TBX20 loss-of-function mutation contributes to double outlet right ventricle

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Abstract. Congenital heart disease (CHD), the most prevalent birth defect in humans worldwide, is still a leading non-infectious cause of infant morbidity and mortality. Increasing evidence demonstrates that genetic risk factors play a key role in the pathogenesis of CHD, and more than 50 genes have been linked to various types of CHD. Nevertheless, CHD is a heterogeneous disorder and the genetic components underpinning CHD in an overwhelming majority of cases remain unknown. In the present study, the entire coding exons and flanking introns of the *TBX20* gene, which codes for a T-box transcription factor essential for the proper development of the heart, were sequenced in a cohort of 146 unrelated patients with CHD. The available relatives of the index patient harboring an identified mutation and 200 unrelated ethnically matched healthy individuals used as the controls were also genotyped for *TBX20*. The functional characteristics of the *TBX20* mutation were assayed by using a dual-luciferase reporter assay system. As a result, a novel heterozygous TBX20 mutation, p.R143W, was identified in an index patient with double outlet right ventricle (DORV). Genetic analyses of the pedigree of the proband revealed that in the family, the mutation co-segregated with DORV transmitted in an autosomal dominant pattern with complete penetrance. The missense mutation was absent in 400 control chromosomes and the altered amino acid was completely conserved evolutionarily across species. Functional analysis revealed that mutant TBX20 had a significantly diminished transcriptional activity compared with its wild-type counterpart. To the best of our knowledge, this study is the first to report the association of TBX20 loss-of-function mutation with increased susceptibility to DORV in humans, which provides novel insight into the molecular mechanisms responsible for CHD, suggesting potential implications for the antenatal prophylaxis of CHD.

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Introduction

Congenital heart disease (CHD), a structural defect that arises from the abnormal formation of the heart or intrathoracic major blood vessel, is the most common form of birth defect in humans, affecting approximately 1% of all live births and accounting for as high as 10% of early miscarriages (1-3). Presently, CHD is still the leading non-infectious cause of infant morbidity and mortality worldwide, with approximately 27% of neonates who die of birth defects having cardiovascular developmental abnormalities (1). Congenital cardiovascular deformities are usually categorized into 25 distinct clinical types, of which 21 designate specific anatomic or hemodynamic lesions, including atrial septal defect, ventricular septal defect, tetraology of Fallot, patent ductus arteriosus, double outlet right ventricle (DORV), endocardial cushion defect, aortic stenosis, coarctation of the aorta, transposition of the great arteries, abnormal pulmonary venous connection, univentricular heart and hypoplastic left heart syndrome (1). Various types of CHD may occur separately or in combination, giving rise to a degraded quality of life, decreased exercise tolerance, delayed fetal brain development or brain injury, thromboembolic events, brain abscess or infective endocarditis, hypothyroidism, pulmonary arterial hypertension or Eisenmenger's syndrome, cardiac dysfunction or heart failure, arrhythmias and even cardiac death (4-27). Although great advances in pediatric cardiovascular care have allowed most newborns with CHD to survive into adulthood, resulting in an increasing number of adults living with CHD, the surviving patients have a significantly increased incidence of late complications and sudden cardiac death (12-28).

Furthermore, due to the continued improvement of health outcomes for children with CHD, substantial mortality rates in CHD have shifted away from the young and towards adults, with a steady increase in age at death (29). Hence, CHD has imposed a vast economic burden on the families of patients and health care systems, and the socioeconomic burden is expected to be heavier in the future with the increasing number of adults with CHD (30). Despite the high prevalence and important clinical significance, the molecular mechanisms underlying CHD in a significant proportion of patients remain poorly understood.

The etiology of CHD is complex and is associated with both environmental and genetic causes (31-34). Non-genetic risk factors for CHD mainly involve paternal characteristics and conditions, the use of maternal therapeutic and non-therapeutic drugs, and parental environmental exposures (31-33), while an increasing number of studies on human genetics have demonstrated that genetic defects play an important role in the pathogenesis of CHD, and to date, a long list of mutations in >60 genes have been linked to isolated CHD or syndromic CHD, where CHD is part of the phenotypes (34-55). Nevertheless, CHD is a heterogeneous disease, and the genetic origin underpinning CHD in an overwhelming majority of patients remains to be unveiled.

