A novel NKX2-5 mutation in familial ventricular septal defect

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Abstract. Ventricular septal defect (VSD) is the most common cardiovascular malformation and an important contributor to the substantial morbidity and mortality in infancy. Growing evidence suggests that genetic defects play important roles in the pathogenesis of congenital VSD. However, VSD is of great genetic heterogeneity and the genetic basis for VSD in the majority of the patients remains largely unhnown. In this study, the entire coding region of the *NKX2-5* gene, which encodes a homeodomain-containing transcription factor crucial to cardiogenesis, was initially sequenced in 136 unrelated patients with VSD. The relatives of a proband harboring the identified mutation and 200 unrelated control individuals were genotyped. The functional characteristic of the mutant transcription factor was analyzed in contrast to its wild-type counterpart using a luciferase reporter assay system. A novel heterozygous NKX2-5 mutation, p.P59A, was identified in a family with autosomal dominant inherited VSD. Absent in the 200 control individuals, the mutation was highly conserved evolutionarily and co-segregated with VSD in the family with complete penetrance. Functional analysis revealed that the p.P59A mutation of NKX2-5 was associated with a decreased transcriptional activity. These findings expand the spectrum of the mutations in *NKX2-5* linked to VSD and provide new insight into the molecular mechanisms involved in VSD. The resuls of the present study may have potential implications in the genetic diagnosis and gene-specific therapy of this common childhood disease.

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Introduction

Congenital heart disease is the most prevalent form of birth defect. It involves changes in the structure of the heart and in major blood vessels of a neonate, affects nearly 1% of newborns, and is the most common non-infectious cause of infant death resulting from birth abnormality. More than 29% of infants who die of a birth defect have a cardiovascular deformity (1). Congenital heart disease, an abnormal development of the heart with dysmorphic features, is comprised of at least 18 distinct types with many additional anatomical variations, of which the ventricular septal defect (VSD) is the most common type. VSD occurs in approximately 50% of all children with congenital cardiovascular malformations, and accounts for 14 to 16% of the defects that require an invasive procedure within the first year of life (1,2). Congenital VSD can occur alone or with other cardiac anomalies, such as atrial septal defect (ASD), Down syndrome, or tetralogy of Fallot. Regardless of other deformations that may accompany VSD, larger VSD may result in cardiac enlargement, congestive heart failure, pulmonary hypertension, Eisenmenger's syndrome, delayed fetal brain development, arrhythmias, and even sudden cardiac death in the absence of surgical or catheter based repair (3-7). Hence VSD is a leading cause of the markedly increased morbidity and mortality in infancy. Nevertheless, the molecular etiology in most VSD patients remains largely unknown (8,9).

Developmentally, VSD is implicated in a heterogeneous, complex biological process associated with environmental and genetic risk factors (8-10). Accumulating evidence underlines the pivotal role of the homeobox-containing transcription factor NKX2-5, in cardiogenesis (11-16). The human *NKX2-5* gene maps to chromosome 5q34 and consists of two exons encoding a protein of 324 amino acids. It is expressed during early cardiac morphogenesis and is indispensible for normal cardiac development (17). Therefore, *NKX2-5* has been one of the preferred candidate genes in identifying the genetic determinants for structural congenital heart defects. Presently, more than 40 mutations of the *NKX2-5* gene have been identified in patients with a variety of congenital heart aberrations including VSD, ASD, conotruncal abnormalities such as tetralogy of Fallot, double outlet right ventricle, L-transposition of the

great arteries, and hypoplastic left heart syndrome (18). 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 (15).

In this study, the coding exons and exon/intron boundaries of *NKX2-5* were initially sequenced in a cohort of 136 unrelated patients with congenital VSD and a novel heterozygous NKX2-5 mutation, p.P59A, was identified in a VSD patient with positive family history. Subsequently, genetic analysis of the family members demonstrated that the mutation co-segregated with an autosomal dominantly inherited form of VSD. Functional analysis revealed that the p.P59A mutation of NKX2-5 was associated with a decreased transcriptional activity. These findings expand the spectrum of mutations in *NKX2-5* linked to VSD and provide new insight into the molecular mechanism involved in the pathogenesis of VSD.

