Prevalence and Spectrum of NKX2-5 Mutations Associated With Sporadic Adult-Onset Dilated Cardiomyopathy

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SUMMARY

Dilated cardiomyopathy (DCM), the most common form of primary myocardial disease, is a leading cause of congestive heart failure and the most common indication for heart transplantation. Recently, NKX2-5 mutations have been involved in the pathogenesis of familial DCM. However, in the prevalence and spectrum of NKX2-5 mutations associated with sporadic DCM remain to be evaluated. In this study, the coding regions and flanking introns of the NKX2-5 gene, which encodes a cardiac transcription factor pivotal for cardiac development and structural remodeling, were sequenced in 210 unrelated patients with sporadic adult-onset DCM. A total of 300 unrelated healthy individuals used as controls were also genotyped for NKX2-5. The functional effect of the mutant NKX2-5 was investigated using a dual-luciferase reporter assay system. As a result, two novel heterozygous NKX2-5 mutations, p.R139W and p.E167X, were identified in 2 unrelated patients with sporadic adult-onset DCM, with a mutational prevalence of approximately 0.95%. The mutations were absent in 600 referential chromosomes and the altered amino acids were completely conserved evolutionarily across species. Functional assays revealed that the NKX2-5 mutants were associated with significantly reduced transcriptional activity. Furthermore, the mutations abrogated the synergistic activation between NKX2-5 and GATA4 as well as TBX20, two other cardiac key transcription factors that have been causally linked to adult-onset DCM. This study is the first to associate NKX2-5 loss-of-function mutations with enhanced susceptibility to sporadic DCM, which provides novel insight into the molecular etiology underpinning DCM, and suggests the potential implications for the genetic counseling and personalized treatment of the DCM patients. (Int Heart J 2017; 58: 521-529)

Key words: Heart failure, Genetics, Transcriptional factor, Mutation, Reporter gene assay

Dilated cardiomyopathy (DCM), which is defined by the presence of progressive left ventricular or biventricular chamber enlargement and contractile dysfunction with normal left ventricular wall thickness in the absence of abnormal loading conditions (arterial hypertension, valve disease) or coronary artery disease sufficient to cause global systolic impairment, represents the most common type of primary myocardial disease, with an estimated prevalence of 1:2500 and annual incidence of 1:15,000–18,000 in adults.1,2 DCM is the most frequent etiology of non-ischemic heart failure, accounting for up to 30%-40% of all heart failure patients, the most common reason for cardiac transplantation in adults and children, and a major cause of sudden cardiac death.3,4 Hence, DCM has imposed a substantial economic burden on patients and society worldwide. Despite important clinical significance, the causes responsible for DCM remain unclear in a majority of cases.

In a subset of patients, DCM may occur as a result of environmental risk factors, such as viral myocardial inflammation, myocardial toxins, and autoimmune disorders.5 However, in approximately 50% of DCM patients, no recognized risk factors can be identified, and such DCM is classified as 'idiopathic' DCM. Among patients with idiopathic DCM, 25%–50% of the cases have a positive family history, giving rise to the term 'familial' DCM, in contrast to 'sporadic' DCM.6 Accumulating evidence has demonstrated the genetic origin of idiopathic DCM, and revealed a pattern of autosomal dominant, autosomal recessive, X-linked, or mitochondrial inheritance in families, of which in over 90% of patients with familial DCM, the pattern of inheritance is autosomal dominant.7 At present, an increasing number of causative mutations in > 50 genes have been causally linked to idiopathic DCM.8,9-20 Among

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METHODS

Recruitment of study subjects: A cohort of 210 unrelated patients with sporadic adult-onset DCM, including 172 patients with idiopathic DCM alone and 38 patients with idiopathic DCM together with congenital atrial septal defect (ASD), was recruited from the Han Chinese population. The available parents of the patients were also included. A total of 300 unrelated healthy individuals, who were matched for ethnicity, gender, and age were enrolled as controls. All subjects underwent comprehensive clinical evaluation, including medical history, physical examination, trans-thoracic echocardiography with color Doppler, 12-lead electrocardiogram, and exercise performance testing as well as the plasma levels of N-terminal pro-brain natriuretic peptide. Cardiac catheterization, coronary angiography, chest X-ray radiography, endomyocardial biopsy, and cardiac magnetic resonance imaging were performed only when required. Idiopathic DCM was diagnosed according to the criteria established by the World Health Organization/International Society and Federation of Cardiology Task Force on the Classification of Cardiomyopathy: a left ventricular end-diastolic diameter > 27 mm/m² and an ejection fraction < 40% or fractional shortening < 25% in the absence of other recognized risk factors, including hypertensive heart disease, ischemic heart disease, valvular heart disease, and viral myocarditis. Individuals with idiopathic DCM in the presence of congenital ASD were also included because NKX2-5 mutations have been involved in familial DCM and ASD. Individuals with a positive family history of DCM were excluded from the present study. The study was carried out in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the local institutional ethics committee. Written informed consent was provided by each study participant prior to investigation.