T-box transcription factors, which are characterized by the presence of a highly conserved sequence-specific DNA-binding domain termed the 'T-box', have been demonstrated to be essential for proper organ development (56). To date, at least 7 members of the T-box gene family, including *TBX20* as an ancient member of the T-box superfamily, have been shown to be highly expressed in embryonic heart tissues in humans and vertebrates (56). In developing mouse embryos, *TBX20* has been shown to be expressed in cardiac progenitor cells, as well as in the developing myocardium and endothelial cells associated with endocardial cushions, the precursor structures for the cardiac valves and the atrioventricular septum (57). In mice, the complete ablation of *TBX20* has been shown to result in the death of embryos at mid-gestation with grossly abnormal heart morphogenesis, while heterozygous *TBX20*-knockout mice have shown mild atrial septal anomalies, including an increased prevalence of patent foramen ovale and primum atrial septal aneurysm, as well as a genetic predisposition to atrial septal defect (58-60). Additionally, in entirely embryonic stem cell-derived mouse embryos, the knockdown of TBX20 using RNA interference has been shown to cause various defects in heart formation, including hypoplasia of the outflow tract, persistent truncus arteriosus and hypoplastic right ventricle (61). In humans, several mutations in *TBX20* have been associated with atrial septal defect, ventricular septal defect and tetralogy of Fallot (62-67). These results warrant the screening of TBX20 for a causative mutation in another subset of patients with CHD.

Materials and methods

Study participants. A cohort of 146 unrelated subjects affected by non-syndromic CHD was enrolled from the Chinese population in this study. The available relatives of the index patient harboring an identified TBX20 mutation were also included. All patients underwent a comprehensive clinical evaluation, including an evaluation of individual and medical histories, a detailed physical examination, 12-lead electrocardiogram, and two-dimensional transthoracic echocardiography with color flow Doppler. A transesophageal echocardiography or a cardiac catheterization examination was performed only when there was a strong clinical indication. The diagnosis of CHD was made by imaging and/or direct view during cardiac surgery. The patients with known chromosomal abnormalities or syndromic CHD, such as Turner syndrome, Down syndrome, Di George syndrome, Marfan syndrome and Holt-Oram syndrome were excluded from this study. The controls comprised 200 unrelated non-CHD individuals from the same geographic area, who were matched to the CHD patients in age, gender and ethnicity.

The present study was performed in accordance with the principles outlined in the Declaration of Helsinki of 1975 and as revised in 2008. The study protocol was reviewed and approved by the Ethics Committee of Tongji Hospital, Tongji University, Shanghai, China (ethical approval number for cases and controls: LL(H)-09-07; date of approval: July 27, 2009). Prior to the commencement of the study, all participants or their guardians provided written informed consent to the use of their blood specimens for genetic analysis.

Mutational scanning of TBX20. Peripheral venous blood samples were obtained from all the study participants. Genomic DNA was isolated from the white blood cells of each participant using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). According to the referential genomic DNA sequence of the *TBX20* gene (GenBank accession no. NG_015805.1), the primers used to amplify the coding exons and flanking introns of *TBX20* by polymerase chain reaction (PCR) were designed as shown in Table I. PCR was performed using HotStar TaqDNA Polymerase (Qiagen, Hilden, Germany) on a Verti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under standard conditions. Both strands of each PCR product were sequenced using a BigDye® Terminator version 3.1 Cycle Sequencing kit under an ABI PRISM 3130 XL DNA Analyzer (both from Applied Biosystems). For an identified sequence variation, the public databases for human sequence variations, including single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/SNP) and human gene mutation (HGM; http://www.hgmd.org) databases, were used to confirm its novelty.

Multiple alignment of TBX20 amino acid sequences among species. Using the online Muscle program (version 3.6; http//www.ebi.ac.uk/Tools/msa/muscle/), the TBX20 human amino acid sequence was aligned with that of the chimpanzee, monkey, dog, cattle, mouse, fowl, zebrafish and frog.

