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Mutations of the *GATA4* and *NKX2.5* genes in Chinese pediatric patients with non-familial congenital heart disease

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Abstract A number of mutations in *GATA4* and *NKX2.5* have been identified to be causative for a subset of familial congenital heart defects (CHDs) and a small number of sporadic CHDs. In this study, we evaluated common *GATA4* and *NKX2.5* mutations in 135 Chinese pediatric patients with non-familial congenital heart defects. Two novel mutations in the coding region of *GATA4* were identified, namely, 487C>T (Pro163Ser) in exon 1 in a child with tetralogy of Fallot and 1220C>A (Pro407Gln) in exon 6 in a pediatric patient with outlet membranous ventricular septal defect. We also found 848C>A (Pro283Gln) in exon 2 of the *NKX2.5* gene in a pediatric patient with ventricular septal defect, patent ductus arteriosus and aortic isthmus stenosis. None of the mutations was detected in healthy control subjects ($n = 114$). This study suggests that *GATA4* and *NKX2.5* missense mutations may be associated with congenital heart defects in pediatric Chinese patients. Further clinical studies with large samples are warranted.

Keywords *GATA4* · NKX2.5 · Congenital heart disease · Mutation · Pediatric patient

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Introduction

Congenital heart defects (CHDs) are the most common developmental anomaly (about 1% in newborns in western countries) and are the leading non-infectious cause of mortality in newborns (Hoffman and Kaplan 2002; Sadowski 2009). Mendelian and chromosomal syndromes account for less than 20% of all CHDs. The genetic mechanism underlying non-chromosomal or non-Mendelian 'sporadic' CHDs accounting for the remaining 80%, is poorly understood (Bentham and Bhattacharya 2008). Transgenic and knockout mouse models have demonstrated that a number of candidate genes (mainly encoding transcriptional factors and receptors) contribute significantly to the etiology of CHDs, and the number of genes that have been shown to be needed for murine heart development probably exceeds 1,700 (Bentham and Bhattacharya 2008). However, so far only a few genes (e.g., *GATA4*, *TBX-5*, *MYH6*, *NOTCH1* and *NKX2.5*/*CSX1*) involved in the pathogenesis of human CHDs have been identified using linkage analysis and candidate-gene approaches (Bentham and Bhattacharya 2008; Clark et al. 2006). *GATA4* has been mapped to chromosome 8p23.1 and encodes a 442-amino acid protein of the GATA family of zinc finger transcription factors (Huang et al. 1995; White et al. 1995). A number of preclinical studies have demonstrated that GATA4 can regulate genes involved in embryogenesis and in myocardial differentiation and function (Brown et al. 2004; Dai et al. 2002; Liang et al. 2001; Molkentin 2000; Pikkarainen et al. 2004; Pu et al. 2004; Rivera-Feliciano et al. 2006; Rojas et al. 2008; Zeisberg et al. 2005). Myocardial expression of *GATA4* is required for proliferation of cardiomyocytes, formation of the endocardial cushions, development of the right ventricle, and septation of the outflow tract. GATA4 binds to the consensus

sequence 5′-AGATAG-3′ and acts as a transcriptional activator of the atrialnatriuretic factor in cooperation with NKX2.5 (Durocher et al. 1997). It appears that small changes in the level of GATA4 protein expression can dramatically influence cardiac morphogenesis and embryonic survival (Pu et al. 2004). Mice lacking Gata4 suffer from defective ventral morphogenesis and heart tube formation (Kuo et al. 1997). GATA binding factor-4 (GATA4) is an important zinc finger-containing transcription factor and its mutations have been found to be causative for a subset of familial atrial and ventricular septal defects (ASD & VSD) and pulmonary stenosis (PS) in several unrelated extended pedigrees (Chen et al. 2010a, b; D'Amato et al. 2010; Garg et al. 2003; Hirayama-Yamada et al. 2005; Okubo et al. 2004; Posch et al. 2010; Posch et al. 2008; Sarkozy et al. 2005). On the other hand, the importance of *GATA4* mutations may be confined to specific subgroups of CHD phenotypes in specific populations among CHD patients without family history (i.e., sporadic CHDs), such as Erdheim–Chester disease (ECD) in Caucasians and double-inlet left ventricle (DILV) in Libyan and tetralogy of Fallot (TOF) in Lebanese (Nemer et al. 2006; Rajagopal et al. 2007). However, previously reported *GATA4* mutations in Chinese patients are mostly associated with cardiac septal defects and TOF (Tang et al. 2006; Zhang et al. 2008; Zhang et al. 2009b).

