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Analysis of *NKX2-5* in 439 Chinese Patients with Sporadic Atrial Septal Defect

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Background: The *NKX2* gene family is made up of core transcription factors that are involved in the morphogenesis of the vertebrate heart. *NKx2-5* plays a pivotal role in mouse cardiogenesis, and mutations in *NKx2-5* result in an abnormal structure and function of the heart, including atrial septal defect and cardiac electrophysiological abnormalities.

Material/Methods: To investigate the genetic variation of *NKX2-5* in Chinese patients with sporadic atrial septal defect, we sequenced the full length of the *NKX2-5* gene in the participants of the study. Four hundred thirty-nine patients and 567 healthy unrelated individuals were recruited. Genomic DNA was extracted from the peripheral blood leukocytes of the participants. DNA samples from the participants were amplified by multiplex PCR and sequenced on an Illumina HiSeq platform. Variations were detected by comparison with a standard reference genome and annotation with a variant effect predictor.

Results: Thirty variations were detected in Chinese patients with sporadic atrial septal defect, and 6 single nucleotide polymorphisms (SNPs) had a frequency greater than 1%. Among the 30 variations, the SNPs rs2277923 and rs3729753 were extremely prominent, with a high frequency and odds ratio in patients.

Conclusions: Single nucleotide variations are the prominent genetic variations of *NKX2-5* in Chinese patients with sporadic atrial septal defect. The SNPs rs2277923 and rs3729753 are prominent single nucleotide variations (SNVs) in Chinese patients with sporadic atrial septal defect.

MeSH Keywords: **Genetic Variation • Heart Diseases • Heart Septal Defects, Atrial • Polymorphism, Single Nucleotide**

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Background

The heart is an extremely important dynamic organ. The development of the heart is complex and involves many signaling pathways and transcription factors [1–3]. Many factors, such as environment factors, maternal factors, special medicine taken during pregnancy, genetic variations and so on [4–9], may disturb these regulated factors and lead to an aberrant structure and function of the heart, potentially resulting in congenital heart disease (CHD). CHD includes atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus, tetralogy of Fallot and so on. Among them, ASD is one of the most common subtypes of CHD [10].

Human *NKX2-5*, murine *Nkx2-5*, and *Drosophila tinman* all belong to the NK homeobox gene family, which includes a conserved homeodomain [11] and plays a crucial role in the development of the heart, regulating the proliferation, differentiation, and electrophysiological properties of cardiac cells. In mice, *Nkx2-5* is expressed in progenitor cells of the heart in the first heart field (FHF), second heart field (SHF), and pharyngeal endoderm [12,13], and it is necessary and sufficient for the development of the SHF [13] and a determinate of differentiation into the myocardial lineage [12]. In the SHF of mice, *NKx2-5* regulates the proliferation of heart progenitors and the morphology of the outflow tract [14]. In zebrafish, *Nkx* genes regulate electrophysiological patterning [15]. Mutations in murine *NKx2-5* result in embryonic death and an abnormal structure and function of heart, including contraction defects, valvular dysmorphogenesis, atrial septal defect, ventricular noncompaction cardiomyopathy, and arrhythmias [16–22].

The aforementioned results demonstrate that *NKx2-5* is a core regulatory transcription factor in cardiogenesis, and that disruption of *NKx2-5* results in an abnormal structure and function of heart. However, the pathogenic mechanism of *NKX2-5* in Chinese patients with sporadic atrial septal defect is unclear. To investigate the genetic variation of *NKX2-5* in Chinese patients with sporadic atrial septal defect, we performed sequence analysis on full length *NKX2-5*.

Material and Methods

Study participants

For this study, 439 patients with sporadic atrial septal defect (157 males and 282 females) and 567 healthy unrelated individuals (274 males and 293 females) were recruited. Patients were recruited from the Department of Cardiovascular Surgery at Yan'an Affiliated Hospital of Kunming Medical University. Healthy unrelated individuals, confirmed by a medical examination and cardiac color Doppler ultrasound, were recruited from our previous epidemiological investigation. The ethnic groups of the participants included Han, Bai, Yi, Dai, and so on. There was no obvious ethnic group difference between the patients and the healthy controls. Diagnosis of ASD was confirmed by cardiac color Doppler ultrasound and findings during cardiac surgery, and other CHDs, such as ventricular septal defect, tetralogy of Fallot, Ebstein anomaly, patent ductus arteriosus, endocardial cushion defect, and Holt-Oram syndromes, were exclusion criteria for this study. This study was approved by the institutional research ethics committee of Yan'an Affiliated Hospital of Kunming Medical University. All participants or their parents provided informed consent. Genomic DNA was extracted from the peripheral blood leukocytes of the participants.

