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Novel NKX2-5 mutations responsible for congenital heart disease

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ABSTRACT. Congenital heart disease (CHD) is the most common birth defect and is the leading cause of infant morbidity and mortality resulting from birth defects. Increasing evidence demonstrates that genetic variation in the *NKX2-5* gene, which encodes a homeobox-containing transcription factor crucial to cardiogenesis, is an important molecular determinant for CHD. Nevertheless, the genetic components underlying CHD remain largely unknown. We screened *NKX2-5* for potential molecular defects in patients with CHD. The entire coding region of *NKX2-5* was initially sequenced in a cohort of 268 unrelated patients with CHD. The relatives of the patients carrying identified mutations and 200 unrelated control individuals were subsequently genotyped. Three novel heterozygous missense *NKX2-5* mutations, p.Q22K, p.R36S, and p.E54K, were identified in three families with autosomal dominantly inherited atrial septal defect, ventricular septal defect, and tetralogy of Fallot, respectively. These mutations, absent in 200 control individuals, appear to be highly conserved evolutionarily and co-segregated with

CHD in the families, with complete penetrance. These findings expand the spectrum of mutations in *NKX2-5* associated with CHD and provide new insight into the molecular etiology involved in the pathogenesis of CHD, which signifies potential implications for genetic diagnosis and gene-specific therapy for this common disease in newborns.

Key words: Congenital heart disease; Transcription factor; Genetics

INTRODUCTION

Congenital heart disease (CHD) is the most prevalent form of birth defect in the structure of the heart or intrathoracic great vessels of a neonate, affecting nearly 1% of newborns, and is the most common cause of infant death resulting from birth abnormality, with more than 29% deaths of infants with a heart birth defect (Lloyd-Jones et al., 2009). Despite its striking prevalence and clinical significance, the etiology of CHD in the majority of cases is unclear (Jenkins et al., 2007; Pierpont et al., 2007). Abnormal cardiac development during embryogenesis seems to occur through a biological process that is heterogeneous and complex, with both environmental and genetic risk factors (Nora and Nora, 1976). Based on molecular genetic studies in CHD pedigrees with Mendelian inheritance patterns, several genes have been identified to be involved in the pathogenesis of CHD, most of which encode cardiac transcription factors such as NKX2-5, TBX5, GATA4, and GATA6 (Li et al., 1997; Schott et al., 1998; Garg et al., 2003; Kodo et al., 2009).

NKX2-5 is the earliest known marker of myocardial progenitor cells in all species in which it has been studied (Akazawa and Komuro, 2005). The human *NKX2-5* gene maps to chromosome 5q34 and consists of two exons encoding a protein of 324 amino acids (Shiojima et al., 1995). This homeobox transcription factor is expressed during early cardiac morphogenesis and functions as a pivotal regulatory protein (Akazawa and Komuro, 2005). Due to its crucial role in normal cardiogenesis, *NKX2-5* has been a prime candidate gene in studies to identify the genetic determinants for congenital structural heart defects. To date, more than 40 mutations within the *NKX2-5* gene have been identified in patients with a variety of congenital heart malformations, including atrial septal defect with normal or abnormal atrioventricular conduction, ventricular septal defect, conotruncal abnormalities as tetralogy of Fallot, double-outlet right ventricle, L-transposition of the great arteries, and hypoplastic left heart syndrome (Stallmeyer et al., 2010). 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 (Prall et al., 2007).

To identify the molecular etiology responsible for CHD, a systematic scan of the *NKX2-5* gene for likely mutations was performed by sequencing it initially in a cohort of 268 unrelated patients with various types of CHD and subsequently in the family members of the patients harboring mutations, in contrast to a total of 200 unrelated control individuals.

