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ORIGINAL ARTICLE

Novel NKX2-5 Mutations in Patients With Familial Atrial Septal Defects

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Abstract Atrial septal defect (ASD) is a common cardiovascular malformation and an important contributor to substantial morbidity and mortality. Increasing evidence demonstrates that mutated *NKX2-5*, a gene encoding a homeobox transcription factor crucial to cardiogenesis, is a significant genetic determinant for congenital ASD. Nevertheless, the genetic basis for ASD in a majority of ASD patients remains largely unknown. In the current study, the entire coding region of *NKX2-5* was sequenced initially for 58 unrelated probands with familial ASD. The relatives of the probands harboring identified mutations and 200 unrelated control individuals were subsequently genotyped. Three novel heterozygous NKX2-5 mutations (p.P43 GfsX59, p.C46 W, and p.S179F) were identified respectively in three families with autosomal dominantly inherited ASD. These mutations, absent in 200 control individuals, cosegregated with ASD in the families that had complete penetrance. The findings expand the spectrum of mutations in NKX2-5 linked to ASD and contribute to genetic counseling, clinical interventions, and prenatal prevention of ASD for individuals with genetic susceptibility.

Keywords Atrial septal defect · Genetics · Transcription factor

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Congenital heart disease, the most prevalent form of birth defect in the structure of a neonate's heart and great vessels, affecting nearly 1% of newborns, is the most common cause of infant death resulting from birth abnormality. More than 29% of the infants who die of a birth defect have a heart defect [24].

Atrial septal defect (ASD), accounting for approximately 33% of all congenital cardiovascular malformations, affects more than 3 in 1,000 live births [25]. Due to an anatomically deficient interatrial septum, ASD allows blood to flow directly between the left and right atria. Categorized by whether they involve other structures of the heart and how they are formed during the cardiac developmental process, ASDs are classified clinically into five types: ostium secundum ASD, patent foramen ovale, ostium primum ASD, sinus venosus ASD, and common or single atrium. Of these five types, ostium secundum ASD is the most frequent defect, representing 85% of all ASDs [18].

Congenital ASD may be isolated or associated with other cardiac anomalies such as ventricular septal defect, pulmonary valve stenosis, and conduction defects. Irrespective of other malformations that may accompany ASD, interatrial communication bidirections and thus persistent blood shunt between two atria may result in cardiac enlargement, congestive heart failure, pulmonary hypertension, Eisenmenger's syndrome, arrhythmias, and even sudden cardiac death in the absence of surgical or catheter-based repair [37].

Obviously, ASD is an important contributor to morbidity and mortality in infancy. Nevertheless, the etiology responsible for ASD in most patients remains largely unknown [19, 30]. An abnormally developed atrial septum is implicated in a heterogeneous, complex biologic process associated with environmental and genetic risk factors [19, 30].

Increasing evidence has highlighted the crucial role of several transcription factors, including NKX2-5, in the septogenesis [30]. The human *NKX2-5* gene maps to chromosome 5q34 and consists of two exons encoding a protein of 324 amino acids. This homeobox transcription factor NKX2-5, expressed during early cardiac morphogenesis, is indispensible for normal cardiac development. Therefore, *NKX2-5* has been a prime candidate gene in studies to identify the genetic determinants of structural congenital heart defects [1, 3].

To date, more than 40 mutations within the *NKX2-5* gene have been identified in patients with a variety of congenital heart malformations including ASDs with normal or abnormal atrioventricular conduction, ventricular septal defects, conotruncal abnormalities such as tetralogy of Fallot, double-outlet right ventricle, L-transposition of the great arteries, and hypoplastic left heart syndrome [34, 38]. These observations strongly suggest that NKX2-5 is important in the later stages of heart development and maturation in addition to its role in cardiac progenitor commitment and patterning in the developing heart [32].

In this study, the coding exons of *NKX2-5* were sequenced in 58 unrelated probands with familial ASD, and three mutations (p.P43GfsX59, p.C46 W, and p.S179F) were identified in three ASD probands, respectively. Genetic analysis of the families carrying mutations demonstrated that in each family, the mutation cosegregated with ASD.