Materials and methods

Study participants. A cohort of 136 unrelated patients with VSD was selected from the Chinese population. Subjects were evaluated by individual and familial history, review of the medical records, complete physical examination, a 12-lead electrocardiogram (ECG) and two-dimensional transthoracic echocardiography with color-flow Doppler. All patients had a classic form of VSD, with a defect diameter of >3 mm and nearly all patients underwent cardiac catheterization and, if required, cardiac surgery. A total of 200 ethnically matched unrelated healthy individuals, which were derived from the general population, were used as controls to screen for the identified mutation in *NKX2-5*. Peripheral venous blood specimens were obtained from subjects and control individuals. The study protocol was reviewed and approved by the local institutional ethics committee and written informed consent was obtained from all participants or their guardians prior to investigation.

Genetic studies. Genomic DNA from all participants was extracted from blood lymphocytes with the Wizard Genomic DNA Purification Kit (Promega). Initially, the candidate gene *NKX2-5* was screened in 136 unrelated patients with VSD and subsequently, genotyping of *NKX2-5* in the available relatives of the index patient carrying an identified mutation and the 200 ethnically matched unrelated healthy control individuals was conducted. The genomic reference DNA sequence of *NKX2-5* was obtained from GenBank (accession No. NT_023133). The primer pairs used to amplify the complete coding region of *NKX2-5* by polymerase chain reaction (PCR) were designed with the on-line Primer 3 software (http://frodo.wi.mit.edu) as follows: primer 1, forward 5'-CACGATGCAGGGAAGCTG-3', and reverse 5'-AGTTTCTTGGGGACGAAAGC-3' (the PCR product was 477 base pairs in size); primer 2, forward 5'-CCTCCACGAGGATCCCTTAC-3', and reverse 5'-CGAGTCCCCTAGGCATGG-3' (the product was 463 base pairs); primer 3, forward 5'-AGAACCGGCGCTACAAGTG-3', and reverse 5'-GAGTCAGGGAGCTGTTGAGG-3' (the product was 473 base pairs). Polymerase chain reaction (PCR) was carried out using HotStarTaq DNA polymerase (Qiagen) on a PE 9700 Thermal Cycler (Applied Biosystems), 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 the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with an ABI PRISM 3130XL DNA Analyzer (Applied Biosystems). The sequencing primers used were previously designed for specific region sequencing. The DNA sequences were viewed and analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The variant was validated by re-sequencing an independent PCR-generated amplicon from the subject and met our quality control thresholds with a call rate >99%.

Multiple sequence alignments. The multiple NKX2-5 protein sequences across mammals were aligned using the program MUSCLE (version 3.6).

Plasmids and site-directed mutagenesis. The recombinant expression plasmids pEFSA-NKX2-5 and atrial natriuretic peptide-luciferase reporter gene, which contains the 2600-bp 5'-flanking region of the atrial natriuretic peptide gene, namely ANP(-2600)-Luc, were kindly provided by Ichiro Shiojima, Chiba University School of Medicine. The identified mutation was introduced into the wild-type *NKX2-5* using a QuickChange II XL site-directed mutagenesis kit (Stratagene) with a complementary pair of primers. The mutant was sequenced to confirm the desired mutation and to exclude any other sequence variations.

Reporter gene assays. COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The ANP(-2600)-Luc reporter construct and an internal control reporter plasmid pGL4.75 (hRluc/CMV, Promega) were used in transient transfection assays to examine the transcriptional activation function of the *NKX2-5* mutant. COS-7 cells were transfected with 0.2 μg of wild-type or mutant pEFSA-NKX2-5 expression vector, 0.2 μg of ANP(-2600)-Luc reporter construct, and 0.04 μg of pGL4.75 control reporter vector using PolyFect transfection reagent (Qiagen). For co-transfection experiments, 0.1 μg of wild-type pEFSA-NKX2-5, 0.1 μg of mutant pEFSA-NKX2-5, 0.2 μg of ANP(-2600)-Luc, and 0.04 μg of pGL4.75 were used. Firefly luciferase and *Renilla* luciferase activities were measured with the Dual-Glo luciferase assay system (Promega) 48 h after transfection. Three independent experiments were performed at minimum for wild-type and mutant *NKX2-5*.

