TBX5 loss-of-function mutation contributes to atrial fibrillation and atypical Holt-Oram syndrome

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Abstract. Previous genome-wide association studies have demonstrated that single nucleotide polymorphisms in T-box (*TBX)5* are associated with increased susceptibility to atrial fibrillation (AF), and a recent study has causally linked a *TBX5* mutation to atypical Holt-Oram syndrome and paroxysmal AF. However, the prevalence and spectrum of *TBX5* mutations in patients with AF remain to be elucidated. In the present study, a cohort of 190 unrelated patients with idiopathic AF were prospectively recruited, with 400 unrelated healthy individuals recruited as controls. The coding exons and flanking introns of the *TBX5* gene were sequenced in the participants. The functional characteristics of the mutant TBX5 were delineated in contrast with its wild-type counterpart using a dual-luciferase reporter assay system. As a result, a novel heterozygous TBX5 mutation, p.P132S, was identified in an index patient with AF, with a mutational prevalence of ~0.53%. Genetic analysis of the proband's family showed that the mutation co-segregated with AF, and was transmitted in an autosomal dominant pattern. The missense mutation was absent in the 800 control chromosomes, and the altered amino acid was completely evolutionarily conserved across species. Functional analyses revealed that the mutant TBX5 had significantly reduced transcriptional activity. Furthermore, the mutation markedly decreased the synergistic activation between TBX5 and NK2 homeobox 5, another transcription factor which has been causatively linked to AF. The present study was the first, to the best of our knowledge, to report on the association between a TBX5 loss-of-function mutation and increased susceptibility to AF. These results provide novel insight into the molecular mechanism underpinning AF, and have potential implications in the development of novel prophylactic and therapeutic strategies for AF, the most common form of sustained cardiac arrhythmia.

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Introduction

Atrial fibrillation (AF), a supraventricular tachyarrhythmia with chaotic atrial electrical activation and consequent ineffective atrial contraction, is the most common form of sustained cardiac arrhythmia, accounting for approximately one-third of hospitalizations for various types of cardiac rhythm disturbances (1). The estimated prevalence of AF is 1-2% in the worldwide population, and the incidence increases rapidly with advancing age, rising from 6% in individuals aged >65 years to 10% in individuals aged ≥80 years (1-3). AF can result in a reduction in quality of life, poor exercise tolerance, thromboembolic stroke, congestive heart failure and increased rates of mortality (1). AF confers a 5-fold increase for the risk of stroke, and a 2-fold increase for the risk of heart failure and succumbing to ortality (1). Therefore, AF represents a substantial socioeconomic burden, which is likely to increase in the future due to the ageing population (4,5). Despite significant morbidity and mortality rates, the etiologies responsible for AF in a considerable proportion of patients remain to be elucidated.

AF is frequently associated with various cardiac disorders and noncardiac comorbidities (1), including valvular heart disease, hypertensive heart disease, ischemic heart disease, renal failure, thyroid dysfunction, diabetes and inflammation (6,7). However, in 15-30% of patients, AF occurs in the absence of previously associated pathology or predisposing factors, defined as idiopathic AF, of which up to 15% exhibits familial clustering and is termed familial AF (8). In previous years, an increasing number of population-based studies have demonstrated that genetic defects are pivotal in the pathogenesis of AF, and mutations in >25 genes, including those coding for ion channels, transcription factors and signaling molecules, have been causally linked to AF (8,9-23). However, AF is a

genetically heterogeneous disorder, and the genetic determinants underpinning AF in a significant number of cases remain to be elucidated.

