A New TBX5 Loss-of-Function Mutation Contributes to Congenital Heart Defect and Atrioventricular Block

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Summary
Congenital heart defect (CHD) represents the most common birth deformity, afflicting 1% of all births worldwide, and accounts for substantial morbidity and mortality. Increasing evidence highlights the pivotal roles of genetic etiologies in the pathogenesis of CHD, and pathogenic mutations in multiple genes, including TBX5 encoding a cardiac core transcription factor key to cardiovascular morphogenesis, have been involved in CHD. However, due to pronounced genetic heterogeneity of CHD, the genetic determinants underlying CHD in most cases remain obscure. In this investigation, by sequencing analysis of the coding exons and flanking introns of the TBX5 gene in 198 unrelated patients affected with CHD, a novel heterozygous mutation, NM_000192.3:c.692C>T; p.(Pro231Leu), was identified in an index patient with familial double outlet right ventricle (DORV), ventricular septal defect (VSD), and atrioventricular block (AVB). Genetic analysis of the proband’s pedigree showed that the mutation co-segregated with the diseases. The missense mutation, which altered the amino acid conserved evolutionarily, was absent from 266 unrelated healthy subjects. Functional analyses with a dual-luciferase reporter assay system unveiled that the Pro231Leu-mutant TBX5 was associated with significantly reduced transcriptional activity on its target genes MYH6 and NPPA. Furthermore, the mutation disrupted the synergistic transactivation between TBX5 and NKX2-5 as well as GATA4, two other transcription factors causally linked to CHD. This study firstly links TBX5 loss-of-function mutation to familial DORV, VSD, and AVB, which provides novel insight into the mechanism underpinning CHD and AVB, suggesting potential implications for genetic evaluation and individualized treatment of patients affected by CHD and AVB.

Key words: Bicuspid aortic valve, Molecular genetics, Transcription factor, Arrhythmia, Reporter gene analysis

C ongenital heart defect (CHD), characterized by structural abnormality of the heart and endothoracic great vessels during embryogenesis, is the most common type of birth malformation in humans, afflicting approximately 1% of all live births.1,2 It accounts for about one-third of all major congenital deformities.2 It is estimated that every year there are 1.35 million neonates born with CHD worldwide.3 According to anatomic and hemodynamic lesions, CHD is clinically categorized into at least 25 different subtypes, such as ventricular septal defect (VSD), atrial septal defect, double outlet right ventricle (DORV), patent ductus arteriosus, tetralogy of Fallot, and transposition of the great arteries.1,4,5 Although minor CHD often resolves spontaneously,6 many serious kinds of CHD, if not treated surgically in the first year of life, may result in degraded health-related quality of life,9 decreased exercise capacity,13–15 delayed central nervous development or brain injury,16–19 cerebral stroke,20–22 pulmonary hypertension,23–27 renal injury or malfunction,28–30 infective endocarditis,31–34 heart fail-
Cardiovascular morphogenesis undergoes a complex biological process, and both heritable and environmental pathogenic factors may disrupt the process, leading to the pathogenesis of CHD. Nevertheless, a growing body of evidence underscores the genetic determinants for CHD, and a long list of causative mutations in more than 60 genes has been linked to CHD in humans, including those encoding cardiac transcription factors, sarcomeric proteins, and signaling molecules. Among these established CHD-related genes, most code for cardiac transcription factors, including GATA4, GATA5, TBX1, GATA6, TBX5, MEF2C, TBX20, NR2F2, HAND2, NKX2-5, and HAND1. However, CHD is a genetically heterogeneous malady, and the genetic components underpinning CHD in most patients remain obscure.

As a member of the T-box transcription factor family, TBX5 plays a critical role in the development of heart and forelimbs. In humans, mutations in TBX5 are mainly reported to cause Holt-Oram syndrome (HOS), showing defects of the heart, cardiac conduction system, and the anterior forelimbs. In animal models with deletion of Tbx5, similar defects are observed. A wide range of cardiovascular defects associated with TBX5 mutations in both humans and animals suggests multiple roles for TBX5 in cardiac development and function, which justifies screening TBX5 for mutations in more patients with various forms of CHD to comprehensively understand the integral roles of TBX5 throughout heart development and adult life.

**Methods**

**Study population:** The current study participants consisted of 198 unrelated patients with various kinds of CHD and 266 unrelated healthy individuals, who were enrolled between January 2014 and December 2018 from the Chinese Han population in the same geographic area. The available family members of the index patients were also recruited. The healthy control individuals were matched to the CHD-affected cases in ethnicity, sex, and age. All the study subjects experienced comprehensive clinical evaluation, including thorough review of familial and medical histories, detailed physical examination, echocardiogram with color Doppler, standard 12-lead electrocardiogram, and routine laboratory test. This research was performed in conformity with the ethical principles outlined in the Declaration of Helsinki. The study protocol was approved by the Medical Ethics Committee of the Shanghai Jing’an District Central Hospital, Fudan University, Shanghai, China. Written informed consent was obtained from patients or legal guardians prior to commencement of the research.