MATERIAL AND METHODS

Study subjects

A cohort of 268 unrelated patients with CHD, who underwent surgical repair or percutaneous closure at hospitals from January 2006 to May 2010, was recruited prospec-

tively. They were clinically evaluated by cardiologists for their individual and familial history, review of the medical records, complete physical examination, standard 12-lead electrocardiogram (ECG), and two-dimensional transthoracic echocardiography with color flow Doppler. Transesophageal echocardiography, cardiac catheterization, electrophysiological investigation, and/or cardiac surgery had previously been conducted in some individuals. Patients with syndromic CHD, such as Marfan, Noonan, Holt-Oram, Alagille, and CHARGE syndromes, as well as chromosomal abnormalities that are highly associated with CHD, such as trisomy 21 (Down syndrome) and 22q11.2 deletion (DiGeorge syndrome), were excluded from the present study. Family members of mutation carriers were subsequently recruited, and their medical records evaluated. We also carried out a physical examination, ECG, and echocardiography, and all individuals were screened for the identified mutations in *NKX2-5* by direct sequencing. A total of 200 unrelated, ethnically matched healthy individuals, who were enrolled from the general population and had no evidence for any type of CHD following the comprehensive evaluation of their individual and familial histories, detailed review of the medical records, and complete physical examination (Stallmeyer et al., 2010), were used as controls to screen for the identified mutations. Peripheral venous blood samples were collected 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 the study.

Genetic studies

Genomic DNA from all participants was extracted from blood lymphocytes with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). First, the candidate gene *NKX2-5* was screened in 268 unrelated patients with CHD. Then, the genotyping *NKX2-5* in the available relatives of the patients carrying the identified mutations and the 200 unrelated, ethnically matched healthy control individuals was carried out subsequently for the presence of mutations identified in the patients. The referential genomic DNA sequence of *NKX2-5* was derived from GenBank (accession No. NT_023133). With the help of the Primer3 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 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); 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). PCR was performed using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a PE 9700 Thermal Cycler (Applied Biosystems, Foster, CA, USA), under standard conditions and concentrations of reagents. Amplified products were analyzed on 1% agarose gels stained with ethidium bromide and purified with QIAquick Gel Extraction Kit (Qiagen). 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 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 various species were aligned using the MUSCLE program (version 3.6, URL: http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=homologene&dopt=MultipleAlignment&list_uids=3230).

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 the MutationTaster program (http://www.mutationtaster.org/), suggesting that the alteration could either be a disease mutation or a harmless polymorphism. Notably, the P value used here is the prediction probability rather than the probability of error as used in Student *t*-test statistics, i.e., a value close to 1 indicates a high prediction probability.

RESULTS

Characteristics of the study subjects

A cohort of 268 unrelated patients with CHD, of which 53 patients had a positive family history, was recruited and clinically evaluated, in contrast to a total of 200 unrelated, ethnically matched healthy individuals as controls. All subjects were of Chinese Han nationality. None of them had overt environmental risk factors for CHD. The clinical characteristics of the 268 unrelated patients with CHD are shown in Table 1.

NKX2-5 mutations

Direct sequencing of the coding exons of the *NKX2-5* gene was performed after PCR amplification of genomic DNA from a cohort of 268 unrelated patients with CHD. Three novel heterozygous mutations in *NKX2-5* were identified in 3 of 268 unrelated patients. The total prevalence of *NKX2-5* mutations based on the cohort population was approximately 1.12%. A substitution of adenine (A) for cytosine (C) in the first nucleotide of codon 22 (alternatively c.64C>A) of the *NKX2-5* gene, corresponding to the transversion of glutamine (Q) to lysine (K) at the amino acid position 22 (p.Q22K), was identified in a patient with an atrial septal defect. A transversion of CGC to AGC at codon 36 (c.106C>A) of the *NKX2-5* gene, predicting the change from arginine (R) to serine (S) at the amino acid position 36 (p.R36S), was identified in a patient with ventricular septal defect. A transition of guanine (G) to A at the nucleotide position 160 from the translation starting point (c.160G>A) of the *NKX2-5* gene, equivalent to the change of the encoded amino acid from glutamic acid (E) to K (p.E54K), was identified in a patient with tetralogy of Fallot. The prevalence of these mutations in patients with atrial septal defect, ventricular septal defect, or tetralogy of Fallot was approximately 2.59%. The sequence chromatograms showing the detected heterozy-

Table 1. Clinical characteristics of the 268 unrelated patients with CHD.