Statistics. Data are given as the mean ± SD. Differences between the two groups were compared with the Student's t-test for continuous variables and a 2-tailed P-value <0.05 was considered to be statistically significant.

Results

Characteristics of the study subjects. A cohort of 136 unrelated patients with VSD was recruited and clinically evaluated against a cohort of 200 ethnically matched unrelated healthy individuals used as controls. None of them had overt traditional risk factors for VSD. The baseline clinical characteristics of the 136 unrelated patients with VSD are shown in Table I.

Table I. Clinical characteristics of 136 unrelated patients with ventricular septal defect.

	Number or mean value	Percentage or range
Male:female	51:85	60:100
Age at the diagnosis of VSD (years)	2.16	0.17-12
Age at the present study (years)	5.40	0.17-16
Positive family history	12	8.82
Distribution of different types of VSDs		
Subpulmonary	6	4.41
Perimembranous	102	75.00
Atrioventricular canal	13	9.56
Muscular	15	11.03
Prevalence of VSDs with other defects		
Isolated VSD	112	82.35
VSD and ASD	18	13.24
VSD, ASD and PDA	2	1.47
VSD, ASD and DORV	2	1.47
VSD and PDA	1	0.74
VSD and PS	1	0.74
Incidence of arrhythmias		
Atrioventricular block	7	5.15
Atrial fibrillation	3	2.21
Treatment		
Surgical repair	115	84.56
Percutaneous closure	4	2.94
Follow-up	17	12.50

VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; DORV, double outlet right ventricle; PS, pulmonary stenosis.

NKX2-5 mutations. Direct sequencing of the coding regions of the *NKX2-5* gene was performed after PCR amplification of genomic DNA from the 136 unrelated VSD patients. A novel heterozygous mutation in *NKX2-5* was identified in 1 out of 136 patients. The total population prevalence of *NKX2-5* mutations based on the cohort patients was approximately 0.74%. A substitution of guanine (G) for cytosine (C) in the first nucleotide of codon 59 of the *NKX2-5* gene (c.175C>G), predicting the transition of proline to alanine at amino acid position 59 (p.P59A), was identified in the proband from family 1. The sequence chromatogram showing the detected heterozygous *NKX2-5* variation of c.175C>G in comparison to control sequence was shown in Fig. 1. The variant was not present in 200 unrelated control individuals. Genetic scan of the family members displayed that the gene variant was present in all affected family members alive, but absent in the unaffected family members examined. Analysis of the pedigree demonstrated that the mutation co-segregated with VSD transmitted as an autosomal dominant trait in the family with complete penetrance. The pedigree structure of the family was illustrated in Fig. 2. The phenotypic characteristics and results of genetic screening of the affected pedigree members is listed in Table II. All 3 living patients underwent surgical repair of the VSDs.

Multiple cross-species alignments of the NKX2-5 protein sequences. A cross-species alignment of the NKX2-5 protein sequences showed that the altered amino acid P59 was highly
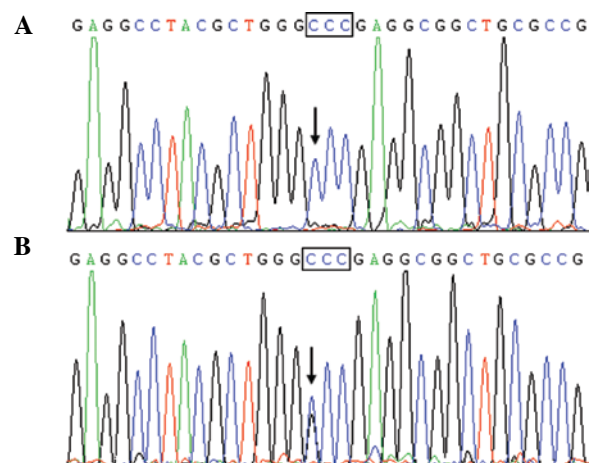

Figure 1. Sequence chromatogram showing the NKX2-5 mutation identified in family 1. The arrow indicates the heterozygous nucleotides of C/G in the proband from family 1 (B) or the homozygous nucleotides of C/C in a control individual (A). The square denotes the nucleotides comprising a codon of NKX2-5.

