ORIGINAL ARTICLE

A Novel *NKX2.6* Mutation Associated with Congenital Ventricular Septal Defect

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Abstract Congenital heart disease (CHD) is the most common birth defect and is the most prevalent non-infectious cause of infant death. Aggregating evidence demonstrates that genetic defects are involved in the pathogenesis of CHD. However, CHD is genetically heterogeneous and the genetic determinants for CHD in an overwhelming majority of patients remain unknown. In this study, the coding regions and splice junctions of the *NKX2.6* gene, which encodes a homeodomain transcription factor crucial for cardiovascular development, were sequenced in 210 unrelated CHD patients. As a result, a novel heterozygous NKX2.6 mutation, p.K152Q, was identified in an index patient with ventricular septal defect (VSD). Genetic analysis of the proband's available family members showed that the mutation cosegregated with VSD transmitted as an autosomal dominant trait with complete penetrance. The missense mutation was absent in 400 control chromosomes and the altered amino acid was completely conserved evolutionarily across species. Due to unknown transcriptional targets of NKX2.6, the functional characteristics of the identified mutation at transcriptional activity were analyzed by using NKX2.5 as a surrogate. Alignment between human NKX2.6 and NKX2.5 proteins displayed that K152Q-mutant NKX2.6 was equivalent to K158Q-mutant NKX2.5, and introduction of K158Q into NKX2.5 significantly reduced its transcriptional activating function when compared with its wild-type counterpart. This study firstly links NKX2.6 loss-of-function mutation with increased susceptibility to isolated VSD, providing novel insight into the molecular mechanism underpinning VSD and contributing to the development of new preventive and therapeutic strategies for this common form of CHD.

Keywords Congenital heart disease · Ventricular septal defect · Genetics · Transcription factor · NKX2.6

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Introduction

Congenital heart disease (CHD) represents the most common form of human birth defect, affecting nearly 1 % of all live births worldwide, and remains a leading cause of morbidity and mortality in childhood, accounting for approximately 10 % of all infant deaths [15, 17]. Congenital

cardiovascular abnormalities are clinically classified into at least 21 different categories with specific anatomic lesions, including ventricular septal defect (VSD), atrial septal defect, tetralogy of Fallot, patent ductus arteriosus, aortic stenosis, pulmonary atresia, endocardial cushion defect, double outlet right ventricle, anomalous coronary arteries, abnormal pulmonary venous return, aorticopulmonary window, truncus arteriosus, transposition of great arteries, coronary artery anomaly, and hypoplastic left heart syndrome, of which VSD is the most prevalent type of CHD in children while in adults atrial septal defect is the most frequently seen developmental malformation [17, 63]. Various kinds of CHDs may occur singly or in combination with each other, resulting in degraded quality of life, limited exercise capacity, delayed brain development, pulmonary hypertension, anomalous lung function, impaired muscle function, autonomic dysfunction, aortic aneurysm or dissection, heart failure, infective endocarditis, thromboembolism, arrhythmias, and even cardiac death [1, 3, 5, 12–14, 16, 21, 27, 32–35, 37, 39–41, 43, 49, 65]. Although great advances made in surgical and medical treatment of children born with CHD have led to a growing number of adult survivors, unfortunately, the incidence of late complications and mortality rate are significantly increased in the survivals [19, 35, 48, 50, 51]. Hence, CHD has imposed a vast economic burden on patients and health care systems, and the socioeconomic burden is projected to increase in the future with increasing CHD adults [52]. Despite the high prevalence and pronounced clinical significance, the molecular mechanism underlying CHD remains poorly understood.

In vertebrates, the heart is the first functional organ that develops during embryogenesis. Cardiac morphogenesis is a complex dynamic biological process that requires the accurate spatial and temporal cooperation of cardiac cell commitment, differentiation, proliferation, and migration, and both environmental and genetic risk factors may disturb this process of cardiogenesis, leading to a large spectrum of CHDs [4, 11, 15, 18, 29, 38]. Recently, a growing body of evidence highlights the genetic basis for CHD, and a great number of mutations in more than 60 genes have been implicated in the pathogenesis of CHD [2, 4, 8, 10, 15, 24, 25, 28, 42, 44, 53–56, 62, 64]. Among these well-established CHD-associated genes, most encode cardiac transcription factors and show autosomal dominant inheritance with incomplete disease penetrance, and the cardiac transcription factor genes NKX2.5 and GATA4 are most frequently associated with non-syndromic CHD [9, 23, 31, 57, 59, 60]. Nevertheless, mutations in these genes only explain the CHDs in a minority of cases and in an overwhelming majority of patients, the genetic components responsible for CHDs are still to be identified.