Primers

Primers (Table 1) were designed to amplify full-length *NKX2-5*. The specificity and efficiency of the primers was validated by polymerase chain reaction (PCR). The amplification system and experimental procedure of PCR are described in the supplementary data (Supplementary Tables 1, 2).

Multiplex PCR and qPCR

Multiplex PCR was performed twice to amplify full-length *NKX2-5*. The amplification system and experimental procedure of multiplex PCR are described in the supplementary data (Supplementary Tables 3–6). The accuracy and efficiency of the multiplex PCR were validated by quantitative polymerase chain reaction (qPCR). The amplification system and experimental procedure of qPCR are described in the supplementary data (Supplementary Tables 7, 8).

Variation detection and statistical analysis

The products of multiplex PCR were mixed to form the sequencing library. An Illumina HiSeq platform was used to sequence the library. Human reference genome sequences (Edition: GRCh38.p7) and Burrows-Wheeler Alignment (BWA) (Edition: 0.1.22) were used to compare the sequences of the samples with the standard reference genome sequences. Detection of variation was processed by a genome analysis toolkit (GATK, Edition: v3.1). An online software variant effect predictor was used to annotate the variation. A square test was used to compare the 2 samples. SPSS 22.0 (IBM, USA) was used to analyze the data. A *P*-value <0.05 was used to indicate a statistically significant difference.

Results

Collation and statistics of raw data

The Q30 for all of the samples was greater than 90%. The total reads of *NKX2-5* were 147 014 508 bp. After filtering, the reads of

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Table 1. Primers for multiplex PCR.

ID of amplicons	Primer sequence	
	Forward primer	Reverse primer
Primer1.1-F	TTCCAGCAAGGGTTAGGTTTT	GCACCCACCCGTATTTATGTTT
Primer1.2-F	ACACGTGCGGGGTCAACG	GAGAGTCCATGGGCACCCC
Primer1.3-F	CCCTGGCTCGCGGAATG	TGCGGAGACCTAGGAACTTTT
Primer1.4-F	CAGGATCACTCATTGCACGC	CCGAGCCTGGTAGGGAAGG
Primer1.5-F	CATGTTGGGAGCCCCTTCTC	CTGCCGCCGCCAACAA
Primer1.6-F	GGAATCCCGGGGCTCTG	GCCTGCCTACGGCGTGG
Primer1.7-F	CGCGCCGCCGTAACC	GCAGGACCAGACTCTGGAGC
Primer1.8-F	CACTGGCACCGCGATCC	TCGCAGGCGCAGGTCTAT
Primer1.9-F	CGTGGACGTGAGTTTCAGCAC	GCGCTGCAGAAGGCGG
Primer1.10-F	GTCCGCCTCTGTCTTCTCCA	TGTCGGTGGCTCCCAGTG
Primer1.11-F	GTAAGAGCGGCTTGACCTACG	CGTCTGTCTCCCTCACCAGG
Primer1.12-F	TAGAAAGTCAGGCTGGCTCAA	TCTGTTCATTTAACTTCTCAAACCA
Primer1.13-F	AAACCAGGTGATGCTCTGGG	CCCGCAGCTTCTTGGACAC
Primer1.14-F	CAAAATTAAGGAATCAAGAAACAGAA	TTTGTTGAGGAGCAGAGGCC
Primer1.15-F	GGCTTCCTCGAATTGGTAGC	CCCCGCTGCAGAAAGGG
Primer1.16-F	TTCGCCCAGCGCTTCG	GGGCGGAGAGGTTCCCTC
Primer1.17-F	CACTCATCTCGTCCACTCCCTT	GGGTTTCTGGTGCCTTTCTT
Primer1.18-F	GGTCCGCAGTATCCCATTTTAA	TCTCCTTTGGATTTACGAATCTTT
Primer1.19-F	GATTGTACGCAAAAGCTGTCTG	GGCCTAACAAAGCCCCGG
Primer1.20-F	GATTTCTCAACTTCCTACCAGACC	GAGACACGCGCCCTTGG
Primer1.21-F	TCTGAACCTCCGATTGGACG	CCAGCCAAGGACCCTAGAGC
Primer1.22-F	GACCCAGGAGGGGAGAAGG	TCAAGCCAGAGGCCTACGCT
Primer1.23-F	GAAAGGCAGACGCACACTTG	TCAAAGACATCCTAAACCTGGAA
Primer1.24-F	GCCAGGGTCGCCTCCA	GCGGCACCATGCAGGG
Primer1.25-F	GGCAGCGCCAGTCTCACA	GGCCCCTGGCCCAATG
Primer1.26-F	AGCAGGTAGCGCTGAGCACA	CCTTCCAAATGCGTCGTGG

