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ORIGINAL ARTICLE

Search of Somatic Mutations of NKX2-5 and GATA4 Genes in Chinese Patients with Sporadic Congenital Heart Disease

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Abstract

Congenital heart disease (CHD) usually occurs sporadically, with only a minority of cases associated with a known genetic mechanism. Cardiac-specific transcription factors NKX2-5 and GATA4 play key roles in the mammalian heart development, and the affected cardiac tissues of CHD patients are prone to somatic mutations which thus participate in the pathogenesis of CHD. We collected 98 patients with sporadic CHD, extracted genomic DNA from cardiac tissues and blood, and then screened NKX2-5 and GATA4 genes using PCR-direct sequence analysis. A novel heterozygous missense mutation (c.907G > A, p.V303I) of NKX2-5 gene was identified in a patient with tetralogy of Fallots. Functional assay revealed that this mutant was associated with significantly reduced transcriptional activity. In addition, we found two known single-nucleotide polymorphisms (SNPs) (rs2277923, rs3729753) in NKX2-5 and two known SNPs (rs56166237, rs3729856) in GATA4. All variations identified in cardiac tissues were consistent with those of peripheral blood, and no somatic mutations were found in cardiac tissues. Our study shows no evidence of NKX2-5 and GATA4 somatic mutations playing a role in the pathogenesis of sporadic CHD.

Keywords Congenital heart disease · Genetics · Transcription factor · Somatic mutation · Reporter gene analysis

Abbreviations

CHD	Congenital heart disease
TOF	Tetralogy of Fallots
ASD	Atrial septal defects
VSD	Ventricular septal defects
TAPVC	Total anomalous pulmonary venous connection
SRVOT	Stenosis of right ventricular outflow tract
DCRV	Double-chambered right ventricle
d-TGA	Transposition of the great arteries
DORV	Double outlet of right ventricle
RVOT	Right ventricular outflow tract
SNP	Single-nucleotide polymorphism.

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Introduction

Congenital heart disease (CHD) is a common human birth defect, with an incidence of 10 in 1000 live births [1]. It mostly occurs sporadically, and the pathogenesis remains unclear. In the past years, an increasing number of germline mutations of cardiac development-related transcription factor genes have been found in familiar and sporadic patients with CHD. NKX2-5 and GATA4, as crucial transcription factors, are involved in the morphogenesis of heart. Considerable mutations of NKX2-5 and GATA4 have been found in a variety of CHDs [2]. However, most of them were identified in familiar cases but not sporadic ones.

In recent years, many somatic mutations of NKX2-5, GATA4, and other cardiac transcription factor genes have been identified in the affected cardiac tissues of patients with CHD, suggesting that such mutations may play key roles in the pathogenesis of CHD [3–8]. Until now, such mutations have been identified in DNA extracted from formalin-fixed

tissues for more than 20 years, without using corresponding peripheral blood as control. Besides, somatic mutations have never been found in the fresh frozen tissues of CHD patients hitherto [9–16].

To investigate whether somatic mutations of NKX2-5 and GATA4 genes participate in the pathogenesis of CHD, we collected the cardiac tissues and corresponding peripheral blood from CHD patients and detected the sequences of these two genes.

Materials and Methods

Patients

We recruited 98 unrelated patients with sporadic CHD who underwent surgery in Nanjing Children's Hospital from June 2010 to December 2012, including tetralogy of Fallots (TOF, $n=26$), atrial septal defects (ASD, $n=17$), ventricular septal defects (VSD, $n=21$), ASD with VSD ($n=15$), total anomalous pulmonary venous connection (TAPVC) with ASD ($n=5$), complete atrioventricular canals ($n=4$), stenosis of right ventricular outflow tract (SRVOT, $n=4$), double-chambered right ventricle (DCRV) with VSD ($n=3$), Ebstein anomaly ($n=1$), transposition of the great arteries (d-TGA, $n=1$), and double outlet of right ventricle (DORV, $n=1$). All patients were evaluated by echocardiography, and the diagnosis was confirmed by echocardiography, 64-slice spiral CT, and surgical intervention. Patients with chromosome anomalies, unknown syndromes, or 22q11.2 microdeletion were excluded from this study. Meanwhile, 200 normal healthy individuals were collected as control. All included subjects had written informed consent, and this study was approved by the institutional ethical committee of Nanjing Medical University.

