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TBX5 loss-of-function mutation contributes to familial dilated cardiomyopathy

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ABSTRACT

The cardiac T-box transcription factor TBX5 is crucial for proper cardiovascular development, and mutations in TBX5 have been associated with various congenital heart diseases and arrhythmias in humans. However, whether mutated TBX5 contributes to dilated cardiomyopathy (DCM) remains unclear. In this study, the coding exons and flanking introns of the *TBX5* gene were sequenced in 190 unrelated patients with idiopathic DCM. The available family members of the index patient carrying an identified mutation and 200 unrelated ethnically matched healthy individuals used as controls were genotyped for *TBX5*. The functional characteristics of the mutant TBX5 were explored in contrast to its wild-type counterpart by using a dual-luciferase reporter assay system. As a result, a novel heterozygous TBX5 mutation, p.S154A, was identified in a family with DCM inherited in an autosomal dominant pattern, which co-segregated with DCM in the family with complete penetrance. The missense mutation was absent in 400 control chromosomes and the altered amino acid was completely conserved evolutionarily across various species. Functional assays revealed that the mutant TBX5 had significantly decreased transcriptional activity. Furthermore, the mutation markedly diminished the synergistic activation of TBX5 with NKX2-5 or GATA4, other two transcription factors causatively linked to DCM. This study firstly associates TBX5 loss-of-function mutation with enhanced susceptibility to DCM, providing novel insight into the molecular mechanisms of DCM, and suggesting the potential implications in the development of new treatment strategies for this common form of myocardial disorder.

1. Introduction

Dilated cardiomyopathy (DCM), characterized by progressive ventricular dilatation and systolic dysfunction in the absence of associated conditions such as hypertension, valvular heart disease and coronary artery disease, is the most prevalent type of primary myocardial disease, affecting approximately 1 in 250 persons [1]. It is one of the most frequent causes of congestive heart failure and is the commonest reason for cardiac transplantation in adults and children [2]. In majority of patients, DCM occurs sporadically, but in 25%–35% of cases, familial transmission of DCM is observed in an autosomal dominant, recessive, or X-linked pattern with variable expressivity and penetrance [3]. Increasing evidence demonstrates that genetic defects play an important role in the pathogenesis of DCM, and a great number of mutations in more than 50 genes have been associated with DCM. Of these well established DCM-associated genes, most code for cardiac structural or regulatory proteins, including myocardial sarcomeric and cytoskeletal proteins [1]. However, DCM is a genetically heterogeneous disorder, and the genetic determinants underpinning DCM in a significant proportion of patients remain unknown.

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It has been found that a group of core cardiac transcription factors, including the homeodomain protein NKX2-5, zinc finger proteins GATA4, GATA5, and GATA6, and T-box factors TBX1, TBX2, TBX3, TBX4, TBX5, TBX18 and TBX20, play a crucial role in the normal cardiovascular development [4]. These core transcription factors physically interact with each other and with an array of other transcription factors, and function in a mutually reinforcing transcriptional network to finely control heart development [4]. Hence, it is not surprising that mutations in most of the core cardiac transcription factors, especially for the most extensively studied cardiac transcription factors NKX2-5, GATA4 and TBX5, have been associated with non-synodromic or synodromic congenital heart diseases as well as arrhythmias [4–22]. Interestingly, mutations in some cardiac transcription factors, such as NKX2-5, GATA4, GATA6 and TBX20, have also been causally linked to DCM in humans [23–28]. Given that the expression profile and functional characteristics of *TBX5* overlap at least partially with those of *NKX2-5*, *GATA4*, *GATA6* and *TBX20* [29–31], it is warranted to make the hypothesis that mutated *TBX5* may contribute to DCM in a subset of patients.

2. Materials and methods

2.1. Study subjects

In this study, a cohort of 190 unrelated patients with DCM was enrolled from the Han Chinese population. The available family members of the index patients were also recruited. A total of 200 ethnically-matched unrelated healthy individuals from a routine physical examination were enlisted as controls. All participants underwent clinical evaluation, chest radiography, electrocardiogram, echocardiography and exercise capacity testing. Cardiac catheterization, coronary angiography, endomyocardial biopsy or cardiac magnetic resonance imaging was performed only if there was a strong clinical indication. The clinical diagnosis of DCM was made in accordance with 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/m^2 and an ejection fraction <40% or fractional shortening <25% in the absence of an apparent secondary cause of cardiomyopathy, such as ischemic heart disease, valvular heart disease, essential hypertension, viral myocarditis and cardiac glycogen storage disease [32]. Familial DCM was defined as the DCM occurring in two or more first-degree relatives of a family. This investigation conformed to the principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the local institutional ethics committee. Written informed consent was obtained from all the participants prior to study.

