.[15-18]

NKX2.5 protein, with 324 amino acids, belongs to the NK-2 family of homeodomain-containing transcription factors conserved from *Drosophila* to humans, and is encoded by the NK2 transcription factor related, locus 5 gene (NKX2.5/CSX1) gene, which has been mapped to chromosome 5q34. This gene includes two exons. TAD, NK2-specific domain, and homeodomain are conserved regions of NKX2.5 that play significant roles in its function. Interaction of NKX2.5 with DNA, which can be regarded as the most important function of NKX2.5 transcription factor, takes place through its 60-amino acid homeodomain. This domain is a helix-tern-helix DNA-binding motif and includes three α-helices (helix 1, helix 2, and helix 3, which provide binding specificity to the domain).[19,20]

Despite considerable advancements in therapy, there are still some patients (about 0.5-6%) who unexpectedly die from this condition.[21] The etiology of TOF is not exactly known. Some evidence suggests that environmental factors may play a role in its etiology.[7] The aim of the present study is to evaluate the existence of mutations in NKX2.5 homeodomain coding region in TOF patients from Iran; so, we screened 27 sporadic Iranian individuals with different TOF phenotypes using DNA sequencing method.

MATERIALS AND METHODS

Study design and participants

The present study was designed as a case series study. Patients were recruited prospectively from April 2012 to September 2013 by Al Zahra Hospital staff at Isfahan University of Medical Sciences, regardless of their sex or ethnicity. All the patients were evaluated by a cardiologist and the diagnosis of TOF was confirmed by echocardiography. Patients with syndromic heart diseases such as DiGeorge, Down, Alagille, Char, Marfan, Noonan, Holt–Oram, or other conditions related to chromosomal anomalies were excluded from the study. The study was approved by the Ethics Committee of Isfahan University of Medical Sciences and written informed consents, considering surrogate decision-making matter, were obtained and all patients' personal health information were kept confidential.

Procedure and variable assessments

Whole blood samples were collected from the patients. DNA was extracted from 200 µl of whole blood of patients by using the PrimePrepTM Genomic DNA Extraction Kit (Genetbio, Daejeon, Korea) according to the manufacturer's protocol. Primers were designed using the DNA sequence available in GenBank. Then, the homeodomain encoding region including 180 bps was polymerase chain reaction (PCR)-amplified. PCR was performed on 25 µl containing 100 ng of genomic DNA, 12.5 µl of Taq DNA Polymerase Master Mix Red (Ampliqon, Odense M, Denmark), and 8.5 µl of ddH2O. The PCR program was started with an initial denaturation at 95°C for 4 min followed by 30 repetitive cycles with a strand separation step at 95°C for 30 s, an annealing step at 66°C for 1 min, and an extension step at 72°C for 35 s, and was finished with a 5-min extension period at 72°C. PCR products were loaded on agarose gel to be visualized. All PCR products were sequenced.

RESULTS

After excluding syndromic patients and confirmation of diagnosis by the cardiologist, 27 patients were included in the study. Twenty-five of them were infants and children (6 days to 11 years of age), one was a teenager (14 years of age), and another was a 33-year-old man [mean ± standard deviation (SD): 5.80 ± 3.90 years]. Thirteen patents were males (mean ± SD: 6.587077 ± 5.02 years) and 14 were females (mean ± SD: 5.0726 ± 2.81 years). General characteristics of the patients are provided in Table 1.

DNA extraction, PCR-amplification, and sequencing of all the specimens were successfully performed. The PCR primers are given in Table 2. All sequences were checked and one synonymous variant was observed in one patient. This variant was c.543G>A; Q181Q [Figure 1].

DISCUSSION

In the present study, we analyzed the sequence encoding homeodomain of NKX2.5 in 27 patients with TOF and found one synonymous variant, i.e., c.543G>A; Q181Q. This silent variant leads to no change in amino acids. This

Table 1: General characteristics of patients affected by tetralogy of Fallot

No	Sex	Age	Symptoms	No	Sex	Age	Symptoms
1	M	5 years	Heart murmur, cyanosis	15	F	6.5 years	Heart murmur
2	M	10 months	Heart murmur	16	M	19 days	Heart murmur
3	F	2 years	Heart murmur	17	M	6 years	Heart murmur
4	F	14 years	Heart murmur	18	M	5 years	Heart murmur
5	M	3.5 years	Heart murmur	19	M	9 months	Heart murmur
6	F	3 years	Heart murmur, cyanosis	20	M	7 years	Heart murmur
7	F	5 years	Heart murmur	21	M	8 years	Heart murmur
8	F	4 years	Heart murmur, cyanosis	22	F	4.5 years	Heart murmur
9	F	11 years	Heart murmur	23	F	2 years	Heart murmur
10	M	33 years	Heart murmur	24	F	3.5 years	Heart murmur
11	F	8.5 years	Heart murmur	25	F	4 years	Heart murmur
12	F	6 days	Heart murmur, cyanosis	26	F	3 years	Heart murmur
13*	M	11 years	Heart murmur	27	M	1.5 years	Heart murmur
14	M	4 years	Heart murmur				