Sample Collection and Storage

Heart tissues were resected during cardiac surgery, cleaned using normal saline, then placed in liquid nitrogen immediately, and stored at − 80 °C at last. Tissues of the right auricle as unaffected tissues were obtained from all patients. Affected tissues including the right ventricular outflow tract (RVOT) were collected from patients with TOF, SRVOT, DCRV/VSD, and DORV; atrial septum tissues were collected from patients with TAPVC/ASD and d-TGA; and atrial tissue was obtained from a patient with Ebstein anomaly. All the tissues resected during surgery were immediately snap frozen in liquid nitrogen and then stored in a freezer at − 80 °C. Corresponding peripheral blood samples were collected prior to surgery, and stored at − 80 °C.

Chemicals, Reagents and Culture Medium

HEK-293 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and penicillin–streptomycin solution were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Trypsin was purchased from Boguang Biotech Co., Ltd. (Shanghai, China). Trizol was purchased from Takara Bio (Dalian, China).

Genetic Analysis

Genomic DNA was extracted from blood samples using TIANamp blood DNA kit (Tiangen, Beijing, China) and from cardiac tissues using QIAmp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturers' instructions. Primers were designed using Primer3 (v. 0.4.0) software (http://frodo.wi.mit.edu/primer3/) based on the cDNA sequences available in GenBank. All coding sequences and flanking introns of NKX2-5 (GenBank NM_004387) and GATA4 (GenBank NM_002052) genes were amplified by PCR. The PCR system contained 1× PCR buffer, 0.2 mmol/L dNTPs, 0.4 μmol/L of each primer, 100 ng genomic DNA, and 1 U Taq DNA polymerase (Takara, Tokyo, Japan) in a final volume of 25 μL. PCR was performed under the following conditions: 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 45 s at 72 °C. The PCR products were purified and directly sequenced using Big Dye Terminator (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Expression Plasmids and Site-directed Mutagenesis

Wild-type full-length human NKX2-5 cDNA (GenBank NM_001166175) was purchased and cloned into pcDNA3.1-3xFlag vector using ClonExpress Entry one-step cloning kit (Vazyme, China). The identified mutation was introduced into the wild-type *NKX2-5* gene using the PCR-based Dpn I treatment method (Vazyme, China). The mutant was confirmed by bidirectional sequencing to exclude any other sequence variations.

Reporter Gene Assays

Human ANF promoter was inserted into pGL4.23 vector (Promega, Madison, USA) to produce ANF-Luc. ANF-Luc and an internal control reporter plasmid pGL4.74 (hRluc/TK; Promega) were used in transient transfection assay to evaluate the transcriptional activity of the *NKX2-5* mutant. HEK-293 cells were seeded onto a 24-well plate and

cultured in DMEM supplemented with 10% fetal bovine serum. The cells were transfected with 0.5 μg of wild-type or mutant NKX2-5, 1.0 μg of ANF-luc, and 0.1 μg of pGL4.74 using PolyJet transfection reagent (SignaGen). Firefly luciferase and *Renilla* luciferase activities were measured using the Dual-Glo luciferase assay system (Promega) 48 h after transfection. The activity of ANF promoter was presented as the fold activation of firefly luciferase relative to *Renilla* luciferase. Three independent experiments were performed.

Statistical Analysis

Statistical analysis was performed using SPSS V16.0. Two-sided χ^2-test was used to evaluate the frequency of genotype and allele between case and control groups. $P < 0.05$ was considered statistically significant.

Table 1 Clinical characterization of patients with CHD in our study

Cardiac phenotype	Number of patients	Cardiac tissue
TOF	26	RVOT, right auricle
SRVOT	4	RVOT, right auricle
DCRV/VSD	3	RVOT, right auricle
DORV	1	RVOT, right auricle
TAPVC/ASD	5	Atrial septum, right auricle
d-TGA	1	Atrial septum, right auricle
Ebstein anomaly	1	Atrial
ASD	17	Right auricle
VSD	21	Right auricle
ASD/VSD	15	Right auricle
CAVC	4	Right auricle

Results

Clinical Features

We collected a total of 98 sporadic, non-syndromic CHD patients consisting of 59 boys and 39 girls, with a mean age of (25.3 ± 32.9) months upon surgery. The youngest was only 2 days, and the oldest was 13.75 years old. This cohort included multiple types of CHD (Table 1).

Somatic Mutations

By direct sequencing, we identified a heterozygous missense mutation in exon 2 of NKX2-5 gene (c.907G > A, p.V303I) in both RVOT and auricula dextra tissues of a patient with TOF, leading to the substitution of valine by isoleucine, which was absent from the 200 controls. This mutation is localized in a conserved sequence and has not been reported before (Fig. 1). Then we performed computational predictive analysis using two algorithms, i.e., SIFT and PolyPhen. The SIFT analysis predicted mutation c.907G > A was TOLERATED with a score of 0.85, while the PolyPhen analysis gave a score of 0.869 (POSSIBLY DAMAGING).