Materials and Methods

Study Subjects

A total of 58 unrelated kindreds with familial ASD were identified among the Han Chinese population. The subjects were evaluated by individual and familial history, review of medical records, complete physical examination, 12-lead electrocardiogram (ECG), and two-dimensional transthoracic echocardiography with color flow Doppler. All the patients had a classic form of ASD with a defect diameter larger than 5 mm. Nearly all the patients underwent cardiac catheterization and, if required, cardiac surgery.

Family history was obtained by multiple interviews with varied family members, and the phenotypes of family members were determined by medical records, physical examination, echocardiogram, and ECG. All the family members recruited underwent an ECG. A total of 200 ethnically matched, unrelated healthy individuals derived from the general Han Chinese population were used as control subjects to screen for identified mutations in *NKX2-5*.

Peripheral venous blood specimens from the subjects and the control individuals were prepared. The study protocol was reviewed and approved by the local institutional ethics committee, and written informed consent was obtained from all the participants or their guardians before the investigation began.

Genetic Studies

Genomic DNA for all the participants was extracted from blood lymphocytes with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Initially, the coding exons and exon/intron boundaries of the candidate gene *NKX2-5* for 58 unrelated probands with familial ASD were screened in their entirety. Subsequently, genotyping *NKX2-5* for the available relatives of probands carrying mutations and the 200 ethnically matched unrelated healthy control individuals was conducted for the presence of mutations identified in the probands.

The referential genomic DNA sequence of *NKX2-5* was derived from GenBank (accession no. NT_023133). By the aid of online Primer 3 software (http://frodo.wi.mit.edu), the primer pairs used to amplify the complete coding region of *NKX2-5* by polymerase chain reaction (PCR) were designed as follows: primer 1 forward 5′-CAC, GAT, GCA, GGG, AAG, CTG-3′ and reverse 5′-AGT, TTC, TTG, GGG, ACG, AAA, GC-3′ (the PCR product was 477 base pairs [bp] in size); primer 2 forward 5′-CCT, CCA, CGA, GGA, TCC, CTT, AC-3′ and reverse 5′-CGA, GTC, CCC, TAG, GCA, TGG-3′ (the product was 463 bp); and primer 3 forward 5′-AGA, ACC, GGC, GCT, ACA, AGT, G-3′ and reverse 5′-GAG, TCA, GGG, AGC, TGT, TGA, GG-3′ (the product was 473 bp).

The PCR was carried out using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a PE 9700 Thermal Cycler (Applied Biosystems, Foster, CA, USA) with

standard conditions and concentrations of reagents. Amplified products were analyzed on 1% agarose gels stained with ethidium bromide and purified with the QIAquick Gel Extraction Kit (Qiagen). Both strands of each PCR product were sequenced with a BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems).

The sequencing primers used had been designed previously for specific region sequencing. The DNA sequences were viewed and analyzed with the DNA Sequencing Analysis Software v 5.1 (Applied Biosystems). The variant was validated by resequencing of an independent PCR-generated amplicon from the subject and met our quality control threshold with a call rate exceeding 99%.

For family 1, the deletion mutation was originally identified as two apparently different sequences overlapping at the deletion point in direct sequencing analysis. To ascertain the actual sequence variation on the mutant allele, the PCR products amplified with primer 1F and 1R were cloned by a TA cloning kit (TaKaRa, Dalian, Liaoning, China) and sequenced. Six individual clones were sequenced for the wild-type allele, and the mutant allele was identified.

Multiple Sequence Alignments

The multiple NKX2-5 protein sequences across various species were aligned using the MUSCLE program, version 3.6 (an online program at http://www.ncbi.nlm.nih.gov/).

Prediction of the Disease-Causing Potential of a Sequence Alteration

The disease-causing potential of a sequence alteration detected in *NKX2-5* was predicted automatically by MutationTaster (an online program at http://www.mutationtaster.org/), which gave a probability for the alteration to be either a disease mutation or a harmless polymorphism. Notably, the *p* value used in this study was the probability of the prediction rather than the probability of error as used in *t* test statistics (i.e., a value close to 1 indicates a high "security" of the prediction).