As a member of the cardiac NK-2 family of transcription factors, which are the vertebrate orthologs of the Drosophila tinman gene, NKX2.5 is crucial to normal cardiac development across vertebrate species, and mutations or altered expression of NKX2.5 may lead to abnormal heart formation in vertebrates [6]. In humans, a long list of mutations in NKX2.5 have been causally linked to a great variety of congenital cardiovascular deformities, including atrial septal defect, ventricular septal defect, and tetralogy of Fallot, as well as atrial fibrillation [9, 22, 23, 31, 58, 61]. NKX2.6 is another member of the NK-2 family of transcription factors and its temporal and spatial expression patterns and functional roles are similar to those of NKX2.5 during cardiovascular development [6]. In mice with targeted deletion of Nkx2.6, it has been revealed that the redundant activity of Nkx2.5 is required for proper cardiac morphogenesis [20]. Therefore, it is warranted to screen NKX2.6 as a preferred candidate gene for human CHD.

Materials and Methods

Ethics

This study was conducted in accordance with the ethical principles of the revised Declaration of Helsinki (Somerset West, Republic of South Africa, 1996). The research protocol was reviewed and approved by the local institutional ethics committee of Tongji Hospital, Tongji University (the ethical approval number for cases and controls: LL(H)-09-07; the date for the approval: July 27, 2009) and written informed consent was obtained from all participants or their guardians prior to commencement of investigation.

Study Subjects

A total of 210 unrelated patients affected with non-syndromic CHD were recruited. The available relatives of the index patient harboring an identified Nkx2.6 mutation were also included. All patients underwent a comprehensive clinical evaluation, including individual and familial histories, medical records, complete physical examination, 12-lead electrocardiogram, and two-dimensional transthoracic echocardiography with color flow Doppler. Cardiac catheterization, angiography, chest X-ray, and cardiac magnetic resonance imaging were performed only if there was a strong clinical indication. Medical records were also reviewed in the case of deceased or unavailable relatives. CHD was confirmed by imaging and/or direct view during cardiac surgery. The patients with known chromosomal abnormalities or syndromic cardiovascular defects, such as Down syndrome, Turner syndrome, Marfan syndrome, Di George syndrome, and Holt–Oram syndrome, were excluded from the study. The controls comprised 200 non-CHD individuals from the same geographic area, who were

Table 1 The intronic primers to amplify the coding exons and exon–intron boundaries of *NKX2.6*

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon (bp)
1	gac,aag,acg,gga,agt,tca,gg	tcg,aac,cca,gga,gat,agg,ag	447
2-a	cca,ggg,aga,gga,aag,tct,tg	cag,gac,ggg,cac,agc,tac,tc	454
2-b	aga,acc,gac,gct,aca,aat,gc	gag,atc,cct,ccg,gaa,aga,ag	500

bp base pairs

matched to the CHD patients in race and gender. All the study subjects were of Chinese Han ethnicity. The ethnic origin of the participants was ascertained by a combination of self-reported ethnicity and a personal questionnaire about the birthplace, language, religion, and ancestry. Approximately 0.5–2 ml of peripheral venous blood sample was collected from each study participant. The clinical studies were performed with investigators blinded to the results of genotypes.