2.2. Screening for TBX5 mutation

Peripheral venous blood samples were taken from all the study participants. Genomic DNA was purified from blood leukocytes of each participant with Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The referential genomic DNA sequence of *TBX5* derived from GenBank (accession no. NG_007373.1). The primers used to amplify the coding exons and intron/exon boundaries of *TBX5* by polymerase chain reaction (PCR) were designed as shown in Table 1. Amplification of genomic DNA fragment by PCR was carried out using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a Veriti Thermal Cycler (Applied Biosystems, Foster, CA, USA) with standard conditions and concentrations of reagents. Both strands of each amplicon were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems). For every identified sequence variant, the single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) and Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS) databases were queried to confirm its novelty.

Table 1
Primer pairs used to amplify the coding exons and flanking introns of the *TBX5* gene.

Coding exon	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Size (bp)
1	TGGAAACTGGGGGCCAAACT	TCTGTCCCCGCAAGAGAAGC	385
2	TTGGGGAAGGAATGCCCACT	ATCCAGATAGCACGGCCTCC	470
3	TACCTACAGTTGCCCGCCTG	GATAGGCGGACAGACGCCTT	477
4	CAGTGCGCTACCTCCAGACT	GGTAGAGGCAGAAAGCGACGA	359
5	ACCCTGGCTTTTTCGGTTGG	CACCCTGGGGTCGAAGTTGG	487
6	GGGCAAACCAAACCCAGGTG	GGGACAGAGGGGGCTCATTC	477
7	CACACCTGGTTCAGCCACTC	CACCCCCAACCCAAGGAAAG	369
8	CACTTTTAGCTGCCTGGTGCC	AGGAAATGTCTGTTGTGAAGCAGG	665

2.3. Alignment of multiple TBX5 protein sequences across species

Multiple TBX5 protein sequences from various species were aligned using the online MUSCLE program, version 3.6 (http://www.ncbi.nlm.nih.gov/).

2.4. Expression plasmids and site-directed mutagenesis

Human *TBX5* containing the whole coding sequence was produced by PCR amplification using the human full-length cDNAs prepared previously [12], digested with *Eco*RI and *Not*I, and subsequently cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The identified mutation was introduced into the wild-type TBX5-pcDNA3.1 construct by PCR-based site-directed mutagenesis using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and verified by sequencing. The expression plasmids NKX2-5-pEFSA, GATA4-pSSRa and ANF-luciferase reporter (ANF-luc), which contains the 2600-bp 5′-flanking region of the *ANF* gene and expresses the Firefly luciferase, were kind gifts provided by Dr. Ichiro Shiojima at Chiba University School of Medicine, Japan.

2.5. Luciferase reporter gene assays

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and maintained in a humidified atmosphere with 5% CO_2. Lipofectamine® 2000 reagent (Invitrogen) was used for transfection of COS-7 cells at about 80% confluency. The internal control vector pGL4.75 (hRluc/CMV, Promega) expressing the Renilla luciferase was used in transient transfection assays to normalize transfection efficiency. In each transfection, the same amount (0.6 μg) of expression plasmid DNA (wild-type TBX5-pcDNA3.1, NKX2-5-pEFSA, GATA4-pSSRa or mutant TBX5-pcDNA3.1) was used alone or together, in combination with 1.0 μg of ANF-luc and 0.04 μg of pGL4.75. Cells were harvested 48 h after transfection, and the Firefly and Renilla luciferase activities were measured with the Dual-Glo luciferase assay system (Promega). The activity of the *ANF* promoter was presented as fold activation of Firefly luciferase relative to Renilla luciferase. Three independent experiments were performed at minimum for each transfection.

2.6. Statistical analysis

Statistical software package SPSS (version 15.0) was used for statistical analysis. Data are expressed as mean ± SD. Differences between means were compared using the Student's unpaired *t* test. Comparison of the categorical variables between two groups was