CLINICAL STUDY

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.

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

Congenital 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.[1,4-8] Although minor CHD often resolves spontaneously,[1] many serious kinds of CHD, if not treated surgically in the first year of life, may result in degraded health-related quality of life,[9-12] 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-

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ure,[35-38] cardiac dysrhythmia,[39-48] and sudden cardiac death.[49-51] Therefore, CHD is associated with remarkable increase in morbidity, mortality, and socioeconomic burden. Despite important clinical significance, the etiologies underlying CHD in the overwhelming majority of cases remain elusive.

Cardiovascular morphogenesis undergoes a complex biological process, and both heritable and environmental pathogenic factors may disrupt the process, leading to the pathogenesis of CHD.[2,3,52,53] 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.[2,3,53-71] Among these established CHD-related genes, most code for cardiac transcription factors, including *GATA4*, *ISL1*, *GATA5*, *TBX1*, *GATA6*, *TBX5*, *MEF2C*, *TBX20*, *NR2F2*, *HAND2*, *NKX2-5*, and *HAND1*.[3,53] 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.[72,73] 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.[72,73] In animal models with deletion of *Tbx5*, similar defects are observed.[72] 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,[72,73] 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.[74] 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 variation: The causative potential of a *TBX5* sequence variation 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.[74] The mutant-type TBX 5-pcDNA3.1 was generated by site-directed mutagenesis with a complimentary pair of primers (forward primer: 5'-AGATTGAGAATAATCTCTTTGCCAAAGGATT-3'; reverse primer: 5'-AATCCTTTGGCAAAGAGATTATTCTCAATCT-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 NKX2-5-pEFSA 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.[74] The reporter plasmid α-myosin heavy chain 6-luciferase (MYH6-luc), which expresses the Firefly luciferase, was constructed as previously described.[75]

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.[74] 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

Table I. 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

of wild-type TBX5-pcDNA3.1, 1.0 μg of mutant TBX5-pcDNA3.1, 0.5 μg of wild-type TBX5-pcDNA3.1 plus 0.5 μg of empty pcDNA3.1, or 0.5 μg of wild-type TBX5-pcDNA3.1 plus 0.5 μg of mutant TBX5-pcDNA3.1, in combination with 1.5 μg of MYH6-luc and 0.04 μg of pGL4.75. To evaluate the synergistic activation, the same amount (0.6 μg) of each expression plasmid (empty pcDNA3.1, wild-type TBX5-pcDNA3.1, mutant TBX5-pcDNA3.1, NKX2-5-pEFSA, GATA4-pSSRa) was used alone or together, in the presence of 1.0 μg of NPPA-luc and 0.04 μg of pGL4.75. The cells were harvested 48 hours after transfection and were then lysed. The Firefly and Renilla luciferase activities were measured on the GloMax-96 Microplate Luminometer (Promega) using the Dual-Glo® Luciferase Assay System (Promega), according to the manufacturer's descriptions. The activity of the promoter was expressed as fold activation of Firefly luciferase relative to Renilla luciferase. Each transfection experiment was carried out in triplicates for three times, and the data for promoter activity were given as mean ± standard deviation of three independent transfection experiments in triplicates.

Statistics: Statistical analysis was made with the SPSS for Windows 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 χ^2 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 cosegregated 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