The NK2 transcription factor related, locus 5 gene (*NKX2.5*/*CSX1*) has been mapped to chromosome 5q34 with 2 exons, encoding a 324-amino acid protein. NKX2.5 belongs to the NK-2 family of homeodomain-containing transcription factors, which are conserved from Drosophila to human (Bartlett et al. 2010; Reamon-Buettner and Borlak 2010). NKX2.5 is the earliest known marker of myocardial progenitor cells in all species studied (Komuro and Izumo 1993; Reamon-Buettner and Borlak 2010; Tanaka et al. 1998; Turbay et al. 1996). Through the 60-amino-acid homeodomain, NKX2.5 interacts with DNA through a helix-turn-helix DNA-binding motif of three α-helices, with helix 3 providing binding specificity. Other conserved regions including the TAD and the NK2-specific domain are also important for its function. Mutations of the Drosophila *NKX2.5* homolog, tinman, results in failure of heart formation (Azpiazu and Frasch 1993; Bodmer 1993), and homozygous inactivation of *NKX2.5* in mammals results in impaired cardiac looping and embryonic lethality (Lyons et al. 1995; Tanaka et al. 1999). In mice, heterozygous loss-of-function Nkx2.5 mutations lead to a mild conduction delay and atrial septal dysmorphogenesis manifest as an increased frequency of patent foramen ovale, atrial septal aneurysm, and ASD (Biben et al. 2000). Mutations in *NKX2.5* have been identified as an important factor responsible for various clinical forms of CHDs. Up to now, more than 126 single nucleotide polymorphisms (SNPs) have been detected in human *GATA4* gene (see http://www.ncbi.nlm.nih.gov/snp), but lesser SNPs and other mutations in *NKX2.5* (<50) have been reported. There are few reports about *NKX2.5* mutations in sporadic Chinese patients (Ding et al. 2009; Liu et al. 2009a, b; Zhang et al. 2009a, b). To evaluate the *GATA4* and *NKX2.5* mutations in CHD patients from China, we have screened 135 sporadic Chinese individuals with different CHD phenotypes and compared to 114 healthy control individuals using denaturing high-performance liquid chromatography (DHPLC) and DNA sequencing approaches.

Results

Clinical phenotypes and frequencies of *GATA4* and *NKX2.5* mutations

GATA4 (accession no. NM_002052) and *NKX2.5* (NM_004387) mutation analysis was performed using DHPLC in a total of 135 unrelated Chinese pediatric CHD patients and 114 healthy subjects. In the patient group, 82 (62.1%) individuals had VSD and 19 (14.4%) suffered from secundum atrial septal defect (ASD; Table 1). TOF was present in 12 pediatric patients and 5 had patent ductus arteriosus (PDA). Altogether, three new non-synonymous SNPs of *GATA4* and *NKX2.5* were detected in the CHD patients but not in healthy controls, with an overall detection frequency of 2.22% in the patient group. We also observed three synonymous SNPs in exons of *GATA4* and *NKX2.5*, which were also detected in healthy subjects.

Phenotypes of *GATA4* mutations

Two probands carried mutations in *GATA4* (frequency = 1.48%). Our direct DNA sequencing analysis revealed the 487C>T (Pro163Ser) mutation in exon 1 and 1220C>A (Pro407Gln) in exon 6. The 1220C>A (Pro407Gln) mutation has not been previously reported in other ethnic groups with various CHDs. Notably, both Pro163 and Pro407 are conserved among several species including the human, mouse and rat. The affected individual in whom the Pro163Ser mutation was detected was a male child with TOF; the Pro407Gln mutation was found in a female patient with outlet membranous VSD (Table 2). However, the parents and siblings of these two patients were healthy. Their parents did not smoke and drink alcohol and had no history of exposure to toxic chemicals. Analysis of 114 healthy Chinese children did not found these two mutations. The odds ratio was 2.200, and 2.285 for 487C>T (Pro163Ser) and 1220C>A (Pro407Gln), respectively, and the *P* was 1.000 and 0.544, respectively (by Fisher's exact test).

