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Original Research

Silencing mutations in JAG1 gene may play crucial roles in the pathogenesis of Tetralogy of Fallot

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Abstract: JAG1 gene through Notch signaling is implicated in cell fate decisions in early cardiac development, and mutations in several proteins in the pathway have been involved in various disorders. Tetralogy of Fallot (TOF) is the most frequent form of complicated congenital heart disease. The abnormality of TOF begins through the first eight weeks of fetal growth and is confused with ventricular septal defects, obstruction to right ventricular outflow tract, aortic dextroposition, and right ventricular hypertrophy. Hence the existence of mutations in JAG1 gene in Iranian patients with TOF is evaluated. The clinical data and peripheral blood samples were collected from 44 sporadic nonsyndromic patients with TOF and compared to 44 healthy individuals. DNA was extracted, and the exon 6 of the JAG1 gene was amplified by PCR then the PCR products were purified and sequenced. The age range in patients and the control group was 2-36 years, and the mean and standard deviation (SD) of the age in patients was (11.69 ± 7.85 years) and in control group (11.63 ± 7.99 years). Finally, the samples were successfully sequenced, then analyzed and one synonymous variant (c.765C>T; p.Y255Y) was observed in 38 patients with frequency (86.4%) and three controls with frequency (6.8%). The c.765C>T variant is significantly associated with the pathogenesis of TOF in Iranian population.

Key words: Mutation; JAG1 gene; Cardiac defect; Tetralogy of Fallot.

Introduction

Tetralogy of Fallot (TOF; OMIM# 187500) is the most frequent form of complicated congenital heart disease (CHD), approximately 3.5% of CHD have TOF, which is repaired by corrective surgery, males and females are equally involved (1, 2). The abnormality of TOF begins through the first eight weeks of fetal growth and is confused with ventricular septal defects (VSD), atrial septal defect (ASD) or abnormalities in the branching pattern of coronary arteries, obstruction to right ventricular outflow tract (RVOT), aortic dextroposition (AD) and right ventricular hypertrophy (RVH) (3). Clinical signs contain cyanosis/clubbing, hypoxia, breathlessness, rejection of food, failure to increase weight and serious congenital heart malformation (4). Hypoxia induced oxidative DNA damage in TOF patients (5). TOF has been seen in some of the syndromes, including Down syndrome, Alagille syndrome, CHARGE syndrome, and DiGeorge syndrome (1, 6, 7). Like most of the other congenital heart diseases, the precise cause of TOF is unknown and also happens sporadically, without any other anomaly. The presence of mutations in genes NK2 Homeobox 5 (NKX2.5), T-Box 1 (TBX1), Binding Protein GATA (GATA), and others, and even the interaction between these genetic factors and the environmental risk factors, causes the pathogenesis of the disease. Dominant mutations resulting from the haploinsufficiency can cause TOF by mutations within the genes encoding cardiac transcription factors. It is assumed that de novo mutations in the jagged1 (JAG1; OMIM# 601920) ligand of transmembrane receptors Notch 1 (NOTCH1), Notch 2 (NOTCH2) and themselves, and other genes that are effective in cardiac development are involved in isolated TOF. JAG1 gene is an extremely conserved ligand that plays a very important role in the developmental stages of the mammals' heart (8-10). JAG1 encodes jagged1, a ligand for the NOTCH family of transmembrane receptors (11). JAG1 through Notch signaling is implicated in cell fate decisions in early cardiac development, and mutations in several proteins in the pathway have been involved in various disorders (12, 13). JAG1 gene is located at 20p12.2, has 26 exons and is transcribed to produce a 5.901 kb mRNA (14). The Jagged1 is a glycosylated transmembrane protein of 180 kDa and 1218 amino acids length with some characteristics, containing an N-terminal 'DSL' motif found in the delta, serrate, and lag-2 ligands in the Notch family, 16 tandem repeated epidermal growth factor (EGF)-like domains, a small intracellular region, cysteine rich region, and a transmembrane region (15-17). Alagille syndrome (AGS) an autosomal dominant disease is a multisystem disorder described by extremely variable expressivity, frequently caused by heterozygous mutations in the JAG1 gene (18, 19). Besides, isolated congenital heart defects with sporadic and a

small number of family cases are related to mutations in the JAG1 gene, cases such as obstructive right heart disease, including TOF, defects in the development of the inner ear leading to deafness and both familial deafness and CHD (20-22). Between the Notch ligands, Exon 6 of JAG1 gene is extremely conserved and is critical for ligand binding to the Notch receptor (9, 23). JAG1 mutations that change the capacity for binding to the receptors are important to identify the pathogenesis of the disease (24). In addition to genetic factors due to the interference of other factors such as environmental factors, the TOF etiology is not accurately identified. So despite many advances in treatment, some patients still die (0.5% to 6%) (25). Due to the lack of studies on JAG1 gene mutations in TOF cases from Iran, this study was conducted to investigate the mutations in the JAG1 gene in the exon 6 region in patients with TOF from Iran using DNA sequencing method.

