European Review for Medical and Pharmacological Sciences 2017; 21: 3441-3446

Correlation between NFATC1 gene polymorphisms and congenital heart disease in children

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Abstract. – OBJECTIVE: To analyze the links between NFATC1 gene polymorphism and congenital heart disease in children.

PATIENTS AND METHODS: In the present study, we selected 85 children patients with congenital heart disease who were hospitalized from February 2013 to February 2015 as research subjects (observation group), and 92 healthy subjects as control group. Restriction fragment length polymorphism (RFLP) was used for analysis of NFATC1 gene in samples from each group.

RESULTS: The distribution of NFATC1 genotype and allele between the observation group (children with congenital heart disease) and the control group showed no significant difference ($p > 0.05$), but AA, GG genotypes, and allele frequency between pathological samples of children with congenital heart disease and the control group displayed significant difference ($p < 0.05$) ($X^2 = 16.04$, $p < 0.05$; $X^2 = 16.29$, $p < 0.05$). Further analyses showed that AA, GG, AG genotype and allele frequency among children with congenital heart disease in observation group also showed a difference, i.e., homozygote (AA, GG) ratio in children with severe congenital heart disease is relatively high.

CONCLUSIONS: There is a correlation between NFATC1 genes and the incidence of congenital heart disease in children, and a correlation between different genotypes and allele frequency and the incidence of the disease.

Key Words:
NFATC1 gene, Congenital heart disease, Children, Restriction fragment length polymorphism (RFLP).

Introduction

Congenital heart disease mainly refers to abnormalities in new blood vessels caused by alloplasia in heart and great vessels during childhood and research suggests that is a common heart disease in children[1,2]. The causes of congenital heart disease in children are complex, so this disease has severely harmed children's healthy growth[3,4]. The incidence of congenital heart disease in children is about 8% in the United States and about 4.2% in the UK, while in China the incidence stands at 10%, and higher if non-viable fetuses are included[5,6].

Congenital heart disease is under the influence of genetic factor(s), environmental factor(s) and many possible unknown factors[7]. Children with congenital heart disease caused by genetic and environmental factors account for about 75-90% of the children with congenital heart disease[8]. But the key genes closely related to congenital heart disease in children and their impact mechanism is not yet clear[9]. The NFATC1 gene, closely related to myocardial cell activity due to the transportation of calcium ions inside myocardial cells, is essential for maintaining the balance of calcium ions inside myocardial cells[10]. Calcium ions play an extremely important role in cardiac osmotic balance and normal activity of myocardial cells. Furthermore, the NFATC1 gene, can take part in T-cell activation and combine with the IL-2 promoter in T-cells to regulate transcription and translation of the downstream-related genes[11,12]. In this study, we looked at the relevance of NFATC1 gene polymorphisms and pediatric congenital heart disease, to provide a theoretical and experimental basis for the genetic mechanisms of congenital heart disease in children.

Patients and Methods

We selected 85 patients with congenital heart disease who were in-patients in our hospital from February 2013 to February 2015 (observation group). A total of 42 males and 43 females, with a mean age of 5.3 ± 4.1 years. The control group of 92 healthy subjects, were 48 males and 44 females, with a mean age of 5.7 ± 4.5 years.

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The heart disease patients were diagnosed according to the diagnostic criteria of congenital heart disease in children. All the subjects in the observation group comply with diagnostic criteria of congenital heart disease and suffer no other related diseases, and children in the control group are in good health. This study was approved by the Ethics Committee of Xuzhou Children's Hospital. Signed written informed consents were obtained from the patients and/or guardians.

Methods

Experimental data acquisition

6 ml of venous blood of children in the control group and the observation group was extracted and centrifuged for 7 min at 3000 rpm/min, then the upper serum isolated and stored at -80°C. Basal cells were stored in liquid nitrogen after being centrifuged and treated with cryopreservation fluid. The main molecular agents used in the present study were purchased from TaKaRa Co. (Dalian, China), and related primers are designed through Primer software and syntheses by the Shanghai Biological Engineering Co., Ltd. (Shanghai, China) (Table I).