Table 1 Distribution of congenital heart defects in 135 Chinese pediatric patients

Cardiovascular anomalies	Patients genotyped ($n = 135$)
Dextrocardia	1 (0.7%)
Aortic isthmus stenosis	4 (3.0%)
Coarctation of the aorta	2 (1.5%)
Complete AVSD	2 (1.5%)
Double outlet right ventricle	1 (0.7%)
Double-chambered right ventricle	2 (1.5%)
Double-outlet of right ventricle	4 (3.0%)
D-transposition of the great arteries	1 (0.7%)
Incomplete AVSD	1 (0.7%)
Partial anomalous pulmonary venous return	2 (1.5%)
PDA	5 (3.8%)
Pulmonary stenosis	14 (10.6%)
Secundum ASD	19 (14.4%)
Single atrium & single ventricle	1 (0.7%)
TOF	12 (9.1%)
VSD	82 (62.1%)

ASD Atrial septal defect, *PDA* patent ductus arteriosus, *TOF* tetralogy of Fallot, *VSD* ventricular septal defect

The synonymous mutation 99G>T (Ala333Al) in *GATA4* was observed in 11 pediatric patients and 6 healthy subjects ($P = 1.000$; odds ratio = 1.583 by Fisher's exact test). Two mutations in the introns, namely 50745A>T and 50946A>C, were also detected in 13 patients and 12 healthy subjects ($P = 0.461$ and 1.000, and odds ratio = 1.015 and 0.634, respectively, by Fisher's exact test; Table 2).

Phenotypes of *NKX2.5* mutations

In the 135 Chinese pediatric patients with CHDs, we only found one non-synonymous mutation in *NKX2.5* exon 2 in a patient with VSD, PDA and ASD (Tables 3, 4). This gave a detection frequency of 0.74%, lower than those reported previously (1.4–4.8%) (Elliott et al. 2003; Esposito et al. 2009; McElhinney et al. 2003). The parents of this patient were non-smokers. Sequence analysis revealed a C to A transition at nucleotide 848 of *NKX2.5* exon 2, which causes Pro283Gln (Table 3). This novel mutation has never been reported in other ethnic populations. This mutation was not found in any healthy control subjects. The odds ratio was 2.286 ($P > 0.05$).

Two known synonymous SNPs (rs2277923 and rs3729753) were detected in the *NKX2.5* gene in both patients and healthy subjects (Table 3). In neither case did the allele frequency differ between the patients and healthy controls ($P > 0.05$ by Fisher's exact test).

Discussion

A number of clinical studies have indicated that mutations of *GATA4* are the cause of many familial and sporadic CHDs (Table 5; Chen et al. 2010a, b; D'Amato et al. 2010; Garg et al. 2003; Hirayama-Yamada et al. 2005; Okubo et al. 2004; Posch et al. 2008, 2010; Sarkozy et al. 2005). Defects in GATA4 are the cause of ASD type 2 (ASD2), which is a congenital heart malformation characterized by incomplete closure of the wall between the atria resulting in blood flow from the left to the right atria. In Chinese CHD patients, we revealed two *GATA4* mutations, namely Pro163Ser mutation in patient with TOF and the other mutation Pro407Gln with a clinical phenotype of VSD. Rajagopal et al. (2007) have identified several mutations of *GATA4*, including Pro163Ser, two mutations in patients with ECD and another one in DILV patients and deduced *GATA4* mutations as a cause of sporadic ECD in Caucasians and DILV in Libyan. However, our study together with other studies in Chinese patients (Tang et al. 2006; Zhang et al. 2008) have found that the mutations of *GATA4* were associated with cardiac septal defects and TOF (Table 5).