Prediction of the causative potential of a TBX20 sequence variation. The disease-causing potential of a *TBX20* sequence variation was predicted using MutationTaster (an online program at http://www.mutationtaster.org), which yielded a probability for a variation to be either a causative mutation or a benign polymorphism. Additionally, another online program PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) was used to predict the possible pathogenic effect of an amino acid substitution occurring in the TBX20 protein.

Table I. The primers used to amplify the coding exons and flanking introns of *TBX20*.

Exon	Forward primer	Reverse primer	Size (bp)
1	5'-tgtgcagctctggagtcgtt-3'	5'-atctcccacccgcgatgtatg-3'	420
2	5'-gggtcatccctacagcctgc-3'	5'-agcaccctcaactacccagg-3'	467
3	5'-agaggggtttgtggaccgga-3'	5'-tgtccaggcttggaatgctct-3'	435
4	5'-gcgtttgatcgaagcagacca-3'	5'-cctcagggaacctgcacagt-3'	482
5	5'-tggttctttcttgcctcactgt-3'	5'-aggcagattggggtaggtgt-3'	497
6	5'-caacctccagcctgtcctca-3'	5'-tgtacaaggaatggggtgcag-3'	446
7	5'-tcatggaatttcatattctt-3'	5'-actggggccacttcctctat-3'	919
8	5'-gccagtctgtgggagtgtac-3'	5'-tggatctggattctcccatt-3'	632

Plasmid constructs and site-directed mutagenesis. Human heart cDNA was prepared as previously described (68). The full-length wild-type cDNA of the human *TBX20* gene was amplified by PCR using PfuUltra High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA, USA) and a pair of specific primers (5'-agtgctagcatggagttcacggcgtcccc-3' and 5'-tgagcggc-cgctcatacaaatggcgtcatca-3'). The PCR fragment with a length of 1,364 base pairs (bp) was doubly digested by endonuclease *Nhe*I and *Not*I (Takara, Liaoning, China). The digested product was fractionated by 1.5% agarose gel electrophoresis, purified using the QIAquick Gel Extraction kit (Qiagen), and then subcloned into pcDNA3.1 (Promega) to construct the recombinant eukaryotic expression vector, pcDNA3.1-TBX20. The atrial natriuretic factor (ANF)-luciferase reporter gene, which contains the 2,600 bp 5'-flanking region of the *ANF* gene, namely ANF-luc, was kindly provided by Dr Ichiro Shiojima, from the Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba, Japan. The identified mutation was introduced into wild-type *TBX20* using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene) with a complementary pair of primers. The mutant was sequenced to confirm the desired mutation and to exclude any other sequence variations.

Reporter gene assay of mutant TBX20. Chinese hamster ovary (CHO) cells (from our cell bank) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and maintained in 12-well plates under standard conditions for 48 h following transfection. One day prior to transfection, the CHO cells were plated at a density of $2x10^5$ cells/well. PolyFect Transfection Reagent (Qiagen) was used for the transfection of the CHO cells according to the manufacturer's instructions. The ANF-luc reporter plasmid and an internal control reporter plasmid pGL4.75 (hRluc/CMV; Promega) were used in the transient transfection assay to evaluate the transcriptional activity of the *TBX20* mutant. Twenty-four hours after plating, the CHO cells were transfected with 0.8 μg of the wild-type or mutant pcDNA3.1-TBX20 expression vector, 1.0 μg of the ANF-luc reporter construct and 0.04 μg of the pGL4.75 control reporter vector. For co-transfection experiments, 0.4 μg of wild-type pcDNA3.1-TBX20 together with 0.4 μg of mutant pcDNA3.1-TBX20 or 0.4 μg of the empty pcDNA3.1 vector were used in the presence of 1.0 μg of ANF-luc and 0.04 μg of pGL4.75. The transfected cells were washed once the following day with medium before being refreshed with new medium. Forty-eight hours after transfection, the cells were lysed, and the Firefly luciferase and *Renilla* luciferase activities were measured using the Dual-Glo luciferase assay system (Promega). The activity of the ANF promoter was presented as the fold activation of Firefly luciferase relative to *Renilla* luciferase. Three independent experiments were conducted at minimum for wild-type and mutant *TBX20*.