Results

Characteristics of the Study Subjects

A total of 58 unrelated kindreds with familial ASD were recruited and clinically evaluated in contrast to a cohort of 200 ethnically matched unrelated healthy individuals used as control subjects. None of them had traditional risk factors for ASD. Among the 58 probands (26 females and 32 males; age range, 0.25–16 years; mean, 3.52 years), 51 (87.93%) had ostium secundum ASD, 5 (8.62%) had ostium primum ASD, and 2 (3.45%) had sinus venosus ASD. Within the proband cohort, 7 (12.07%) also had a single first-degree atrioventricular conduction block, 1 (1.72%) had first-degree atrioventricular conduction block and paroxysmal atrial fibrillation, and 9 (15.52%) had some other form of congenital heart disease. The clinical characteristics of the 58 unrelated probands with familial ASD are summarized in Table 1.

NKX2-5 Mutations

The probands in 58 kindreds with familial ASD were genetically evaluated. Direct sequencing of the coding regions of the *NKX2-5* gene was performed after PCR amplification of genomic DNA from the 58 index patients. Three heterozygous mutations in *NKX2-5* were identified in 3 of 58 unrelated probands. The total population prevalence of *NKX2-5* mutations based on the probands was approximately 5.17%.

A 17-bp (GCCCTCCTCCTGCATGC) deletion in exon 1 from coding nucleotides 126 to 142 (alternatively, c.126_142del) of the *NKX2-5* gene, resulting in a frameshift from amino acid 43 of downstream codons, was identified in the proband from family 1. The deletion frameshift arises in amino acid 43, with proline 43 as the

Table 1 Clinical characteristics of the 58 unrelated probands with familial atrial septal defects (ASDs)

	n	Percentage or range
Male:female	32:26	55:45
Age at current study: years (range)	3.52	0.25–16
Distribution of different types of ASDs (%)		
Ostium secundum ASD	51	87.93
Ostium primum ASD	5	8.62
Sinus venosus ASD	2	3.45
Prevalence of ASDs with other defects (%)		
Isolated ASD	49	84.48
ASD and VSD	6	10.34
ASD and VSD and PDA	1	1.72
ASD and VSD and DORV	1	1.72
ASD and PDA	1	1.72
Incidence of arrhythmias (%)		
Single AVB	7	12.07
AVB and paroxysmal AF	1	1.72
Treatment		
Surgical repair	42	72.41
Percutaneous closure	16	27.59

VSD ventricular septal defect, *PDA* patent ductus arteriosus, *DORV* double-outlet right ventricle, *AVB* atrioventricular block, *AF* atrial fibrillation

first affected amino acid, changing into a glycine, and the new reading frame ending in a stop codon at position 59. The position of the stop in the new reading frame is calculated starting at the first amino acid changed by the frameshift and ending at the first stop codon. This also may be described as a p.P43GfsX59 mutation, predicting a truncated protein (100 amino acids) without a homeodomain.

A substitution of guanine (G) for cytosine (C) in the third nucleotide of codon 46 of the *NKX2-5* gene (c.138C > G) corresponding to the transversion of cysteine to tryptophane at amino acid position 46 (p.C46 W) was identified in the proband from family 2.

A change of C into thymine (T) at nucleotide position 536 from the translation starting point (c.536C > T) of the *NKX2-5* gene, equivalent to the transition of serine into phenylalanine at amino acid 179 (p.S179F), was identified in the proband from family 3.

The sequence chromatograms showing the detected heterozygous *NKX2-5* variations of c.126_142del, c.175C > G, and c.536C > T compared with control sequences are shown in Fig. 1. A diagram of NKX2-5 showing the locations of novel mutations relative to the amino-terminus, homeodomain, and carboxyl-terminus is presented in Fig. 2.

All three probands carrying identified mutations presented with isolated ostium secundum ASD and without evidence of ECG-documented atrioventicular conduction block or atrial fibrillation. The three variants were neither found in the control population nor reported in the SNP database at the Web site (http://www.ncbi.nlm.nih.gov/SNP). The genetic scan of the three unrelated families showed that the gene variant was present in all the affected family members alive but absent in the unaffected family members tested in each family. Analysis of the pedigrees demonstrated that the mutation cosegregated with ASD transmitted as an autosomal dominant trait in the three families with complete penetrance. The pedigree structures of the three families are shown in Fig. 3.

Clinical evaluation of the family members from the three studied families showed that 10 (38.46%) of 26 individuals had ostium secundum ASD and that half of the affected individuals also had ECG-documented first-degree atrioventricular conduction block. Other structural cardiovascular malformations identified in affected family members included ventricular septal defect (I-1 in family 2), pulmonary artery stenosis (I-2 in family 3), and left ventricular hypertrophy and mitral valve fenestration (I-1 in family 1).