Table 2 Frequencies of *GATA4* mutations in Chinese pediatric patients with CHDs and healthy controls

Position	Nucleotide variation	Amino acid variation	Patients*			Healthy control		
			Mutated chromosome number	Total chromosome number	Mutant allele frequency	Mutated chromosome number	Total chromosome number	Mutant allele frequency
Exon 1	99G>T	Ala33Ala	11	264	0.042	6	228	0.026
Exon 1	487C>T	Pro163Ser	1	262	0.038	0	192	0.000
Intron	50745A>T	–	8	268	0.052	6	204	0.049
Intron	50946A>C	–	5	268	0.019	6	204	0.029
Exon 6	1220C>A	Pro407Gln	1	268	0.004	0	204	0.000

* $P > 0.05$ patient group vs. healthy control by Fisher's exact test; the results are for all SNPs tested. For the analysis of various SNPs in the *GATA4* gene, the total number of chromosomes examined was slightly different in both patient and healthy subject groups

Table 3 Frequencies of *NKX2.5* mutations in Chinese pediatric patients with CHDs and healthy controls

DbSNP	Position	Nucleotide variation	Amino acid variation	Patients*			Healthy control		
				Mutated chromosome number	Total chromosome number	Mutant allele frequency	Mutated chromosome number	Total chromosome number	Mutant allele frequency
rs2277923	Exon 1	63G>A	Glu21Glu	56	252	0.222	52	228	0.228
rs3729753	Exon 2	606C>G	Leu202Leu	5	268	0.018	4	218	0.018
	Exon 2	848C>A	Pro283Gln	1	270	0.004	0	204	0.000

* $P > 0.05$ patient group vs. healthy control by Fisher's exact test; the results are for all SNPs tested. For the analysis of various SNPs in the *NKX2.5* gene, the total number of chromosomes examined was slightly different in both patient and healthy subject groups

Table 4 A summary of three novel mutations identified among 135 pediatric Chinese patients and associated congenital heart defects

Nucleotide variation	Amino acid change	Patient no.	Type of congenital heart defect
NKX2.5			
848C>A	Pro283Gln	1	VSD, PDA, aortic isthmus stenosis
GATA4			
487C>T	Pro163Ser	1	TOF
1220C>A	Pro407Gln	1	Outlet membranous VSD

PDA Patent ductus arteriosus, *TOF* tetralogy of Fallot, *VSD* ventricular septal defect

The reasons for the distinct clinical phenotypes of *GATA4* mutations are unknown, but may be related to complicated gene–gene and gene–environment interactions.

GATA family members recognize the consensus motif (A/T)GATA(A/G) through a conserved multifunctional DNA-binding domain (Ko and Engel 1993). The DNA-binding domain of GATAs comprises two zinc fingers of the $CX_2CX_{17}CX_2C$ type and an adjacent basic domain. Like other GATA members, GATA4 is composed of two transactivation domains (TADs), two IV zinc fingers (N-terminal zinc finger, NZf; and C-terminal zinc finger, CZf), and a nuclear localization signal (Molkentin 2000). NZf is highly conserved that can interact with FOG2 and CZf may interact with other transcription factors such as NKX2.5, NF-At3, MEF2C, and HAND2 (Durocher et al. 1997; Lee et al. 1998; Molkentin et al. 1998; Morin et al. 2000). Until now, only one missense mutation, Pro163Ser, was reported in TADs of GATA4. The mutation 487C>T (Pro163Ser) is located in TAD2 of GATA4 (Rajagopal et al. 2007). Hirayama-Yamada et al. (2005) have found a mutation 155C>T in *GATA4* exon 1, leading to a missense mutation, S52F, in TAD1. The identification and functional characterization of two evolutionarily conserved TADs suggests that each of these domains modulates critical functions in the transcriptional regulatory programs encoded by GATA4 during vertebrate development (Morrisey et al. 1997).

Another mutation, Pro407Gln, is located in the C-terminal domain of the GATA4 protein. Okubo et al. (2004) and Garg et al. (2003) have separately reported another two frameshift mutations S358X (1074delC) and E359X (1075delG) in the C-terminal of GATA4 and these two frameshifts may inactivate transcription of downstream genes and thus result in haploinsufficiency. As GATA4 functionally interacts with TBX5 and NKX2.5, the missense mutations may interfere with the coordinated interaction between *GATA4* and other transcription factors in cardiogenesis (Durocher et al. 1997; Garg et al. 2003; Sepulveda et al. 2002).