NKX2-5 were 145502856 bp. The total reads of *NKX2-5* matched to the reference sequence were 79 266 998 bp. After filtering, the corrected reads were 66 473 696 bp. The average depth of the sequencing of each amplicon was approximately 5800-fold.

Thirty variations detected in 439 patients

Through comparison with the standard reference genome sequences, 30 variations (Supplementary Table 9) were detected in patients. The 30 variations included 18 single nucleotide polymorphisms (SNPs) and 12 novel variations (without rs number). The 18 SNPs were rs2277923, rs703752, rs3131915, rs3729753, rs376790353, rs549757001, rs562842387, rs531139209, rs892817393, rs1004442524, rs540202205, rs375010127, rs202071628, rs553775519, rs759221178, rs767243751, rs72554029, and rs551687864. The 12 novel variations, described as chromosomal locations, are 173233943, 173234153, 173233940, 173233733, 173232329, 173233307, 173234249, 173235293, 173234696, 173234963, 173235001, and 173233307. Six SNPs of the 30 variations had a high frequency greater than 1%. The 6 high-frequency SNPs in the 439 patients are listed in Table 2.

Two SNPs detected in 567 healthy controls

Two SNPs (Table 3) were detected in healthy controls. These SNPs were rs703752 and rs2277923 and had frequencies of 68.78% and 15.17%, respectively.

rs2277923 and rs3729753 are prominent SNVs with a high frequency and odds ratio in patients

Among the 30 variations, rs2277923 and rs3729753 were prominent with a high frequency and odds ratio (Table 4) in patients.

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Table 2. Six high-frequency SNPs detected in 439 patients.

LOC1	rs number	REF	ALT	AAC	LOC2	SNP (n=439)	Frequency (%)
173235021	rs2277923	T	C	Glu > Glu	Extron	352	80.18
173232508	rs703752	C	A	None	3/UTR	73	16.63
173233891	rs3131915	C	G	None	Intron	68	15.49
173232938	rs3729753	C	G	Leu > Leu	Extron	21	4.78
173233982	rs376790353	C	A	None	Intron	9	2.05
173233290	rs549757001	G	A	None	Intron	8	1.82

LOC1 – position in the chromosome; REF – reference genomic locus information; ALT – locus variation information in the sample; AAC – amino acid change; LOC2 – position in the *NKX2-5*; SNPs – single nucleotide polymorphisms.

Table 3. Two SNPs detected in healthy controls.

LOC1	rs number	REF	ALT	AAC	LOC2	SNP (n=567)	Frequency (%)
173232508	rs703752	C	A	none	3/UTR	390	68.78
173235021	rs2277923	G	A	Glu>Glu	Extron	86	15.17

LOC1 – position in the chromosome; REF – reference genomic locus information; ALT – locus variation information in the sample; AAC – amino acid change; LOC2 – position in the *NKX2-5*; SNPs – single nucleotide polymorphisms.

Table 4. χ^2, *P*-value and OR of SNPs in ASD versus control.

SNP	ASD (n=439)	Control (n=567)	χ^2, *P*-value	OR, 95%CI	
rs2277923	352	86	χ^2=425.45, P<0.001	5.286	(4.328–6.458)
rs3729753	21	0	χ^2=27.70, P<0.001	27.123	(3.663–200.858)
rs703752	73	390	χ^2=270.92, P<0.001	0.242	(0.195–0.300)
rs202071628	1	0	χ^2=1.293, P=0.256	0.998	(0.993–1.002)

OR – odds ratio; ASD – atrial septal defect; SNP – single nucleotide polymorphisms.

