218

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Research Paper

Missense mutations in CSX/NKX$_{2.5}$ are associated with atrial septal defects

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Abstract

Objective: To study the gene mutations of homeobox transcription factor (CSX/NKX$_{2.5}$) associated with a Chinese family with secundum atrial septal defect (ASD). **Methods:** Polymerase chain reaction and DNA sequencing were used to check all the members in the family with ASD, and single strand conformation polymorphism analysis (SSCP) was used to check 126 normal control people for detecting the mutations of CSX/NKX$_{2.5}$ gene. **Results:** Three mutations, G270A(Glu32Lys), G378A (Glu68Lys) andG390A (Glu72Lys) were identified in CSX/NKX$_{2.5}$ gene of ASD patients. However, the other members in the family with ASD and the control did not have such gene mutations. **Conclusion:** These mutations of CSX/NKX$_{2.5}$ gene, which were identified in a Chinese family, may be one of the secundum ASD etiologic causes.

Keywords: secundum atrial septal defect; homeobox transcription factor; gene mutation

INTRODUCTION

Congenital heart malformations are the most common form of birth defect [1]. Atrial septal defects (ASD) are a subset of malformations that result in a persistent common atrial canal. There is considerable evidence for genetic heterogeneity. However although a growing number of the molecular determinants that govern the process of cardiogenesis have been identified in the past decade [2], the role of these molecules in the development of congenital cardiac defect is largely unknown. Recent research reports that point mutations in the human cardiac homeobox gene CSX/NKX$_{2.5}$ are associated with cases of familial atrial septal defect (ASD) [3]. Here we investigate the familial secundum ASD associated with three mutations in CSX/NKX2.5 in a Chinese family.

MATERRIALS AND METHODS

All affected individuals had an ASD of the secundum type. This was defined as an unrestrictive ASD, with increased right ventricular preload and increased pulmonary blood flow of more than 1.5 systemic flows (according to the Doppler continuity equation). And four patients had surgical closure of ASD. We carried out echocardiography on all obligate carriers and siblings of affected individuals. Of all unaffected siblings of patients, none had evidence of cardiac disease or indirect evidence for left-to-right shunt. And, no other cardiac defect was detected in any individual; in particular, left ventricular mass was normal in all individuals with no evidence of any echocardiography abnormality related to familial hypertrophic cardiomyopathy (using the echocardiography criteria). No additional malformations were ob-

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served. We obtained consent from adult subjects and from parents on behalf of their children. Blood samples were collected from patients (Ⅲ3, Ⅲ7, Ⅳ1, Ⅳ7), and unaffected individuals (Ⅲ2, Ⅲ5 Ⅲ6, Ⅲ8, Ⅲ9, Ⅲ10, Ⅳ2, Ⅳ5, Ⅳ6, Ⅳ9). Genomic DNA was prepared from cell lines by standard procedures(***Fig 1***).

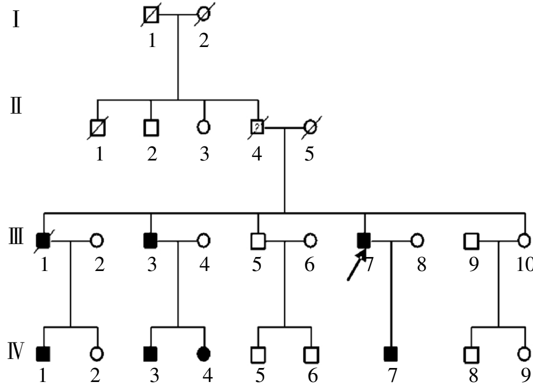

Fig 1 Pedigrees of proband available (Subjects filled in with hatching were clinically affected, subjects blank were unaffected individuals, subjects with arrow was the proband of this family, and subject with oblique line were dead individuals)

We selected 126 health normal individuals (53 females and 73 males). All normal individuals had echocardiography performed, and none had evidence of secundum ASD. We obtained consent from all individuals and blood samples were collected from them. Genomic DNA was prepared from cell lines by standard procedures.

Exons 1 and 2 of the CSX/$NKX_{2.5}$ were amplified by PCR . The primer sets used were listed in ***Tab 1***.

Tab 1 **Primer sets of exons(F: the forward primers; R: the reverse primers)**

Exon	primer
Exon1F	5′CTACCTGCTGCCCGGACAC 3′;
Exons1R	5′CCTGGCCCTGAGTTTCTTGG 3;
Exon 2aF	5′TCCGTAGGTCAAGCCGCTCT 3′;
Exon 2aR	5′CGTAGGCAGGCGCGTAGG 3′;
Exon 2bF	5′CAGAACCGGCGCTACAAGT 3′;
Exon 2bR	5′GGAGCTGTTGAGGTGGGATC 3′;

All PCRs were performed using 50 ng genomic DNA, 200 Um dNTP and 10 pmol of each primer. PCR conditions were: pre-denaturing at 94℃ for 5 min; 32 cycles of denaturing (94℃ for 30 s), annealing (exon1: 55℃ for 30 s; exon2a: 53℃ for 30 s; exon2b: 57℃ for 30 s). and chain extending (72℃ for 30 s); at last ending by extension step of 72℃ for 10 min.