Sequence analysis of NKX2-5: Peripheral venous blood specimens were collected from all the study participants. Genomic DNA was isolated from whole blood samples with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer’s protocol. The genomic DNA sequence of the human NKX2-5 gene (accession number: NC_000005.10) was derived from the Gene database (http://www.ncbi.nlm.nih.gov/gene/). With the help of the online Primer 3 software (http://primer3.ut.ee/), the primer pairs to amplify the coding exons and flanking introns of NKX2-5 by polymerase chain reaction (PCR) were designed as shown in Table I. Direct PCR-sequencing of the NKX2-5 gene was performed in 210 unrelated patients with sporadic adult-onset DCM and 300 unrelated control individuals as previously described. For an identified sequence variance, the single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) database and human gene mutation database (HGMD; http://www.hgmd.org) were consulted to check its novelty.

Multiple alignments of NKX2-5 proteins across species: The amino acid sequences of the NKX2-5 protein from human were aligned with those from chimpanzee, monkey, dog, cattle, mouse, rat, zebrafish, and frog using the MUSCLE program (http://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&dopt=MultipleAlignment&list_uids=3230).

Prediction of the causative potential of novel NKX2-5 sequence variations: The disease-causing potential of the novel NKX2-5 sequence variations was predicted by MutationTaster (http://www.mutationtaster.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), PROVEAN (http://provean.jcvi.org/index.php), and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), when appropriate.

Expression plasmids and site-directed mutagenesis: The recombinant expression plasmids of ANF-luciferase (ANF-luc) reporter plasmid, which contains the 2600-bp 5'-flanking region of the ANF gene and expresses Firefly luciferase, were kind gifts from Dr. Ichiro Shiojima of Chiba University School of Medicine, Japan. The eukaryotic expression plasmid TBX20-pcDNA3.1 was constructed as described elsewhere. The identified mutation was introduced into the wild-type NKX2-5 using a Quick-Change II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with a complementary pair of primers, and was validated by sequencing.

Cell culture and reporter gene assay: COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, and plated at a density of 1x10⁴ cells per well on 6-well plates 24 hours before transfection. Transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s standard protocol. The internal control reporter plasmid pGL4.75 (hRluc/CMV, Promega), which expresses Renilla luciferase, was used in transient transfection experiments to normalize transfection efficiency. Cells were transfected with 0.4 μg of empty vector, 0.4 μg of wild-type NKX2-5, and 0.4 μg of

| Table 1. Primer Pairs Used to Amplify the Coding Exons and Flanking Introns of the NKX2-5 Gene |
|---|---|---|
| Exon | Forward primer (5´ to 3´) | Reverse primer (5´ to 3´) | Amplicon (bp) |
| 1 | CTTTGGTCGCTACGGCTACCTG | TCTTGGGGAGCAAGGCGACCA | 543 |
| 2-a | TGCTAGTGAGAGGGATCGTGCGG | CCGTGAGGATTGAGGCCAC | 592 |
| 2-b | AAATCAGCTCAGGCGAGGT | GCTGTTGAGGATCGGT | 497 |
R139W-mutant NKX2-5, 0.4 μg of E167X-mutant NKX2-5, 0.2 μg of wild-type NKX2-5, 0.2 μg of wild-type NKX2-5 plus 0.2 μg of R139W-mutant NKX2-5, or 0.2 μg of wild-type NKX2-5 plus 0.2 μg of E167X-mutant NKX2-5, in combination with 1.0 μg of ANF-luc and 0.04 μg of pGL4.75. For synergistic activation experiments, the same amount (0.2 μg) of expression plasmid (empty plasmid, wild-type NKX2-5, wild-type GATA4, wild-type TBX20, R139W-mutant NKX2-5, or E167X-mutant NKX2-5) was used alone or together in the presence of 1.0 μg of ANF-luc and 0.04 μg of pGL4.75. Cells were lysed 48 hours after transfection, and the Firefly luciferase and Renilla luciferase activities were measured using a GloMax® 96 Luminometer (Promega) with the Dual-Glo luciferase assay system (Promega) following the manufacturer’s manual. The activity of the ANF promoter was expressed as a fold of Firefly luciferase activity relative to Renilla luciferase activity. All experiments were carried out 3 times independently and each experiment was performed in triplicate.