Materials and Methods

Study subjects

The study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The informed written consent was obtained from the patients or their parents prior to the procedure, and all patient's personal health information was kept confidential. In a case-control study, 44 (24 males, 20 females) sporadic nonsyndromic patients with TOF in the age group of 2–36 years were included in the study group. And 44 healthy individuals (sex and age matched with the study group) with no family history of CHD, and other related genetic diseases formed the control group. Cases and controls were recruited from December 2015 to January 2017 by Afshar Hospital, Yazd, Iran. TOF patients were evaluated by a cardiologist and confirmed by echocardiography.

Procedure and variable assessments

Human genomic DNA was isolated from Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood of all samples (n=88). DNA was extracted from the blood by High pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. A polymerase chain reaction (PCR) primer pair flanking the exon 6 were designed using Primer3Plus to produce a PCR amplicon of 500 bp and ordered from SinaClon BioScience Co., Iran (Table 1). PCR was performed in a final volume of 35 µL containing 17.5 µL Master Mix (Ampliqon, Odense M, Denmark), 0.8 µL of each primer, 12.9 µL ddH2O and 100 ng of genomic DNA. Amplification was done in T100™ Thermal Cycler (Bio-Rad, CA, USA) with an initial denaturation at 95 °C for 5 min, followed by 35 repetitive cycles of denaturation at 95 °C for 30 sec, annealing at 63 °C for 30 sec, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. PCR products were electrophoresed on agarose gel, and visualized under ultraviolet (UV) light after electrophoresis. All PCR products were purified and sequenced by the Macrogen Sequencing Team (Macrogen Inc., Seoul, Korea).

Table 1. The sequences of primers for exon 6 of JAG1 gene.

Primer	Sequence (5'->3')	Length (bp)
Forward	CTTTTGTCAGGAGTCGGCTG	20
Reverse	ATGTTTCTAGCCCCAGTCGT	20

Bioinformatical and statistical analysis

The DNA sequence alignments were assembled using CodonCode Aligner software (CodonCode Corporation) and Geneious software (Biomatters Ltd.) with the reference sequence published on the NCBI website. The data were analyzed using mean ± SD, Independent two-sample t –tests and Chi-square, by the SPSS software, version 16.0 (SPSS Inc., Chicago IL., USA). A P-value of less than 0.05 was considered significant.

Results

The statistical analysis showed that the frequency of gender (24 males, 20 females) distribution in the case and control group is quite similar (P=1.000, Table 2). The age range in patients and the control group was 2-36 years, and the mean and standard deviation (SD) of the age in patients was 11.69 ± 7.85 years and in control group was 11.63 ± 7.99 years, so there was no significant difference between the means (P=0.968, Table 2). Finally the samples were successfully sequenced, then analyzed and one synonymous variant, c.765C>T; p.Y255Y, was observed in 38 patients with frequency 86.4% and three controls with frequency 6.8%. Fig. 1 illustrates a JAG1 heterozygous variant c.765 C>T. So statistical analysis of data showed a significant dif-

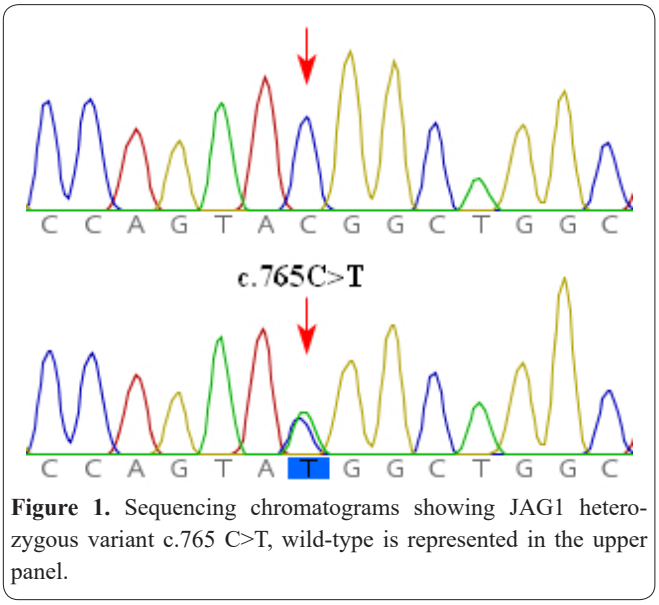

Figure 1. Sequencing chromatograms showing JAG1 heterozygous variant c.765 C>T, wild-type is represented in the upper panel.

Table 2. General and variant characteristics of TOF patients and controls.