Statistical analysis. Quantitative data are expressed as the means ± standard deviation (SD). The Student's unpaired t-test was used to compare numeric variables between 2 groups. Comparisons of the categorical variables between 2 groups were made using Pearson's χ^2 test or Fisher's exact test where appropriate. A two-tailed value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the study population. A cohort of 146 unrelated patients with CHD (75 males and 71 females, with 32 having a family history of CHD) was clinically evaluated in contrast to a total of 200 unrelated non-CHD control individuals (102 males and 98 females, with no family history of CHD). All the patients were confirmed to suffer from CHD, while the control individuals had no evidence of structural cardiac abnormalities. None of them had established environmental risk factors for CHD, such as maternal illness and drug use in the first trimester of pregnancy, parental smoking and long-term exposure to toxicants and ionizing radiation. There was no difference in either gender or ethnicity between the patient and the control groups. The baseline clinical characteristics of the 146 patients with CHD are summarized in Table II.

Identification of a novel TBX20 mutation. By direct PCR sequencing of the *TBX20* gene in the 146 unrelated patients with CHD, a novel mutation was identified in 1 patient, with a mutational prevalence of approximately 0.68%. Specifically, a substitution of thymine for guanine in the first nucleotide of codon 134 (c.400G>T), predicting the transition of arginine into tryptophane at amino acid position 134 (p.R134W), was detected in the index patient from family 1. The sequence chromatograms showing the discovered heterozygous *TBX20* mutation compared with its control sequence are presented in

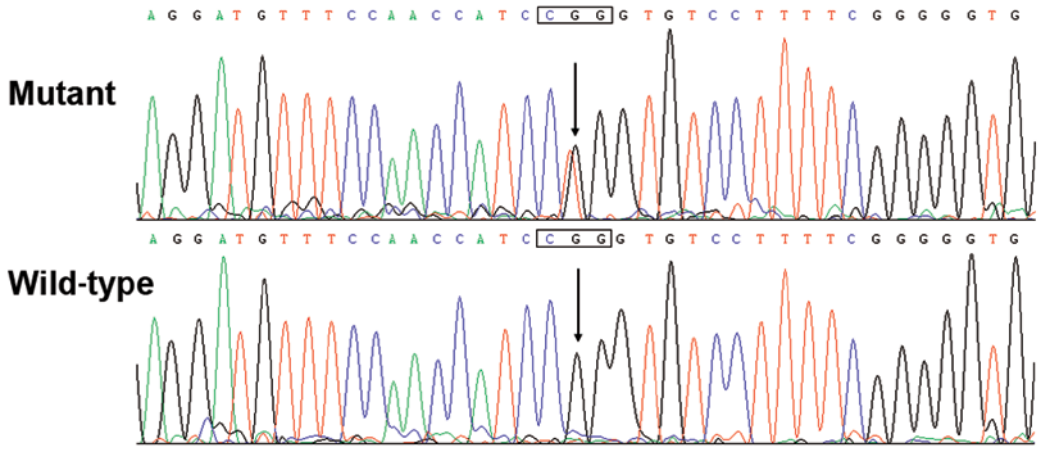

Figure 1. Sequence electropherograms displaying the *TBX20* mutation and the corresponding control. The arrows point to the heterozygous nucleotides of C/T in the proband from family 1 (mutant) or the homozygous nucleotides of C/C in the corresponding control individual (wild-type). The rectangle indicates the nucleotides comprising a codon of TBX20.

Table II. Clinical characteristics of the 146 unrelated patients with CHD.