**Statistical analysis:** Variables are presented as numbers (percentage) or mean ± standard deviation (SD), when appropriate. Categorical variables were compared between two groups using chi-square (χ²) test or Fisher exact test. Continuous variables were compared using unpaired Student’s t-test or the Mann-Whitney U test. Statistical significance was accepted at a two-tailed P value of < 0.05. All statistical analyses were conducted using SPSS version 21.0 software (SPSS, Chicago, IL, USA).

**RESULTS**

**Clinical features of the study participants:** Baseline clinical characteristics and demographic data on the 210 DCM patients and 300 controls are summarized in Table II. All the patients manifested with typical DCM phenotype as described previously. None of them had other known risk factors for DCM except for ASD. The available parents of the patients were excluded from the DCM phenotype by echocardiograms. The control individuals had normal echocardiogram results without evidence of structural heart diseases. There were no significant differences in sex ratio or mean age between DCM patients and controls (P > 0.05). Compared with those in the control group, in the patient group the blood pressure levels, left ventricular ejection fraction, and left ventricular fractional shortening were significantly decreased (P < 0.05), whereas the heart rate, left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and the plasma concentration of N-terminal pro-brain natriuretic peptide were significantly increased (P < 0.05).

**Novel NKX2-5 mutations:** Direct PCR-sequencing of NKX2-5 led to identification of two heterozygous mutations in two of 210 unrelated patients with sporadic DCM, respectively, with a mutational prevalence of approximately 0.95%. Specifically, a substitution of thymine (T) for adenine (A) in the first nucleotide of codon 139 (c.415A>T), predicting the change of the codon encoding glutamic acid (E) into a stop codon (X) at amino acid position 139 (p.R139W), was discovered in a female patient aged 47 years, who was initially diagnosed with DCM at age of 42 years. A transversion of guanine (G) into T at coding nucleotide 498 (c.499G>T), predicting the change of the codon encoding glutamic acid (E) into a stop codon (X) at amino acid position 167 (p.E167X), was discovered in a male patient aged 42 years, who was first diagnosed with DCM at age of 38 years. Additionally, both of the mutation carriers also had documented congenital ASD and progressive atrioventricular block (AVB). At enrollment for this study, the R139W-mutation carrier had a left ventricular end-systolic diameter of 54 mm, left ventricular end-diastolic diameter of 64 mm, and left ventricular ejection fraction of 35% as well as a secondum ASD of 7 mm in maximum diameter and first-degree AVB; while the E167X-mutation carrier had a left ventricular end-systolic diameter of 62 mm, left ventricular end-diastolic diameter of 74 mm, and left ventricular ejection fraction of 28% as well as a secondum ASD of 12 mm in maximum diameter and second-degree AVB. The parents of the two mutation carriers had neither DCM phenotype nor NKX2-5 mutations, indicating that these mutations were de novo. The sequence chromatograms displaying the heterozygous NKX2-5 mutations compared with

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (n = 210)</th>
<th>Controls (n = 300)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47.5 ± 8.3</td>
<td>48.2 ± 9.7</td>
<td>0.3956</td>
</tr>
<tr>
<td>Male (%)</td>
<td>112 (53.3)</td>
<td>158 (52.7)</td>
<td>0.8820</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118.4 ± 12.6</td>
<td>126.0 ± 10.4</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.2 ± 9.3</td>
<td>85.2 ± 8.5</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>79.9 ± 14.1</td>
<td>74.5 ± 8.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>67.5 ± 8.0</td>
<td>46.6 ± 6.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>56.9 ± 7.8</td>
<td>35.1 ± 5.6</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>36.3 ± 11.1</td>
<td>64.6 ± 8.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>92.2 ± 4.7</td>
<td>42.5 ± 5.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>NT-proBNP (ng/L)</td>
<td>5208 ± 5139</td>
<td>85 ± 72</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>NYHA function class (%)</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>25 (11.9)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>II</td>
<td>74 (35.2)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>III</td>
<td>90 (42.9)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>IV</td>
<td>21 (10.0)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Data represent the mean ± standard deviation or numbers (percentages). SBP indicates systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; and NA, not applicable or not available.
their control sequences are shown in Figure 1. A schematic diagram of NKX2-5 protein showing the structural domains and locations of the mutations found in the present study is presented in Figure 2. The two mutations were neither observed in the control chromosomes nor found in the SNP and HGM databases (consulted again on September 8, 2016).