A previous study by Sinner *et al* (24) identified five novel AF susceptibility loci by using a combination of genotyping, expression quantitative trait loci mapping and functional analysis, including a locus on chromosome 12q24 intronic to *TBX5*. The AF-associated single nucleotide polymorphism (SNP) at the *TBX5* locus, rs10507248, which was also significantly associated with ischemic stroke, was shown to modulate the expression of *TBX5* in human atrial tissues. In a genome-wide association investigation, Holm *et al* (25) found that the SNP in *TBX5* was positively associated with the electrocardiograph PR interval, QRS duration, QT interval, and with common arrhythmias, including AF and advanced atrioventricular block. In addition, the associations between the SNP in *TBX5* and the electrocardiographic parameters (PR interval, QRS duration and QT interval) and AF were replicated independently in a Chinese Han population (26). As *TBX5* is widely expressed in the heart, including the atria, atrioventricular node and ventricular bundle branches, and mutations in *TBX5* have been reported to underlie Holt-Oram syndrome, features of which include forelimb malformations and congenital heart defects, atrioventricular conduction abnormalities and AF, of which AF is the predominant phenotype (27,28), the present study hypothesized that genetically defective *TBX5* may predispose to AF in a subset of patients. Therefore, the present study aimed to identify novel mutations in *TBX5* responsible for AF, which may have potential implications for genetic counseling of AF patients.

Materials and methods

Study subjects. In the present study, subjects were recruited from the Chinese Han population at the Shanghai Gongli Hospital and Shanghai Chest Hospital (Shanghai, China), and included 190 unrelated patients with idiopathic AF (98 males; 92 females; age range, 38-57 years) and 400 unrelated healthy individuals (206 males; 194 females; age range, 38-59 years), which were used as controls. Whenever available, the index patient's first- and second-degree relatives were also enrolled. All participants underwent detailed clinical evaluation, including family history, medical history, physical examination, routine biological tests, a standard 12-lead electrocardiogram and a transthoracic echocardiogram. Subjects with structural heart disease, ischemic heart diseases, hypertension, diabetes, or any other known risk factor for AF were excluded from the investigation. The patients were clinically classified in accordance with the 2014 AHA/ACC/HRS Atrial Fibrillation Guideline (1). The classification was as follows: Idiopathic AF, AF occurring in individuals without other cardiac or systemic diseases; familial AF, idiopathic AF occurring in two or more first-degree relatives; paroxysmal AF, AF that terminates spontaneously or with intervention within 7 days of onset; persistent AF, AF with a duration of >7 days; longstanding persistent AF, continuous AF for a duration of >12 months; permanent AF, when a joint decision was made by the patient and clinician to cease further attempts to restore and/or maintain sinus rhythm. The present study conformed to the principles of the Declaration of Helsinki (29). The experimental protocol was reviewed and approved by the ethics committee of Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China). Prior to the investigation, all the participants provided written informed consent.

Genetic screening for TBX5 mutations. Peripheral venous blood samples and clinical data were collected from all participating subjects (Table I). Genomic DNA was isolated from blood leukocytes using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The primers used for amplification of the coding exons and splice junctions of *TBX5* by polymerase chain reaction (PCR) were designed, as described previously (30), and manufactured by Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences were as follows: Exon 1 (428 bp) forward (F), 5'-GACGCC ATAATCCTCTGGGC-3' and reverse (R), 5'-AAGAGCTGC CTCCACCTACT-3'; exon 2 (598 bp) F, 5'-GTCATGATC TCCGCCGTGTC-3' and R, 5'-GAACAGCGAAGGAGG CAGCG-3'; exon 3 (493 bp) F, 5'-AGGGCGAGGCCGAGT TTATG-3' and R, 5'-ACGACCCTTGGAGTTGGGTC-3'; exon 4 (462 bp) F, 5'-GGCACTTTTAGGGTTCGCCC-3' and R, 5'-TCTCCTCATCGGCACACCAG-3'; exon 5 (480 bp) F, 5'-GAGTCCAGGCCAGTGAGGTC-3' and R, 5'-CCGCTT TTCCAGAGGCGTTG-3'; exons 6 and 7 (675 bp) F, 5'-TGG TGCGCTTCTCCTAACACT-3' and R, 5'-CTCCGACGCCCC ATGCGAGG-3'; exon 8a (487 bp) F, 5'-CCCTGATCCGAC GTCTTTCC-3' and R, 5'-AACACGACAACTCCATGTGC-3'; exon 8b (437 bp) F, 5'-CTGAGTGGGTGCACACTGGA-3' and R, 5'-AGGGCTGGAGGATTCGCTTC-3'; and exon 8c (676 bp) F, 5'-ACTTGGGGTCTCGGGCACGC-3' and R, 5'-CGAACTTCGGGGCTGTGCAG-3'.