Parameter	Statistics
Male gender (%)	75 (51)
Age (years)	5±2
Positive family history (%)	32 (22)
Distribution of different types of CHD	
Isolated CHD (%)	66 (45)
VSD (%)	22 (15)
ASD (%)	20 (14)
PDA (%)	16 (11)
ECD (%)	5 (3)
DORV (%)	3 (2)
Complex CHD (%)	80 (55)
TOF (%)	19 (13)
ASD + VSD (%)	11 (8)
DORV + VSD (%)	10 (7)
ASD + PDA (%)	9 (6)
VSD + PDA (%)	9 (6)
ECD + TGA (%)	8 (5)
TGA + VSD (%)	4 (3)
Other complication of CHD (%)	10 (7)
Incidence of arrhythmia	
Atrial fibrillation (%)	3 (2)
Atrioventricular block (%)	5 (3)
Treatment	
Surgical repair (%)	93 (64)
Catheter-based closure (%)	35 (24)
Follow-up (%)	18 (12)

CHD, congenital heart disease; VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; ECD, endocardial cushion defect; DORV, double outlet of right ventricle; TOF, tetralogy of Fallot; TGA, transposition of great arteries.

Fig. 1. A schematic diagram depicting the location of the identified mutation in T-box structural domain of the TBX20 protein is presented in Fig. 2. The missense mutation was neither found in the 400 control chromosomes nor reported in the SNP and HGM databases (consulted again on November 25, 2014). Genetic screening of the family of the mutation carrier revealed that in the family, the mutation was present in all affected family members available, but absent in the unaffected family members examined. Analysis of the pedigree displayed that in the family, the mutation co-segregated with CHD transmitted in an autosomal dominant mode with complete penetrance (Fig. 3). In addition, the grandfather (I-1) and father (II-5) of the proband also had congenital ventricular septal defect. The phenotypic characteristics and status of the TBX20 mutation of the affected family members are listed in Table III.

Alignment of multiple TBX20 amino acid sequences across species. As illustrated in Fig. 4, a cross-species alignment of the TBX20 amino acid sequences revealed that the altered amino acid arginine at position 134 was completely conserved evolutionarily.

Disease-causing potential of the TBX20 variation. The *TBX20* sequence variation of c.400G>T was predicted to be causative by MutationTaster, with a P-value of 1.000. No SNPs in the altered region were found in the MutationTaster database. Moreover, the amino acid substitution, p.R134W, which occurred in TBX20 was also predicted to be possibly damaging by PolyPhen-2, with a score of 1.000 (sensitivity, 0.00; specificity, 1.00).

Diminished transcriptional activity of the R134W mutant of TBX20. The wild-type TBX20 (WT) and the R134W-mutant TBX20 (R134W) activated the *ANF* promoter by approximately 8- and 2-fold, respectively (Fig. 5). When wild-type TBX20 was co-expressed with the same amount of R134W-mutant TBX20, the induced activation of the *ANF* promoter was approximately 3-fold. These results indicate that the

Table III. Phenotypic characteristics and status of the TBX20 mutation in the affected family members.

Individual	Gender	Age (years)	Cardiac phenotype	TBX20 mutation
Family 1				R134W
I-1	M	49[a]	DORV, VSD	NA
II-5	M	26	DORV, VSD	+/-
III-3	F	0	DORV	+/-

[a]Age at death. M, male; F, female; DORV, double outlet right ventricle; VSD, ventricular septal defect; NA, not available or not applicable; +/-, heterozygoty.

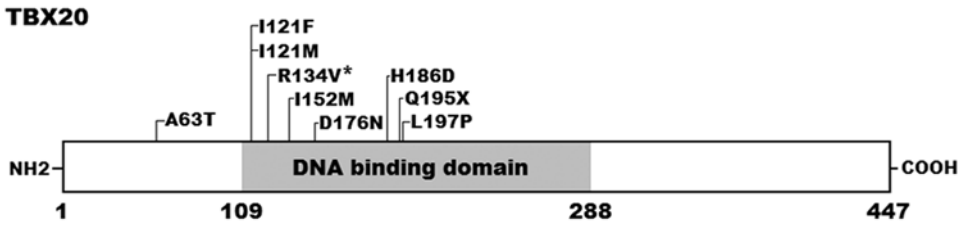

Figure 2. Schematic diagram of the TBX20 protein structure with the mutations responsible for congenital heart disease shown. The mutations associated with congenital heart disease are shown above the T-box structural domain. The mutation identified in this study is marked by asterisk (*). NH2, amino-terminus; COOH, carboxyl-terminus.