To date, more than 42 mutations have been detected in *NKX2.5*, including 33 SNPs, 6 deletions and 2 insertions. A number of missense and nonsense mutations in *NKX2.5* have been found in families with inherited autosomal-dominant ASD and atrioventricular conduction block (Table 6; Akcaboy et al. 2008; Benson et al. 1999; Ding et al. 2009; Elliott et al. 2003; Esposito et al. 2009; Gioli-Pereira et al. 2010; Goldmuntz et al. 2001; Gutierrez-Roelens et al. 2002; Hobbs et al. 2005; Hosoda et al. 1999; Ikeda et al. 2002; Liu et al. 2009a, b; McElhinney et al. 2003; Schott et al. 1998; Stallmeyer et al. 2010; Watanabe et al. 2002; Zhang et al. 2009a, b). Other congenital heart abnormalities have been observed at low penetrance in these families, including VSD, Ebstein's anomaly, TOF, subvalvular aortic stenosis, and tricuspid valve abnormality. Many of these mutations are also found in sporadic CHDs. Studies in Chinese CHD patients have indicated *NKX2.5* mutations are associated with CHDs, but are very rare in Chinese (Ding et al. 2009; Liu et al. 2009a, b; Zhang et al. 2009a, b). For Chinese patients, *NKX2.5* mutation investigation should be limited within a number of familial and special phenotype of CHDs.

A novel mutation in the coding region of *NKX2.5* (848C>A) was found in the C-terminal portion in this study. Experimental studies have shown that the

Table 5 Critical missense and nonsense mutations of *GATA4* with clinical phenotypes of congenital heart defects identified from previously reported and present studies

Mutation	Amino acid change	Location	Phenotype	Population	Familial/ sporadic (F/S)	References
82C>T	His28Tyr	TAD1	VSD	Chinese	S	Chen et al. (2010a)
155C>T	Ser52Phe	TAD1	ASD	Japanese	F	Hirayama-Yamada et al. (2005)
196G>T	Ala66Thr		VSD	Chinese	S	Chen et al. (2010a)
278G>C	Gly93Ala	TAD1-TAD2 gap	Secundum ASD	Caucasian	S	Tomita-Mitchell et al. (2007)
487C>T	Pro163Ser	TAD2	TOF	Chinese	S	This study and Rajagopal et al. (2007)
			ECD	Caucasian		
622T>C	Phe208Leu	NZf-CZf gap	VSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
631T>C	Phe211Leu	NZf-CZf gap	VSD, AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
640G>A	Gly214Ser	NZf-CZf gap	VSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
648C>G	Glu216Asp	NZf	TOF	Libyan	S	Nemer et al. (2006) and Poirier et al. (2003)
668T>C	Met223Thr	NZf	VSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
677delC	Pro226X	NZf	AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
687G>T	Arg229Ser	NZf	ASD, VSD, AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
700G>A	Gly234Ser	NZf	AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
715A>G	Asn239Asp	NZf	CHD	Caucasian	S	Reamon-Buettner and Borlak (2005)
715A>G	Asn239Ser/ Asp	NZf	VSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
731A>G	Tyr244Cys	Basic region	VSD, AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
742C>T	Met247Thr	Basic region	Persistent atrial fibrillation	Caucasian	S	Posch et al. (2010)
743A>G	Asn248Ser	Basic region	ASD, AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
755G>C	Arg252Pro	Basic region	AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
764T>C	Ile255Thr	Basic region	ASD	Caucasian	S	Reamon-Buettner and Borlak (2005)
779G>A	Arg260Gln	Basic region	VSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
782T>C	Leu261Pro	Basic region	ASD, VSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
796C>T	Arg266Thr	NZf-CZf gap	AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
799G>A	Val267Met	NZf-CZf gap	PDA	Chinese	S	Tang et al. (2006)
818A>G	Asn273Ser	CZf	AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
830C>T	Thr277Ile	CZf	CHD	Caucasian	S	Reamon-Buettner and Borlak (2005)
839C>T	Thr280Met	CZf	ASD	Chinese	F	Chen et al. (2010b)
848G>A	Arg283His	CZf	ASD, AVSD, overriding aorta	Caucasian	S	Reamon-Buettner and Borlak (2005)
855T>C	Asn285Lys	CZf	AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
874T>C	Cys292Arg	CZf	ASD, VSD, AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
881C>T	Ala294Val	CZf	ASD	Caucasian	S	Reamon-Buettner and Borlak (2005)
886G>A	Gly296Ser	CZf	ASD, VSD, PDA, AR, PS, MR	Caucasian	F	Garg et al. (2003), Rajagopal et al. (2007), and Sarkozy et al. (2005)
905A>G	His302Arg	Basic region & nuclear importing region	AVSD	Caucasian	S	Reamon-Buettner and Borlak (2005)
946C>G	Gln316Glu	Nuclear importing region	ASD, mild PS, small muscular VSDs	Caucasian	S	Tomita-Mitchell et al. (2007)
1037C>T	Ala346Val	C-terminal domain	ECD	Caucasian	S	Rajagopal et al. (2007)
1074delC	Ser358X	C-terminal domain	ASD ± PS	Japanese	F	Okubo et al. (2004)
1075delG	Glu359X	C-terminal domain	ASD, dextrocardia	Caucasian Japanese	F	Garg et al. (2003) and Hirayama-Yamada et al. (2005)
1081A>G	Met361Val	C-terminal domain	VSD	Caucasian	S	Reamon-Buettner et al. (2007)