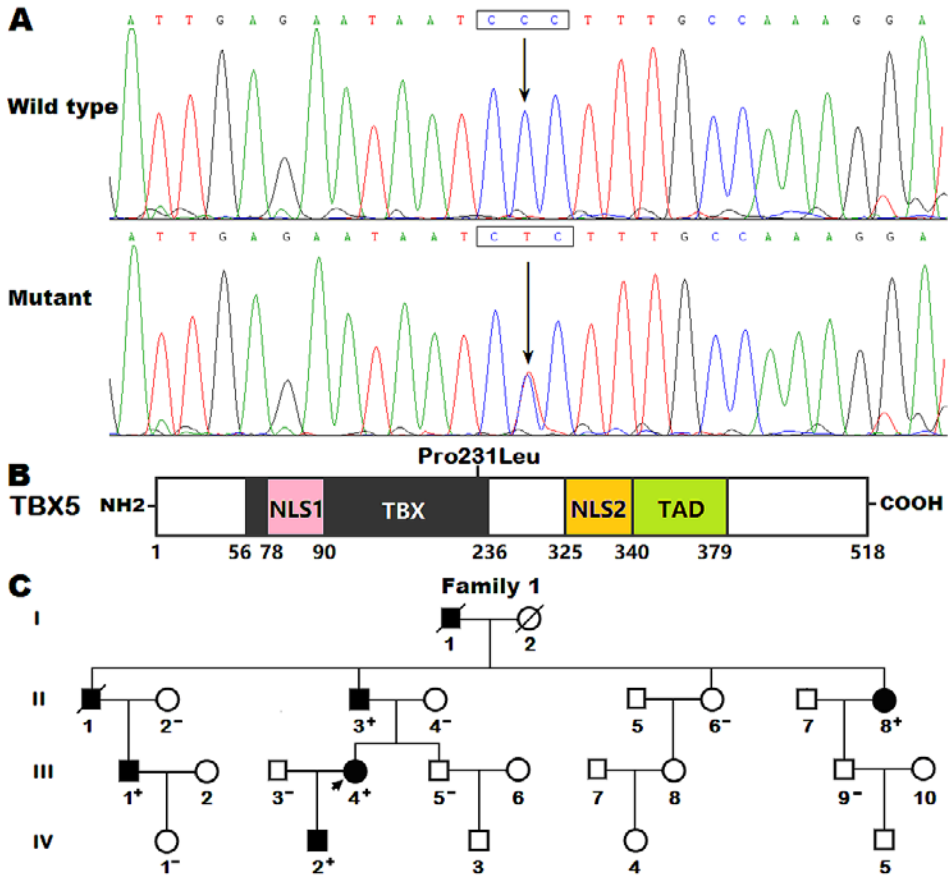

Figure 1. Novel *TBX5* mutation underlying familial heart defect and atrioventricular block. **A:** Sequence electropherograms showing the *TBX5* mutation and its wild-type control. The arrow points to the homozygous nucleotides of C/C in a healthy control subject (wild type) or the heterozygous nucleotides of T/C in the index patient (mutant). A rectangle marks the nucleotides comprising a codon of *TBX5*. **B:** Schematic diagram illustrating the structural domains of the TBX5 protein and the location of the identified mutation. NH2 indicates amino-terminus; TBX, T-box; TAD, transcriptional activation domain; NLS1, nuclear location signal 1; NLS2, nuclear location signal 2; COOH, carboxyl-terminus. **C:** Pedigree structure of the family affected by congenital heart defect and atrioventricular block. The family was arbitrarily designated as family 1. Family members are identified by generations and numbers. Open symbols represent unaffected family members; closed symbols, affected family members; squares, male member; circles, female members; symbols with a slash, the deceased members; an arrow beside the closed circle, the proband; " + ", carriers of the heterozygous *TBX5* mutation; "-", non-carriers.

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.

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 NKX2-5 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 NKX2-5, 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

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				Pro231Leu
I-1	M	61*	DORV, VSD, III^0 AVB	NA
II-1	M	49*	DORV, VSD, III^0 AVB	NA
II-3	M	47	DORV, VSD, II^0 AVB	+/-
II-8	F	41	DORV, VSD, II^0 AVB	+/-
III-1	M	25	DORV, VSD, I^0 AVB	+/-
III-4	F	23	DORV, VSD, I^0 AVB	+/-
IV-2	M	1	DORV, VSD, I^0 AVB	+/-

M indicates male; F, female; DORV, double outlet right ventricle; VSD, ventricular septal defect; ASD, atrial septal defect; AVB, atrioventricular block; III^0, third-degree; II^0, second-degree; I^0, first-degree; and NA, not available; and +/-, heterozygote.
* Age at death.

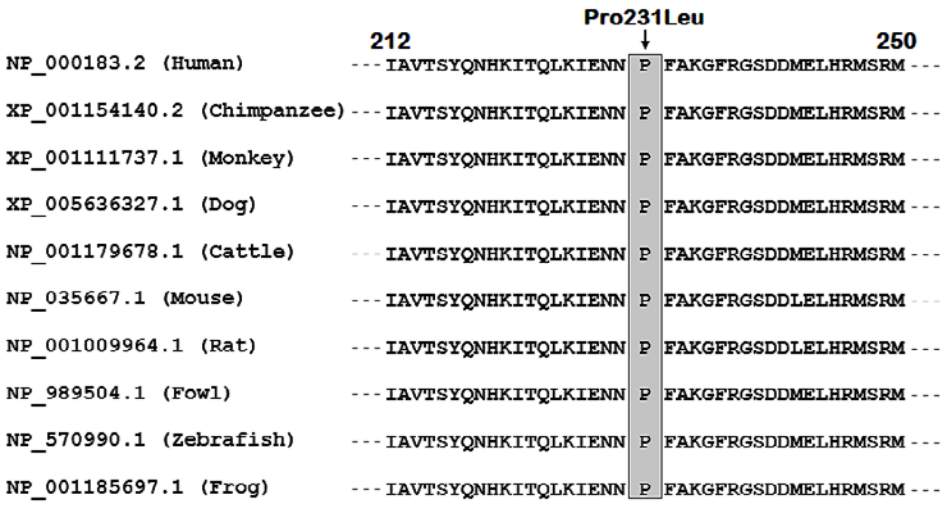

Figure 2. Alignment of multiple TBX5 proteins across species. The altered proline at amino acid 231 is completely conserved evolutionarily among various species.