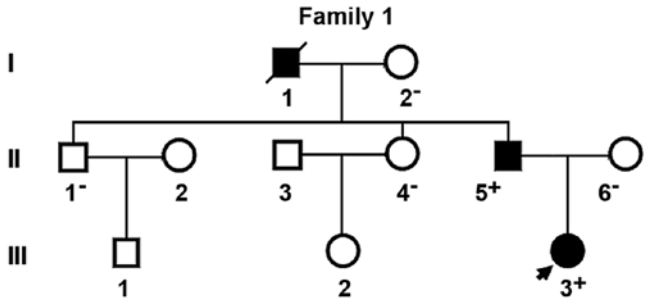

Figure 3. Pedigree structure of the family with congenital heart disease. Family is designated as family 1. Family members are identified by generations and numbers. Squares indicate male family members; circles, female members; closed symbols, affected members; open symbols, unaffected members; symbol with a slash, the deceased member; arrow, proband; +, carriers of the heterozygous mutation; -, non-carriers.

mutant TBX20 has a significantly reduced transcriptional activity compared with its wild-type counterpart.

Discussion

In the present study, a novel heterozygous mutation in TBX20, p.R134W, was identified in a family with DORV. In the family, the mutant allele was present in all the affected living family members, but absent in the unaffected relatives examined and 400 referral chromosomes from an ethnically-matched control population. A cross-species alignment of the TBX20 amino acid sequences revealed that the affected amino acid was completely conserved evolutionarily. The identified TBX20 sequence variation was predicted to be a pathogenic mutation, and functional analysis revealed that the mutant TBX20 was associated with a significantly decreased transcriptional activity. Therefore, it is very likely that functionally impaired TBX20 contributes to DORV in this family.

The human *TBX20* gene maps on chromosome 7p14.3, which contains 8 exons and spans approximately 52 kb of the genomic DNA sequence, encoding a T-box transcription factor with 447 amino acids (GenBank accession no. NG_015805.1; NP_001071121.1). The T-box domain is a novel type of DNA-binding domain, which is responsible for DNA sequence recognition and binding to the consensus motif of GGTGT within the promoters of target genes, and is also required for interaction with co-factors (57). The identified mutation (p.R134W) is located in the T-box DNA binding domain (109-288 AA) of TBX20 (Fig. 2), and it is thus anticipated to exert an influence on the transcriptional activity of TBX20, possibly by interfering with the specific binding to target DNA sites of TBX20.

It has previously been verified that TBX20 regulates multiple downstream genes expressed during cardiac morphogenesis, including *ANF*, fibroblast growth factor 10 (*FGF10*), connexin 40 (*Cx40*) and connexin 45 (*Cx45*), of which *ANF* is the most extensively characterized T-box target gene in the heart (69). Hence, the functional characteristics of the *TBX20* mutation may be explored by assaying the transcriptional activity of the *ANF* promoter. In this study, the functional effect of the novel p.R134W mutation of TBX20 identified in patients with familial DORV were deciphered by transcriptional activity assay and the results revealed a diminished transcriptional activation of the *ANF* promoter. These data indicate that genetically compromised TBX20 is potentially an alternative molecular pathogenesis of DORV.

It has been substantiated that TBX20 physically interacts with multiple cardiac transcription factors, including NK2

Table IV. Summary of the TBX20 mutations previously identified in patients with congenital heart disease.

Authors/(Refs.)	Nucleotide change	Amino acid change	Cardiac defect
Kirk *et al* (62)	c.456C>G	p.I152M	ASD, VSD, PFO
	c.583C>T	p.Q195X	ASD, CoA, MVP, MR
Liu *et al* (63)	c.187G>A	p.A63T	ASD
	c.361A>T	p.I121F	TAPVC, ASD
	c.785C>T	p.T262M	TOF, PFO, PDA
Qian *et al* (64)	c.597C>G	p.H186D	ASD, TOF, CMV, MR
	c.601T>C	p.L197P	ASD, TOF
Posch *et al* (65)	c.374C>G	p.I121M	ASD, TOF, CVD
Liu *et al* (67)	c.526G>A	p.D176N	ASD

ASD, atrial septal defect; VSD, ventricular septal defect; PFO, patent foramen ovale; CoA, coarctation of the aorta; MVP, mitral valve prolapse; MR, mitral regurgitation; TAPVC, total anomalous pulmonary venous connection; TOF, tetralogy of Fallot; PDA, patent ductus arteriosus; CMV, cleft mitral valve; CVD, cardiac valve defect.

