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RNT4 3′-UTR Insertion/Deletion Polymorphisms Are Not Associated with Atrial Septal Defect in Chinese Han Population: A Brief Communication

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Atrial septal defect (ASD) is a common type of congenital heart disease, which is defined as any communication through atrial septum. Several studies have revealed that genetic factors may influence the susceptibility of ASD. Recent studies have shown that *reticulon 4* (*RTN4*) gene might be involved in some processes relevant to heart development, such as regulation of cell migration and vascular remodeling. This study aimed to evaluate *RTN4* gene polymorphisms of CAA and TATC insertion/deletion in relation to the risk of ASD in Chinese Han population. A total of 175 ASD patients and 308 unrelated healthy controls were successfully investigated. The polymorphisms of patients were determined by polymerase chain reaction–polyacrylamide gel electrophoresis. There was no significant difference in the allele frequencies of CAA and TATC insertion/deletion in *RNT4* gene between ASD patients and controls. The same results were seen in their genotypes. The present study suggests that CAA and TATC insertion/deletion polymorphisms of *RNT4* gene may not be a useful marker to predict the susceptibility of ASD in Chinese Han population.

Introduction

Congenital heart disease (CHD) is a common cardiovascular malformation in infants and children. In Chinese Han population, the morbidity of CHD is approximately 6.7 out of every 1000 live births and 1.7 per 10 stillbirths (Yang *et al.*, 2009). CHD is a class of malformations including atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus, tetralogy of Fallot, and so forth. ASD is a common type of CHD, characterized as any communication within the atrial septum that results in shunting between the atria. By the heterogeneous anatomical structures, ASD is classified into five categories: patent foramen ovale, atrial secundum defect, atrial primum defect, sinus venous defect, and coronary sinus defect. The ASD patients are usually asymptomatic in their early life and gradually develop such complications as heart failure and atrial arrhythmia in later life caused by the persistent blood shunting.

The pathogenesis of CHD is unclear while some studies have indicated that genetic risk factors are responsible for this disease. The transcription factors *NKX2-5*, *GATA4*, and *TBX5* were shown to be involved in the pathogenesis of CHD (Basson *et al.*, 1999; Elliott *et al.*, 2003; Garg *et al.*, 2003; Liu *et al.*, 2009). A family study reported an association of mutated *NKX2-5* gene with familial ASD (Liu *et al.*, 2009), and the mutations of *GATA4* gene were detected in sporadic ASD patients (Garg *et al.*, 2003). Moreover, the dysfunction of *TBX5* gene caused the Holt-Oram syndrome, which is characterized by congenital forelimb and cardiac malformations, such as ASD (Basson *et al.*, 1999). *Cited2* and *Fog2/Zfpm2* have been also found to take part in the pathogenesis of ASD in mouse models (Svensson *et al.*, 2000; Yin *et al.*, 2002).

Nogo, encoded by *RTN4* gene, is a group of transmembrane proteins corresponding to three protein variants, Nogo-A, Nogo-B, and Nogo-C (Oertle *et al.*, 2003). For the correlation with neuronal regeneration, Nogo protein expression has been intensively studied in nervous system (Chen *et al.*, 2000; Huber *et al.*, 2002). Recent protein analyses found that Nogo protein was expressed ubiquitously in human tissues. Nogo-B was found in both adult human heart and embryonic heart (O'Neill *et al.*, 2004; Nath *et al.*, 2009). A number of studies demonstrated that Nogo-B maybe involved in a variety of processes relevant to heart development, such as cell apoptosis and metabolism, regulation of cell migration, and vascular remodeling (Acevedo *et al.*, 2004; Tambe *et al.*, 2004; Kuang *et al.*, 2006). These results indicate that Nogo protein expression may affect the cell physiologic morphogenesis throughout the cardiovascular development, and draw our attention to the relationship between *RTN4* gene and heart development disease.

The human *RTN4* gene spans over 75 kb and is located on chromosome 2p13-14 (Yang *et al.*, 2000), which has been

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Table 1. Polymorphic Single-Nucleotide Polymorphism Markers, Location, Polymerase Chain Reaction Primers, and Corresponding Alleles

Marker	*Location*	*Primer sequence*	*Product (bp)*	*Allele (bp)*
TATC	3′-UTR	5′-CCTGTCTGACTGCCATGTG-3′	150	$(TATC)_1$ (146)
		5′-CGGCAAGACTATCTGCAACA-3′		$(TATC)_2$ (150)
CAA	3′-UTR	5′-TCAACATGAAATGCCACACA-3′	127	$(CAA)_1$ (124)
		5′-GCAAACAAACAACATTTTTGGA-3′		$(CAA)_2$ (127)

UTR, untranslated region.