Discussion

In this study, we sequenced full-length *NKX2-5* and detected 30 variations of *NKX2-5* in Chinese patients with sporadic atrial septal defect. Six of the 30 variations had a frequency greater than 1%. The SNPs rs2277923 and rs3729753 were prominent SNVs with a high frequency and odds ratio in Chinese patients with sporadic atrial septal defect.

Human *NKX2-5* consisted of 3218 bp located at 5q35.1 and encoded 3 isoforms through pre-RNA splicing. The 3 isoforms were isoform 1, isoform 2, and isoform 3. Isoform 1 has the homeodomain, which is strictly expressed in the heart, and is the most abundant isoform in heart tissue. Isoform 2 and isoform 3 lack the homeodomain and have a low abundance in heart tissue [23]. Isoform 1 consists of 324 amino acids, which contain an evolutionarily conserved homeodomain-containing domain (HD, amino acids 138–197) in the middle, a tinman domain (TN, amino acids 10–21) at the N-terminus, a nuclear localization signal (NLS) near the N-terminus of the homeodomain, a NK2-specific domain (NK2, amino acids 212–234) at the C-terminus [11,24-26], and a tyrosine-rich homophilic interaction domain (YRD) near the NK2 domain [27,28]. The HD domain is involved in DNA binding activity and transcriptional activation [29]. The NLS domain could influence the location of NKX2-5 by phosphorylation and dephosphorylation [30]. The NK2 domain functions as a regulator of transcriptional activation [31]. The YRD domain is essential for the function of NKX2-5 in cardiogenesis and embryonic development [27]. The function of the TN domain is still unclear. As a crucial transcriptional factor, NKX2-5 is a regulator of the development of the heart [1–3,32,33] that functions by binding its downstream targets through its functional domain, such as the HD domain, TN domain, NK domain, and YRD domain [28,31,34,35].

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In this study, we detected 30 genetic variations of *NKX2-5* in patients with sporadic atrial septal defect, and no frameshift mutations or insertion/deletion mutations were detected. These results demonstrate that single nucleotide variations (SNVs) are the prominent genetic variations of *NKX2-5* in Chinese patients with sporadic atrial septal defect. Among them, 6 SNPs had a high frequency greater than 1%. These SNPs were distributed in the exons, introns, and 3′UTR of *NKX2-5*. Although these SNPs did not change the amino acid sequence and structure of the domains of NKX2-5, we still considered these variations, especially the SNPs rs2277923 and rs3729753, to have the potential and ability to cause sporadic atrial septal defect for the following reasons. First, compared with healthy people, patients with sporadic atrial septal defect had a high frequency of and odds ratio for rs2277923 and rs3729753. Second, although synonymous SNVs did not change the structure of the protein, a synonymous SNV can influence the structure of the pre-RNA and the binding to RNA binding protein [36,37]. Pre-RNA of *NKX2-5* was spliced through an alternative splicing mechanism to generate 3 isoforms. The presence of an SNV may influence the recognition and binding of the RNA binding protein and spliceosomes. The presence of an SNV may also influence the stability and degradation of RNA in the cytoplasm [36,37]. Third, as a crucial transcription factor, the regulatory functions of NKX2-5 depended on its quality and abundance. SNV might influence the RNA level, resulting in changes in the abundance and distribution of the transcript of *NKX2-5*, which then disturbs the downstream targets of NKX2-5.

Conclusions

Single nucleotide variations are the prominent genetic variations of *NKX2-5* in Chinese patients with sporadic atrial septal defect. The SNPs rs2277923 and rs3729753 are prominent SNVs in Chinese patients with sporadic atrial septal defect. A pathogenic mechanism of rs2277923 and rs3729753 may exist at the RNA level. Further studies are needed to validate this possibility.

Supplementary Tables

Supplementary Table 1. Amplification system of PCR.

Reagent component	Volume	Concentration
Buffer (10×)	1 uL	
Primer (1 uM)	2 uL	
dNTP (2.5 mM)	0.8 uL	
DNA polymerase (5 U/uL)	0.1 uL	1×
Template DNA	2 uL	
Mg_2+ (100 mM)	1 uL	
ddH_2O	3.2 uL	
Paraffin oil	10 uL	

Supplementary Table 2. Experimental procedure of PCR.

