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Research paper

Genetic variations of NKX2-5 in sporadic atrial septal defect and ventricular septal defect in Chinese Yunnan population

Yu Cao [a,1], Junqiang Wang [a,b,1], Chuanyu Wei [a], Zongliu Hou [a], Yaxiong Li [a], Honglin Zou [a], Mingyao Meng [a], Wenju Wang [a,*], Lihong Jiang [a,*]

[a] Yan'an Affiliated Hospital of Kunming Medical University, Kunming Medical University, Kunming, Yunnan, PR China
[b] The People's Hospital of Wenshan Prefecture, Wenshan, Yunnan, PR China

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ABSTRACT

Congenital heart disease (CHD) is the most common birth abnormality, and more than 40% CHD subtypes are sporadic atrial septal defect (ASD) and ventricular septal defect (VSD). The etiology of ASD and VSD remains largely unknown. NKX2-5 gene is a highly conserved homeobox protein gene and expressed in the developing heart. Its mutations can cause sporadic ASD and VSD. This study aimed to investigate the genetic variations of NKX2-5 in ASD and VSD in Chinese Yunnan population. The whole 2 coding exon and partial flanking intron sequences of NKX2-5 gene were screened using DNA sequencing in 107 ASD patients and 391 VSD patients as well as 487 healthy individuals (control) who had parental origin (three generations) from the Yunnan province in China. Results found that, 4 reported single nucleotide polymorphisms (SNPs) (rs2277923, rs3729753, rs703752 and rs202071628) were detected. A novel heterozygous DNA sequence variant (DSV) (1500G > C) in the 3′UTR region of NKX2-5 gene were identified in 2 VSD patients, but none in ASD and controls. One single nucleotide polymorphism (rs2277923), the frequency of which was significantly higher in ASD group, and the allele and genotype were associated with the occurrence of ASD. Besides, a weak statistical association existed between rs703752 and VSD (uncorrected $P = 0.028$). The novel DSV (1500G > C) of NKX2-5 gene may contribute to a small number of VSD, and rs2277923 SNP may contribute to the risk of sporadic ASD in Chinese Yunnan population.

1. Introduction

Congenital heart disease (CHD) is the main cause of death in infants, which affects 4–10 per 1000 live births in the world (Go et al., 2013). Epidemiological study shows that, there is a high incidence of CHD in Yunnan province in China, and the main subtypes of CHD are sporadic atrial septal defect (ASD) and ventricular septal defect (VSD), which account for approximately 40% of all congenital heart malformation in Yunnan province (Jiang et al., 2005). Although about 80% of CHD is multi-factorial and arises through various combinations of genetic and environmental contributors, the etiology of sporadic ASD and VSD are largely unknown (Blue et al., 2012).

To date, more than 40 genes have been reported to be involved into congenital cardiac malformation (Bruneau, 2008; McCulley and Black, 2012). NKX2-5 gene is a highly conserved homeobox protein gene and expressed in the developing heart, as well as in adult heart. It is the first known marker associated with CHD, and is initially validated in pedigrees with autosomal dominant inheritance of cardiac septal defects (Wessels and Willems, 2010). So far, approximately 30 mutations in the NKX2-5 gene have been found in ASD and VSD patients in different regions. In this study, the NKX2-5 gene mutations in 391 VSD patients and 107 ASD patients as well as 487 healthy individuals were detected, and the correlations between genotypes and phenotypes were investigated. The objective is to provide a foundation for a more integrated understanding of the molecular basis of ASD and VSD.

2. Subjects and methods

2.1. Subjects

From August 2011 to April 2014, 391 unrelated VSD patients (204 males and 187 females) and 107 unrelated ASD patients (49 males and 58 females) were recruited from Department of Cardiac Surgery, Yan'an Affiliated Hospital of Kunming Medical University (Kunming,

Abbreviations: CHD, Congenital heart disease; ASD, atrial septal defect; VSD, ventricular septal defect; SNPs, single nucleotide polymorphisms; DSV, DNA sequence variant; OR, odds ratio; CI, confidence interval; TOF, tetralogy of Fallot; PDA, patent ductus arteriosus; HRHS, Hypoplastic Right Heart Syndrome.

* Corresponding authors at: Yan'an Affiliated Hospital of Kunming Medical University, Kunming Medical University, Kunming 650051, Yunnan, PR China.
E-mail addresses: wang_wenju@yeah.net (W. Wang), lihongjiangchinayn@163.com (L. Jiang).

[1] These authors contributed equally to this work.

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China). All patients had parental origin (three generations) from the Yunnan province in China, and had no familiar history. The diagnosis was made through physical examination and echocardiography by an experienced cardiologist. All patients had experienced cardiac surgical operation (ASD repair or VSD repair). In addition, 487 unrelated healthy individuals (274 males and 213 females) from the Department of Physical Examination in Yan'an Affiliated Hospital of Kunming Medical University were selected as control. Signed written informed consents were obtained from all patients and controls. This study was approved by the Institutional Review Boards of Kunming Medical University.

2.2. Extraction of genomic DNA

2 mL of peripheral blood was collected from all subjects. The DNA was extracted using Axyprep Blood Genomic DNA Miniprep kit (Axygen Scientific Inc., CA, USA) according to manufacturer's instructions.