NFATC1 Gene cCloning

Testicular tissue was cut into 1 mm3, and added 5 times equal volume of 1 mg/ml collagenase IV, then performed vibration at 37°C for 15 min. 10 times equal volume of 2.5 mg/ml trypsin was added, then vibrated at 37°C for 15 min; removed the upper cell suspension to centrifugal tube containing 10% fetal bovine serum Dulbecco's Modified Eagle Medium (DMEM)/F12 to terminate the digestion. After centrifugation in 200 g/min for 10 min, the supernatant was removed and washed twice with PBS (phosphate-buffered saline). 10% fetal bovine serum DMEM/F12 was added and cell mass was disrupted, filtered by 300 mesh strainer to isolate testicular tissue cell suspension. The final concentration was adjusted to 1×106/ml.

To obtain NFATC1 gene, TaKaRa animal cell genome extraction agent is used to extract and purify the genome in samples of the control group and the observation group. Then the genomes obtained from different samples are used as template to amplify NFATC1 gene, the specific steps are as follows: PCR reaction system (50 μl of) are as follows: ddH2O 38 μl, 10 × Buffer 5 μl, dNTP 4 μl (250 μmol/l), F 1 μl (0.5 mmol/l), R 1 μl (0.5 mmol/l), DNA 1 μl, LA 1 μl. The PCR (Polymerase Chain Reaction) reaction procedure is 95°C 5 min, 95°C 30s, 62°C 30s, 72°C 30s, 30 cycles, 72°C for 10 min, and reserved at 4°C. PCR products were identified through a 1% agarose gel and then observed through gel imager1[3].

NFATC1 Gene Polymorphism

To determine the relationship between gene mutations and congenital heart disease in children, 10 μl of PCR product obtained through PCR amplification is added 2 μl AvrII restriction enzymes (Thermo, Shanghai, China), followed by addition of 5 μl 10 × buffer and 331 μl sterile distilled water, then placed in 37°C water bath for 1h. 5 ul product after restriction enzyme digestion is taken and added 1 μl 6 × buffer, then goes through electrophoresis for 30 min in 1% a non-deformed polyacrylamide gel (100V, 40 mA), and then observed by gel imaging system[14].

NFATC1 Gene Sequencing

PCR products were sequenced after connection and conversion, and the specific operation is as follows: In this study, the PCR product is connected to the T19 carrier after restriction enzyme digestion and recovery, and then transformed into *E. coli* DH5α competent cells (the operation is in accordance with Molecular Cloning Manual). Three cases of PCR product in wild-type, heterozygous and muted homozygous of genes randomly selected form transformants are sent to the Shanghai Sangon Company for sequencing and analysis.

Classification Congenital Heart Disease in Children

We divided congenital heart disease into three groups based on the hemodynamic changes.

No shunt (non-cyanotic type): It mainly refers to the absence of abnormal path and shunt at the left and right side of the heart or between artery and vein, and there is no cyanosis during this process. The main symptoms include: coarctation of the aorta, aortic valve stenosis and pulmonary stenosis, pulmonary valve stenosis, primary pulmonary hypertension.

Left to right shunt group (latent cyanotic type): patients with this type of congenital heart disease have an abnormal path between the blood circulation routes along both sides of the heart. In the early stage, systemic circulation pressure on the left side of the heart is higher than the pulmonary circulation pressure on the right side of the lung, so usually, there are no cyanoses when the blood shunts from left to right. Such as atrial septal defect, ventricular septal defect, patent ductus arte-

Table I. NFATC1 exon primer sequence.

Primers		Product length
F	TGCGATCGTGCCGTAGCTGGC	462bp
R	GCTGGTACGTAGCTTGCCTAGTCG	

F = Forward; R = Reverse

Table II. Comparison of distribution of NFATC1 genotype and allele frequency in the control group and the observation group.

		Genotype frequency (%)				
Group	N	AA	GG	AG	X^2	p
Observation	85	28 (33)	30 (35.3)	27 (31.7)	16.04	0.000
Control	92	37 (40.2)	32 (34.8)	22 (25)		
		Allele frequency (%)				
Group	N	A	G		X^2	p
Observation	170	105 (48.8)	47 (51.2)		16.29	0.000
Control	184	113 (52.2)	33 (47.8)			

riosus, primary pulmonary septal defect and aortic sinus aneurysm ruptured into the right heart or pulmonary artery and so on.