Table 5 continued

Mutation	Amino acid change	Location	Phenotype	Population	Familial/ sporadic (F/S)	References
1129A>G	Ser377Gly	C-terminal domain	VSD, cardiac hypertrophy	Caucasian	S	Poirier et al. (2003), Posch et al. (2008), and Reamon-Buettner et al. (2007)
1138G>A	Val380Met	C-terminal domain	ASD + VSD	Chinese	S	Tang et al. (2006)
1207C>A	Leu403Met	C-terminal domain	Hypoplastic right ventricule, sinus venous ASD	Lebanese	S	Rajagopal et al. (2007)
1220C>A	Pro407Gln	C-terminal domain	VSD, TOF	Chinese	S	This study and Zhang et al. (2009b)
1232C>T	Ala411Val	C-terminal domain	VSD, cardiac hypertrophy, atrial fibrillation	Caucasian	S	Poirier et al. (2003), Posch et al. (2010), Posch et al. (2008), and Tomita-Mitchell et al. (2007)
1273G>A	Asp425Asn	C-terminal domain	ASD, VSD, TOF	Caucasian	S	Tomita-Mitchell et al. (2007) and Zhang et al. (2009b)
1288C>G	Leu430Val	C-terminal domain	ASD, AVSD	Caucasian	S	Reamon-Buettner et al. (2007)
1295T>C	Leu432Ser	C-terminal domain	ASD	Caucasian	S	Reamon-Buettner et al. (2007)
1306C>T	His436Tyr	C-terminal domain	VSD, AVSD, VSD + ASD + PFO	Chinese	S	Chen et al. (2010a)
1324G>A	Ala442Thr	C-terminal domain	VSD	Caucasian	S	Reamon-Buettner et al. (2007)

AR Aortic regurgitation, *ASD* atrial septal defect, *AVSD* atrioventricular septal defect, *CHD* congenital heart defect, *CZf* C-terminal zinc finger, *ECD* endocardial cushion defect, *MR* mitral regurgitation, *NZf* N-terminal zinc finger, *PDA* patent ductus arteriosus, *PFO* patent foramen ovale, *PS* pulmonary stenosis, *TOF* tetralogy of Fallot, *VSD* ventricular septal defect

C-terminal portion of NKX2.5 is important for its function distinct from the role of the homeodomain (Kasahara et al. 2000; Kasahara et al. 2001). Previous functional studies of *NKX2.5* mutations have demonstrated decreased binding of mutant NKX2.5 proteins with mutations outside the homeodomain to dimeric DNA binding sites despite normal binding to monomeric sites (Kasahara et al. 2000). Kasahara et al. (2000) have suggested that this portion of the gene is critical for the cooperative homodimerization and heterodimerization (with other transcription factors such as GATA4) of the NKX2.5 protein on dimeric DNA binding sites. Similarly, the mutation Pro283Gln (848C>A) may also affect NKX2.5-DNA binding.