Genetic Screening of *NKX2.6*

Genomic DNA was extracted from each participant's peripheral blood leukocytes with Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The coding exons and flanking exon–intron boundaries of the *NKX2.6* gene were sequenced in 210 unrelated patients with CHD. The available relatives of the index patient carrying an identified *NKX2.6* mutation and 200 unrelated control individuals were subsequently genotyped for *NKX2.6*. The referential genomic DNA sequence of *NKX2.6* was derived from GenBank (accession no. NG_030636), a gene sequence database at the National Center for Biotechnical Information (NCBI; http://www.ncbi.nlm.nih.gov/). With the aid of the online Primer 3 program (http://frodo.wi.mit.edu/), the primer pairs used to amplify the coding regions and splice junction sites of *NKX2.6* by polymerase chain reaction (PCR) are designed as shown in Table 1. The PCR was carried out using HotStar *Taq* DNA Polymerase (Qiagen GmbH, Hilden, Germany) on a Veriti Thermal Cycler (Applied Biosystems, Foster, CA, USA), with standard conditions and concentrations of reagents. Both strands of each PCR product were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems). The sequencing primers were the same as those designed for specific region amplifications. The DNA sequences were analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The identified *NKX2.6* variant was confirmed by resequencing an independent PCR-generated amplicon from the same case. Additionally, an identified sequence variation was queried in the single nucleotide polymorphism (SNP) database at NCBI (http://www.ncbi.nlm.nih.gov/), the human gene mutation (HGM) database (http://www.hgmd.org/), and the 1000 Genome Project (1000 GP) database (http://www.1000genomes.org/) to confirm its novelty.

Alignment of NKX2.6 Amino Acid Sequences Among Various Species

Conservation of the amino acid altered by missense mutation was estimated by aligning human NKX2.6 to chimpanzee, monkey, dog, cattle, mouse, rat, fowl, zebrafish, and frog NKX2.6 using the HomoloGene and Show Multiple Alignment links on the NCBI's website (http://www.ncbi.nlm.nih.gov/homologene).

Prediction of the Causative Potential of a Novel *NKX2.6* Sequence Variation

The disease-causing potential of a novel *NKX2.6* sequence variation was predicted by MutationTaster (an online program at http://www.mutationtaster.org), which automatically gave a probability for the variation to be either a deleterious mutation or a benign polymorphism. Notably, the p value used here is the probability of the correct prediction rather than the probability of error as used in t test statistics (i.e., a value close to 1 indicates high accuracy of the prediction). Additionally, another online program PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) was also used to evaluate the possible pathogenic effect of an amino acid substitution on the structure and function of NKX2.6 protein.

Plasmids and Site-Directed Mutagenesis

The recombinant expression plasmid NKX2.5-pEFSA and the ANF–luciferase (ANF–Luc) reporter vector, which contains the 2600-bp 5′-flanking region of the *ANF* gene, were kindly provided by Dr. Ichiro Shiojima from Chiba University School of Medicine, Japan. Owing to unknown downstream genes of NKX2.6, NKX2.5 was used as a surrogate in transcriptional analysis to assess functional consequences of the NKX2.6 homeodomain substitution as previously described [20]. Alignment between human NKX2.6 and NKX2.5 proteins displayed that K152Q-

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NKX2.6    1    MLLSP-VTSTPFSVKDILRLERERSCPAASPHPRVRK----SPENFQYLRMDAEPRGSEV    55
               M  SP +T TPFSVKDIL LE+++    AA+     R     +P +         E
NKX2.5    1    MFPSPALTPTPFSVKDILNLEQQQRSLAAAGELSARLEATLAPSSCMLAAFKPEAYAGPE    60


NKX2.6   56    HNAGGGGGDRKLDGSEPPGGPCEAVLEMDAERM----GEPQPGLNAASPLGGGTRVPERG   111
                A G    R   G  P    C  +          +P P  A  P          +
NKX2.5   61    AAAPGLPELRAELGRAPSPAKCASAFPAAPAFYPRAYSDPDP---AKDPRAEKKELCALQ   117
                                                     ↓
NKX2.6  112    VGNSGDSVRGGRSEQPKARQRRKPRVLFSQAQVLALERRFKQQRYLSAPEREHLASALQL   171
                  +      +E+P+AR+RRKPRVLFSQAQV  LERRFKQQRYLSAPER+ LAS L+L
NKX2.5  118    KAVELEKTEADNAERPRARRRRKPRVLFSQAQVYELERRFKQQRYLSAPERDQLASVLKL   177


NKX2.6  172    TSTQVKIWFQNRRYKCKRQRQDKSLELAG----HPLTPRRVAVPVLVRDGKPCLG-PGPG   226
               TSTQVKIWFQNRRYKCKRQRQD++LEL G       P   RR+AVPVLVRDGKPCLG   P
NKX2.5  178    TSTQVKIWFQNRRYKCKRQRQDQTLELVGLPPPPPPPARRIAVPVLVRDGKPCLGDSAPY   237