Amplification of the genomic DNA fragment by PCR was performed on a Veriti Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using a 25 μl reaction mixture consisting of 2 μl genomic DNA (100 ng/μl), 2.5 μl 10X Taq Buffer (Qiagen, Hilden, Germany), 5 μl 5X Q Solution (Qiagen), 2 μl dNTP Mixture (2.5 mM each; Takara Biotechnology Co., Ltd., Dalian, China), 0.5 μl of each primer (20 mM each), 0.25 μl HotStar TaqDNA polymerase (5 U/μl; Qiagen) and 12.25 μl deionized H_2O. The thermal cycling conditions were as follows: An initial pre-denaturation at 95˚C for 15 min, followed by 35 cycles of denaturation at 95˚C for 1 min, annealing at 62˚C for 30 sec and extension at 72˚C for 1 min, with a final extension at 72˚C for 5 min. Each amplicon was sequenced using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) under an ABI PRISM 3130 XL DNA analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The identified variant was validated by the resequencing of a second PCR product, and queried in the SNP (http://www.ncbi.nlm.nih.gov/SNP), 1000 Genomes (www.1000genomes.org), and Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS) databases to confirm it as novel.

Alignment of multiple TBX5 protein sequences across species. The amino acid sequences of multiple TBX5 proteins from various species, including human (NP_000183.2), chimpanzee (XP_001154140.2), monkey (XP_001111737.1), dog (XP_005636327.1), cattle (NP_001179678.1), mouse (NP_035667.1), rat (NP_001009964.1), fowl (NP_989504.1),

Table I. Baseline characteristics of the patients with idiopathic AF and control individuals.

Variable	Patients (n=190)	Controls (n=400)	P-value
Demographics			
Age (years)	53±9	54±8	0.1738
Male, n (%)	98 (52)	206 (52)	0.9857
BMI (kg/m^2)	24±4	24±3	1.0000
Positive family history of AF (%)	82 (43)	0 (0)	<0.0001
Type of AF			
Paroxysmal AF (%)	72 (38)	0 (0)	<0.0001
Persistent AF (%)	55 (29)	0 (0)	<0.0001
Long-standing persistent AF (%)	37 (19)	0 (0)	<0.0001
Permanent AF (%)	26 (14)	0 (0)	<0.0001
Echocardiographic parameters			
LAD (mm)	38±6	35±5	<0.0001
LVEF (%)	63±5	63±6	1.0000
Medical history			
Stroke or TIA (%)	10 (5)	0 (0)	<0.0001
ICD (%)	5 (3)	0 (0)	0.0033
Treatment of AF			
Catheter based ablation (%)	86 (45)	0 (0)	<0.0001
Pharmacological cardioversion (%)	51 (27)	0 (0)	<0.0001
Electrical cardioversion (%)	28 (15)	0 (0)	<0.0001
Follow-up (%)	25 (13)	0 (0)	<0.0001

AF, atrial fibrillation; BMI, body mass index; LAD, left atrial diameter; LVEF, left ventricular ejection fraction; TIA, transient ischemic attack; ICD, implanted cardiac defibrillator.

zebrafish (NP_570990.1) and frog (NP_001185697.1), were aligned using the online Multiple Sequence Comparison by Log-Expectation program (http://www.ebi.ac.uk/Tools/msa/muscle/).