The genetic architecture of non-familial CHDs probably includes accumulation of rare nonsynonymous variants in cardiac developmental genes leading to mutational loading of cardiac developmental networks, copy number variation in cardiac developmental genes, and common variants that may not be obviously associated with cardiac development but may modulate genetic buffering pathways (e.g., folate and arachidonic acid metabolism) (Bentham and Bhattacharya 2008). GATA4 can interact with NKX2.5 and several other proteins which may directly and indirectly modify cardiac development and morphogenesis. For example, the cytochrome P450s CYP2C9 and 2C19 are regulated by GATA4 (Mwinyi et al. 2010a, b), and both CYP2C9 and 2C19 are involved in arachidonic acid metabolism, resulting in active metabolites that may modulate the activity and proliferation of cardiac endothelial cells.

In conclusion, this study has shown that genomic *GATA4* and *NKX2.5* missense mutations may be associated with non-familial CHDs with diverse clinical phenotypes in Chinese pediatric patients. However, the small sample size of this study does not allow us to establish such an association. A larger sample should be analyzed to determine how common *GATA4* mutations are among patients with sporadic CHDs and the functional impact.

Materials and methods

Subjects

From September 2005 to April 2006, we recruited prospectively 135 cases diagnosed with CHD. All the subjects were assessed with regard to clinical and family history. All these patients had no family history of CHDs. Physical examination and echocardiogram were performed in Children's Hospital of Fudan University (Shanghai, China). The clinical diagnosis of these patients included VSD, ASD, PDA, pulmonary artery stenosis (PAS) and others (Table 1). In addition, 114 unrelated healthy individuals

Table 6 Critical missense and nonsense mutations of *NKX2.5* with clinical phenotypes of congenital heart defects identified from previously reported and present studies

Amino acid change	Position	Clinical phenotype	References
Lys15Ile	TAD	ASD	McElhinney et al. (2003)
Glu21Gln	5′-Coding region	TOF, other CHDs	Goldmuntz et al. (2001) and McElhinney et al. (2003)
Gln22Pro	5′-Coding region	TOF	McElhinney et al. (2003)
Arg25Cys	5′-Coding region	ASD, TOF	Akcaboy et al. (2008), Gioli-Pereira et al. (2010), Goldmuntz et al. (2001), and McElhinney et al. (2003)
Ala42Pro	5′-Coding region	Ebstein's anomaly	Gioli-Pereira et al. (2010)
Ala63Val	5′-Coding region	CHDs	McElhinney et al. (2003)
Cys82Ser	5′-Coding region	Wolff–Parkinson–White syndrome	Esposito et al. (2009)
Ala88X	5′-Coding region	ASD, AV block	Hirayama-Yamada et al. (2005)
Glu109X	5′-Coding region	CHDs	Reamon-Buettner and Borlak (2010)
Ala127Glu	5′-Coding region	ASD	McElhinney et al. (2003)
Arg142Cys	Homeodomain	AV block, ASD, VSD, TOF	Gutierrez-Roelens et al. (2002)
Gln149X	Homeodomain	AV block, ASD, VSD, TOF	Benson et al. (1999)
Gln160Pro	Homeodomain	CHDs	Reamon-Buettner and Borlak (2010)
Gln170X	Homeodomain	AV block, ASD	Schott et al. (1998)
Leu171Pro	Homeodomain	CHDs	Reamon-Buettner and Borlak (2010)
Thr178Met	Homeodomain	AV block, ASD	Elliott et al. (2003) and Schott et al. (1998)
Trp185Leu	Homeodomain	CHDs	Reamon-Buettner and Borlak (2010)
Gln187His	Homeodomain	AV block, ASD	Gutierrez-Roelens et al. (2002)
Asn188Lys	Homeodomain	AV block, ASD	Benson et al. (1999)
Arg189Gly	Homeodomain	AV block, ASD	Benson et al. (1999)
Arg190Cys	Homeodomain	AV block, ASD	Hirayama-Yamada et al. (2005)
Arg190Leu	Homeodomain	ASD	Stallmeyer et al. (2010)
Tyr191Cys	Homeodomain	AV block, ASD	Benson et al. (1999)
Gln198X	3′-Coding region	AV block, ASD	Schott et al. (1998)
Arg216Cys	NK2-specific domain	TOF	Goldmuntz et al. (2001) and McElhinney et al. (2003)
Ala219Val	NK2-specific domain	TOF	Goldmuntz et al. (2001) and McElhinney et al. (2003)
Tyr256X	3′-Coding region	CHDs	Reamon-Buettner and Borlak (2010)
Tyr259X	3′-Coding region	AV block, ASD, VSD	Benson et al. (1999)
Cys264X	3′-Coding region	AV block, ASD	McElhinney et al. 2003)
Pro275Thr	3′-Coding region	CHDs	Ikeda et al. (2002)
Pro280Leu	3′-Coding region	Wolff–Parkinson–White syndrome	Esposito et al. (2009)
Pro283Gln	3′-Coding region	VSD, PDA, aortic isthmus stenosis	This study
Asn291del	3′-Coding region	CHDs	McElhinney et al. (2003)
Ala323Thr	3′-Coding region	TOF	McElhinney et al. (2003)
Pro407Gln	3′-Coding region	TOF	Zhang et al. (2009b)
Asp425Asn	3′-Coding region	VSD	Zhang et al. (2009b)