**Genetic analyses:** Peripheral venous whole blood specimens were collected from every study participant. Genomic DNA was extracted from blood leukocyte with the PureLink® Genomic DNA Mini Kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer’s manual. The entire coding exons and flanking introns of TBX5 were amplified by polymerase chain reaction (PCR) with HotStar Taq DNA Polymerase (TaKaRa, Dalian, Liaoning, China) on a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Foster, CA, USA) with standard concentrations of reagents. The primers for PCR amplification were designed as described elsewhere. The amplified fragments were purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and were then subjected to PCR sequencing under an ABI 3730 XL DNA Analyzer (Applied Biosystems) with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer’s protocol. An identified TBX5 mutation was verified by bidirectional sequencing of an independent PCR-generated amplicon using the mutation carrier’s DNA sample. For each identified TBX5 variation, the Genome Aggregation Database (https://gnomad.broadinstitute.org), the 1000 Genomes Project database (http://www.1000genomes.org), and the Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/snp) were retrieved to check its novelty.

**Alignment of multiple TBX5 protein sequences across species:** To estimate whether an altered amino acid was conserved evolutionarily, the amino acid sequences of TBX5 from human were aligned with those from chimpanzee, monkey, dog, cattle, mouse, rat, fowl, zebrafish, and frog, using the online MUSCLE program (https://www.ebi.ac.uk/Tools/msa/muscle/).

**Prediction of the pathogenic potential of a TBX5 sequence variant:** The causative potential of a TBX5 sequence variant was predicted by the online software of MutationTaster (http://www.mutationtaster.org), PROVEAN (http://provean.jcvi.org/index.php), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2).

**Expression plasmids and site-directed mutagenesis:** The wild-type expression plasmid TBX5-pcDNA3.1 was constructed as previously described. The mutant-type TBX5 5-pcDNA3.1 was generated by site-directed mutagenesis with a complimentary pair of primers (forward primer: 5'-AGATTGAGAATAATCTTGGCAAGATTGAGAAGATATTG-3'; reverse primer: 5'-ATCCCTGTGGCAGAAGAGATTATCTTCATCTCT-3') and the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), following the manufacturer’s product instructions, and was confirmed by sequencing. The expression plasmids of NXX2-5-pEFa and GATA4-pSSRa, as well as the reporter plasmid of natriuretic peptide precursor A-luciferase (NPPA-luc), which expresses the Firefly luciferase, were described previously.

**Cell transfection and reporter gene assay:** COS-7 cells were cultured and transfected with various plasmids by using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) as previously described. The internal control plasmid pGL4.75 (Promega, Madison, WI, USA), which expresses the Renilla luciferase, was used to normalize transfection efficiency. Briefly, COS-7 cells were transfected with 1.0 μg of empty pcDNA3.1, 1.0 μg...
Statistics: Statistical analysis was made with the SPSS software package (SPSS, Chicago, IL, USA). Continuous variables were compared between two groups with Student’s unpaired t-test. Comparison of the categorical variables between two groups was made using Pearson’s χ² test or Fisher’s exact test, when appropriate. A two-sided P-value of < 0.05 indicated significant difference.

Results

Baseline clinical and demographic characteristics of the study population: In the present investigation, a cohort of 198 unrelated patients with CHD (116 males and 82 females, with a mean age of 24 years) was clinically investigated in contrast to a total of 266 unrelated control persons (157 males and 109 females, with an average age of 24 years). All patients had echocardiogram-documented CHD, while the control people had normal echocardiograms, with no evidence of cardiac diseases. Among the 198 cases, 62 had positive family history of CHD, whereas among the 266 controls, none had positive family history of CHD. There were no significant differences in ethnicity, gender, age, and geographical area between case and control groups. The baseline clinical and demographic features of the 198 patients affected with CHD are summarized in Table I.

Identification of a pathogenic TBX5 mutation: By sequencing analysis of the whole coding regions and splicing junction sites of the TBX5 gene, a heterozygous mutation, NM_000192.3: c.692C>T; p.(Pro231Leu), was identified in one female index patient with familial DORV, VSD, and atrioventricular block (AVB). Genetic analysis of the proband’s pedigree showed that the mutation co-segregated with the diseases, which were transmitted in an autosomal dominant pattern, with complete penetrance. The missense mutation, which altered the amino acid conserved evolutionarily, was absent from 266 unrelated healthy subjects. The sequence chromatograms illustrating the heterozygous TBX5 mutation of c.692C>T and its wild-type control sequence are shown in Figure 1A. The schematic diagram exhibiting the structural domains of the TBX5 protein and the location of the identified mutation is given in Figure 1B. The pedigree structure of the family with CHD and AVB is shown in Figure 1C. The phenotypic characteristics and TBX5 mutation status of the affected pedigree members are shown in Table II. The missense mutation was neither detected in 266 control individuals nor found in the Genome Aggregation Database, the 1000 Genomes Project database, and the Single Nucleotide Polymorphism database (accessed again on December 2, 2019), indicating its novelty. In addition, as shown in Table I, there were 30 patients with VSD + DORV, of whom five other patients with VSD + DORV had also AVB except for the index patient. Among the five patients with VSD + DORV + AVB who did not have TBX5 mutation, there were two patients with the positive family history of CHD.