studied in detail from several aspects recently, particularly in gene expression and regulation. The 3′-untranslated regions (UTRs) of eukaryotic mRNA, which is among noncoding regions, have been shown to be involved in regulating mRNA stability, cellular and subcellular localization, and translation efficiency (Mendell and Dietz, 2001; Kuersten and Goodwin, 2003). Myotonic dystrophy, neuroblastoma, and α-thalassemia have been found to be associated with the mutations in 3′-UTR by Conne *et al.* (2000). The TATC and CAA insertion/deletion are located at 4068–4071 and 4548–4554 in 3′-UTR of *RNT4* gene mRNA (Gen-Bank AY102279), respectively. In recent years the research on the TATC and CAA insertion/deletion in the 3′-UTR of eukaryotic mRNAs has become popular. A research revealed that the CAA insertion polymorphism contributed to the occurrence of schizophrenia (Novak *et al.*, 2002). Furthermore, a population-based study indicated that the homozygote of TATC deletion in *RTN4* gene was related with dilated cardiomyopathy (Zhou *et al.*, 2009).

Based on these studies, we considered that the polymorphisms of TATC and CAA insertion/deletion in the *RTN4* 3′-UTR may be one of the genetic factors that influence the susceptibility to CHDs. However, our previous observations were not able to detect any correlation between the selected polymorphisms and VSD in Chinese Han population (Chen *et al.*, 2011). In the present study, we further explore the association between the TATC and CAA insertion/deletion polymorphisms of *RTN4* gene and ASD in Chinese Han population.

Materials and Methods

Subjects

A total of 175 sporadic unrelated ASD patients who underwent percutaneous closure or operation in the West China Hospital of Sichuan University (Chengdu, China) were recruited between September 2008 and September 2010. The diagnosis of each patient was identified by transthoracic or transesophageal echocardiographic examination and cardiac catheterization. The patients with other congenital or acquired heart diseases, noncardiovascular abnormalities, or family history of ASD were excluded. Control subjects were 308 unrelated healthy individuals who volunteered to participate in our study while receiving a routine health survey at our hospital during the same time. They had no evidences of organic cardiac disease and cardiac dysfunction, nor any genetic diseases, and their echocardiogram results were normal. All subjects were from Han population living in Sichuan Province of southwestern China. Information on demographic characteristics was collected and blood samples were obtained following a resting period after the echocardiographic examination. The written informed consent was given by each participant or their parents after the nature of study had been fully explained. The protocol was approved by the medical ethics committee of the West China Hospital of Sichuan University.

Determination of genotypes

Two hundred microliters of peripheral blood sample was collected into EDTA-anticoagulated tube from each subject and the genomic DNA was extracted by a DNA isolation kit (Bioteke) following the manufacturer's instructions. Genotyping of the TATC and CAA insertion/deletion polymorphisms of *RTN4* gene was determined by polymerase chain reaction (PCR) followed by polyacrylamide gel electrophoresis method. The information of gene location, alleles, and sequences of primers is presented in Table 1. Amplification was performed in a total of 25 μL reaction mixture containing 75 mM Tris HCl (pH 9), 1.5 mM $MgCl_2$, 150 mM KCl, 2 mM $(NH_4)_2SO_4$, 200 pmol dNTP, 10 pmol of each primer, 50 ng of genomic DNA, and 1.5 U of Taq DNA polymerase (Sangon Biotech). Briefly, the PCR conditions were consisted of initial denaturation 5 min at 94°C, 36 cycles of denaturation 30 s at 94°C, annealing 45 s at 61°C, extension at 72°C for 55 s, and a final elongation at 72°C for 10 min. Three microliters of PCR products were separated by electrophoresis on 6% polyacrylamide gel and silver nitrate staining was used for visualizing the bands. At least 20% of the samples were randomly run twice to reveal 100% concordance of genotype determination and further verified by DNA sequencing analysis (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems).

Statistical analysis

Independent *t*-test or Chi-square test was used to test the significant difference of age or gender using SPSS software (version 16.0; SPSS, Inc.). The genotype and allele frequencies were obtained by direct counting and the odds ratios (ORs) with respective 95% confidence intervals (CIs) were reported to estimate the effects of different alleles and genotypes. All the statistical analyses of Hardy–Weinberg equilibrium test and the association between genotypes and alleles were performed using SHEsis online program (http://analysis.bio-x.cn/myAnalysis.php) (Shi and He, 2005). Additionally, in order to explore the association between polymorphisms and ASD accident, the study power was estimated using the QUANTO 1.1.1 program. The test was two sided and a p-value of < 0.05 was considered statistically significant.

