A novel TBX5 loss-of-function mutation associated with sporadic dilated cardiomyopathy

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Abstract. Dilated cardiomyopathy (DCM) represents the most prevalent form of primary cardiomyopathy, and is the most common reason for heart transplantation and a major cause of congestive heart failure. Aggregating evidence demonstrates that genetic defects are associated with DCM, and a great number of mutations in >50 genes have been linked to DCM. However, DCM is a genetically heterogeneous disorder and the genetic components underpinning DCM in a significant proportion of patients remain unknown. In the present study, the coding exons and flanking exon-intron boundaries of the T-Box 5 (TBX5) gene, which encodes a T-box transcription factor required for normal cardiac development, were sequenced in 146 unrelated patients with sporadic DCM. The functional characteristics of the mutant TBX5 were assayed in contrast to its wild-type counterpart by using a dual-luciferase reporter assay system. As a result, a novel heterozygous TBX5 mutation, p.A143T, was identified in a patient with sporadic DCM. The missense mutation, which was absent in 400 control chromosomes, altered the amino acid that was completely conserved evolutionarily among species. Biological analyses revealed that the A143T mutation of TBX5 was associated with significantly decreased transcriptional activity on the promoter of the target gene atrial natriuretic factor (ANF), when compared to its wild-type counterpart. Furthermore, the A143T mutation abolished the synergistic activation of the ANF promoter between TBX5 and GATA binding protein 4 (GATA4), another crucial transcriptional factor for heart development. To the best of our knowledge, this is the first report on the association of a TBX5 loss-of-function mutation with an enhanced susceptibility to sporadic DCM, providing novel insight into the molecular mechanisms of the pathogenesis of DCM and suggesting potential implications for the prenatal prophylaxis and personalized treatment of this commonest primary myocardial disease.

Introduction

Dilated cardiomyopathy (DCM), clinically characterized by ventricular chamber enlargement and an impaired contraction of the left ventricle or both ventricles, represents the most prevalent type of heart muscle disease, with an estimated prevalence of 1 in 250 individuals (1). It is the most common indication for heart transplantation and a major cause of chronic congestive heart failure and sudden cardiac death (2-4). DCM has diverse etiologies with both genetic defects and environmental risk factors implicated in the pathogenesis of DCM (1-3,5). In approximately half of the cases, DCM occurs in the absence of an identifiable cause, such as coronary artery disease, viral myocarditis, cardiac valve disease, hypertension, toxic exposure, alcohol abuse, nutritional deficiency, autoimmune abnormality or metabolic disorder, and this type of DCM is defined as idiopathic or primary DCM. However, in 25-50% of cases, the familial transmission of idiopathic DCM is observed in an autosomal dominant, recessive, X-linked, or mitochondrial pattern with variable expressivity and penetrance, hence termed familial DCM, which is in contrast to sporadic DCM which is not associated with a family history (5,6). Aggregating evidence has indicated that genetic pathogenic factors play a crucial role in the pathogenesis of idiopathic DCM, and genetic mutations in >50 genes have been causally linked to idiopathic or familial DCM (1-3,7-12). Among these established DCM-associated genes, the majority encode sarcomeric, cytoskeletal, nucleoskeletal and calcium-handling proteins (2). Nevertheless, DCM is of remarkable genetic heterogeneity and the genetic basis underlying DCM in a significant proportion of patients remains unclear.

Previous studies have underlined the pivotal roles of several core cardiac transcription factors in cardiac development and structural remodeling, including the GATA zinc finger-containing transcription factor, NK homeodomain tran-
scription factor and T-box transcription factor families (13-17), and mutations in GATA binding protein (GATA)4, GATA5, GATA6, NK2 homeobox (NKX2)-5, NKX2-6 and T-Box 5 (TBX5) have been associated with various congenital heart diseases and arrhythmias, including cardiac septal defect, tetralogy of Fallot, double outlets of the right ventricle and atrial fibrillation (18-49). Recently, mutations in GATA4, GATA5, GATA6, NKX2-5 and TBX5 have been associated with familial DCM (50-56). Additionally, mutated GATA4 has also been shown to be involved in sporadic DCM (57). However, the prevalence and spectrum of TBX5 mutations in patients with sporadic DCM remain to be investigated.