Multiple alignments of TBX5 proteins from various species: As shown in Figure 2, alignment of TBX5 proteins across species displayed that the altered proline at amino acid position 231 was completely conserved evolutionarily.

Disease-causing potential of TBX5 variation: The c.692 C>T mutation in TBX5 was predicted to be disease-causing by MutationTaster, with a P-value of approximately 1.000. The amino acid substitution p.Pro231Leu in TBX5 was predicted to be probably damaging by

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### Table 1. Demographic and Baseline Clinical Features of the Patients Suffering from Congenital Heart Defect (n = 198)

| Variable | n or mean with SD | % or range |
|----------|------------------|-----------|
| **Demographics** | | |
| Male | 116 | 59 |
| Age (years) | 24 ± 11 | 1-45 |
| Positive family history of CHD | 62 | 31 |
| **Distribution of various types of CHD** | | |
| Isolated CHD | 92 | 46 |
| ASD | 38 | 19 |
| VSD | 36 | 18 |
| PDA | 16 | 8 |
| DORV | 2 | 1 |
| Complex CHD | 106 | 54 |
| TOF | 32 | 16 |
| VSD + DORV | 30 | 15 |
| VSD + ASD | 21 | 11 |
| VSD + PDA | 18 | 9 |
| VSD + TGA | 5 | 3 |
| **Incidence of cardiac arrhythmias** | | |
| Atrioventricular block | 29 | 15 |
| Atrial fibrillation | 14 | 7 |
| **Medical treatment** | | |
| Cardiac surgery | 118 | 60 |
| Catheter-based repair | 62 | 31 |
| Follow-up | 18 | 9 |

SD indicates standard deviation; CHD, congenital heart defect; ASD, atrial septal defect; VSD, ventricular septal defect; PDA, patent ductus arteriosus; DORV, double outlet right ventricle; TOF, tetralogy of Fallot; and TGA, transposition of the great arteries.
Reduced transcriptional activity of the mutant TBX5 protein: As shown in Figure 3, wild-type and Pro231Leu-mutant TBX5 plasmids (each 1.0 μg) transcriptionally activated the MYH6 promoter by ~13 folds and ~2 folds, respectively (wild type versus mutant: $t = 8.09854$, $P = 0.00126$). When the same amount of wild-type and Pro231Leu-mutant TBX5 plasmids (each 0.5 μg) were used in combination, the induced transcriptional activity was ~5-fold (wild type + empty plasmid versus wild type + mutant: $t = 5.36215$, $P = 0.00584$).

Diminished synergistic transactivation between mutant TBX5 and NKKX2-5 as well as GATA4: As shown in Figure 4, the same amount of wild-type and Pro231Leu-mutant TBX5 plasmids transcriptionally activated the NPPA promoter by ~8 folds and ~2 folds, respectively (wild type versus mutant: $t = 9.25716$, $P = 0.00076$). In the presence of wild-type NKKX2-5, the same amount of wild-type and Pro231Leu-mutant TBX5 plasmids transcriptionally activated the NPPA promoter by ~28 folds and ~10 folds, respectively (wild type versus mutant: $t = 6.12604$, $P = 0.00360$), while in the presence of wild-type GATA4, the same amount of wild-type and Pro231Leu-mutant TBX5 plasmids transcriptionally activated the NPPA promoter by ~21 folds and ~7 folds, respectively (wild type versus mutant: $t = 7.09868$, $P = 0.00208$).

Discussion

In this study, a novel heterozygous TBX5 mutation, NM_000192.3: c.692C>T; p.(Pro231Leu), was identified in a family with DORV, VSD, and AVB. The mutation, which co-segregated with the diseases in the family, was neither observed in the 532 reference chromosomes nor PolyPhen-2, with a score of 1.000 (sensitivity, 0.00; specificity, 1.00) and was predicted to be deleterious by PROVEAN, with a PROVEAN score of ~9.607.