Table 2. Genotype and Allele Frequencies of TATC and CAA Insertion/Deletion Polymorphisms in *RTN4* Gene in Atrial Septal Defect Patients and Healthy Controls

Marker	*Genotype*[a]			χ^2; p-*value*	*Allele*[a]		χ^2; p-*value*	*Odds ratio (95% CI)*
TATC	$(TATC)_1/(TATC)_1$	$(TATC)_1/(TATC)_2$	$(TATC)_2/(TATC)_2$		$(TATC)_1$	$(TATC)_2$		
Patients	59 (0.337)	89 (0.509)	27 (0.154)	$\chi^2=2.654$	207 (0.591)	143 (0.409)	$\chi^2=0.280$	0.930 (0.712–1.216)
Controls	121 (0.393)	133 (0.432)	54 (0.175)	$p=0.265$	375 (0.609)	241 (0.391)	$p=0.597$	
CAA	$(CAA)_1/(CAA)_1$	$(CAA)_1/(CAA)_2$	$(CAA)_2/(CAA)_2$		$(CAA)_1$	$(CAA)_2$		
Patients	13 (0.074)	86 (0.491)	76 (0.434)	$\chi^2=3.013$	112 (0.320)	238 (0.680)	$\chi^2=0.071$	1.039 (0.784–1.378)
Controls	32 (0.104)	128 (0.416)	148 (0.481)	$p=0.222$	192 (0.312)	424 (0.688)	$p=0.789$	

[a]Frequencies are displayed in parenthesis.
CI, confidence intervals.

Results

In the cases, the age at diagnosis ranged from 1.0 to 59.8 years and the mean age was 24.6 ± 15.8 years. In the controls, the mean age was 27.4 ± 15.5 years. The gender distribution (men to women) in cases was 1.69:1, and was 1.85:1 in controls. There were no significant differences between the patients and controls with respect to age or gender distribution (data are not shown). Genotyping data for each polymorphism were successfully obtained for 100% of the 483 subjects. The genotype and allele frequency distributions of both polymorphisms between the ASD patients and controls met the requirements of the Hardy–Weinberg equilibrium (CAA-controls: $p=0.581$, CAA-cases: $p=0.087$; TATC-controls: $p=0.101$, TATC-cases: $p=0.489$).

The distributions of *RTN4* gene 3'-UTR TATC and CAA insertion/deletion alleles and genotypes are summarized in Table 2. Overall, no significant differences were observed in genotypic polymorphism between ASD patients and controls (CAA: $p=0.222$; TATC: $p=0.256$). We also assessed the hypothetical risk of ASD associated with *RNT4* gene allele by calculating ORs with their 95% CIs. In accordance with the findings of Zhou *et al.* (2009), the frequencies of $(TATC)_1$ alleles were found to be decreased in patients when compared with controls although the difference did not reach statistical significance ($p=0.587$, OR = 0.930, 95% CI = 0.712–1.216).

Similarly, there was no relationship at CAA allelic polymorphism between patients and controls ($p=0.789$, OR = 1.039, 95% CI = 0.784–1.378). Furthermore, at the 0.05 level of significance with the two-sided test for these two polymorphisms, the power calculations showed that 175 cases and 308 controls were sufficient for 91% and 94% power to detect an association between CAA and TATC insertion/deletion polymorphisms and the disease with an effect size OR of 2.0.

Discussion

In the vertebrate embryo, heart is the first functional organ. Cardiogenesis is a very complicated process. First, the mesenchymal cells differentiate from endothelial cells within mesenchymal transition of the primitive heart (Person *et al.*, 2005). Then, the mesenchymal cells form endocardial cushion (De la Cruz *et al.*, 1983; Eisenberg and Markwald, 1995; Angelique *et al.*, 1999). From the fourth week of the embryonic development, the atrial septum develops by the fusion of the septa primum and the secundum, which is related to the endocardial cushions. During this process, the cardiac development is associated with the expression of several genes in order, which play a key role in cell migration, differentiation, proliferation, as well as cell–cell interactions (Brown *et al.*, 2005). Thus, ASD may result from dysfunction of certain genes that affect cell biology during cardiovascular development and morphogenesis (Srivastava and Olson, 2000; Pierpont *et al.*, 2007).

To our best knowledge, this is the first study to investigate the association between the *RTN4* 3'-UTR insertion/deletion polymorphisms and the susceptibility of ASD. However, no significant difference was observed in either the allele or the genotype frequency distribution of CAA and TATC polymorphisms in *RNT4* gene between ASD patients and controls. The possible reasons are as follows: first, as the gene variation may have a minor effect on the susceptibility of ASD, a large number of studied patients are needed to avoid a false negative result. Second, genetic heterogeneity and genetic polymorphisms vary greatly among ethnic populations. The association between *RNT4* gene polymorphisms and ASD should be tested in groups of different ethnic origin. In addition, the study of more polymorphisms in the *RNT4* gene is needed to exclude their possible effect on ASD in the Chinese Han population. Moreover, the gene transcription and protein expression of *RNT4* gene may play an important part in the pathogenesis of disease. Future studies should regard Nogo protein expression during the human embryological development and try to establish animal models to provide the underlying possible mechanisms.

In conclusion, our results showed that there were no significant differences in the risk of ASD associated with CAA and TATC insertion/deletion polymorphisms of *RNT4* gene. However, our present study may set a ground for further studies of *RTN4* gene and its possible role in the etiology of heart diseases.

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Disclosure Statement

The authors claim no conflicts of interest.

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