**Multiple alignments of NKX2-5 protein sequences:** As shown in Figure 3, a cross-species alignment of NKX2-5 protein se-

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**Figure 1.** Sequence electropherograms showing the heterozygous NKX2-5 mutations as well as their corresponding control sequences. The arrows indicate the heterozygous nucleotides of A/T (A) and G/T (B) in the patients (mutant) or the homozygous nucleotides of A/A (A) and G/G (B) in the corresponding control individuals (wild type). The rectangle marks the nucleotides comprising a codon of NKX2-5.

**Figure 2.** Schematic diagram showing the structural domains of NKX2-5 and the mutations associated with dilated cardiomyopathy. The mutations identified in the patients with sporadic adult-onset dilated cardiomyopathy are noted above the structural domains. NH2, amino-terminus; TN, tinman domain; HD, homeodomain; NK, nucleotide kinase domain; COOH, carboxyl-terminus.
sequences showed that the altered amino acids p.R139 and p.E167 were completely conserved evolutionarily.

**Novel NKX2-5 sequence variations predicted to be pathogenic:**
The identified NKX2-5 sequence variations c.415A>T and c.499G>T were both predicted to be disease-causing with a P value of 1.000 by MutationTaster. Additionally, the amino acid substitution p.R139W was predicted to be probably damaging with a score of 1.000 (sensitivity: 0.00; specificity: 1.00) by PolyPhen-2, deleterious with a PROVEAN score of -7.564 by PROVEAN, and damaging with a SIFT score of 0 and a median information content of 1.53 by SIFT.

**Diminished transcriptional activity of the NKX2-5 mutants:**
As shown in Figure 4, the same amount (0.4 μg) of wild-type, R139W-mutant and E167X-mutant NKX2-5 activated the ANF promoter by ~11-fold, ~2-fold and ~1-fold, respectively (wild-type NKX2-5 versus R139W-mutant NKX2-5: t = 11.9232, P = 0.0003; wild-type NKX2-5 versus E167X-mutant NKX2-5: t = 14.3451, P = 0.0001). When the same amount of wild-type NKX2-5 (0.2 μg) was co-transfected with R139W-mutant or E167X-mutant NKX2-5 (0.2 μg), the induced activation of the ANF promoter was ~4-fold and ~5-fold, respectively (wild-type NKX2-5 versus R139W-mutant NKX2-5 versus E167X-mutant NKX2-5: t = 14.3451, P = 0.0001).

![Figure 3. Multiple alignments of NKX2-5 proteins across species. Alignment of multiple NKX2-5 proteins among various species displayed that the altered amino acids of p.R139 and p.E167 are completely conserved evolutionarily across species.](image-url)
plus wild-type NKX2-5: $t = 9.6367, P = 0.0006$; wild-type NKX2-5 versus E167X-mutant NKX2-5 plus wild-type NKX2-5: $t = 8.0972, P = 0.0013$).

**Figure 4.** Functional defects caused by NKX2-5 mutations. Activation of atrial natriuretic factor promoter driven luciferase reporter in COS-7 cells by wild-type or mutant NKX2-5 (R139W or E167X), alone or together, showed significantly diminished transcriptional activation by the mutations. Experiments were performed in triplicate, with the mean and standard deviations shown. ** and * indicate $P < 0.001$ and $P < 0.005$, respectively, when compared with wild-type NKX2-5 (0.4 μg).

Abrogated synergistic activation between NKX2-5 and GATA4 as well as TBX20 by the mutations: As shown in Figure 5, in the presence of 0.2 μg of wild-type GATA4, the same amount (0.2 μg) of wild-type, R139W-mutant, and E167X-mutant NKX2-5 activated the ANF promoter by ~23-fold, ~10-fold, and ~3-fold, respectively (wild-type NKX2-5 versus R139W-mutant NKX2-5: $t = 7.1140, P = 0.0021$; wild-type NKX2-5 versus E167X-mutant NKX2-5: $t = 12.0678, P = 0.0003$); while in the presence of 0.2 μg of wild-type TBX20, the same amount (0.2 μg) of wild-type, R139W-mutant, and E167X-mutant NKX2-5 activated the ANF promoter by ~18-fold, ~8-fold, and ~2-fold, respectively (wild-type NKX2-5 versus R139W-mutant NKX2-5: $t = 5.6285, P = 0.0049$; wild-type NKX2-5 versus E167X-mutant NKX2-5: $t = 9.8672, P = 0.0006$).