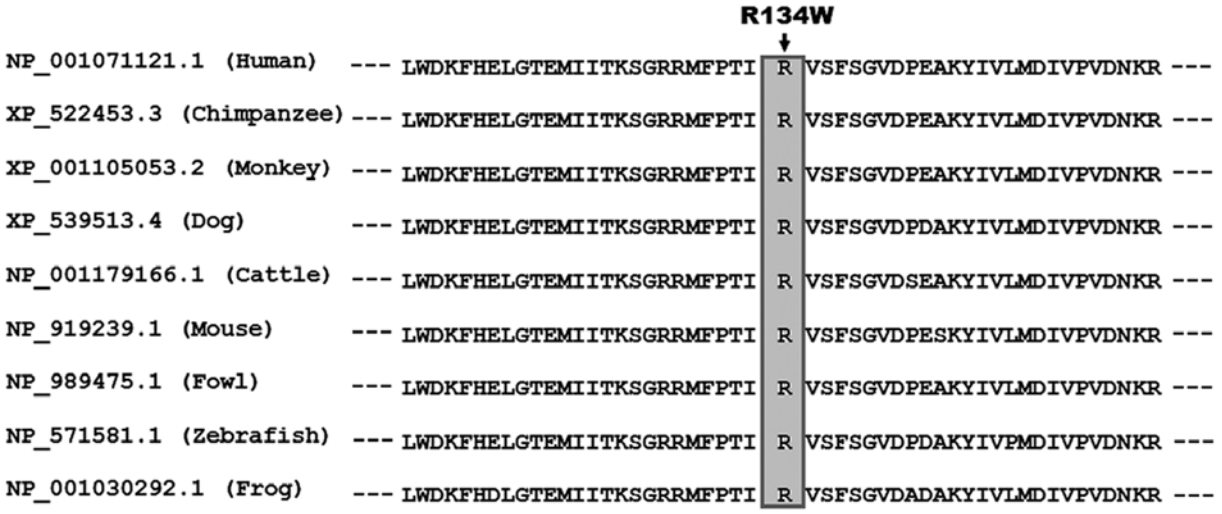

Figure 4. Alignment of multiple TBX20 amino acid sequences across species. The altered arginine at amino acid position 143 (p.R143) of TBX20 is completely conserved evolutionarily among various species.

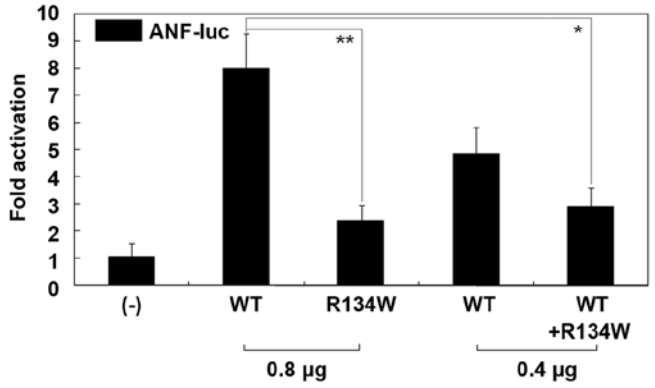

Figure 5. Diminished transcriptional activity of TBX20 caused by the mutation. Activation of the ANF-luciferase reporter in cultured Chinese hamster ovary (CHO) cells by wild-type TBX20 (WT) or the mutant (R143W), alone or in combination, showed significantly diminished transcriptional activity by the mutant protein. Experiments were performed in triplicate with mean and standard deviations presented. *,**P<0.005 when compared with wild-type TBX20.

homeobox 5 (NKX2-5), GATA binding protein (GATA)4 and GATA5, collaborating to synergistically activate cardiac gene expression during embryogenesis (57), and multiple mutations in NKX2-5 (44,70-73), GATA4 (37,40,41,48,74-79) and GATA5 (42,43,46,49,50) have been causally linked to various types of CHD, which suggests that TBX20 loss-of-function mutation predisposes to CHD, possibly by reducing the synergistic activation of cardiac target genes with these partners.