NKX2.6  227    APAF-----PSPYSAAVSPYSCYGGYSGAPYGAGYGTCYAGAPSGPAPHTPLASAG----   277
               APA+      P Y+A    Y  Y GY GA    GY +C A P+GP+P  P    +A
NKX2.5  238    APAYGVGLNPYGYNA----YPAYPGYGGAACSPGY-SCTAAYPAGPSPAQPATAAANNNF   292


NKX2.6  278    --FGHGGQNAT-----PQGHL-AATLQGVRAW   301
                 FG G  NA       PQ +   +TL G+RAW
NKX2.5  293    VNFGVGDLNAVQSPGIPQSNSGVSTLHGIRAW   324
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Fig. 1 Alignment of human NKX2.6 protein sequence with that of NKX2.5. Alignment between human NKX2.6 and NKX2.5 protein sequences indicates that K152Q-mutant NKX2.6 is equivalent to K158Q-mutant NKX2.5. The *arrow* points to the altered amino acid, lysine

mutant NKX2.6 was equivalent to K158Q-mutant NKX2.5 (as shown in Fig. 1). The c.472A>C transition, which was predicted to generate the p.K158Q mutation, was introduced into the wild-type *NKX2.5* using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with a complementary pair of primers. The mutant was sequenced to confirm the desired mutation and to exclude any other sequence variations.

Reporter Gene Analysis of Mutant NKX2.5

COS-7 cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum and seeded in 12-well plates (1×10^5) before transfection. The ANF(-2600)–Luc reporter construct and an internal control reporter plasmid pGL4.75 (hRluc/CMV, Promega) were used in transient transfection assay to assess the transcriptional activity of the NKX2.5 mutant. Twenty-four hours after plating, the COS-7 cells at about 70 % confluence were transfected with 0.4 μg of wild-type

or mutant NKX2.5-pEFSA, 1.0 μg of ANF(-2600)–Luc reporter construct, and 0.04 μg of pGL4.75 using PolyFect Transfection Reagent (Qiagen). For co-transfection experiments, 0.2 μg of wild-type NKX2.5-pEFSA together with 0.2 μg of mutant NKX2.5-pEFSA or 0.2 μg of empty vector were used in the presence of 0.4 μg of ANF(-2600)–Luc and 0.04 μg of pGL4.75. Firefly luciferase and Renilla luciferase activities were measured with the Dual-Glo luciferase assay system (Promega) 48 h after transfection. The activity of the *ANF* promoter was presented as fold activation of Firefly luciferase relative to Renilla luciferase. At least three independent experiments were conducted in triplicate for wild-type and mutant *NKX2.5*.

Statistical Analysis

Data are expressed as means ± standard deviations. Continuous variables were tested for normal distribution and Student's unpaired *t* test was used to compare the numeric variables between two groups. Comparison of the

Table 2 Baseline clinical characteristics of the 210 unrelated patients with CHD

Variable	Statistic
Male gender (%)	115 (54.8)
Age (years)	12.7 ± 6.8
Positive family history (%)	25 (11.9)
Prevalence of different types of CHD	
Isolated CHD (%)	162 (77.1)
VSD (%)	66 (31.4)
ASD (%)	51 (24.3)
PDA (%)	20 (9.5)
PA (%)	8 (3.8)
CoA (%)	7 (3.3)
PS (%)	6 (2.9)
TAPVC (%)	3 (1.4)
SV (%)	1 (0.5)
Complex CHD (%)	48 (22.9)
TOF (%)	18 (8.6)
DORV + VSD (%)	7 (3.3)
ASD + VSD (%)	7 (3.3)
TA + VSD (%)	6 (2.9)
TGA +VSD (%)	1 (0.5)
Others (%)	9 (4.3)
Incidence of arrhythmia	
Atrial fibrillation (%)	13 (6.2)
Atrioventricular block (%)	9 (4.3)
Treatment	
Surgical repair (%)	109 (51.9)
Catheter-based closure (%)	85 (40.5)
Follow-up (%)	16 (7.6)

CHD congenital heart disease, *VSD* ventricular septal defect, *ASD* atrial septal defect, *PDA* patent ductus arteriosus, *PA* pulmonary atresia, *CoA* coarctation of the aorta, *PS* pulmonary stenosis, *TAPVC* total abnormal pulmonary venous connection, *SV* single ventricle, *TOF* tetralogy of Fallot, *DORV* double outlet of right ventricle, *TA* truncus arteriosus, *TGA* transposition of great arteries

categorical variables between two groups was completed using Pearson's χ^2 test or Fisher's exact test when appropriate. A two-tailed *p* value less than 0.05 was considered to indicate statistical difference.