**Figure 5.** Disrupted synergistic activation between NKX2-5 and GATA4 or between NKX2-5 and TBX20 resulted from mutations. The synergistic activation of the atrial natriuretic factor promoter in COS-7 cells by GATA4 and mutant NKX2-5 or TBX20 and mutant NKX2-5 was disrupted when compared with that by GATA4 and wild-type NKX2-5 or TBX20 and wild-type NKX2-5. Experiments were performed in triplicate, and the mean and standard deviations are given. ** represents $P < 0.001$ and * represents $P < 0.005$, when compared with their wild-type counterparts.

Abrogated synergistic activation between NKX2-5 and GATA4 as well as TBX20 by the mutations: As shown in Figure 5, in the presence of 0.2 μg of wild-type GATA4, the same amount (0.2 μg) of wild-type, R139W-mutant, and E167X-mutant NKX2-5 activated the ANF promoter by ~23-fold, ~10-fold, and ~3-fold, respectively (wild-type NKX2-5 versus R139W-mutant NKX2-5: $t = 7.1140, P = 0.0021$; wild-type NKX2-5 versus E167X-mutant NKX2-5: $t = 12.0678, P = 0.0003$); while in the presence of 0.2 μg of wild-type TBX20, the same amount (0.2 μg) of wild-type, R139W-mutant, and E167X-mutant NKX2-5 activated the ANF promoter by ~18-fold, ~8-fold, and ~2-fold, respectively (wild-type NKX2-5 versus R139W-mutant NKX2-5: $t = 5.6285, P = 0.0049$; wild-type NKX2-5 versus E167X-mutant NKX2-5: $t = 9.8672, P = 0.0006$).

**Discussion**

In the current study, two novel heterozygous NKX2-5 mutations of p.R139W and p.E167X were identified in two unrelated patients with sporadic adult-onset DCM as well as congenital ASD and progressive AVB, respectively. A cross-species alignment of multiple NKX2-5 proteins showed that the altered amino acids were completely conserved evolutionarily. The two non-synonymous mutations, which were absent in the 600 referential chromosomes from an ethnically-matched control population, were predicted to be pathogenic by automatic tools such as MutationTaster, PolyPhen-2,
PROVEAN, and SIFT. Biological analyses unveiled that the mutants were both associated with significantly reduced transcriptional activity. Furthermore, the mutations consistently disrupted the synergistic activation between NKX2-5 and GATA4 as well as TBX20. These results support that NKX2-5 loss-of-function mutations predispose to DCM, in addition to ASD and AVB.

It has been verified that NKX2-5 can interact physically with other cardiac core transcriptional factors to form complexes, including GATA4 and TBX20 with profiles of expression and function in the heart overlapping with those of NKX2-5, and regulate synergetically the expression of multiple critical cardiac genes, including those coding for ANF, CX40, and Ca²⁺ channels. In this study, the NKX2-5 mutations identified in the patients with sporadic adult-onset DCM and congenital ASD were shown to result in significantly diminished transcriptional activation of the ANF promoter alone or in synergy with GATA4 or TBX20, suggesting that haplo-insufficient or dominant-negative effect caused by the NKX2-5 mutations is potentially an alternative pathological mechanism of DCM as well as ASD and AVB.