Expression plasmids and site-directed mutagenesis. The *TBX5*-pcDNA3.1 expression plasmid was constructed, as described previously (30). Briefly, the full-length wild-type cDNA of the human *TBX5* gene were amplified by PCR using the cDNA prepared in our previous study (9), digested with EcoRI (Takara Biotechnology Co., Ltd.) and NotI (Takara Biotechnology Co., Ltd.), and subsequently inserted into the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.). The mutant *TBX5*-pcDNA3.1 was generated by PCR-mediated site-directed mutagenesis using a QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), and verified by sequencing. The NK2 homeobox 5 (NKX2-5)-pEFSA expression plasmid and atrial natriuretic factor (ANF)-luciferase (ANF-luc) reporter, which harbors the 2,600 bp 5'-flanking region of the *ANF* gene and expresses Firefly luciferase, were provided by Dr Ichiro Shiojima (Chiba University School of Medicine, Chiba, Japan).

Luciferase reporter gene assays. COS-7 cells (provided by the Cardiovascular Laboratory at the Shanghai Chest Hospital) were plated in 12-well Costar culture plates (BD Biosciences, Franklin Lakes, NJ, USA) at a density of 1x10^5 cells/well, and maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum. Tranfections were performed on the second day of plating using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The pGL4.75 internal control vector, which expressed Renilla luciferase (hRluc/cytomegalovirus; Promega), was used in the transient transfection assays to normalize transfection efficiency. In each transfection experiment, the same quantity (0.5 μg) of expression plasmid DNA (wild-type *TBX5*-pcDNA3.1, NKX2-5-pEFSA or mutant *TBX5*-pcDNA3.1) was used, either alone or in combination with 1.0 μg ANF-luc and 0.04 μg pGL4.75. The cells were harvested 48 h following transfection, and the activities of Firefly and Renilla luciferase were measured using the Dual-Glo luciferase assay system (Promega). The activity of the *ANF* promoter was determined and expressed as the fold activation of Firefly luciferase relative to Renilla luciferase. The experiments were repeated at least three times in triplicate.

Statistical analysis. Data are expressed as the mean ± standard deviation, unless otherwise indicated. Student's unpaired *t*-test or Fisher's exact test were used to determine significant differences. Two-tailed P<0.05 was considered to indicate a statistically significant difference.

Table II. Phenotypic characteristics and status of the TBX5 mutation of the affected living family members in the pedigree.

	Subject information			Phenotype	Electrocardiogram			Echocardiogram		Genotype
Identity	Gender	Age at time of study (years)	Age at diagnosis of AF (years)	AF (classification)	Heart rate (bpm)	QRS interval (ms)	QT/QTc	LAD (mm)	LVEF (%)	TBX5 mutation
Family 1										P132S
II-1	M	56	39	Permanent	69	100	412/441	42	58	+/-
II-3	M	53	34	Persistent	63	96	376/384	36	64	+/-
II-8	F	48	42	Paroxysmal	72	114	422/462	40	60	+/-
III-2	F	30	30	Paroxysmal	75	90	420/469	32	62	+/-

AF, atrial fibrillation; M, male; F, female; QTc, corrected QT interval; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; +/-, heterozygote for P132S mutation of TBX5.

Results

Clinical characteristics of the recruited subjects. In the present study, a cohort of 190 unrelated patients with idiopathic AF was clinically evaluated and compared with 400 unrelated control individuals. All the patients had an electrocardiogram-documented AF phenotype, without known secondary causes of AF. The average age of the patients at initial diagnosis of idiopathic AF was 46±9 years. The control individuals had normal electrocardiographic results with no history of AF occurrence. No significant differences were identified between the patient and control groups in ethnicity, gender or age. The baseline clinical characteristics of the subjects are summarized in Table I.