Right to left shunt group (cyanotic type): The main symptom of patients with this type of congenital heart disease includes malformations that constitute abnormal transportation between right and left side of the cardiovascular cavity. Venous blood from the right cardiovascular cavity flows into the left cardiovascular cavity through abnormal traffic, which causes the flow of a great deal of blood into the systemic circulation, hence the presence of lasting cyanoze.

Statistical Analysis

Measurement data obtained in this study are represented with $X^2 \pm s$. Distribution of allele and genotype frequency is represented with relevant technical data. Allele frequency = (case number of homozygous × 2 + case number of heterozygous) × 2-1 × n-1. All data are analyzed by statistical software SPSS 20.0 (SPSS Inc., Chicago, IL, USA). $p<0.05$ was considered statistically significant.

Results

NFATC1 gene PCR-RFLP

GNAS2-G/G genotype cut by AvrII restriction enzyme, results in two bands: 185 bp and 309 bp. NFATC1 genes cannot be cut by AvrII restriction enzyme, so it has only one band, 494bp; NFATC1 produces three bands, 494 bp, 185 bp and 309 bp after being cut by Pml1 restriction endonuclease (Figure 1). The RE digestion results of PCR products was consistent with our sequencing results.

Genotype and Allele Frequencies of Observation Group Versus Control Groups

We carried out statistical tests on allele A, G frequency, genotype AA, GG frequency in the observation and control group, and the experimental result showed significant differences between the control group and the observation group ($X^2 = 16.04$, $p <0.05$; $X^2 = 16.29$, $p <0.05$). X^2 test and verification results of genotype and allele frequencies of the control group and the observation group showed that it complied with Hardy-Weinberg equilibrium (Table II).

Genotype and Allele Frequencies from children with Cardiac Insufficiency

We divided children with cardiac insufficiency into three subtypes: sinus arrhythmia, ectopic beats and ectopic rhythm and conduction abnormalities. We carried out statistical tests on genotype (AA, GG) and allele (A, G) frequency of the three types. The experimental result showed significant differences in genotype and allele frequencies

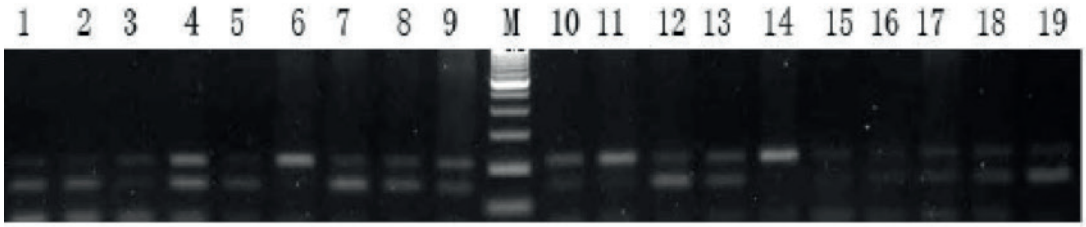

Figure 1. Electrophoresis result of NFATC1 gene PCR-RFLP. Lanes 1, 2 = NFATC1 gene AA homozygote; Lanes 3-5, 7-10, 12,13 = NFATC1 gene A/G heterozygote genotype; Lanes 6, 11, 14 = NFATC1 gene homozygous GG