At the time of the study, no surviving family members available had atrial fibrillation during monitoring of 12-lead ECG. All the affected family members underwent surgical repair except for individual I-1 in family 1, who died of ASD complications before the study and had medical records of ASD and atrioventricular block. The phenotypic characteristics and the results of the genetic screening for the affected pedigree members are listed in Table 2.

Multiple Alignments of the NKX2-5 Protein Sequences Across Species

A cross-species alignment of NKX2-5 protein sequences showed that the altered amino acids were highly conserved evolutionarily, as presented in Fig. 4, suggesting that these amino acids are functionally important.

Disease-Causing Potential of a Sequence Alteration

The sequence alterations of c.175C > G and c.536C > T detected in *NKX2-5* were predicted to be disease-causing, with p values of 0.99953 for c.175C > G and 0.99999 for c.536C > T, providing evidence that the two alterations were disease-causing mutations rather than benign polymorphisms. The sequence alteration of c.126_142de could not be handled because of an excessively long deletion (currently, MutationTaster handles only Ins/Del up to 12 bases).

Discussion

This report describes three previously unrecognized mutations of *NKX2-5* identified in three families with familial ASD. Half of the affected family members had atrioventricular block. These novel heterozygous mutations were present in all the affected family members alive but absent in unaffected relatives tested and 400 normal chromosomes from a matched control population.

A cross-species alignment of NKX2-5 protein sequences showed that the altered amino acids were highly conserved evolutionarily. Prediction of the causative potential of a sequence alteration demonstrated that two missense mutations of p.C46 W and p.S179F were disease-causing with probability values as high as 1. The pathogenic likelihood of the deletion mutation could not be predicted by the MutationTaster due to the excessively long deletion. However, this sequence variation resulted in a frameshift starting from amino acid 43 and a premature termination of the translation (p.P43GfsX59). This would predict a truncated protein for only 100 amino acids lacking all known domains important for its functionality and thus probably a disease-causing mutation with haploinsufficiency, with loss of function being the pathophysiologic mechanism. Therefore, it is very likely that the three mutations were responsible for the ASD in these families.