PCR products were purified using Qiagen PCR purification and were sequenced using an ABI 3730 by Union Gene Company(ShangHai).

Polymerase Chain Reaction-s ingle Strand Conformation Polymorphism (SSCP)

PCR products (4 μl) were mixed with 3 μl loading buffer. After 5min denaturation at 95℃, this was cooled in the ice water until performing the eletrophoresis with 8% of denatured polyacrylamide gel. The gel was visualized with silver staining and then photoed by camera.

RESULTS

The direct sequence result of four patients showed: four patients (Ⅲ3, Ⅲ7, Ⅳ1, Ⅳ7) were identified to be hemizygotes of novel CSX/NKX2-5 missense mutations: (1)G94A(GAG-AAG) mutations in Exon 1, resulting in the substitution of amino acid from glutamic acid to lysine(E32K), were shown in ***Fig 2a***; (2)G202A(GAG-AAG) mutation in Exon 1, resulting in the substitution of amino acid from glutamic acid to-lysine (E68K), were shown in ***Fig 2b***; (3) G214A(GAG-AAG) mutations in Exon 1, resulting in the substitution of amino acid from glutamic acid to lysine(E72K), were shown in ***Fig 2c***.

We used SSCP to search for these mutations. The results showed that abnormal bands were found in four patients with ASD of the experimental family group. But, the abnormal band was not detected in the normal individuals of this family, and not found in the 126 healthy unrelated Chinese control group (***Fig 3***).

DISCUSSION

During the past decade, an emerging body of evidence has accumulated that cardiac transcription factors control a cardiac gene program and play a critical role in transcriptional regulation during cadiogenesis and during the adaptive process in adult hearts [4-6]. Especially, an evolutionally conserved homeobox transcriptional factor CSX/NKX2-5 has recently been in the forefront in the field of congenital heart malformations. CSX/$NKX_{2.5}$ is a homeobox-containing gene, which was first isolated from the murine heart[7]. It is predominantly expressed in the heart, and targeted gene disruption revealed its essential role in normal heart development and morphogenesis [8], which indicates the critical role of CSX/NKX2.5 not only in the morphogenesis of the heart, but also in the physiological function of the cardiac system[9-12].

In human, mutations in the CSX/nkx2-5 gene provide the evidence that the genetic factors are etiologically crucial in syndromic congenital heart diseases[13-14]. In the majority of patients with secumdum

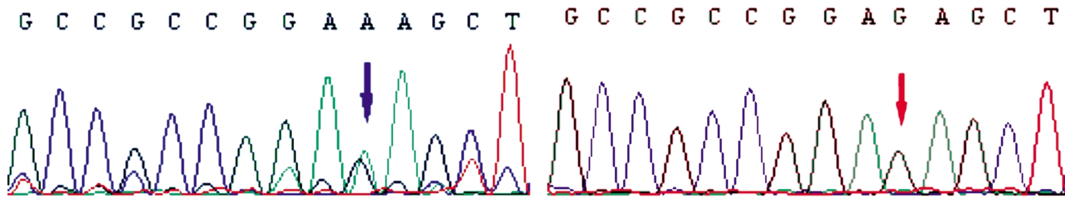

Fig 2a Sequence analysis identifying missense mutation in Exon 1 of CSX/NKX2-5. The blue arrow on the sequencing photogram indicated the hemizygous variance (G94A), with the wild-type sequence shown by red arrow. The mutation resulted in substitution of lysine for glutamic acid(E32K)

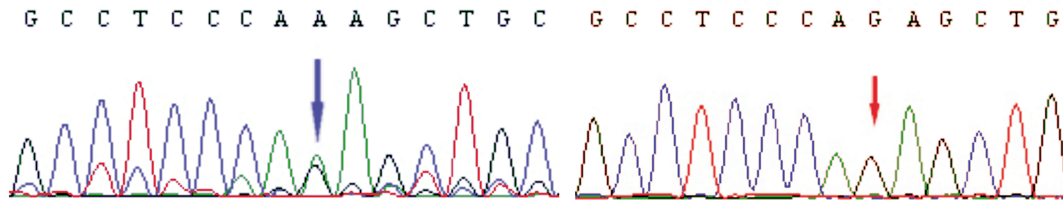

Fig 2b Sequence analysis identifying missense mutation in Exon 1 of CSX/NKX2-5. The blue arrow on the sequencing photogram indicated the hemizygous variance (G202A), with the wild-type sequence shown by red arrow. The mutation resulted in substitution of lysine for glutamic acid(E68K).