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Table II. Phenotypic Characteristics and TBX5 Mutation Status of the Family Members with Congenital Heart Defect and Atrioventricular Block  

| Individuals | Gender | Age (years) | Cardiac phenotype | TBX5 mutation |
|-------------|--------|-------------|-------------------|---------------|
| Family 1    |        |             |                   |               |
| I-1         | M      | 61*         | DORV, VSD, III° AVB | NA            |
| II-1        | M      | 49*         | DORV, VSD, III° AVB | NA            |
| II-3        | M      | 47          | DORV, VSD, II° AVB | +/-           |
| II-8        | F      | 41          | DORV, VSD, II° AVB | +/-           |
| III-1       | M      | 25          | DORV, VSD, F° AVB  | +/-           |
| III-4       | F      | 23          | DORV, VSD, F° AVB  | +/-           |
| IV-2        | M      | 1           | DORV, VSD, F° AVB  | +/-           |

M indicates male; F, female; DORV, double outlet right ventricle; VSD, ventricular septal defect; ASD, atrial septal defect; AVB, atrioventricular block; III°, third-degree; II°, second-degree; I°, first-degree; and NA, not available; and +/-, heterozygote.  
* Age at death.

The human TBX5 gene maps on chromosome 12q 24.1, coding for a transcription factor protein consisting of 518 amino acids. The TBX5 protein possesses four functionally important structural domains: a T-box domain, a transcriptional activation domain, and two nuclear localization signal domains. The T-box domain is responsible for target DNA binding and protein-protein interactions, while the transcriptional activation domain functions to transactivate target genes, and nuclear localization signals serve to drive nuclear localization. Previous investigations have substantiated that TBX5 is amply expressed in the hearts of vertebrates and humans, encompassing the endocardium, myocardium, epicardium, and conduction system of embryonic and adult hearts, where it plays a crucial role in cardiovascular development and postnatal cardiac remodeling. Recent researches have validated that TBX5 transcriptionally mediates expression of multiple target genes key to cardiac structure and function, encompassing MYH6, NPPA, SCN5A, and GJA1, alone or in synergy with GATA4, NKX2-5, MEF2C, GATA6, and TBX20, and mutations in TBX5 and its target genes and transcriptionally cooperative partners have been associated with CHD and/or AVB in humans. In the present study, the mutation identified in patients with familial CHD and AVB was located in the T-box domain, and functional analyses demonstrated that the mutant was associated with significantly diminished transactivation of target genes, alone or synergistically with NKX2-5 or GATA4. These findings indicate that TBX5 haploinsuffi-
Figure 3. Functional impairment of TBX5 resulted from the mutation. Analysis of the activation of the MYH6 promoter-driven luciferase in COS-7 cells by wild-type or Pro231Leu-mutant TBX5, alone or in combination, unveiled that the Pro231Leu-mutant TBX5 protein had significantly reduced transcriptional activity on the MYH6 promoter. Experiments were conducted in triplicates and the results are given as means with standard deviations. Here ** and * indicate $P = 0.00126$ and $P = 0.00584$, respectively, when compared with the same amount of wild-type TBX5.

Figure 4. Diminished synergistic transactivation between mutant TBX5 and NKX2-5 as well as GATA4. Measurement of the NPPA promoter-driven luciferase in COS-7 cells by TBX5 plus NKX2-5 or TBX5 plus GATA4 revealed that the Pro231Leu mutation disrupted the synergistic transactivation between TBX5 and NKX2-5 as well as GATA4. Experiments were done in triplicates. Here *, **, and *** indicate $P = 0.00076$, $P = 0.00360$, and $P = 0.00208$, respectively, in comparison with their wild-type counterparts.

ciency is an alternative molecular mechanism underlying CHD and AVB in a subgroup of patients.

It may be attributable to aberrant cardiovascular development that genetically compromised TBX5 predisposes to CHD and AVB. During murine embryogenesis, TBX5 is highly expressed throughout the cardiac crescent and linear heart tube, in the left ventricle and ventricular septum, and in trabeculae, common atrium, as well as cardiac conduction system, including atrioventricular bundle and Purkinje fibers. In mice, homozygous disruption of Tbx5 resulted in embryonic death because of failure to undergo cardiac looping and left ventricular and sinoatrial hypoplasia, while heterozygous ablation of Tbx5 led to VSD, ASD, endocardial cushion defect, left ventricular hypoplasia, and morphological and functional abnormalities in the conduction system, including atrioventricular and bundle branch blocks. In humans, TBX5 is abundantly expressed in embryonic and postnatal hearts, and an increasing number of TBX5 mutations have been associated with HOS, including CHD and AVB. Taken collectively, these results indicate that functionally abnormal TBX5 confers increased susceptibility to CHD and AVB in humans.

In conclusion, this investigation firstly links TBX5 loss-of-function mutation to familial DORV and AVB, which provides new insight into the molecular pathogenesis of DORV and AVB, suggesting potential implications for genetic evaluation personalized management of patients affected with CHD and AVB.
Disclosure

Conflicts of interest: None.

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