	TOF (n=44)	Control (n=44)	p
Male / Female, n (%)	24 (54.5)/20	24/20 (45.5)	1.000
Age (Mean±SD) (year)	11.69±7.85	11.63±7.99	0.968
765C>T / wild-type, n (%)	38 (86.4)/ 6 (13.6)	3 (6.8)/41 (93.2)	<0.001

Table 3. Distributions of genotypes and alleles at c.765 C>T variant in JAG1 gene between TOF patients and controls.

Frequency	TOF n (%)	Control n (%)	p
Genotype			
CC	6 (13.6%)	41 (93.2%)	<0.001
CT	23 (52.3%)	1 (2.3%)	
TT	15 (34.1%)	2 (4.5%)	
Allele			
C	35 (39.8%)	83 (94.3%)	<0.001
T	53 (60.2%)	5 (5.7%)	

ference between patients and controls (P<0.001, Table 2). The allele and genotype distribution of c.765 C>T in JAG1 was identified and compared between TOF patients and control group. Based on the results, the frequency of T allele was significantly higher in TOF patients in comparison to controls (60.2% versus 5.7%, Table 3).

Discussion

For the first time in CHD patients with pulmonary stenosis and TOF, mutations in the JAG1 gene were identified (20). The exon 6 of JAG1 gene is much conserved between the Notch ligands (9, 23). In the present study, in the 38 patients with TOF and three controls one synonymous variant, i.e., c.765C>T; p.Y255Y was found. Since the variant at nucleotide 765 C→T is silent, the tyrosine amino acid does not change at 255 in the protein. For the first time, this variant was reported in 1999 in patients with Alagille syndrome (AGS) (26). Warthen *et al.,* studied 247 patients with AGS in a cohort study. They revealed that 232 (94%) of the patients with AGS had mutations in the JAG1 gene, hence, they concluded that heterozygous mutations in JAG1 cause AGS. They also observed the c.765C>T mutation in some patients (27). In 2014, Vazquez-Martinez *et al.,* analyzed nine Mexican patients with AGS. Interestingly, they found several mutations including c.765C>T variant (with the frequency of 0.53) in the JAG1 gene in 20–89% of the patients (14). Although the prevalence and effect of JAG1 gene mutations on cardiac demonstrations in AGS have been documented, reports on JAG1 mutations and correlation with phenotype are not well known in TOF (28).

In studies that were conducted on patients with TOF, other types of variants were reported for the exon 6 of JAG1 gene. For example, Eldadah *et al.*, reported a missense mutation c.821G>A; G274D in JAG1 gene in a large kindred segregating TOF patients with reduced penetrance. Nine of the eleven mutation carriers showed cardiac defect, including TOF, VSD and isolated peripheral pulmonic stenosis (PPS) (1).

Similarly, in 2009, a particular G274D mutation in the second EGF repeat of the JAG1 was identified to correlate with TOF manifestations but not with AGS phenotypes (29). Kola and colleagues identified four novel variations in JAG1 gene in Indian patients with TOF which were two missense c.814G>T; V272F and c.834 G>T; E278D, one nonsense c.765C>A; Y255X, and one silent variation c.861C>T; N287N in TOF patients (28).

Mutations in some genes are associated with TOF, including JAG1 (1, 28-30), NKX2.5 (31-35), NKX2.6 (36), GATA6 (37-39), GATA4 (40, 41), GATA5 (42, 43), Zinc Finger Protein, FOG Family Member 2 (ZFPM2/FOG2) (33, 44), Growth Differentiation Factor 1 (GDF1) (45), T-Box 1 (TBX1) (46), TBX5 (47), NOTCH1 (48), Gap Junction Protein Alpha 5 (GJA5) (49), Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2 (CITED2) (50, 51), Blood Vessel Epicardial Substance (BVES) (52), Paired Like Homeodomain 2 (PITX2) (53).

In conclusion, although synonymous variants exchange only codons, not amino acids, studies have shown that they can change gene expression and alter structure, stability, and the protein function and play a major role in human disorders pathogenesis (54-60). The synonymous variant c.765C>T does not change the amino acid, hence, based on the effects of the silencing variants on the protein functions, it may be concluded that, the variant affects the JAG1 function as a ligand binding form. Accordingly, functional study of this domain and its associated ligands can more clearly illuminate the effects of the variant and its pathogenicity. In the Middle East as well as Iran, limited genetic studies have been conducted on congenital heart disease such as TOF. According to the population-specific distribution of genetic variants for CHD, present study displays the outcomes of mutational analysis in patients with CHD in Iran. Therefore, this variant has a significant role in Iranian patients with TOF.

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Statement of Ethics
The participants and their parents have signed written informed consent. This study was approved by the local ethical committee of the Shahid Sadoughi University of Medical Sciences in Yazd, Iran.

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