Results

Clinical Characteristics of the Study Subjects

A cohort of 210 unrelated patients with CHD was clinically evaluated in contrast to a total of 200 unrelated non-CHD control individuals. All the study subjects had no established environmental risk factors for CHD, such as

maternal illness and drug use in the first trimester of pregnancy, parental smoking, and long-term exposure to toxicants and ionizing radiation. The baseline clinical characteristics of the 210 unrelated CHD patients are summarized in Table 2.

Identification of a Novel Nkx2.6 Mutation

By sequence analysis of the coding exons and flanking introns of *NKX2.6*, a heterozygous sequence variation was identified in 1 of 210 unrelated CHD patients, with a mutational prevalence of about 0.48 %. Specifically, a substitution of cytosine for adenine in the first nucleotide of codon 152 (c.454A>C), predicting the transition of lysine into glutamine at amino acid position 152 (p.K152Q), was identified in an index patient with VSD. The sequence electropherograms showing the identified heterozygous *NKX2.6* variation in contrast to its corresponding control sequence are shown in Fig. 2. The schematic diagrams of NKX2.6 and NKX2.5 proteins showing the structural domains and location of the mutation identified in this study are presented in Fig. 3. The variation was neither observed in 400 control chromosomes nor found in the SNP, HGM, and 1000 GP databases, which were consulted again on August 9, 2014, indicating a novel mutation.

Genetic screening of the proband's relatives showed that the mutation was present in all the affected living family members, but absent in unaffected family members who were examined. Analysis of the pedigree demonstrated that the mutation cosegregated with VSD transmitted in an autosomal dominant pattern and with complete penetrance. The pedigree structure of the family is shown in Fig. 4. The phenotypic characteristics and status of NKX2.6 mutation of the affected family members are listed in Table 3.

Multiple Alignments of NKX2.6 Protein Sequences Among Various Species

As shown in Fig. 5, a cross-species alignment of multiple NKX2.6 protein sequences displayed that the affected amino acid of p.K152 was completely conserved evolutionarily, suggesting that the amino acid is functionally important.

### Disease-Causing Potential of the Identified *NKX2.6* Sequence Variation

The *NKX2.6* sequence variation of c.454A>C was predicted to be pathogenic, with a *p* value of 0.956766. The amino acid substitution of p.K152Q was also predicted to be probably damaging by another software PolyPhen-2, with a score of 0.964 (sensitivity: 0.62; specificity: 0.92),
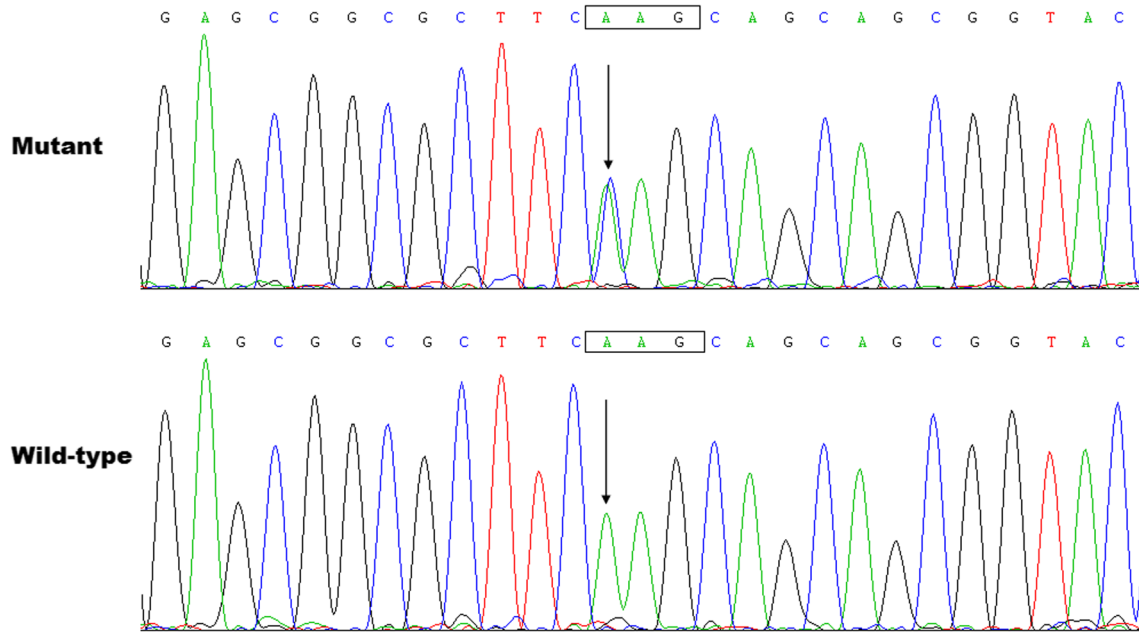
Fig. 2 Sequence electropherograms showing the *NKX2.6* mutation in contrast to its corresponding control. The *arrow* indicates the heterozygous nucleotides of A/C in the proband from family 1 (mutant); or the homozygous nucleotides of A/A in the corresponding control individual (wild-type). The *rectangle* means the nucleotides constituting a codon of *NKX2.6*