**Subjects and methods**

**Study subjects.** A cohort of 146 unrelated patients with sporadic DCM was recruited from the Han Chinese population. The controls consisted of 200 unrelated healthy individuals, who were matched to the patients with DCM in ethnicity and gender. All study participants underwent a comprehensive clinical evaluation, including a report of individual and familial histories, medical records, physical examination, two-dimensional transthoracic echocardiography with color flow Doppler, X-ray, standard 12-lead electrocardiogram and exercise tolerance testing. Cardiac catheterization, angiography, or cardiac magnetic resonance imaging was performed only if there was a strong clinical indication. The diagnosis of idiopathic DCM was made as previously described: a left ventricular end-diastolic diameter of >27 mm/m² and an ejection fraction of <40% or fractional shortening <25% in the absence of abnormal loading conditions, coronary artery disease, congenital heart lesions and other systemic diseases (50-53,58,59). The patients with co-existent conditions that may result in ventricular systolic dysfunction, such as coronary heart disease, hypertensive heart disease, valvular heart disease and viral myocarditis, were excluded from the study. The clinical studies were performed by investigators blinded to the genotypic results. This study was performed in conformity with the principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of Shanghai Eighth People's Hospital, Shanghai, China. Written informed consent was obtained from all participants prior to the enrollment in the study.

**Mutational screening of TBX5.** A peripheral venous blood sample was prepared from each study participant, and the genomic DNA was extracted using a Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI, USA). The coding exons and flanking introns of the TBX5 gene were sequenced in the 146 unrelated patients with sporadic DCM. A total of 200 unrelated healthy control individuals were also genotyped for TBX5. The primer pairs used to amplify the coding regions and splicing junctions of TBX5 by polymerase chain reaction (PCR) were designed as previously described (56). PCR was carried out using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a Veriti Thermal Cycler (Applied Biosystems, Foster, CA, USA). Both strands of each PCR product were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing kit under an ABI PRISM 3130x1 DNA Analyzer (both from Applied Biosystems). The identified TBX5 variant was validated by re-sequencing an independent PCR-generated amplicon from the same individual, and was queried in the single nucleotide polymorphism (SNP) database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), the human gene mutation database (HGMD; http://www.hgmd.org/) and the 1000 Genomes Project (1000 GP) database (http://www.1000genomes.org/) to confirm its novelty.

**Alignment of multiple TBX5 protein sequences across species.** The conservation of an affected amino acid was shown by using the online MUSCLE program on the NCBI website (http://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&doctype=MultipleAlignment&list_uids=160).

**Plasmids and site-targeted mutagenesis.** The recombinant expression plasmid, TBX5-pcDNA3.1, was constructed as previously described (56). The identified mutation was introduced into the wild-type TBX5-pcDNA3.1 construct by PCR-based site-directed mutagenesis using a QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and confirmed by sequencing. The expression vector, GATA4-pSSRa, and the atrial natriuretic factor (ANF) reporter plasmid (ANF-luc) reporter, which contains the 2600-bp 5'-flanking region of the ANF gene and expresses Firefly luciferase, were kindly provided by Dr Ichiro Shiojima from Chiba University School of Medicine (Chiba, Japan).

**Biological analysis of mutated TBX5.** COS-7 cells (a fibroblast-like cell line derived from monkey kidney tissue) from our cell bank were seeded in 24-well plates and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells at approximately 90% confluency were transfected using Lipofectamine™ 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The internal control reporter plasmid pGL4.75 (hRluc/CMV; Promega Corp.), which expresses the Renilla luciferase, were used in transient transfection assays to normalize transfection efficiency. For each transfection, the COS-7 cells were transfected with 0.4 µg of wild-type or mutant TBX5-pcDNA3.1, 1.0 µg of ANF-luc reporter construct and 0.04 µg of pGL4.75. For co-transfection experiment, 0.2 µg of wild-type TBX5-pcDNA3.1 and 0.2 µg of mutant TBX5-pcDNA3.1 were used in the presence of 0.4 µg of ANF-luc and 0.04 µg of pGL4.75. For the synergistic activation experiment, 0.4 µg of wild-type GATA4-pSSRa was added. Firefly and Renilla luciferase activities were measured with the Dual-Glo Luciferase Assay system (Promega Corp.) 48 h after transfection. The activity of the ANF promoter was expressed as the fold activation of Firefly luciferase relative to Renilla luciferase. Three independent experiments were conducted at minimum for each transfection.