Table II. Phenotypic characteristics and status of the NKX2-5 mutation in the affected pedigree members.

Subject Information				Phenotype			Genotype
Identity	Gender	Age at time of study (years)	Age at diagnosis of VSD (years)	VSD (mm)	Other structural defects	AVB	P59A
II-2	F	58[a]	56	14	ASD	+	N/A
II-3	M	53[a]	51	22	PAS	+	N/A
III-3	M	32	12	19	-	-	+/-
IV-3	M	6	6	5	-	-	+/-
IV-4	F	4	4	8	-	-	+/-

F, female; M, male; VSD, ventricular septal defect; N/A, not available; AVB, atrioventricular block; ASD, atrial septal defect; PAS, pulmonary artery stenosis. + indicates present and – denotes absent. [a]Age at death.
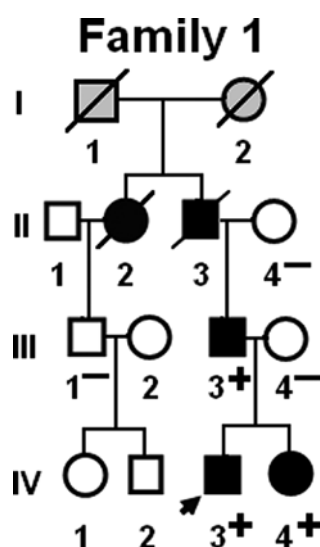

Figure 2. Pedigree structure of the family with the ventricular septal defect. Family members are identified by generations and numbers. Squares indicate male family members; circles, female members; symbols with a slash, the deceased members; closed symbols, affected members; open symbols, unaffected members; stippled symbols, members with phenotype undetermined; arrow, proband; +, carriers of the heterozygous mutations;–, non-carriers.
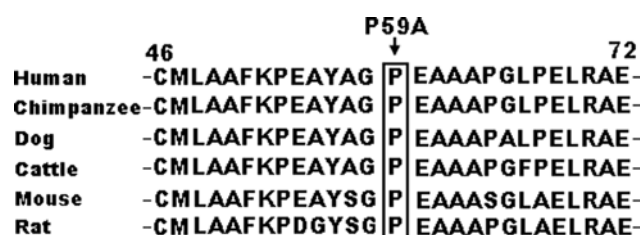

Figure 3. Alignment of multiple NKX2-5 protein sequences across species. The altered amino acid of P59 is completely conserved evolutionarily among mammals.
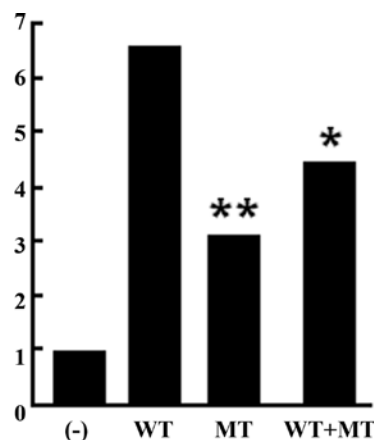

Figure 4. Transcriptional activity of the mutant NKX2-5 on the *ANP* promoter. COS-7 cells were transfected with 0.2 μg of wild-type or mutant pEFSA-NKX2-5 expression vector, 0.2 μg of ANP(-2600)-Luc reporter construct, and 0.04 μg of pGL4.75 control reporter vector. For co-transfection experiments, 0.1 μg of wild-type pEFSA-NKX2-5, 0.1 μg of mutant pEFSA-NKX2-5, 0.2 μg of ANP(-2600)-Luc, and 0.04 μg of pGL4.75 were used. The activity of the *ANP* promoter was presented as fold activation of the firefly luciferase relative to the *Renilla* luciferase. Values are the mean ± SD of data from three independent experiments performed in triplicate. *P<0.001 and **P<0.0001, respectively, when compared with the wild-type NKX2-5.

conserved evolutionarily among mammals as shown in Fig. 3, suggesting that the amino acid is functionally important.