*This patient has variant c.543G>A; Q181Q

Table 2: The sequences of primers for the homeodomain region of NKX2.5 gene

Primer	Sequence	Length (bp)
Forward	5´- CCTTACCATTACTGTGCGGC -3´	20
Reverse	5´- GCCGAGTCCCCTAGGCAT -3´	18

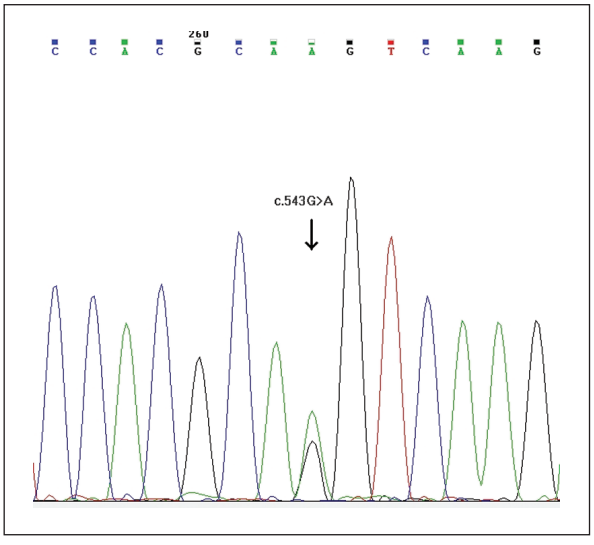

Figure 1: NKX2.5 heterozygous variant 543G>A

sequence variant was reported in 2009 for the first time[22] and was observed in one patient with secundum atrial septal defect (ASD). In 2013, Beffagna *et al*. reported this variant in three out of 100 patients affected by syndromic and nonsyndromic congenital heart diseases (CHDs).[23] They also observed that p.Q181Q existed as a relatively common variant in the control population consisting of 250 healthy unrelated individuals (with the frequency of 1.6%). Moreover, in another study c.543G>A variant was observed in two patients affected by Down syndrome with congenital heart defects and also in two out of 113 control individuals (with the frequency of 1.8%).[24]

In another study by Reamon-Buettner *et al*., c.543G>A was observed in one patient who was also heterozygous for one other sequence alteration (i.e., c356C>A; p.A119E).[25] They did not observe this variant in the 100 healthy controls. In addition, by using the Vienna RNA folding algorithm, they predicted that this synonymous variant affects NKX2.5 mRNA folding. They also took the advantage of a yeast-based assay to see if the observed variants had clinical significance and observed that p.A119E variant, with the presence of c.543G>A, and c.63A>G in cis, reduced transactivation activities of the protein. These observations show that even for synonymous variants, which are expected to have no functional effect, there are ways to influence gene functions.

Some genes are responsible for TOF, including mutations in NKX2.5,[15,22,26,27] GATA4 interacts physically with NKX2.5,[28] GATA6,[29-31] JAG1,[11,32-34] JAG5,[35] TBX20,[36] BVES,[37] mitochondrial ATP8 gene,[38] epigenetic changes of some genes such as NKX2.5,[39] HAND1,[39] VANGL2,[40] and single nucleotide polymorphisms of some genes such as PTPN11[41] and MTHFR.[42] In addition, TOF has been observed to be concomitant with some syndromes and associations such as Down, Alagille, DiGeorge, and CHARGE syndromes, and VACTERL association.[8-14]

CONCLUSION

In conclusion, although we did not find any pathological mutation in this group of TOF patients, the significant role of NKX2.5 gene in normal development of the heart and also its mutations in the occurrence of different phenotypes of CHDs have been proved in other studies. In our knowledge, there are limited studies on the genetic bases of CHDs in the Middle East. According to the population-specific distribution of mutations for this disease, this study presents the results of mutation analysis in patients with CHDs in Iran. Apparently, the homeodomain of NKX2.5 gene may not have an outstanding role in Iranian patients with TOF.

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Conflicts of interest

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTION

MK contributed in the conception of the work, conducting the study, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. FK contributed in the conception of the work, drafting and revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. SA contributed in the conception of the work, conducting the study, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. AA contributed in the conception of the work, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work.

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