reported in such population genetic databases as the Genome Aggregation Database, the 1000 Genomes Project database, and the Single Nucleotide Polymorphism database. The missense mutation altered the evolutionarily conserved amino acid and was predicted to be disease-causing by MutationTaster, PolyPhen-2, and PROVEAN. Biological assays revealed that Pro231Leu-mutant TBX5 was associated with significantly decreased transcriptional activity on the *MYH6* and *NPPA* promoters. Furthermore, the mutation abrogated the synergistic transactivation between TBX5 and NKX2-5 as well as GATA4, two other transcriptional factors that have been causally linked to CHD.[3] Therefore, it is very likely that the genetically defective *TBX5* gene contributes to DORV, VSD, and AVB in this family.

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.[74] 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.[74] 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.[72] Recent researches have validated that TBX5 transcriptionally mediates expression of multiple target genes key to cardiac structure and function, encompassing *MYH6*, *NPPA*, *SCN5A*, and *GJA5*, alone or in synergy with *GATA4*, *NKX2-5*, *MEF2C, GATA6*, and *TBX 20*,[72] and mutations in *TBX5* and its target genes and transcriptionally cooperative partners have been associated with CHD and/or AVB in humans.[72,73] 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-

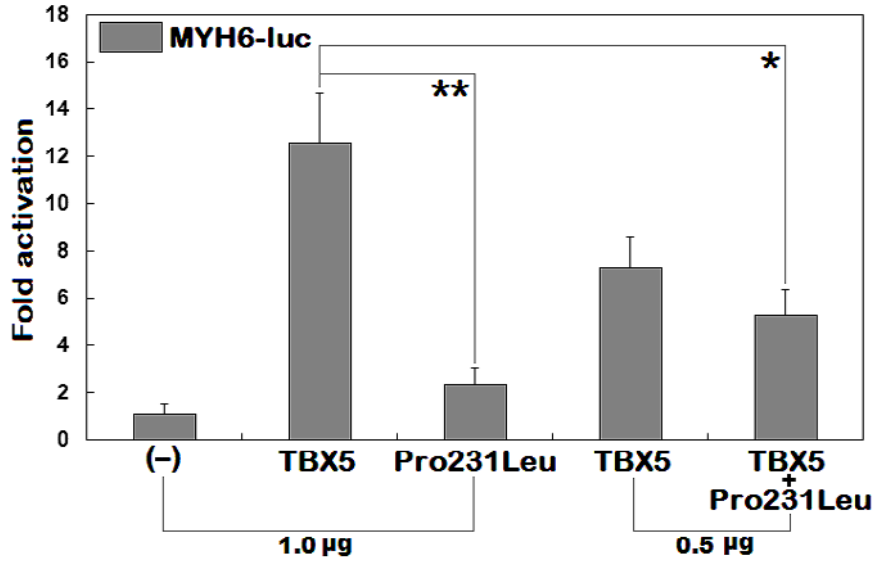

Figure 3. Functional impairment of TBX5 resulted from the mutation. Analysis of the activation of the *MYH6* promoter-driven luciferase in COS-7 cells by wild-type or Pro231Leu-mutant TBX5, alone or in combination, unveiled that the Pro231Leu-mutant TBX5 protein had significantly reduced transcriptional 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.

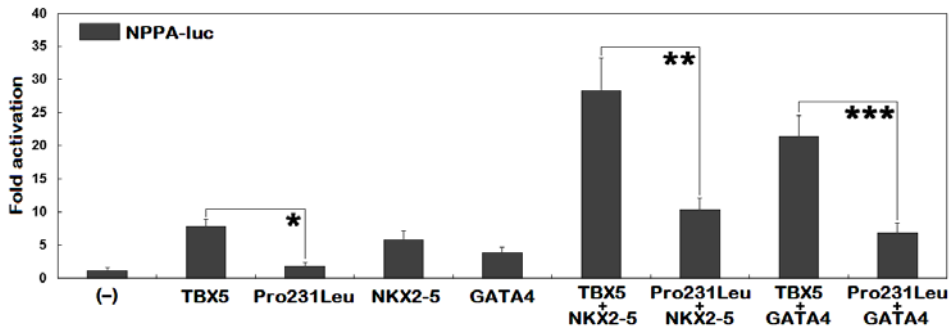

Figure 4. Diminished synergistic transactivation between mutant TBX5 and NKX2-5 as well as GATA4. Measurement of the *NPPA* promoter-driven luciferase in COS-7 cells by TBX5 plus NKX2-5 or TBX5 plus GATA4 revealed that the Pro231Leu mutation disrupted the synergistic transactivation between TBX5 and 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.[76,77] 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.[76-78] In humans, TBX5 is abundantly expressed in embryonic and postnatal hearts,[79] and an increasing number of *TBX5* mutations have been associated with HOS, including CHD and AVB.[72,73] 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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