In humans, NKX2-5 loss-of-function mutations have been previously implicated in familial adult-onset DCM, ASD, and arrhythmias, of which the most commonly reported phenotypes are ASD and AVB in 68.4% and 65.7% of the cases, respectively. Costa and colleagues screened NKX2-5 in 220 probands with adult-onset DCM, and found a novel missense mutation (p.I184M) in a proband, as well as two previously reported functional polymorphisms (p.R25C and p.A119S) in 2 other probands, with a mutational prevalence of about 1.36%. Genetic analysis of the mutation carrier’s family showed that the mutation was present in all affected living adult family members. Furthermore, the proband also suffered from patent foramen ovale and complete heart block; his elder brother also suffered from ASD, right bundle-branch block, and atrial fibrillation; and the proband’s living nephew also suffered from ASD and right bundle-branch block. Functional deciphers of p.I184M in vitro revealed a significant decrease in DNA-binding activity despite increased expression levels of mutant protein due to reduced degradation via the ubiquitin-proteasome system, resulting in diminished activation of target genes. Yuan and co-workers sequenced NKX2-5 in 130 unrelated patients with idiopathic DCM, and identified a novel missense mutation (p.S146W) in an index patient, with a mutational prevalence of about 0.77%. Genetic analysis of the pedigree demonstrated that the mutation co-segregated with DCM in the family with complete penetrance. Additionally, the proband and his niece also had premature ventricular contractions; the proband’s father also had third-degree atrioventricular block, atrial fibrillation, and frequent premature ventricular contractions; and the proband’s brother also had paroxysmal atrial fibrillation, second-degree atrioventricular block, and premature ventricular contractions. These clinical investigations together with the current study indicate that NKX2-5 mutations may be an uncommon cause of DCM as well as ASD and arrhythmias.

Association of NKX2-5 loss-of-function mutations with enhanced susceptibility to DCM as well as congenital heart disease and arrhythmia may be partially ascribed to the developmental and regenerative defects of the myocardium as well as cardiac maladaptation. NKX2-5, which is highly expressed in cardiac precursor cells during embryogenesis and continues high expression in the heart throughout adulthood, is essential for proper cardiac development and determination of myocardial cell fate. Targeted deletion of Nkx2-5 in mice led to a deranged gene regulatory network, retarded cardiac growth, arrested chamber formation, and early embryonic demise, while conditional deletion at postnatal stages resulted in cardiovascular malformations, progressive cardiomyopathy, and cardiac conduction block. In a feline model of right ventricular pressure overload established by banding the pulmonary artery, or in adult mice with adrenergic-induced cardiac hypertrophy, the expression of NKX2-5 was up-regulated, implying that NKX2-5 participates in the cardiac hypertrophic response during pressure overload or stress stimulation. In contrast, heart-restricted expression of a dominant-negative human NKX2-5 mutant in the mouse induced progressive atrioventricular conduction defects and heart failure, and injection of doxorubicin promoted more severe cardiac dysfunction and increased cardiomyocyte apoptosis. Moreover, NKX2-5 has been demonstrated to promote cardiomyocyte differentiation and mediate adult cardiac hypertrophic response through interacting with other important cardiac transcription factors, such as TBX5, GATA4 and SRF. Furthermore, NKX2-5 also regulates expression of gap junction protein connexin43 and sarcomere organization in postnatal cardiomyocytes. In humans, an increasing number of NKX2-5 mutations have been associated with various congenital heart diseases including ASD, ventricular septal defect, Fallot’s tetralogy, hypoplastic left ventricle transposition of the great arteries and valvular deformities, cardiac arrhythmias including cardiac conduction block and atrial fibrillation, and DCM. Collectively, these findings along with the present study indicate that NKX2-5 plays important roles not only in early cardiovascular morphogenesis, but also in the postnatal maturation and homeostasis of cardiomyocytes and the adaptive remodeling of adult heart.

Notably, NKX2-5 physically interacts with such cooperative partners as GATA4, TBX5, and TBX20, and has been shown to cooperatively regulate the transcription of several essential cardiac target genes, such as troponin I, troponin C, α-actin, and α-myosin heavy chain, and furthermore, mutations in these cooperative partners and cardiac target genes have been causally related to DCM. Hence, it is likely that genetically compromised NKX2-5 confers increased susceptibility to DCM by down-regulating expressions of cardiac target genes.

Previously, mutations in more than 50 genes have been involved in the pathogenesis of DCM. Although we made a genetic analysis of several cardiac core transcriptional factor genes in the two mutation carriers with DCM, including GATA4, GATA5, GATA6, TBX5, TBX20, and HAND1, as described previously, and found no mutations, we cannot rule out the possibility that the genetic variants in other genes may also contribute to DCM in these two patients. Genome sequencing analysis may help to explain the possibility for these patients.

In conclusion, the current study is the first to associate NKX2-5 loss-of-function mutations with sporadic DCM, which expands the mutational spectrum of NKX2-5 linked to DCM and provides novel insight into the molecular mechanism of DCM, suggesting potential implications for genetic counseling and personalized treatment of DCM patients.