Step	Temperature	Time	Cycle
1	95°C	15 min	1 cycle
2	94°C	30 s	
3	60°C	90 s	35 cycles
4	72°C	30 s	
5	72°C	10 min	1 cycle

Supplementary Table 3. Amplification system of the first turn multiplex PCR.

Reagent component	Volume	Concentration
Buffer (10×)	1 uL	
Primer (50 nM)	2 uL	
dNTP (2.5 mM)	0.8 uL	
DNA polymerase (5 U/uL)	0.1 uL	1×
Template DNA	2 uL	
Mg_2+ (100 mM)	1 uL	
ddH_2O	3.2 uL	
Paraffin oil	10 uL	

Supplementary Table 4. Experimental procedure of the first turn multiplex PCR.

Step	Temperature	Time	Cycle
1	95°C	15 min	1 cycle
2	94°C	30 s	
3	60°C	10 min	4 cycles
4	72°C	30 s	
5	94°C	30 s	
6	60°C	1 min	20 cycle
7	72°C	30 s	

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Supplementary Table 5. Amplification system of the second turn multiplex PCR.

Reagent component	Volume	Concentration
Buffer (10×)	2 uL	
Barcode (2 uM)	3.6 uL	
dNTP (2.5 mM)	0.8 uL	
DNA polymerase (5 U/uL)	0.1 uL	1×
Template DNA	10 uL	
Mg_2+ (100 mM)	1 uL	
ddH_2O	3.6 uL	
Paraffin oil	20 uL	

Supplementary Table 6. Experimental procedure of the second turn multiplex PCR.

Step	Temperature	Time	Cycle
1	95°C	15 min	1 cycle
2	94°C	30 s	
3	60°C	4 min	5 cycles
4	72°C	30 s	
5	94°C	30 s	
6	65°C	1 min	10 cycle
7	72°C	30 s	

Supplementary Table 7. Amplification system of qPCR.

Reagent component	Volume	Concentration
Mix	6 uL	
Primer (1 uM)	2 uL	
Template DNA	1.8 uL	1×
ROX	0.24 uL	
ddH_2O	2 uL	

Supplementary Table 8. Experimental procedure of qPCR.

Step	Temperature	Time	Cycle
1	95°C	2 min	1 cycle
2	94°C	30 s	
3	60°C	30 s	35 cycles
4	72°C	30 s	

Supplementary Table 9. 30 variations detected in 439 patients.

LOC	rs number	REF	ALT	SNP (n=439)	Frequency (%)
173235021	rs2277923	T	C	352	80.18
173232508	rs703752	C	A	73	16.63
173233891	rs3131915	C	G	68	15.49
173232938	rs3729753	C	G	21	4.78
173233982	rs376790353	C	A	9	2.05
173233290	rs549757001	G	A	8	1.82
173233943	None	C	A	4	0.91
173232370	rs562842387	C	T	3	0.68
173234153	None	A	G	2	0.46
173234425	rs531139209	C	G	2	0.46
173233698	rs892817393	A	G	2	0.46
173233940	None	G	T	2	0.46
173234640	rs1004442524	C	T	2	0.46

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LOC	rs number	REF	ALT	SNP (n=439)	Frequency (%)
173232273	rs540202205	C	G	2	0.46
173233733	None	G	A	1	0.23
173232340	rs375010127	T	C	1	0.23
173232839	rs202071628	C	G	1	0.23
173232329	None	G	T	1	0.23
173232316	rs553775519	G	T	1	0.23
173233307	None	A	T	1	0.23
173234249	None	C	T	1	0.23
173235293	None	G	C	1	0.23
173234696	None	T	C	1	0.23
173234775	rs759221178	G	A	1	0.23
173234784	rs767243751	G	T	1	0.23
173234847	rs72554029	C	G	1	0.23
173234963	None	G	A	1	0.23
173235001	None	G	A	1	0.23
173233307	None	A	T	1	0.23
173233409	rs551687864	G	A	1	0.23

LOC – position in the chromosome; REF – reference genomic locus information; ALT – locus variation information in the sample.

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