**Statistical analysis.** Continuous variables are presented as the means ± SD and the Student’s unpaired t-test was used
to compare the continuous variables between 2 groups. Non-continuous and categorical variables are presented as frequencies or percentages and were compared using Pearson’s $\chi^2$ test or Fisher’s exact test where appropriate. All statistical analyses were two-sided and P-values $<0.05$ were considered to indicate a statistically significant difference.

**Results**

**Baseline clinical characteristics of the study population.** In this case-control study, 146 unrelated patients with sporadic DCM were clinically evaluated in contrast to 200 unrelated healthy control individuals. The patients presented with the typical phenotype of DCM as previously described by Elliott *et al* (58). The control individuals had normal echocardiographic parameters without any evidence of heart disease. All the study participants stated to have no family history of DCM, and none of them had known risk factors or comorbidities for DCM, such as coronary artery disease, hypertension, viral myocarditis, valvular heart disease, congenital heart disease, drug abuse or exposure to toxicants. The baseline clinical characteristics of the study subjects are summarized in Table I.

**Identification of a novel TBX5 mutation.** By sequence analysis of TBX5, a heterozygous variation was identified in 1 of the 146 unrelated patients with sporadic DCM, with a mutational prevalence of approximately 0.68%. Specifically, a transversion of guanine into adenine at nucleotide position 143 (c.427G>A), predicting the substitution of threonine for alanine at amino acid 143 (p.A143T), was identified in a 47-year-old female with DCM. She stated to have no positive family history of DCM, and had no overt congenital cardiovascular abnormalities or forelimb malformations. The sequence electropherograms showing the detected heterozygous TBX5 variation in contrast to its corresponding control sequence are presented in Fig. 1. The schematic diagram of TBX5 protein showing the T-box structural domain and the location of the mutation identified in this study is illustrated in Fig. 2. The mutation was not observed in the 400 control chromosomes and it was not found in the SNP, HGM or 1000 GP databases, which were consulted again on March 19, 2015, suggesting a novel mutation.

**Alignment of multiple TBX5 protein sequences across species.** As shown in Fig. 3, a cross-species alignment of multiple TBX5 protein sequences displayed that the alanine at amino acid

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Table I. Baseline clinical characteristics of the study subjects and control individuals.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (n=146)</th>
<th>Controls (n=200)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>54±10</td>
<td>55±9</td>
<td>0.3309</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>72 (49)</td>
<td>98 (49)</td>
<td>0.9538</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>119±13</td>
<td>126±12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80±8</td>
<td>83±7</td>
<td>0.0002</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>87±14</td>
<td>75±12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>69±7</td>
<td>46±6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>59±7</td>
<td>36±6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>38±8</td>
<td>63±7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>19±6</td>
<td>31±5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NYHA function class (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>17 (12)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>II</td>
<td>45 (31)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>III</td>
<td>62 (43)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>IV</td>
<td>21 (14)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

SBP, 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; NYHA, New York Heart Association; NA, not applicable or not available.
acid position 143 of human TBX5 was completely conserved evolutionarily among species, implying that the amino acid is functionally important.

Causative potential of a TBX5 variation. The TBX5 sequence variation of c.427G>A was predicted to be pathogenic by MutationTaster, with a P-value of 0.9999999932. No SNPs in the altered region were reported in the MutationTaster database. The amino acid substitution of p.A143T was also predicted to be possibly damaging by another online program, PolyPhen-2, with a score of 0.994 (sensitivity, 0.46; specificity, 0.96). These results indicate that the TBX5 mutation contributes to DCM in this mutation carrier.

Reduced transcriptional activity of the mutant TBX5. As shown in Fig. 4, the same amount (0.4 µg) of wild-type TBX5 and its mutant counterpart, A143T, activated the ANF promoter by approximately 11- and 2-fold, respectively. When wild-type TBX5 was co-expressed with the same amount (0.2 µg) of the TBX5 mutant, A143T, the induced activation of the ANF promoter was approximately 4-fold; while 0.2 µg of wild-type TBX5 alone activated the ANF promoter by approximately 7-fold. These functional data reveal that the TBX5 mutant, A143T, had a significantly reduced transcriptional activity and a dominant-negative effect on its wild-type counterpart.