Identification of a novel TBX5 mutation. Through the use of sequencing, a heterozygous missense mutation in *TBX5* was identified in one of the 146 unrelated patients with AF, with a mutational prevalence of ~0.53%. Specifically, a substitution of thymine (T) for cytosine (C) was identified in the first nucleotide of codon 132 (c.394C>T), predicting the change of proline (P) to serine (S) at amino acid position 132 (p.P132S). This mutation was in an index patient, who was diagnosed with lone AF at the age of 39 years. The DNA sequencing chromatograms, showing the heterozygous mutation of c.394C>T in *TBX5* and its control sequence, are shown in Fig. 1A. A schematic diagram of TBX5, showing the T-box structural domain and location of the mutation identified in the present study is presented in Fig. 1B. The missense mutation, which was absent in the 200 control individuals, was not found in the SNP, 1000 Genome and EVS databases (accessed on May 9, 2015). Genetic analyses of the proband's family members showed that the mutation was present in all the affected living family members, but was absent in the unaffected family members examined. Analysis of the pedigree revealed that the mutation co-segregated with AF, and was transmitted in an autosomal dominant pattern in the family with complete penetrance. Additionally, the proband's sister (II-8) had mild bilateral forelimb deformities, a secundum atrial septal defect and atrioventricular conduction block, a phenotype of atypical Holt-Oram syndrome. The pedigree structure of the family is shown in Fig. 1C, and the phenotypic characteristics of the affected living family members are presented in Table II.

Multiple alignments of TBX5 protein sequences among various species. The alignment of multiple amino acid sequences of TBX5 proteins across species, including human, chimpanzee, monkey, dog, cattle, mouse, rat, fowl, zebrafish and frog, showed that the altered proline at amino acid residue 132 of TBX5 was completely conserved evolutionarily, suggesting that this amino acid is of functional importance (Fig. 2).

Mutant TBX5 exhibits decreased transcriptional activity As shown in Fig. 3, the same quantity (0.5 μg) of the wild-type and P132S-mutant TBX5 constructs transcriptionally activated the *ANF* promoter by ~8-fold and ~4-fold, respectively (wild-type, vs. mutant: t=6.0007, P=0.0039). This indicated that the P132S-mutant TBX5 had significantly decreased