It is interesting to note that ventricular septal defect was also observed in 2 mutation carriers with DORV. In fact, 9 mutations in the coding region of *TBX20* (Fig. 2) have been previously associated with various congenital cardiovascular deformities. In a previous study, Kirk *et al* (62) screened the entire coding region of *TBX20* by direct DNA sequencing in 353 probands with CHD, and found 2 heterozygous mutations (p.I152M and p.Q195X) within exons encoding the T-box DNA-binding domain in 2 probands with familial CHD, with

a mutational prevalence of approximately 0.57%. Specifically, in family 1 carrying the missense change, p.I152M, which segregated with CHD over 3 generations, the proband had atrial septal defect; her grandmother had a small ventricular septal defect, and her mother had a large patent foramen ovale with a permanent left-to-right blood shunt. In family 2, the nonsense change, p.Q195X, which truncates TBX20 within the T-box DNA-binding domain, was present in 2 living affected individuals, of whom the proband had a small atrial septal defect, as well as mild coarctation of the aorta, and his mother had marked mitral valve prolapse with mild regurgitation. Functional assays revealed that I152M-mutant TBX20 had reduced function while Q195X-mutant TBX20 was severely compromised and most likely effectively null (62). In another study, using PCR sequencing, Liu *et al* (63) screened the exons 2 to 6 of *TBX20* (which code for the T-box DNA-binding domain) in 203 unrelated patients with CHD, and discovered 3 non-synonymous mutations of p.A63T, p.I121F and p.T262M in 3 patients atrial septal defect, total anomalous pulmonary venous connection and tetralogy of Fallot, respectively, with a mutational prevalence of approximately 1.48%. However, a functional analysis of these new sequence variants remains to be carried out. In their study, Qian *et al* (64) scanned *TBX20* in 192 unrelated children with CHD, and identified 2 mutations in 4 children, with a mutational prevalence of approximately 2.08%, including p.H186D found in 2 unrelated children (1 child had an atrioventicular canal with secundum and primum atrial septal defects, as well as a cleft mitral valve with moderate mitral regurgitation; another child had pentalogy of Fallot) and p.L197P identified in a female with pentalogy of Fallot and in another female with isolated atrial septal defect. However, the functional effects of the 2 mutations have not been characterized. Previously, Posch *et al* (65) sequenced the coding region of *TBX20* in 170 unrelated patients with secundum atrial septal defect, and found a novel mutation, p.I121M in an index patient, with a mutational prevalence of approximately 0.59%. Genetic analysis of the pedigree of the proband revealed that the mutation co-segregated with CHD in the third generation kindred. A functional analysis demonstrated that TBX20-I121M resulted in a significantly enhanced transcriptional activity, which was further increased in the presence of co-transcription factors GATA4/5 and NKX2-5. By whole-exome sequencing in combination with a CHD-related gene filter, Liu *et al* (67) analyzed a family of 3 generations with atrial septal defect, and identified a novel TBX20 mutation, p.D176N. This mutation was predicted to be deleterious by bioinformatics programs (SIFT, PolyPhen-2 and MutationTaster), but experimental research of the mutation was not performed. These CHD-associated TBX20 mutations mentioned above are summarized in Table IV. Taken together, these findings highlight the exquisite sensitivity of the developing cardiovascular system to the level of TBX20, suggesting the pivotal role of TBX20 in the cardiovascular development.

In conclusion, to the very best of our knowledge, this is the first study on the association of TBX20 loss-of-function mutation with increased susceptibility to DORV in humans, which provides novel insight into the molecular mechanisms underlying DORV and suggests the potential implications for genetic counseling and medical care of the families affected by DORV.

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