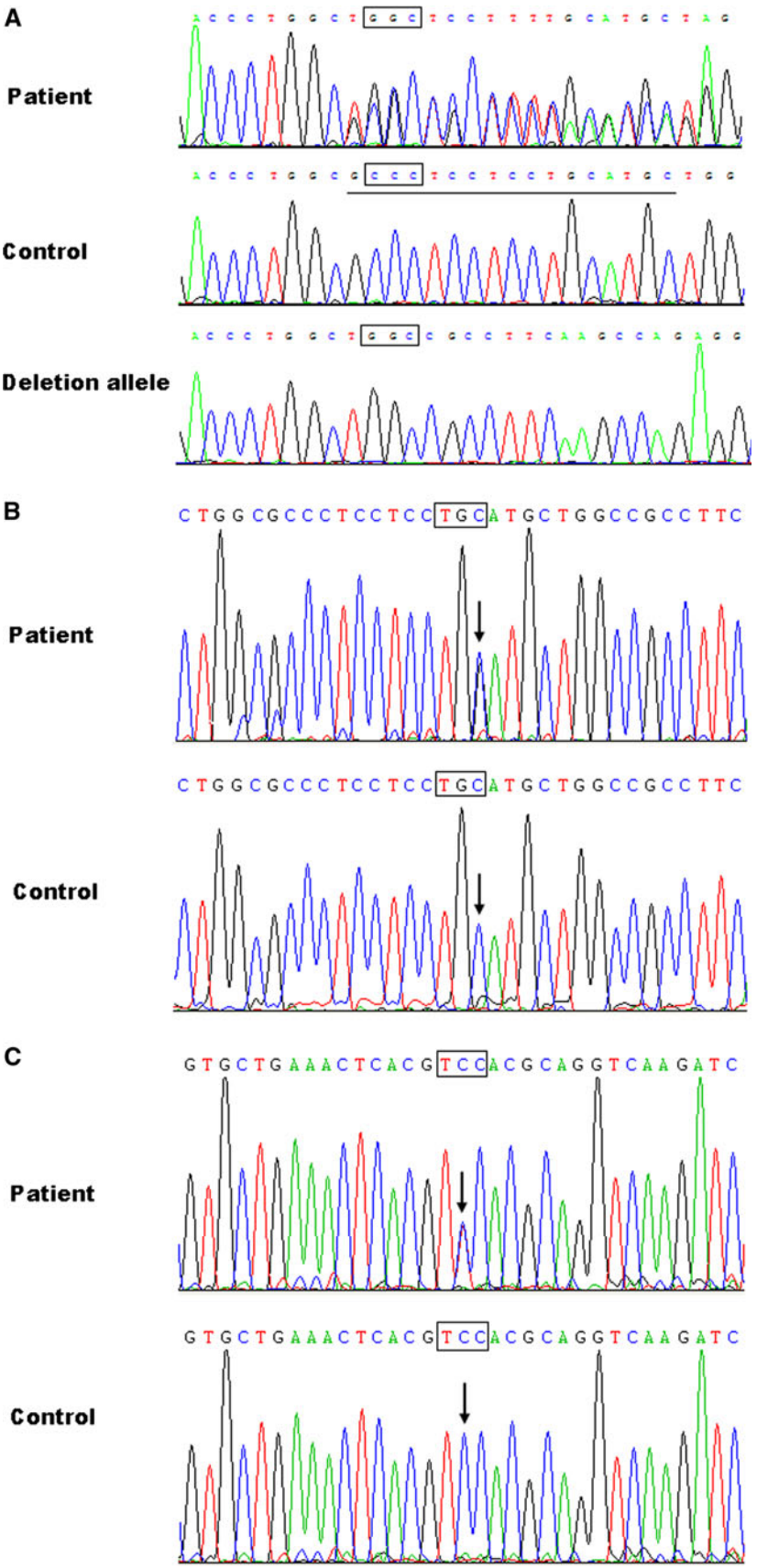

Fig. 1 Sequence chromatograms of *NKX2-5* in index patients and control subjects. The heterozygous *NKX2-5* mutations of c.126_142del (alternatively p.P43GfsX59), c.175C > G (p.C46 W), and c.536C > T (p.S179F) compared with control sequences are shown in **a**, **b**, and **c**, respectively. The *underlined* nucleotides of GCCCTCCTCCTGCATGC in an allele of the control subject are those deleted in a counterpart of the patient (**a**). The *arrow* indicates the heterozygous nucleotides of C/G (**b**) and C/T (**c**) in the probands from families 2 and 3 (patients), respectively, or the homozygous nucleotides of C/C (**b**) and C/C (**c**) in the corresponding control subjects (controls). The *square* denotes the nucleotides comprising a codon of NKX2-5

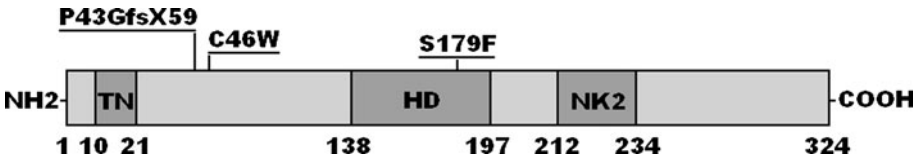

Fig. 2 Diagram of NKX2-5 depicting the locations of novel mutations. The mutations identified in this study are noted above the diagram of the NKX2-5 protein. NH_2 amino-terminus, *TN* tinman domain, *HD* homeodomain, *NK* nucleotide kinase domain, *COOH* carboxyl-terminus

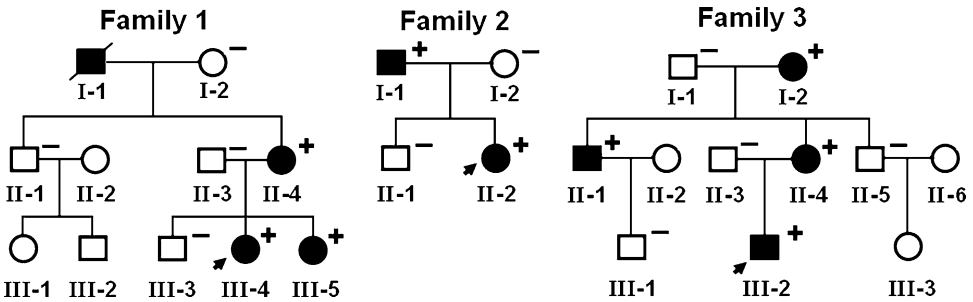

Fig. 3 Pedigree structures of families with atrial septal defects, designated as families 1, 2, and 3, respectively. Family members are identified by generations and numbers. Squares = male family members; circles = female members; symbols with a slash = the deceased members; closed symbols = affected members; open symbols = unaffected members; arrow = proband; + = carriers of the heterozygous mutations; − = noncarriers