Fig. 3 Schematic diagrams of human NKX2.6 and NKX2.5 protein structures with the mutation responsible for congenital ventricular defect shown. The mutation associated with congenital ventricular defect is shown above the structural domains. *NH₂* amino-terminus, *TN* tinman domain, *HD* homeodomain, *NK* nucleotide kinase domain, and *COOH* carboxyl-terminus
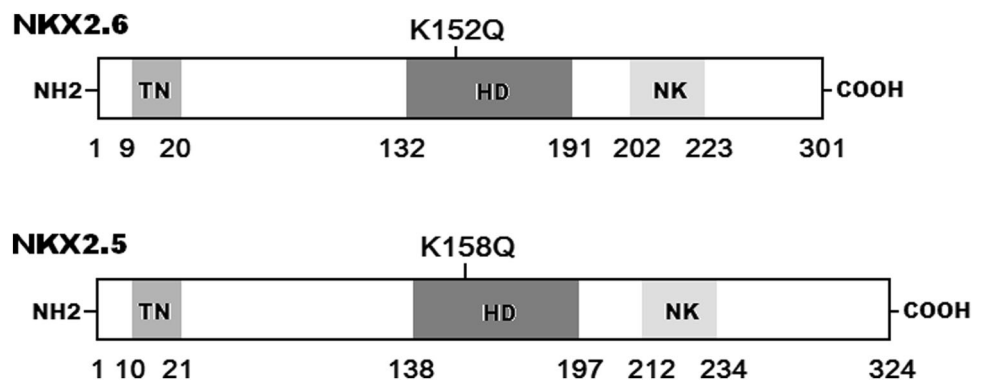
implying that mutated NKX2.6 contributes to the development of VSD in these mutation carriers.

Diminished Transcriptional Activity of the Mutant NKX2.5

As shown in Fig. 6, the wild-type NKX2.5 and the K158Q-mutant NKX2.5 activated the *ANF* promoter by ∼11- and ∼3-fold, respectively. When wild-type NKX2.5 was co-expressed with the same amount of K158Q-mutant NKX2.5, the induced activation of the *ANF* promoter was ∼fivefold. These results reveal that the K158Q-mutant NKX2.5 is associated with significantly diminished trans-activational activity compared with its wild-type counterpart.

Discussion

In the present study, a novel heterozygous mutation in NKX2.6, p.K152Q, was detected in a family with VSD. The mutant allele was present in all affected family members available but absent in unaffected relatives examined and 400 referral chromosomes from an ethnically matched control population. A cross-species alignment of multiple NKX2.6 protein sequences showed that the altered amino acid was completely conserved evolutionarily. The p.K152Q variation was predicted to be pathogenic by both MutationTaster and PolyPhen-2, and the functional analysis indicated that the homeobox substitution resulted in functional impairment. Therefore, it is very likely that mutant NKX2.6 predisposes these mutation
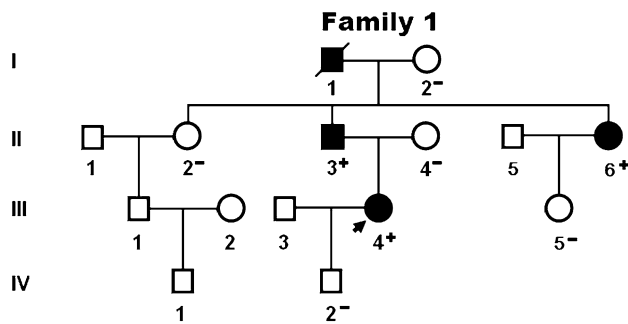
Family 1