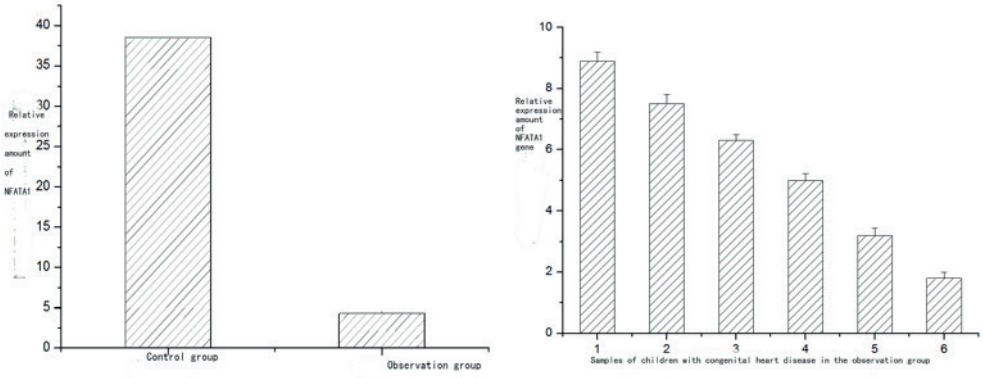

Figure 2. Expression testing of different genotypes NFATC1. ***A,*** Relative gene expression quantity of NFATC1 in the control group and the observation group; ***B,*** NFATC1 gene expression quantity of patients with different NFATC1 genotypes in the observation group.

(X^2 = 15.43 p <0.05; X^2 = 16.27, p <0.05) among patients with different degrees of illness in the observation group. X^2-test and verification result of genotype and allele frequencies of the control group and the observation group showed that it complied with Hardy-Weinberg equilibrium (Table III).

Expression Detection of Different Genotype of NFATC1

To explore the impact of different gene polymorphisms of NFATC1 on the expression of the NFATC1 gene, we used ELISA to detect NFATC1 gene expression in the observation and the control groups, as well as NFATC1 gene expression of different patients (Figure 2). Expression of NFATC1 in the observation group was relatively low, and the amount of NFATC1 in different genotypes among patients in the observation group showed significant differences (p <0.05). The results were consistent, indicating a relatively close correlation between NFATC1 genes and congenital heart disease in children.

Discussion

Congenital heart disease is a common birth defect, with many complications, that causes severe harm to children's lives[15]. Although research on congenital heart disease in children has made some success, there are no well-defined studies on the pathogenesis and related causes of congenital heart disease in children. Therefore, the pathogenesis of this disease is not yet clear, which directly leads to the current absence of specific medicine for the disease. Jones et al. showed that, among the diverse factors causing congenital heart disease, gene-environment interactions could lead to gene mutations which lead to different types of congenital heart diseases[16]. They concluded that toxic substances in the environment, ingested through food or water, results in congenital heart defects when the immune system of the pregnant women and fetus are low.

The NFATC1 gene can participate in the transport of calcium ions in the body and transport

Table III. Comparison of distribution of NFATC1 genotype and allele frequencies in children with varied degrees of arrhythmia in the observation group.

Group	N	Genotype frequency (%) AA	GG	AG	X^2	p	H-W equilibrium
No shunt (non-cyanotic type)	30	8	10	12	15.75	$p<0.05$	0.121
Left to right shunt group (latent cyanotic type)	29	7	8	14			
Right to left shunt group (cyanotic type)	26	13	12	1			

Group	N	Allele frequency (%) A	G		X^2	p	H-W equilibrium
No shunt (non-cyanotic type)	60	28 (47)	32 (53)	16.49	$p<0.05$	0.152	
Left to right shunt group (latent cyanotic type)	58	28 (48.3)	30 (51.7)				
Right to left shunt group (cyanotic type)	52	27 (52)	25 (48)				

of calcium ions in myocardial cells[17]. As an intracellular signal transduction mechanism, calcium signaling system plays an important role in the normal physiological activities of cells[18]. Concentration, distribution and real-time changes of calcium ions can directly affect the contractility, heart rate, growth and apoptosis of myocardial cells[19-21]. Children with congenital heart disease (and low calcium ion concentrations) have weak myocardial contractility and are thus unable to provide enough blood and nutrients[22] throughout the body.

Conclusions

In this study, we explored the correlation between NFATC1 gene polymorphisms and congenital heart disease. Our results show no significant difference in the distribution of NFATC1 genotypes and alleles in children with congenital heart disease. However, significant differences in AA, GG genotype, and allele frequency between samples of children with congenital heart disease and of those in the control group were observed. Additional analyses showed homozygous AA, GG genotype frequency and allele frequency in children with severe congenital heart disease is relatively high. ELISA results were consistent with the above, indicating that there exists a correlation between NFATC1 gene polymorphism and congenital heart disease in children. Our results may provide a theoretical and experimental basis for the treatment of congenital heart disease in children.

Conflict of interest

The authors declare no conflicts of interest.

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