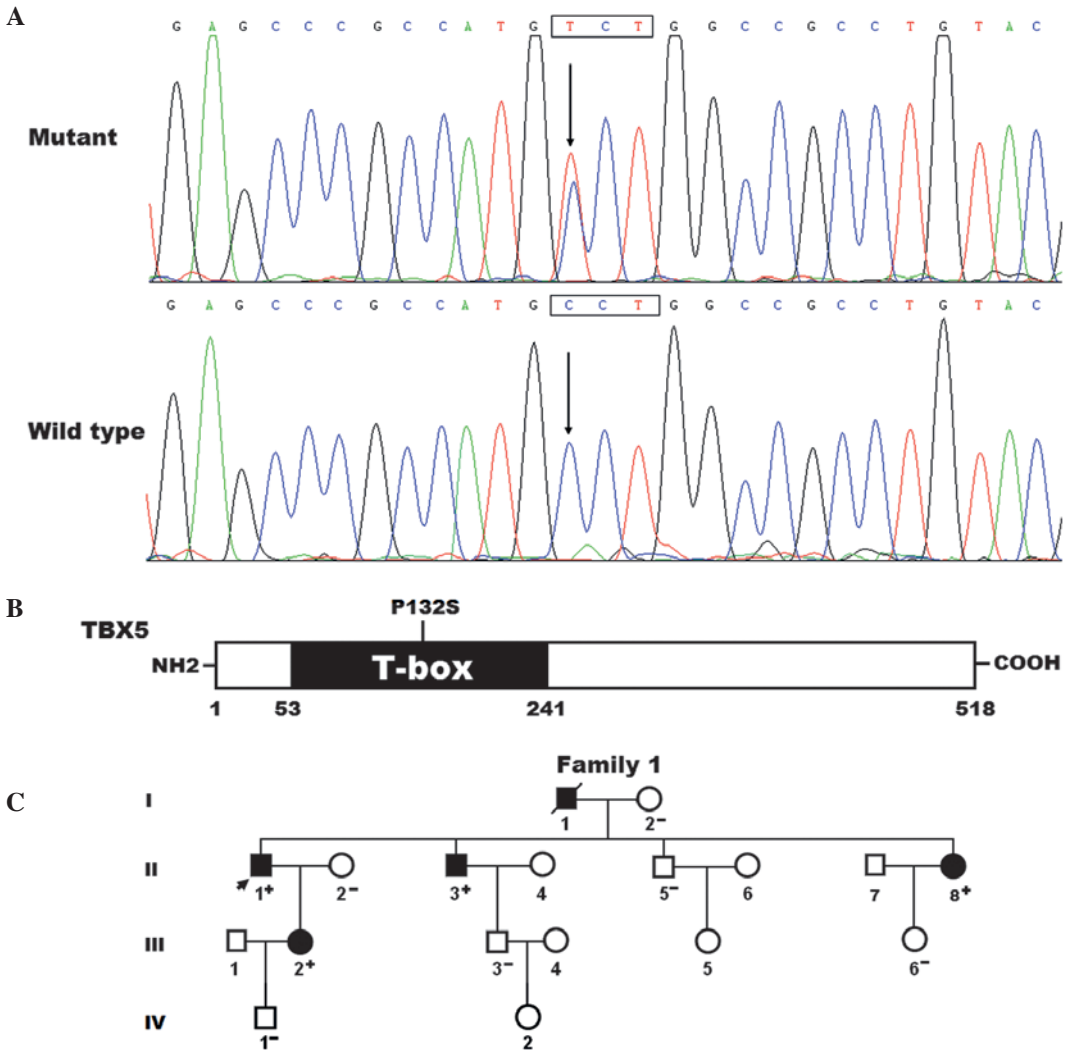

Figure 1. Novel TBX5 mutation is associated with familial AF. (A) Electropherogram output showing the heterozygous *TBX5* mutation and its wild-type control. The arrows point to the heterozygous nucleotides of C/T in the proband (mutant) and the homozygous nucleotides of C/C in the control individual (wild-type). The rectangle indicate the nucleotides, which comprise the codon of *TBX5*. (B) Schematic diagram of the TBX5 protein structure with the AF-associated mutation shown. The mutation identified in patients with familial AF is shown above the T-box structural domain. NH2 indicates an amino-terminus and COOH indicates a carboxyl-terminus. (C) Pedigree of a family containing individuals with AF-associated TBX5 mutation. The family was designated as family 1. Family members are identified by generations and numbers. Square, male family member; circle, female member; symbol with a slash, deceased member; closed symbols, affected members; open symbols, unaffected members; arrows, proband; +, carrier of TBX5 mutation; -, non-carrier. TBX, T-box; AF, atrial fibrillation.

transcriptional activity, compared with its wild-type counterpart.

Synergistic activation is reduced between mutant TBX5 and NKX2-5. As shown in Fig. 3, in the presence of 0.5 μg of wild-type NKX2-5, the same quantity (0.5 μg) of wild-type and P132S-mutant TBX5 induced the activation of the *ANF* promoter by ~26-fold and ~9-fold, respectively (wild type, vs. mutant: t=10.7419, P=0.0004), suggesting that the mutant TBX5 had reduced synergistic transcriptional activation with NKX2-5, compared with the wild-type.

Discussion

In the present study, a novel heterozygous mutation of p.P132S in TBX5 was identified in a family comprising individuals diagnosed with AF. The missense mutation, which co-segregated with AF in the family with complete penetrance, was absent in the 800 reference chromosomes from a matched control population. The alignment of multiple TBX5 protein sequences across species revealed that the altered amino acid was completely conserved evolutionarily. Functional analysis revealed that the P132S-mutant TBX5 was associated with significantly decreased transcriptional activation, which was shown when alone or in synergy with NKX2-5. Therefore, it is likely that mutated TBX5 predisposes individuals carrying this mutation to AF.