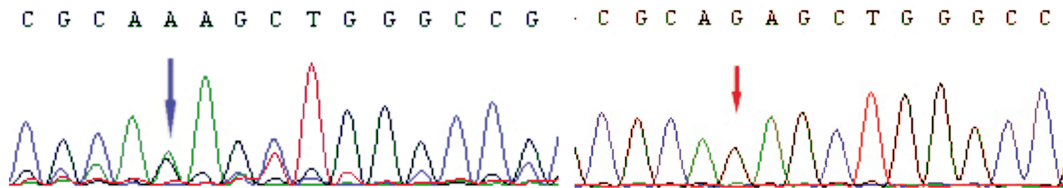

Fig 2c Sequence analysis identifying missense mutation in CSX/NKX2-5. The blue arrow on the sequencing photogram indicated the hemizygous variance(G214A), with the wild-type sequence shown the by red arrow. The mutation resulted in substitution of lysine for glutamic acid(E72K).

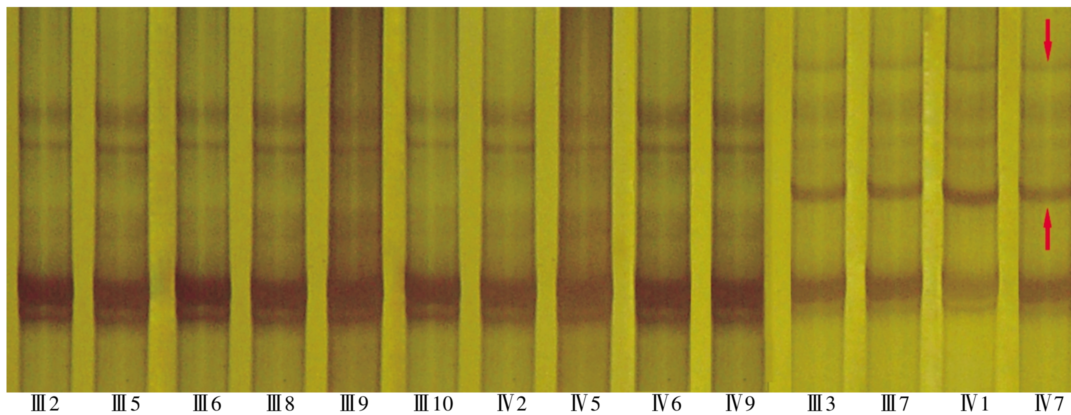

Fig 3 The abnormal bands were found in four patients (showed by red arrow). Contrasted to electropherogram of affected individuals, the abnormal bands were not found in familial normal individuals and the normal control.

ASD associated with AV block, CSX/nkx2-5 mutations are situated within the homeodomain [15,16]. Some reports illustrate the transcriptional activities of most CSX/nkx2-5 mutant proteins with mutations in the homeodomain being impaired due to reduced DNA-binding affinity[17,18].

From our research, the affected individuals in the famliy displayed the secundum atrial septal defect, while other evidence of cardiac disease was not found. It is suggested that this could be described in isolated secundum atrial septal family, other than the syndomic ASD family. Three novel mutations (E32K, E68K and E72K) were identified in all affected individuals in which we obtained blood samples(Ⅲ3,Ⅳ1, Ⅲ7, Ⅳ7)in this isolated ASD family. As highlighted in the results, these mutations were not found in the normal individuals of the family or the 126 health -normal controls. It was initially

suggested that these mutations play an important role in the pathogenesis of ASD. On the other hand, there were three mutations identified in the present study which were outside the homeodomain. This finding suggested these novel mutations may not disrupt the DNA-binding affinity and the transcriptional activity of CSX/nkx2-5. Therefore, further study is needed to identify impact of these mutations on the expression and function of the CSX/nkx2-5 gene.

Some data also indicated that CSX/nkx2-5 play a crucial role in the transcriptional regulation of several sets of cardiac-specific genes, such as MEF-2C, GATA4, ANP, BNP and so on [19-21], which are essential for embryonic myocardium to differentiate beyond the stage of looping morphogenesis. Therefore, it needs advanced study whether these mutations of CSX/nkx2-5 affect the expression of other genes.

The present study increasingly illustrates the importance of single-gene defects in nonsyndromic congenital heart diseases. Moreover, identification of specific genetic causes for congenital cardiac malformations, will allow for improved family counseling and provide insight into the developmental mechanisms that result in normal and abnor mal cardiac development.

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