	Number or mean value	Percentage or range
Male:female	152:116	57:43
Age at the present study (years)	5.62	0.25-38
Age at the diagnosis of CHD (years)	3.74	0-16
Positive family history	53	19.78
Distribution of different types of CHD		
Isolated CHD	211	78.73
ASD	105	39.18
VSD	86	32.09
PDA	5	1.87
DORV	3	1.12
PS	3	1.12
TAPVC	3	1.12
COA	2	0.75
TGA	2	0.75
CAVC	1	0.37
Cor triatriatum	1	0.37
Complex CHD	57	21.27
ASD+VSD	17	6.34
TOF	15	5.60
VSD+PDA	6	2.24
VSD+PFO	5	1.87
VSD+DORV	4	1.49
VSD+TGA	3	1.12
VSD+PFO+PDA	2	0.75
ASD+PDA	1	0.37
ASD+TGA	1	0.37
ASD+VSD+DORV	1	0.37
ASD+VSD+PDA	1	0.37
COA+PDA	1	0.37
Incidence of arrhythmias		
Atrioventricular block	12	4.48
Atrial fibrillation	3	1.12
Treatment		
Surgical repair	165	61.57
Percutaneous closure	78	29.10

CHD = congenital heart disease; ASD = atrial septal defect; VSD = ventricular septal defect; PDA = patent ductus arteriosus; DORV = double-outlet right ventricle; PS = pulmonary stenosis; TAPVC = total abnormal pulmonary venous connection; COA = coarctation of the aorta; TGA = transposition of great arteries; CAVC = common arteriovenous canal; TOF = tetralogy of Fallot; PFO = patent foramen ovale.

gous *NKX2-5* variations of c.64C>A, c.106C>A, and c.160G>A in comparison to control sequences are shown in Figure 1. These three variants were not present in the 200 unrelated control individuals. Genetic scan of the family members of the three unrelated patients harboring the identified mutations showed that the gene variant was present in all affected family members alive, but absent in unaffected family members tested in each family. Analysis of the pedigrees demonstrated that the mutation co-segregated with CHD was transmitted as an autosomal dominant trait in the three families with complete penetrance. The pedigree structures of the three families are illustrated in Figure 2. The phenotypic characteristics and results of genetic screening of the affected pedigree members are summarized 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 shown in Figure 3, suggesting that these amino acids are functionally important.