Since the sequences of NKX2-5 and GATA4 fragments from the blood samples and fresh frozen tissues of the 98 CHD cases were identical, there was no somatic mutation.

Sequence Variations

Four reported single-nucleotide polymorphisms (SNPs) were found, i.e., rs2277923 c.63 A > G (p.E21E) and rs3729753 c.606 G > C (p.L202L) in *NKX2-5* gene, and rs56166237 c.99G > T (p.A33A) and rs3729856 c.1129A > G (p.S377G) in GATA4 gene. All the variations were detected in the genomic DNA from both cardiac tissues and blood (Table 2). No significant differences were found in the

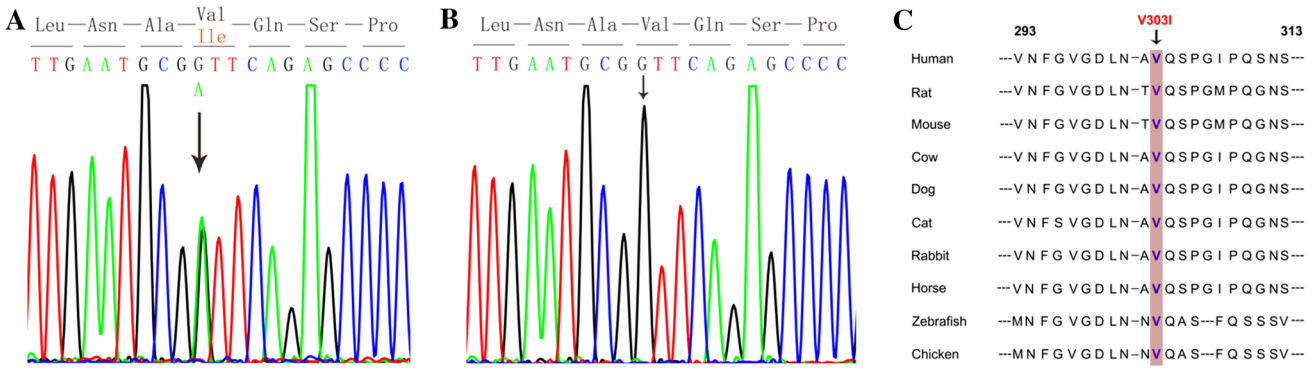

Fig. 1 A novel heterozygous mutation detected in NKX2-5 gene. **a** A heterozygous c.907G > A mutation of NKX2-5 detected in both cardiac tissue and peripheral blood of a patient with TOF. **b** Normal sequence of NKX2-5 as control. **c** Amino acid sequence comparison shows that Val 303 of NKX2-5 has high conservation between different species

Table 2 Sequence variations identified in CHD patients

Gene	Nucleotide change	Amino acid change	Gene position	Cardiac phenotype (number of patients)	Status (refSNP)
NKX2-5	c.907G > A	p.V303I	Exon2	TOF (1)	Unreported
	c.63A > G	p.E21E	Exon1	VSD (11), ASD (7), ASD/VSD (13), TOF (16), PS (1), TAPVC/ASD (3), d-TGA (1), DORV (1), CAVC (1)	rs2277923
	c.606G > C	p.L202L	Exon2	VSD (3), ASD/VSD (2), DCRV/VSD (1)	rs3729753
GATA4	c.99G > T	p.A33A	Exon2	VSD (1), TOF (1)	rs56166237
	c.1129A > G	p.S377G	Exon6	VSD (2)	rs3729856

Table 3 Frequencies of genotypes and alleles in CHD patients

Gene	SNP	Frequency (%) of genotypes			Frequency (%) of alleles	
	c.63 A > G	AA	AG	GG	A	G
NKX2-5	CHD	11.2	50	38.8	36.2	63.8
	Control	18.5	49	32.5	43	57
	c.606 G > C	GG	GC	CC	G	C
	CHD	93.9	6.1	0	96.9	3.1
	Control	94.5	5.5	0	97.3	2.7
	c.99G > T	GG	GT	TT	G	T
GATA4	CHD	98	2	0	99	1
	Control	99.5	0.5	0	99.8	0.2
	c.1129A > G	AA	AG	GG	A	G
	CHD	98	2	0	99	1
	Control	96.5	3.5	0	98.3	1.7

allele frequencies of these SNPs between CHD patients and normal controls, accompanied by similar frequencies in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) (Table 3).

Diminished Transcriptional Activity of NKX2-5 Mutant

To determine whether p.V303I affected the transcriptional activity of NKX2-5, ANF promoter luciferase reporter together with either wide-type or mutant NKX2-5 plasmid were co-transfected to HEK-293 cells. The transfection efficiency was quantified by Western blot. As shown in Fig. 2, p.V303I plasmid significantly attenuates the transactivation of ANF promoter ($P < 0.05$), demonstrating that target gene-induced activation of the mutant protein was suppressed.