2.3. Primer design, PCR amplification and genotyping

The sequences of whole 2 coding exons and partial flanking introns in NKX2-5 gene were amplified by PCR using the following system: 2 μL DNA sample, 0.15 μL Taq polymerase (5 U/μL), 1 μL forward and reverse primers (5 μM for each), 2 μL dNTP mixture, 2.5 μL 10 × PCR buffer (Mg^{2+} plus), and 17.35 μL ddH_2O to total volume of 25 μL. The 10 × PCR buffer (Mg^{2+} plus) contained 100 mM Tris–HCl, 500 mM KCL and 15 mM $MgCl_2$. The amplification conditions were as follows: 95 °C for 5 min; 36 cycles at 95 °C for 30 s, X °C for 30 s, 72 °C for 40 s; final extension at 72 °C for 5 min (X: N1, 61; N2, 63; N3, 58). PCR kits were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). Three primers (N1–N3) were designed by Prime 5.0 (http://www.premierbiosoft.com) to amplify the sequences of whole 2 exons and partial flanking introns in NKX2-5 gene. Primer sequences were presented in Table 1.

The genotypes of 4 SNPs of NKX2-5 gene (rs2277923, rs3729753, rs703752 and rs202071628) were determined using DNA direct sequencing method (Beijing Liuhe Huada Gene Polytron Technologies Inc., Beijing, China). The DNAstar software (DNASTAR Corp., WI, USA) was used to compare DNA sequencing diagrams and find the related polymorphism sites.

2.4. Statistical analysis

All SNPs were assessed for Hardy–Weinberg equilibrium (HWE) by χ^2 statistics. The comparisons of genotype and allele frequencies were evaluated by χ^2 test. The association of NKX2-5 gene polymorphisms with CHD risk was estimated by computing odds ratio (OR) and 95% confidence interval (CI) from the multivariate logistic regression analysis. All statistical analysis was performed by SPSS 17.0 software (SPSS Inc., IL, USA). In addition, Haplotype constructions and Mendel linkage disequilibrium were analyzed using online computer platform SHEsis and Haploview software (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He, 2005). Haplotypes with frequencies >3% in the combined cases and controls were examined. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Sequencing results

A total of 107 ASD patients and 391 VSD patients as well as 487 controls were analyzed for mutation screening with NKX2-5 gene using DNA sequencing. 4 reported single nucleotide polymorphisms (SNPs) (rs2277923, rs3729753, rs703752 and rs202071628) were detected. The information of them was shown in Table 2. A new heterozygous mutation in NKX2-5 gene was identified in 2 VSD patients, but none in ASD and controls. Its 1500th base G mutated into C. The sequencing diagram was shown in Fig. 1.

3.2. Results of statistical analysis

rs2277923, rs3729753, rs703752 and rs202071628 SNPs of ASD and VSD as well as controls were in line with the genetic balance Hardy–Weinberg test. GG, GA and AA genotypes were detected in rs2277923 site. The frequency distribution of three genotypes had significant difference between ASD and control group ($P = 0.009$), and logistic regression analysis revealed that, the AA genotype was positively correlated with ASD ($P = 0.002$; OR = 2.124; 95% CI: 1.203–3.750). The frequency distribution of 2 alleles (G, A) had significant difference ASD and control group ($P = 0.008$), and logistic regression analysis revealed that A allele was positively correlated with ASD ($P = 0.008$, OR = 1.503, 95% CI: 1.113–2.029). In addition, a weak statistical association existed between rs703752 and VSD group (uncorrected, $P = 0.028$). GG, GT and TT genotypes were detected in rs703752 site. Logistic regression analysis revealed that GG genotype was positively correlated with VSD ($P = 0.045$; OR = 7.615; 95% CI: 0.960–60.42). The frequency distribution of 2 alleles (G, T) had significant difference between groups ($P = 0.016$), and logistic regression analysis revealed that the G allele was positively correlated with VSD ($P = 0.016$; OR = 1.507; 95% CI: 1.078–2.107).

The differences of rs3729753 and rs202071628 sites polymorphism distribution were not statistically significant between ASD and control group or between VSD and control group ($P > 0.05$). There was no significant difference of rs2277923 site polymorphism distribution between VSD and control group, with no significant difference of rs703752 site polymorphism distribution between ASD and control group. The details were listed in Tables 3–4.

3.3. Haplotype and linkage disequilibrium analysis

The linkage disequilibrium map was constructed according to the distribution of the rs2277923, rs3729753, rs703752 and rs202071628 sites (Fig. 2). rs2277923 and rs703752 as well as rs2277923 and rs3729753 were linked, respectively ($D' = 0.86$, $r^2 = 0.096$; $D' = 1.0$, $r^2 = 0.019$). There were 7 haplotypes according to haplotype analysis of these 4 SNPs. One of the most common haplotypes, GGGG, showed obvious protective effect in ASD patients ($P = 0.005$; OR = 0.648; 95% CI: 0.477–0.881). AGGG haplotype showed obviously risky effects in ASD occurrence ($P = 0.001$; OR = 1.263; 95% CI: 1.033–1.546) (Table 5).

4. Discussion

The human NKX2-5 gene, locating on human chromosome 5q34, contains two exons and 5′UTR as well as 3′UTR regions. It encodes a

Table 1
NKX2-5-specific primers.

Primer	Forward (5′–3′)	Reverse (5′–3′)	Product (bp)	Annealing temperature (°C)
N1	TCTCCTGCCCCTTGTGCTCA	CGTAGGCCTCTGGCTTGAAGG	332	61
N2	TGGAGAAGACAGAGGCGGACAA	CGTAGGCGTTATAACCGTAGGGAT	396	63
N3	ACAACAACTTCGTGAACTTCGG	ATCGTCATTTCTTACAGCAATAGGT	542	58