Fig. 4 Pedigree structure of the family with congenital ventricular defect. Family is designated as family 1. Family members are identified by generations and numbers. *Square* indicates male family member, *circle* female member, *closed symbol* affected member, *open symbol* unaffected member, *symbol with a slash* the deceased member, *arrow* proband, "+" carrier of the heterozygous mutation, and "−" non-carrier

Table 3 Phenotypic characteristics and status of NKX2.6 mutation of the affected pedigree members

Subject information				Phenotype	Genotype
Identity	Gender	Age at time of study (years)	Age at diagnosis of VSD (years)	Cardiac structural defects	NKX2.6 mutation
Family 1					K152Q
I-1	M	62[a]	46	VSD	NA
II-3	M	50	22	VSD	±
II-6	F	46	16	VSD	±
III-4	F	25	2	VSD	±

M male, *F* female, *VSD* ventricular septal defect, *NA* not available or not applicable, ± presence of heterozygous mutation

[a] Age at death

carriers to VSD. To our knowledge, this is the first to associate NKX2.6 loss-of-function mutation with enhanced susceptibility to isolated VSD in humans.

The official name of NKX2.6 is NK-2 transcription factor-related locus 6. This term refers to the grouping of homeodomain-containing proteins into 20 different classes, one of which is the NK-2 class. The abbreviation NK refers to Nirenberg and Kim, who completed a search for *Drosophila* genes containing homeodomain-encoding motifs. The "X" in NKX was added to designate vertebrate members of the family. Among the NK-2 genes expressed in vertebrates, a subset, referred to as the "cardiac group", includes *Nkx2.3*, *Nkx2.5*, *Nkx2.6*, *Nkx2.7*, *Nkx2.8*, and *Nkx2.10* [6]. The human *NKX2.6* gene maps to chromosome 8p21.2 and consists of two exons encoding a protein of 301 amino acids, which is expressed in early embryonic heart progenitor cells, playing an important role in proper

cardiovascular development [7, 46]. The NKX2.6 protein contains an evolutionarily conserved homeodomain that recognizes and binds to a consensus DNA motif, AAGTG. The homeodomain is centrally located at amino acid positions 132–191 and is predominantly involved in target DNA binding, nuclear translocation as well as interaction with other transcription factors [20, 36]. The NKX2.6 mutation of p.K152Q identified in this study is located in homeodomain, and thus may be anticipated to exert influence on the transcriptional activity of NKX2.6 by interfering with its nuclear distribution or DNA-binding ability.

In order to determine the functional consequence of the K152Q homeodomain substitution in NKX2.6, NKX2.5 was chosen as a surrogate mainly based on the following reasons. Firstly, neither a target gene nor a binding site recognition sequence for NKX2.6 has been verified. Secondly, both NKX2.5 and NKX2.6 belong to members of the NK family, and they share highly conserved structural motifs, especially in the homeodomain. Thirdly, biochemical analyses of NKX2.5 have been well performed repeatedly. Finally, there are in vivo studies substantiating that NKX2.5 may compensate for the lack of NKX2.6 during embryogenesis, and therefore implying that some DNA binding and transcriptional regulatory activities are shared [7, 20, 46]. Consequently, functional assays of K158Q-mutant NKX2.5 instead of K152Q-mutant NKX2.6 revealed that the homeodomain substitution significantly reduced transcriptional activity on a target gene, *ANF*. These findings suggest that haploinsufficiency or dominant-negative effect caused by *NKX2.6* mutation is potentially an alternative pathological mechanism of VSD. Nevertheless, the direct molecular mechanism by which mutant NKX2.6 predisposes to VSD needs to be explored in order to overcome the limitation to the current indirect functional analysis.