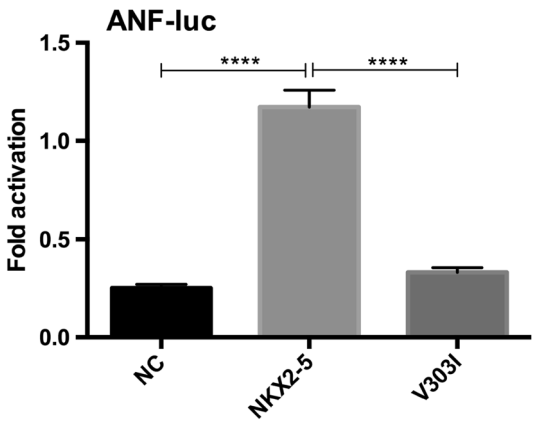

Fig. 2 Decreased transcriptional activity of NKX2-5 mutant protein. Relative luciferase activation of ANF-Luc in HEK-293 cells showed significantly reduced transcriptional activity of the p.V303I mutant protein. (****, $P < 0.0001$)

Discussion

Cardiac transcription factors NKX2-5 and GATA4 play crucial roles in cardiac morphogenesis. Up to now, many germline mutations of NKX2-5 and GATA4 genes have been identified in CHD patients, especially in familial ones, but rare in sporadic CHD cases [17–20].

Somatic mosaicism means genetic variation in somatic cell occurs after fertilization, which is well-established

to dominate in cancer, aging, and other diseases [21]. In recent years, somatic mutation has been involved in variety of cardiovascular diseases, including long-QT syndrome [22], vascular malformative/overgrowth disorders [23], and idiopathic atrial fibrillation [24, 25]. Interestingly, Reamon-Buettner et al. reported frequent somatic mutations of cardiac transcription factors NKX2.5, TBX5, GATA4, HEY2, and HAND1 in malformed regions but not unaffected ones of the heart, as a novel mechanism for CHD [3–8]. However, all the somatic mutations have been identified in cardiac tissues fixed by formalin for over 20 years, and no corresponding blood samples have been employed as control.

In this study, we used fresh frozen tissues instead of formalin-fixed tissues. RVOT, atrial septum, and atrial tissues were considered to be malformed, whereas the right atrial appendage was considered unaffected. We failed to found any somatic mutations of NKX2-5 or GATA4 gene. In addition, we identified a novel missense mutation of NKX2-5 gene in RVOT tissue of a patient with TOF, which was confirmed in the right atrial appendage and peripheral blood of the same case. Additionally, two known SNPs were identified in cardiac tissues and the peripheral blood DNA of corresponding patients. All the variations identified in cardiac tissues were confirmed in blood samples. Overall, there was no somatic variant.

Similarly, several recent studies were unable to find any somatic mutation in a series of transcription factors NKX2-5, GATA4, TBX20, and HAND1 using fresh frozen heart tissues of patients with CHD [9–16]. The most important difference between these studies and those of Reamon-Buettner et al. is the storage method of heart tissues, i.e., fresh frozen or formalin fixation. Long-term fixation in formalin causes DNA degradation and jeopardizes its quality. Furthermore, formalin may remain in the DNA solution, affecting PCR results [26]. Quach et al. reported that formalin damaged bases and affected PCR, which may induce mutation [27]. Therefore, a large number of mutations detected in the studies of Reamon-Buettner et al. may be ascribed to low-quality DNA [26, 27].

Given that mosaicism may reduce the detection rate of somatic mutations in affected tissue, the technique of sequence analysis and the clone area of mutant cells are determinative [28]. Therefore, whether somatic mutation exists in cardiac tissue upon CHD is still controversial, requiring further in-depth studies. In our CHD cohort, NKX2-5 or GATA4 gene had no somatic mutations. More sensitive mutation detection approaches may be necessary to find low-frequency somatic variants.

Although there were no somatic mutations of transcription factor NKX2-5 or GATA4, we identified a novel heterozygous missense mutation of NKX2-5 gene, with weakened transcriptional activity.

Conclusion

In summary, we sequenced the variations of NKX2-5 and GATA4 genes in the cardiac tissues of CHD patients using PCR-direct sequencing technique, finding no somatic mutations though. Given the detection methods and the sizes of tissues, further studies are needed to confirm whether somatic mutations are the pathogenesis of CHD. Moreover, a novel heterozygous missense mutation (c.907G > A, p.V303I) was identified in NKX2-5 in a TOF patient. Our findings expand the mutation spectrum of NKX2-5 and provide a new clue for clarifying the mechanism of CHD.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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