Transcriptional activity of the NKX2-5 mutant. The transcriptional activation function of NKX2-5 in COS-7 cells was examined using one of its direct cardiac downstream target genes, *ANP*, as a luciferase reporter, and the activity of the *ANP* promoter was presented as fold-activation of the firefly luciferase relative to *Renilla* luciferase. The same amounts of wild-type (0.2 μg) and mutant *NKX2-5* (0.2 μg) activated the *ANP* promoter by ~6.6- and ~3.2-fold, respectively. When the same amount of wild-type *NKX2-5* (0.1 μg) was co-transfected with mutant *NKX2-5* (0.1 μg), the induced activation of the *ANP* promoter was ~4.5-fold. These results suggest that mutant NKX2-5 has a significantly reduced activation activity compared with wild-type NKX2-5 (Fig. 4).

Discussion

In the present study, we report a previously unrecognized *NKX2-5* missense mutation identified in a family with congenital VSD. The novel heterozygous mutation was present in all

the affected family members alive but absent in the unaffected relatives examined and in the 400 normal chromosomes from a matched control population. A cross-species alignment of the NKX2-5 protein sequences demonstrated that the altered amino acid was evolutionarily highly conserved among mammals. Functional analysis displayed that the p.P59A mutation of NKX2-5 was associated with decreased transcriptional activity. Therefore, it is very likely that mutated NKX2-5 is responsible for the VSD in this family.

Our results are supported by the discoveries of other *NKX2-5* mutations predisposing to congenital VSD. To date, at least 16 mutations in NKX2-5 (p.E21Q, p.Q22P, p.Q25C, p.E109X, p.T178M, p.Q149X, p.R142C, p.W185L, p.Y191C, p.L202fs, p.R216C, p.A219V, p.Y259X, p.A323T, p.L171P, and p.R190H) have been detected in patients with isolated or syndromic VSD, showing that although *NKX2-5* mutations are involved in a long list of cardiac malformations, one of frequent phenotypes ascribed to a mutation in *NKX2-5* is VSD (11,19-24). In most of these patients, the VSD-related mutations are familial, whereas sporadic cases remain relatively rare (11,19-24). Similar to these findings, the mutation prevalence of over 8% (1/12) in our patients with positive family history suggests that the NKX2-5 mutations could be a major cause of familial VSD. Notably, remarkable genetic heterogeneity of VSD was substantiated by an inability to observe mutations in roughly 99% of our cohort patients, despite somatic *NKX2-5* mutations as a potential mechanism of VSD in some patients (25-27). Therefore, the contribution of genes other than *NKX2-5* to VSD pathogenesis seems likely.

Mutations in other transcription factors associated with cardiogenesis, such as GATA4, TBX5, and GATA6 (28-33), as well as mutations in cardiac structural proteins such as troponin I type 3 (TNNI3) and α myosin heavy chain (MYH6) have been identified in VSD patients (34,35). However, so far, only *NKX2-5* mutations have been reported to cause a cardiac septal defect phenotype and the development of an atrioventricular block (18). The most common two phenotypes caused by mutated *NKX2-5* are cardiac septal defect and atrioventricular conduction disturbance (17), suggesting the pivotal role of *NKX2-5* not only in the morphogenesis of the heart, but also in the development of the cardiac conduction system. In the present study, a compound phenotype of VSD and an atrioventricular block were observed in 2/5 family members who carried the identified mutation of *NKX2-5*. The atrioventricular block appeared to be progressive with increasing age in each individual as previously described (11,14,23,36,37). As atrioventricular block is a possible cause of sudden cardiac death, a molecular genetic scanning will appear to be most favorable in identifying individuals at risk for life-threatening dysrhythmias. When an individual carrying an *NKX2-5* mutation is identified, it is imperative for health care professionals to carefully monitor such a case that may currently present no symptoms, or to spontaneously close or surgically correct VSD, offering accurate and timely therapy.