Diminished synergistic transcriptional activity of the TBX5 mutant. As shown in Fig. 5, in the presence of 0.4 µg of GATA4, the same amount (0.4 µg) of wild-type TBX5 and the TBX5 mutant, A143T, activated the ANF promoter by approximately 25- and 7-fold, respectively. When wild-type TBX5 was co-expressed with the same amount (0.2 µg) of the TBX5 mutant, A143T, the induced activation of the ANF promoter was approximately 14-fold. These functional data reveal that the TBX5 mutant, A143T, had a significantly diminished synergistic transcriptional activity with GATA4.

Discussion
In this study, a novel heterozygous mutation in TBX5, p.A143T, was identified in a patient with sporadic DCM. The mutation was absent in the 400 referral chromosomes, and altered the amino
acid that was completely conserved evolutionarily. Functional analyses revealed that the TBX5 mutant, A143T, was associated with a significantly decreased transcriptional activity and had a dominant-negative effect on its wild-type counterpart. Furthermore, the mutation diminished the synergistic activation between TBX5 and GATA4. Therefore, it is likely that mutant TBX5 predisposes the mutation carrier to DCM. To the best of our knowledge, this is the first clinical study that demonstrates the association of TBX5 loss-of-function mutation with an enhanced susceptibility to sporadic DCM.

To date, 17 members of the T-box containing transcription factor family have been discovered in mammals, of which 6 members, including TBX1, TBX18 and TBX20 of the TBX1 subfamily, and TBX2, TBX3 and TBX5 of the TBX2 subfamily, are expressed in the heart (16). In humans, the TBX5 gene, which maps to chromosome 12q24.1 and contains 8 coding exons, encodes a protein of 518 amino acids. The TBX5 protein has an important structural domain termed T-box, which is highly conserved throughout members of the T-box family and across various species. The T-box motif is essential for the specific binding to target DNA and the protein-protein interactions, and previous studies have substantiated that TBX5 regulates the expression of multiple important target genes expressed in the heart during embryogenesis, including ANF, CX40 and serum response factor (SRF), alone or in synergy with transcriptionally cooperative partners, such as GATA4 and NKX2-5 (16,18,38,60,61). In the present study, the TBX5 protein, which maps to chromosome 12q24.1 and contains 8 coding exons, encodes a protein of 518 amino acids. The TBX5 protein has an important structural domain termed T-box, which is highly conserved throughout members of the T-box family and across various species. The T-box motif is essential for the specific binding to target DNA and the protein-protein interactions, and previous studies have substantiated that TBX5 regulates the expression of multiple important target genes expressed in the heart during embryogenesis, including ANF, CX40 and serum response factor (SRF), alone or in synergy with transcriptionally cooperative partners, such as GATA4 and NKX2-5 (16,18,38,60,61). In the present study, the TBX5 mutation identified in a patient with sporadic DCM is located in the T-box domain, and functional analyses unveiled that the mutation led to a significantly reduced transcriptional activity of TBX5, alone or synergistically with GATA4, and exerted a dominant-negative effect on wild-type TBX5. These findings suggest that haploinsufficiency or a dominant-negative effect resulted from the TBX5 mutation may be an alternative pathological mechanism of the pathogenesis of sporadic DCM.

The association of TBX5 loss-of-function mutation with an increased vulnerability to familial DCM has been reported previously. Zhang et al (56) sequenced the coding regions and splice junction sites of TBX5 in a cohort of 190 unrelated patients with idiopathic DCM, and found a novel heterozygous mutation (p.S154A) in an index patient with a positive family history, with a mutational prevalence of approximately 0.53%. Genetic analysis of the proband's pedigree revealed that the mutation co-segregated with DCM transmitted in an autosomal dominant pattern with complete penetrance. Biological assays revealed that the mutation significantly decreased the transcription activating function of TBX5, as well as its synergism with partners GATA4 and NKX2-5 at the ANF promoter. Similarly, in the present study, a novel TBX5 loss-of-function mutation, p.A143T, was linked to sporadic DCM. These observational results suggest that genetically compromised TBX5 contributes to the pathogenesis of DCM in a subset of patients.