Previously *NKX2.6* mutations were associated with truncus arteriosus, another type of CHD. Heathcote et al. made a genetic analysis of a large consanguineous family with truncus arteriosus, and identified an F151L mutation in the homeodomain of NKX2.6. Due to unknown transcriptional targets of NKX2.6, the functional characteristics of the F151L-mutant NKX2.6 were investigated by using F157L-mutant NKX2.5 as a surrogate, and introduction of F157L into human NKX2.5 substantially reduced its transcription activating function, its synergism with partners at the *ANF* promoter and its specific DNA-binding affinity [20]. Ta-Shma et al. performed a whole exome analysis of a three-patient family with complex CHD, including the proband with VSD and aortic arch hypoplasia, his dead brother with truncus arteriosus, stenosis of right pulmonary artery and right aortic arc, and his sister with truncus arteriosus and right aortic arc, and discovered a homozygous insertion mutation of

Fig. 5 Alignment of multiple NKX2.6 protein sequences among species. The altered amino acid of p.K152 in NKX2.6 is completely conserved evolutionarily across various species
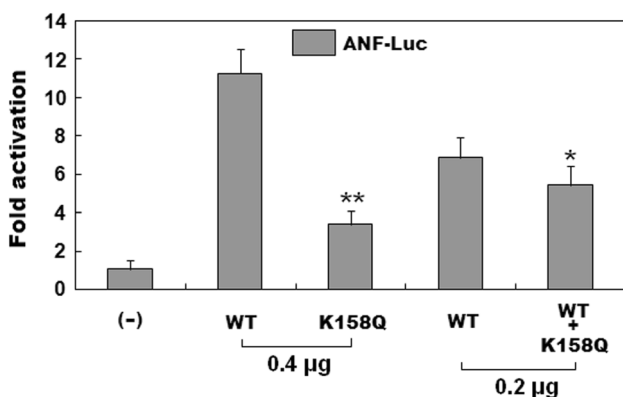
Fig. 6 Functional impairment of K158Q-mutant NKX2.5. Activation of the ANF–Luc reporter in cultured COS-7 cells by wild-type NKX2.5 (WT) or mutant (K158Q), alone or in combination, showed significantly decreased transcriptional activity by mutant protein. Experiments were performed in triplicate and mean and standard deviations are given. ** $p < 0.001$ and * $p < 0.005$, respectively, when compared with wild-type NKX2.5

c.453_454insT in *NKX2.6*, which was predicted to generate a truncated protein with only N-terminal 152 amino acids, namely p.K152X mutation. However, functional analysis of this truncation mutation was not made [47]. Khetyar et al. screened the coding regions and splice signal sequences of the *NKX2.6* gene in 12 unrelated CHD patients, but found no non-synonymous mutation [26]. The discrepancy in the mutational prevalence of these reports including the present study may be partially explained by different sample size and ethnicity as well as

great genetic heterogeneity. Taken together, these findings support that NKX2.6 plays a key role in human heart development.

Association of genetically defective *Nkx2.6* with enhanced susceptibility to CHD has been revealed in experimental animals. In mice, targeted deletion of *Nkx2.5* led to abnormal heart morphogenesis and embryonic lethality; although a linear heart tube formed normally in mutant embryos, the looping morphogenesis was not initiated [30]. In contrast, *Nkx2.6*-null mice were viable with no obvious abnormalities in the heart [45]. Nevertheless, in mutant embryos homozygous for *Nkx2.6*, *Nkx2.5* mRNA expression expanded to the pharyngeal pouch endoderm, suggesting functional compensation for loss of Nkx2.6 in the pharyngeal pouches of these embryos [45]. Furthermore, overlapping functions for *Nkx2.5* and *Nkx2.6* have been demonstrated in the *Nkx2.5* and *Nkx2.6* double-knockout mouse embryos, which lacked pharyngeal pouches and had less advanced atrium, mainly due to enhanced apoptosis and reduced proliferation of pharyngeal endodermal cells [46]. Given the high degree of homology between Nkx2.5 and Nkx2.6 proteins over the homeodomain and their overlapping expression patterns, these data indicate that Nkx2.5 acts at a subset of Nkx2.6 target genes [20].

In conclusion, this study firstly associates functionally compromised NKX2.6 with increased vulnerability to isolated VSD, highlighting the essential role of NKX2.6 in human cardiovascular development, and implying the potential implications for genetic counseling and clinical care of the patients with VSD.

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