Association of impaired *NKX2-5* with increased susceptibility to VSD and atrioventricular block has been revealed in animal models. The homeobox-containing transcription factor encoded by the *tinman* gene, a homologue of *NKX2-5*, is expressed in the dorsal vessel (an insect equivalent of the vertebrate heart) of the fruit fly *Drosophila melanogaster* and targeted disruption of *tinman* leads to the lethal failure of the vessel formation (38). In *Xenopus*, expression of a similar DNA-non-binding mutant of *NKX2-5* has been shown to exert dominant negative effects on embryos, exhibiting small heart or no heart formation (39). In mice, *NKX2-5* is highly expressed in the early heart progenitor cells in both primary and secondary heart fields during embryogenesis and continues to be expressed at a high level in the heart through adulthood. A transiently elevated expression of *NKX2-5* is observed in specialized myocardial conduction cells during the development of the cardiac conduction system (17). In transgenic mice expressing a DNA binding-impaired mutant of the mouse NKX2-5 (I183P), under the β-myosin heavy chain promoter, the accumulation of mutant protein in the embryo, neonate, and adult myocardium resulted in progressive and profound cardiac conduction defects and heart failure (40). Targeted disruption of *NKX2-5* in mice results in embryonic lethality at about ED9.5, with aberrant heart morphogenesis and growth retardation (41,42). Mice heterozygous for *NKX2-5*-null alleles were predisposed to cardiac septal dysmorphogenesis and abnormal atrioventricular conduction (12). Ventricular-restricted NKX2-5 knockout around ED8.0 to ED8.5, created by crossing floxed-NKX2-5 mice with myosin light chain 2v-Cre knock-in mice, postnatally resulted in progressive and advanced conduction defects and in left ventricular hypertrophy (14). In addition, perinatal loss of NKX2-5 leads to rapid conduction and contraction defects by regulating the expression of several ion channel genes (43). Together, these experimental results from animals imply that *NKX2-5* mutations are involved in a variety of congenital cardiac abnormalities including VSD and progressive conduction defects in humans.

Previous investigations have demonstrated that NKX2-5 is an upstream regulator or transcriptional activator of other genes expressed during cardiac development including atrial natriuretic factor gene, brain natriuretic peptide gene, and α-cardiac actin gene (42,44,45). NKX2-5 binding to target DNA may occur in conjunction with other partners including transcription factor GATA4 and serum response factor (45-47), and the synergistic transcriptional activation mediated by NKX2-5 and GATA4 has been ascertained (48). Therefore, the functional characteristics of the *NKX2-5* mutations can be explored by analysis of the transcriptional activity of the *ANP* promoter in cells transfected with *NKX2-5* mutants in contrast to wild-type. Up to now, a total of 8 VSD-related NKX2-5 mutations (p.Q25C, p.T178M, p.Q149X, p.R142C, p.Y191C, p.Y259X, p.L171P, and p.R190H) have been characterized and all the mutations have loss-of function effects on the transcriptional activity of the *ANP* promoter except for p.Q25C, which has little effect (22,49-51). Similarly, the novel p.P59A mutation of NKX2-5 identified in our familial VSD patients demonstrates a decreased transcriptional activity on the downstream gene. These findings indicate that haploinsufficiency or the dominant negative effect of NKX2-5 mutations are the major pathophysiological mechanisms underlying congenital VSD.

In conclusion, the current study links a novel mutation in the cardiac transcription factor NKX2-5 to familial VSD as well as to atrioventricular block. Furthermore, it provides new insight into the molecular mechanism implicated in the pathogenesis of VSD, implying potential implications for the

genetic diagnosis and gene-specific therapy for this common disease in infancy.

Acknowledgements

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