The discovery that functionally impaired TBX5 predisposes to DCM may be partially attributed to the abnormal development and structural remodeling of the heart. In vertebrates and humans, TBX5 is amply expressed in the embryonic and adult hearts, and is required for normal cardiac development and structural remodeling, including cellular specification, proliferation, differentiation and migration, tissue patterning and morphogenesis (62). In mice, TBX5 is highly expressed in the cardiac crescent, linear heart tube, common atrium, left ventricle, left-side ventricular septum, trabeculae of the right ventricle, and the atrial aspect of the atrioventricular valves (63). Mice with a homozygous deletion of two Tbx5 alleles died by E10.5, due to failure of cardiac looping, hypoplasia of sinuatria and left ventricle; while mice with a heterozygous deletion of one Tbx5 allele presented with atrial septal defects, ventricular septal defects, endocardial cushion defects, hypoplastic left heart, aberrant trabeculation, and atrioventricular block, similar with what were observed in patients with Holt-Oram syndrome (64). Moreover, in mice TBX5 and GATA4 are co-expressed and interact physically in the developing atria and ventricles, and mice with doubly heterozygous deletion of Tbx5 and Gata4 suffered embryonic lethality, thin atrial and ventricular myocardium with reduced cell proliferation and atrioventricular septation defects (65). Taken together, these results from experimental animals support that TBX5 loss-of-function mutation is involved in DCM in humans.

It has been substantiated that TBX5 physically interacts with multiple core cardiac transcriptional factors, including GATA4, NKX2-5, MEF2c and TBX20, to form a transcriptional complex resulting in the synergistic activation of target gene expression (16). In the present study, the TBX5 mutation was linked to decreased synergistic activation of the atrial natriuretic factor (ANF) promoter-driven luciferase in COS-7 cells by wild-type TBX5 or A143T-mutant TBX5 (A143T), alone or in combination, showed significantly diminished transcriptional activity by mutant protein. Experiments were performed in triplicate, and the mean ± standard deviations are shown. **P=0.0003 (t=12.1366), *P=0.0025 (t=6.7513), respectively, when compared with wild-type TBX5. ANF-luc, ANF-luciferase.
genes required for proper cardiac development, including ANF, CX40, SRF, MHY6 and ID2 (16), and loss-of-function mutations in several transcriptional cooperative partners of TBX5, including NKK2-5, GATA4, GATA5, GATA6 and TBX20, have been reported to be responsible for DCM in humans (50-57,66). Therefore, genetically defective TBX5 may contribute to DCM by decreasing the expression of some target genes important for cardiac development and structural remodeling.

Notably, TBX5 mutations have previously been related to Holt-Oram syndrome, an autosomal dominant disease characteristic of congenital cardiac defects and anterior upper limb deformities. Cardiac phenotypes constitute a wide spectrum of cardiovascular developmental anomalies, including atrial septal defect, ventricular septal defect, tetralogy of Fallot, atriocentric ventricular defect, patent ductus arteriosus, mitral valve defect and aberrant pulmonary vein, as well as cardiac conduction block and atrial fibrillation (37,48,67). There are three variations of Holt-Oram syndrome which have been encountered in clinical practice: patients may have only cardiovascular abnormalities (4%), only forelimb malformations (27%), or both (69%), and these cardiac and skeletal deformations vary widely ranging from mild to severe, even within families (48).

In the present study, in addition to DCM, the mutation carrier had no apparent cardiac or skeletal anomaly. Different genetic backgrounds, epigenetic modifiers and functional characteristics of gene mutations (loss-of-function, dominant-negative, or gain-of-function effect) as well as its temporal and spatial effects during cardiac development (germline or somatic) are potential explanations for the pronounced variability in clinical presentation (46).

In conclusion, to the best of our knowledge, this study is the first to present an association of a TBX5 loss-of-function mutation with an increased susceptibility to sporadic DCM, providing novel insight into the molecular mechanisms of the pathogenesis DCM, and suggesting potential implications for prenatal prophylaxis and personalized treatment of the most common type of primary cardiomyopathy.

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