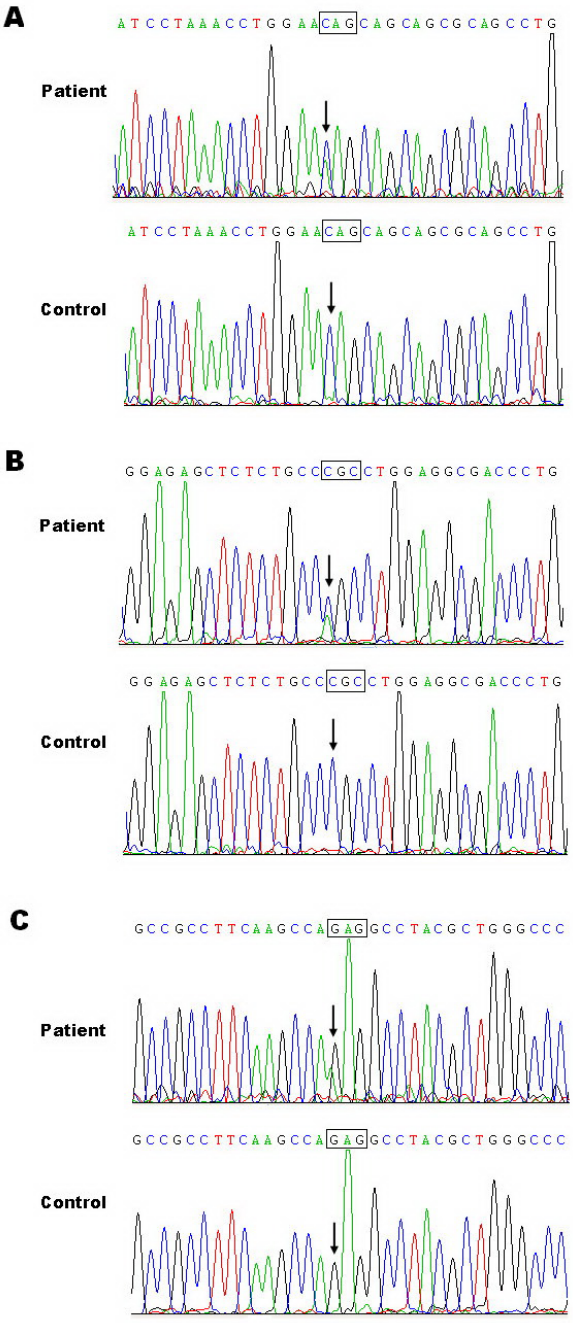

Figure 1. Sequence chromatograms of NKX2-5 in index patients and controls. Arrows indicate the heterozygous nucleotides of C/A (**A**), C/A (**B**) and G/A (**C**), in the probands from families 1, 2 and 3 (Patient), respectively, or the homozygous nucleotides of C/C (**A**), C/C (**B**) and G/G (**C**) in the corresponding controls (Control). Squares denote the nucleotides comprising a codon of NKX2-5.

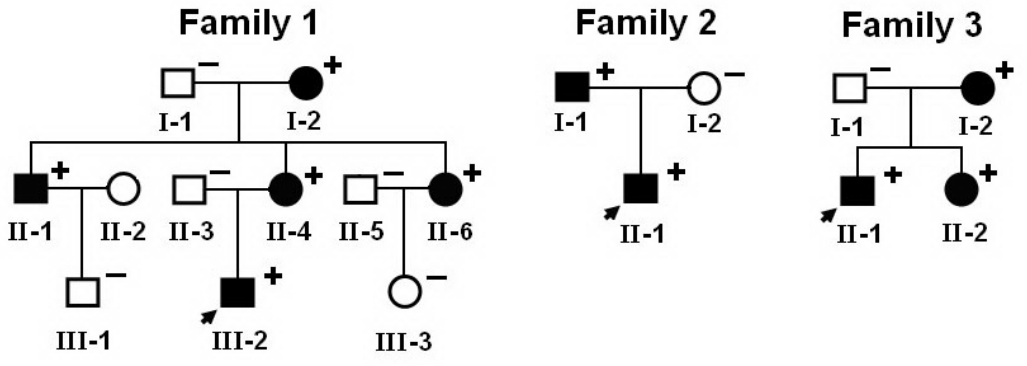

Figure 2. Pedigree structures of families with atrial septal defects. Families are designated as family 1, 2, and 3. Family members are identified by generations and numbers. Squares = males; circles = females; closed symbols = affected members; open symbols = unaffected members; arrows = proband; + = carriers of the heterozygous mutations; - = non-carriers.