ASD Atrial septal defect, *AV* atrioventricular, *CHD* congenital heart defect, *PDA* patent ductus arteriosus, *TAD* transactivation domain, *TOF* tetralogy of Fallot, *VSD* ventricular septal defect

served as the control. After informed consent was obtained, 5 mL blood sample was collected from each participant.

Genomic DNA extraction

Genomic DNA was isolated from peripheral blood leukocytes of participants using the DNA isolation Kit from Genebase (Vancouver, Canada), according to the manufacture's protocol.

Polymerase chain reaction

The exons of *GATA4* (accession no. NM_002052) and *NKX2.5* (NM_004387) were amplified using Polymerase

chain reaction (PCR). PCR was performed for exons 1–6 of *GATA4*, and exons 1 and 2 of *NKX2.5*. Primers were designed by Primer premier 5.1 or as described by previous studies (Tang et al. 2006; Zhang et al. 2009a). Primer sequences are presented in Supplementary Table 1. We divided *GATA4* exon 1 and *NKX2.5* exon 2 into two amplicons, as these exons were large, and used 2× GC buffer (Takara, Otsu, Japan) instead of 10× GC buffer for exon 1 of *GATA4*.

The PCR was performed in 25 μl standard PCR buffer, containing 50 ng genomic DNA, 0.2 mol/l forward and reverse primers, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each *d*NTP, and 1 U Taq DNA polymerase. The amplification program was one cycle of an initial denaturation step at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s; 51–65°C for 30 s (different anneal temperatures with different primers); 72°C for 30 min, and a final extension at 72°C for 7 min. Reactions were performed on ABI9700 PCR machine (Applied Biosystems Inc., Carlsbad, CA).

For the analysis of various SNPs in *GATA4* and *NKX2.5* genes, the total number of chromosomes examined was slightly different in both patient and healthy subject groups. For the 99G>T SNP in *GATA4*, 264 and 228 chromosomes were analyzed in the patient and healthy subject groups, respectively. For 50745A>T, 50946A>C, and 1220C>A mutations in *GATA4*, 268 and 204 chromosomes were checked in the patient and healthy subject groups, respectively. For 63G>A, 606C>G, and 848C>A mutations in *NKX2.5*, 252, 268 and 270 chromosomes were tested in the patient group.

DHPLC analysis

Polymerase chain reaction products were denatured for 10 min at 95°C and subject to denaturing HPLC analysis system (Transgenomic Inc., San Jose, CA). The fragments were eluted at temperatures calculated by the DHPLC melting program for the successful resolution of heteroduplexes. Samples with double or triple-peaked DHPLC chromatograms were selected to run repeated PCR and DHPLC analysis. If there was a similarity between the two chromatograms, the samples were defined as "mutants".

DNA sequence analysis

For samples with melting profiles different from the wild-type, PCR fragments were reamplified. PCR products with bidirectional extraction and DNA sequencing were performed using the BigDye Teminator Cycle Sequencing v3.1 Kit and an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems Inc., Carlsbad, CA).

Statistical analysis

Comparison of allele frequencies between two groups was conducted by Fisher's exact test. A $P < 0.05$ was considered statistically significant.

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