As a member of the TBX transcription factor family, *TBX5* is located on human chromosome 12q24.1, encoding a protein of 518 amino acids. The TBX5 protein contains a functionally important structural domain, termed T-box, which is essential for DNA-binding affinity and specificity, and for protein-protein interactions (27). In the present study, the TBX5 mutation identified in the patients with AF was located in the T-box, and biological analyses demonstrated that the mutation impaired the transactivational function of

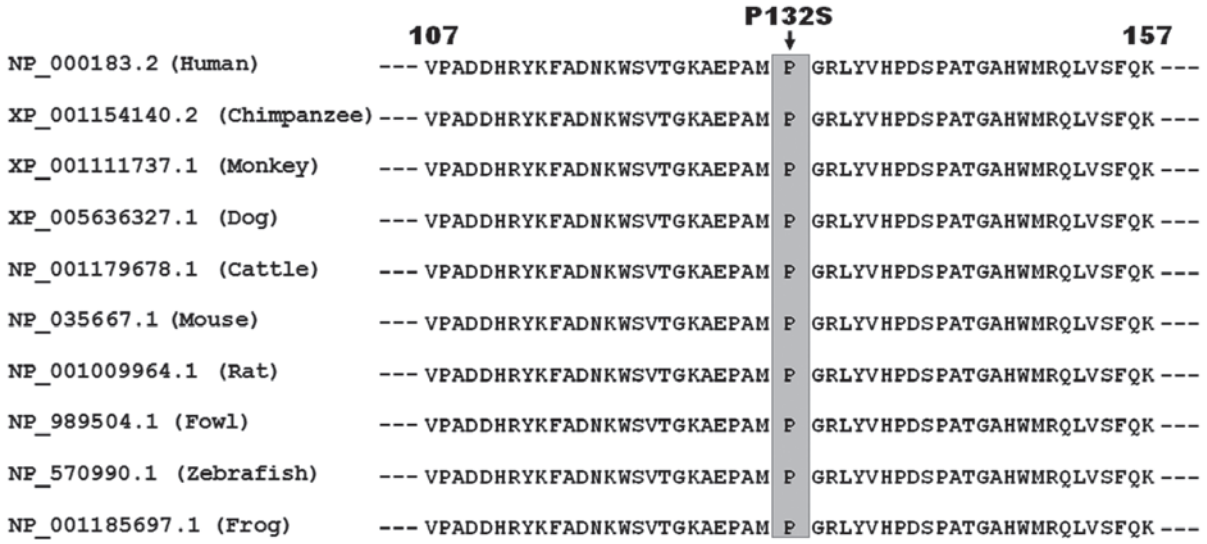

Figure 2. Multiple alignments of the TBX5 protein sequences from various species. The altered proline at amino acid position 132 of TBX5 shows complete evolutionary conservation across species. TBX, T-box.