Table 2 Phenotypic characteristics and status of the NKX2-5 mutations of the affected pedigree members

Subject information				Phenotype			Genotype
Identity	Gender	Age at study (years)	Age at diagnosis of ASD (years)	ASD (mm)	Other structural defects	AVB[a]	NKX2-5 mutations
Family 1							P43GfsX59
I-1	M	68[b]	24	14 × 16	LVH, MVF	+	NA
II-4	F	38	16	20 × 25	−	+	±
III-4	F	18	15	10 × 16	−	−	±
III-5	F	15	12	12 × 14	−	−	±
Family 2							C46 W
I-1	M	32	16	15 × 20	VSD	+	±
II-2	F	5	2	20 × 28	−	−	±
Family 3							S179F
I-2	F	59	46	10 × 20	PAS	+	±
II-1	M	36	34	25 × 32	−	−	±
II-4	F	30	28	12 × 17	−	+	±
III-2	M	5	3	20 × 24	−	−	±

ASD atrial septal defect, *AVB* atrioventricular block, *M* male, *F* female, *LVH* left ventricular hypertrophy, *MVF* mitral valve fenestration, *NA* not available or not applicable, *VSD* ventricular septal defect, *PAS* pulmonary artery stenosis

[a] + indicates present and – denotes absent

[b] Age at death

Although the mechanisms by which the three novel *NKX2-5* mutations cause ASD with or without atrioventricular block have not been defined, previous functional studies suggest that these mutations may exert loss of function or a dominant negative effect [8, 17, 20, 21, 28, 41]. Notably, the NKX2-5 mutations affecting the homeodomain resulted in reduced or loss of DNA binding, transcriptional activation activity, and protein-protein interactions, particularly with GATA4 and TBX5, in all the biochemical assays. The mutations also gave rise to an anomalous nuclear location in some functional analyses [17, 20, 21, 28, 41].

In contrast, the mutations with an intact homeodomain exhibited normal DNA binding to the monomeric binding

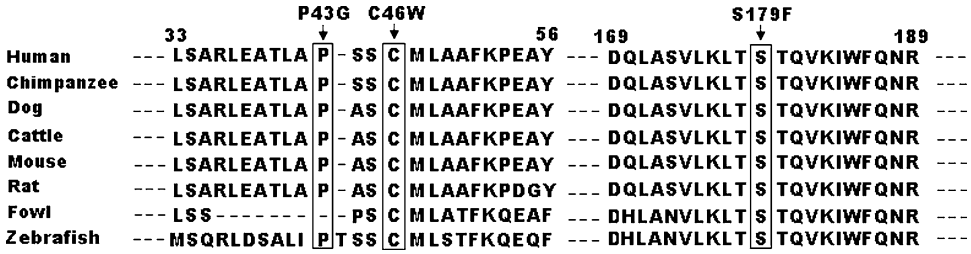

Fig. 4 Alignment of multiple NKX2-5 protein sequences across species. The altered amino acids of P43 (the first amino acid altered by a deletion frameshift mutation), C46, and S179 are completely conserved evolutionarily

site but displayed remarkable reduction in DNA binding to the dimeric sites, thus reducing the transcriptional activation activity [8, 21]. However, the mutations located at the carboxyl-terminus to the homeodomain of the NKX2-5 protein may have enhanced transcriptional activation activity, inducing apoptosis of cardiomyocytes by upregulating *NKX2-5*-target genes such as *ANP* and *MEF2* [28, 41]. Therefore, the mutations p.P43GfsX59 and p.S179F, which affect the homeodomain of the NKX2-5, probably have little or no DNA binding activity or transcriptional activation function, whereas the mutation p.C46 W located at the amino-terminus to the homeodomain of the NKX2-5 likely has normal DNA-binding activity but reduced transcriptional activation activity.