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

Identity	Gender	Age at time of study (years)	Age at diagnosis of CHD (years)	Cardiac structural defects	Other structural abnormalities	Arrhythmias	*NKX2-5* mutations
Family 1							Q22K
I-2	F	59	23	ASD	PS, RVH	Second-degree AVB	+/-
II-1	M	36	19	ASD	-	First-degree AVB	+/-
II-4	F	32	15	ASD	-	First-degree AVB	+/-
II-6	F	29	12	ASD	-	-	+/-
III-2	M	5	5	ASD		-	+/-
Family 2							R36S
I-1	M	30	9	VSD	-	Paroxysmal AF	+/-
II-1	M	6	3	VSD	-	-	+/-
Family 3							E54K
I-2	F	57	9	TOF	BPV	-	+/-
II-1	M	22	2	TOF	-	-	+/-
II-2	F	19	1	TOF	-	-	+/-

F = female; M = male; CHD = congenital heart disease; ASD = atrial septal defect; VSD = ventricular septal defect; TOF = tetralogy of Fallot; PS = pulmonary stenosis; RVH = right ventricular hypertrophy; BPV = bicuspid pulmonary valve; AVB = atrioventricular block; + = present; - = absent.

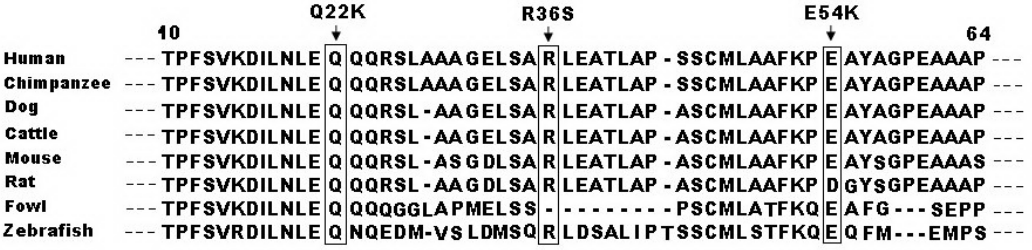

Figure 3. Alignment of multiple NKX2-5 protein sequences across species. The altered amino acids of Q22, R36, and E54 are highly conserved evolutionarily.

Disease-causing potential of a sequence alteration

The sequence alterations of c.64C>A, c.106C>A, and c.160G>A detected in *NKX2-5* were all predicted to be disease-causing, with P values of 0.99743, 0.99098, and 0.99769 for c.64C>A, c.106C>A, and c.160G>A, respectively, providing evidence for the three alterations to be malicious disease mutations rather than benign polymorphisms.

DISCUSSION

In the present study, we report three previously unrecognized missense mutations of *NKX2-5* identified in patients with familial CHD. 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 causative potential of a sequence alteration demonstrated that all three mutations of p.Q22K, p.R36S, and p.E54K were disease-causing with probability values as high as close to 1. Therefore, it is very likely that the three mutations are associated with the CHD in these three families.

Our results are supported by the finding of other *NKX2-5* mutations predisposing to congenital CHD by impairing or reducing cardiac transcription factor *NKX2-5* (Kasahara et al., 2000; Kasahara and Benson, 2004). To date, more than 40 mutations in *NKX2-5* have been described, of which more than 30 mutations have been observed in patients with atrial septal defect, showing that although *NKX2-5* mutations are involved in a long list of cardiac malformations, the most frequent phenotype resulted is atrial septal defect (Stallmeyer et al., 2010). In most of these patients, the CHD-causing mutations are familial, whereas sporadic cases remain relatively infrequent (Elliott et al., 20003; Sarkozy et al., 2005). Similar to these findings, the figure of 3/53 mutations (approximately 6%) in our patient cohort with positive family history suggests that the *NKX2-5* mutations could be a major cause of familial CHD. Notably, the remarkable genetic heterogeneity of CHD was proven by an inability to detect mutations in nearly 99% of our cohort patients, despite somatic NKX2-5 mutations being a likely mechanism of CHD in some patients (Reamon-Buettner and Borlak, 2004). Hence, the contribution of genes other than *NKX2-5* to CHD pathogenesis seems probable.