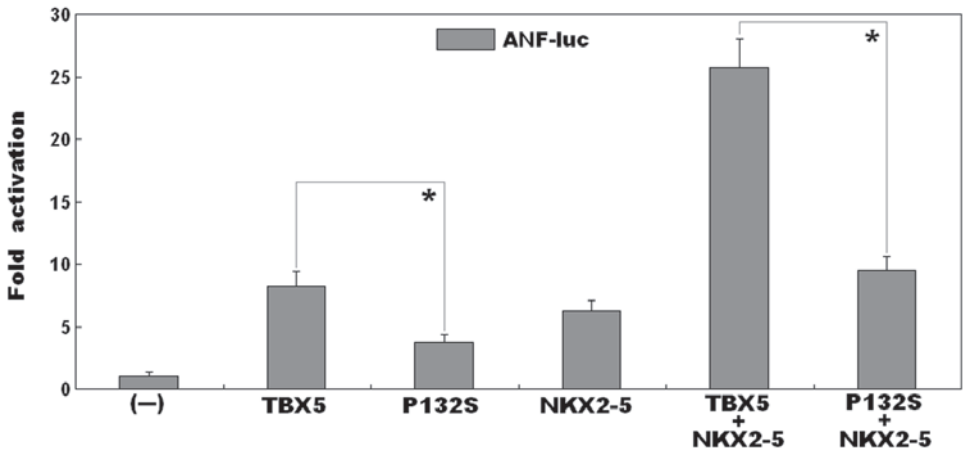

Figure 3. Effects of the TBX5 mutation on transcriptional activation in the presence and absence of NKX2-5. Transcriptional activation of ANF promoter-driven luciferase in COS-7 cells by transfection with wild-type TBX5 or P132S-mutant TBX5 (P132S), alone or together with NKX2-5. The results showed significantly decreased transcriptional activity by the mutant protein. Experiments were performed in triplicate. Results are normalized and expressed as the mean ± standard deviation of three independent transfections. *P<0.0.5, between P132S-mutant TBX5 and wild-type TBX5. TBX, T-box; NKX2-5, NK2 homeobox 5.

TBX5 in the absence and presence of NKX2-5. These findings suggested that haploinsufficiency or dominant-negative effects resulting from the *TBX5* mutation may be an alternative pathological mechanism of AF in a minority of patients.

The fact that the TBX5 loss-of-function mutation confers enhanced susceptibility to AF may be partially attributed to developmental defects of the heart. In humans and vertebrates, TBX5 is expressed at high levels in the embryonic heart, with a crucial role in cardiovascular development, including myocardial cell proliferation, specification, differentiation, migration, tissue patterning and morphogenesis (27). In mice, *TBX5* is expressed in the cardiac crescent, linear heart tube, common atrium, ventricles, inferior and superior vena cavae, and throughout the central conduction system, including the atrioventricular node and ventricular bundle branches (31,32). The homozygous deletion of *TBX5* in mice leads to embryonic death, predominantly as a result of failure of cardiac looping, hypoplasia of sinuatria and left ventricle; whereas heterozygous *TBX5*-null mice suffer from atrial septal defects, ventricular septal defects, endocardial cushion defects, left heart hypoplasia, and distinct morphological and functional defects in the atrioventricular and bundle branch conduction systems, similar to what has been observed in patients with Holt-Oram syndrome (32,33). In humans, multiple longitudinal studies have shown that abnormal cardiac conduction is an independent risk factor of AF (28,34-37). Taken together, these observational results indicate that genetically compromised *TBX5* increases the susceptibility of humans to AF, most likely by causing hypoplasia of the heart, in particular within the cardiac conduction system.

Previous studies have shown that TBX5 physically interacts with other cardiac transcriptional factors, including NKX2-5, GATA4, GATA5 and GATA6, and forms a transcriptional complex to synergistically activate multiple downstream genes

that are crucial for cardiovascular development, including *ANF* and *CX40* (27,38-41). In addition, loss-of-function mutations in several transcriptionally cooperative partners and target molecules of TBX5, including NKX2-5, GATA4, GATA5, GATA6, ANF and CX40, have been implicated in the pathogenesis of AF in humans (8-14,42-44). Therefore, functionally impaired TBX5 may contribute to AF by reducing the expression of target genes.

Of note, Postma *et al* (28) reported that a gain-of-function mutation in *TBX5* resulted in atypical Holt-Oram syndrome and AF, which was similar to prior reports showing that cytogenetic abnormalities, which produced *TBX5* duplication with presumed overexpression of TBX5, caused Holt-Oram syndrome-associated anomalies (45-48). In addition, several studies in experimental models have demonstrated that the biologic consequences in cells with reduced and augmented expression levels of TBX5 are similar (45,49-52). Taken together, these previous findings and those from the present study indicate that the dose of TBX5 requires fine regulation to avoid cardiovascular pathology (53).

In conclusion, to the best of our knowledge, the present study was the first to report the association of a TBX5 loss-of-function mutation with AF, which provides a novel insight into the molecular mechanisms underlying AF and has potential implications for the development of novel therapeutic strategies for AF. Furthermore the present study demonstrated that AF may be the only clinical presentation of Holt-Oram syndrome associated with a *TBX5* mutation, thus suggesting that there is a requirement for electrocardiographic monitoring in patients with Holt-Oram syndrome.

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