In addition to production of truncated proteins, mRNAs harboring premature termination codons may be selectively degraded by a surveillance mechanism called nonsense-mediated mRNA decay, resulting in decreased abundance of mutant mRNA transcripts [12]. Hence, the p.P43GfsX59 mutation of NKX2-5 also may be implicated with haploinsufficiency by failure to yield protein.

Since the first report of the *NKX2-5* mutations leading to congenital heart disease [36], at least 41 different heterozygous germline *NKX2-5* mutations have been identified in patients with congenital cardiovascular disease [34, 38]. Of these 41 mutations, 33 are single-nucleotide substitutions, 6 are deletions, and 2 are insertions.

The *NKX2-5* mutations are spread along the gene, and except for one at a splice site, all are located in the coding region, 18 of which affect conserved regions, including 2 in the TN domain, 14 in the homeodomain, and 2 in the NK2 domain [34, 38]. Moreover, *NKX2-5* mutations have been identified in patients with either familial or sporadic cardiac defects. Of the 41 mutations, 25 are familial, 14 are sporadic, and 2 are sporadic/familial cases.

Although *NKX2-5* mutations are involved in a long list of cardiac malformations, the most frequent phenotype in patients with *NKX2-5* mutations is ASD with or without atrioventricular block. Specifically, 29 of the 41 mutations were detected in patients with ASD: 26 in the atrioventricular block and 25 in the ASD combined with atrioventricular block [34, 38]. However, the prevalence of *NKX2-5* mutations varies significantly in different cohorts of individuals with congenital cardiovascular diseases [2, 4, 7, 8, 10, 11, 13, 14, 16, 27, 31, 38–40]. According to the 14 reports on the prevalence of *NKX2-5* mutations in different cohorts of patients with cardiac defects, the detection frequencies of *NKX2-5* mutations are 20% (7/35) [4], 18.75% (3/16) [13], 4.80% (6/126) [8], 2.96% (18/608) [27], 2.48% (3/121) [38], 1.39% (1/72) [2], 1.37% (2/146) [7], 1.26% (2/159) [10], 0.92% (1/109) [16], 0% (0/230) [39], 0% (0/227) [14], 0% (0/205) [31], 0% (0/104) [11], and 0% (0/62) [39], respectively, and the prevalence in the compound population is 1.94% (43/2219).

Similar to these findings, the mutational prevalence of 5.17% (3/58) in our cohort suggests that *NKX2-5* mutations are an infrequent cause of ASD. Furthermore, remarkable genetic heterogeneity of ASD was proved by an inability to detect mutations in nearly 95% of our index patients despite somatic *NKX2-5* mutations as a likely mechanism of ASD in some patients [33]. Hence, the contribution of genes other than *NKX2-5* to ASD pathogenesis appears likely.

Mutations in other transcription factors associated with cardiogenesis, such as TBX5 [22], GATA4 [5, 9], and GATA6 [23], also have been detected in patients with ASD. Also, mutations in cardiac structural proteins such as alpha myosin heavy chain (MYH6) and alpha cardiac actin (ACTC1) were identified in familial ASD [6, 26]. However, to date, only *NKX2-5* mutations are reported to cause an ASD phenotype and development of atrioventricular block [34, 38]. The most common two phenotypes caused by mutated *NKX2-5* are ASD and atrioventricular conduction disturbance [1, 34, 38], indicating the pivotal role of *NKX2-5* not only in the morphogenesis of the heart but also in the construction of the cardiac conduction system.

In the current study, a compound phenotype of ASD and atrioventricular block was observed in half of family members who carried the identified mutations of *NKX2-5*. Moreover, the atrioventricular block seems to be a progredient with increasing age in each individual, as described previously [13, 15, 29, 35, 36], whereas it is not observed soon after birth.

Atrioventricular block is a possible cause of sudden death, and molecular genetic screening appears to be the most helpful in identifying individuals at risk for the life-threatening arrhythmia. When a person harboring a NKX2-5 mutation is identified, it is necessary for medical

staff to monitor this patient carefully, who may currently present with no symptoms or may have spontaneously closed or surgically corrected ASD. Monitoring enables offering accurate and early therapy.

In conclusion, the current study links novel mutations in the cardiac transcription factor NKX2-5 to familial ASD as well as atrioventricular block, which has potential implications in gene-specific prophylaxis and therapy of this common congenital heart disease.

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