Mutations in other transcription factors associated with cardiogenesis, such as TBX5, GATA4, GATA6, and NOTCH1 (Li et al., 1997; Garg et al., 2003, 2005; Kodo et al., 2009), and mutations in genes, such as *RAF1*, *SOS1* and *BRAF* within signal transduction pathways (Rodriguez-Viciana et al., 2006; Razzaque et al., 2007; Roberts et al., 2007), have also been detected in patients with CHD. Also, mutations in cardiac structural proteins as alpha myosin heavy chain (MYH6) and alpha cardiac actin (ACTC1) were identified in familial CHD (Ching et al., 2005; Matsson et al., 2008). However, so far, only *NKX2-5* mutations were reported to cause an atrial septal defect phenotype and development of atrioventricular block (Stallmeyer et al., 2010). The two most common phenotypes caused by mutated *NKX2-5* are atrial septal defect and atrioventricular conduction disturbance (Akazawa and Komuro, 2005), indicating the crucial role of *NKX2-5* not only in the morphogenesis of the heart, but also in the construction of cardiac conduction system. In the present study, a compound phenotype of atrial septal defect and atrioventricular block was observed in three fifths of the members of family 1 who carried the identified mutations of *NKX2-5*, and the atrioventricular block seemed to be

progressive with increasing age in each individual as described previously (Schott et al., 1998; Hosoda et al., 1999; Pashmforoush et al., 2004; Hirayama-Yamada et al., 2005; Sarkozy et al., 2005), while it is not observed soon after birth. As atrioventricular block is a possible cause of sudden death, molecular genetic screening appears to be of great help in identifying individuals at risk for the life-threatening heart conduction disturbances. When a person harboring an *NKX2-5* mutation is identified, it is necessary for medical staff to carefully monitor this patient who initially may present no symptoms, or may have spontaneously closed or surgically corrected atrial septal defect, which enables offering accurate and early therapy.

Association of compromised *NKX2-5* with increased predisposition to CHD and atrioventricular block has been reported in animal models. The homeobox-containing transcription factor encoded by the *tinman* gene, a counterpart of *NKX2-5*, was expressed in the dorsal vessel (an equivalent to the heart of vertebrates) of the fruit fly *Drosophila melanogaster* and the deletion of the transcription factor led to lethal failure of vessel formation (Benson et al., 1999). In *Xenopus*, expression of a similar DNA-nonbinding mutant of Nkx2.5 was demonstrated to cause dominant negative effect on embryos, showing small heart or no heart formation (Grow and Krieg, 1998). In mice, *NKX2-5* was highly expressed in the early heart progenitor cells in both primary and secondary heart fields during embryogenesis and continued to be expressed at a high level in the heart through adulthood. In particular, a transiently elevated expression of *NKX2-5* was observed in specialized myocardial conduction cells during the development of cardiac conduction system (Akazawa and Komuro, 2005). In transgenic mice expressing a DNA binding-impaired mutant of 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 (Kasahara et al., 2001). Targeted disruption of *NKX2-5* in mice caused embryonic lethality around ED10.5, with retarded cardiac development (Lyons et al., 1995; Tanaka et al., 1999). Mice heterozygous for *NKX2-5*-null alleles were predisposed to atrial septal defect and abnormal atrioventricular conduction (Biben et al., 2000). 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, gave rise to progressive and advanced conduction defects and left ventricular hypertrophy postnatally (Pashmforoush et al., 2004). In addition, perinatal loss of Nkx2-5 brought about rapid conduction and contraction defects by regulating expression of several ion channel genes (Briggs et al., 2008). Taken together, these experimental findings in animals suggest that *NKX2-5* mutations underlie a variety of congenital cardiac abnormalities in humans, including atrial septal defect with or without progressive conduction anomaly, ventricular septal defect, and tetralogy of Fallot.

CONCLUSIONS

The findings of the present study link novel mutations in the cardiac transcription factor NKX2-5 to CHD and provide a new insight into the molecular mechanism implicated in the pathogenesis of CHD, representing an important step in progress toward molecular phenotyping and thus directed rather than empiric therapy for this common disease in newborns.

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