HAND1 loss-of-function mutation contributes to congenital double outlet right ventricle

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Abstract. Congenital heart defects (CHDs), a wide variety of developmental abnormalities in the structures of the heart and the great thoracic blood vessels, are the most common form of birth defect in humans worldwide. CHDs are accountable for substantial morbidity and are still the leading cause of birth defect-related deaths. Recent studies have demonstrated the pivotal roles of genetic defects in the pathogenesis of CHDs, and a great number of genetic mutations have been associated with CHDs. Nevertheless, CHDs are a genetically heterogeneous disorder and the genetic basis underlying CHDs in an overwhelming majority of cases remains unclear. In the present study, the coding exons and flanking introns of the heart and neural crest derivatives expressed transcript 1 (*HAND1*) gene, which encodes a basic helix-loop-helix transcription factor crucial for cardiovascular development, were sequenced in 158 unrelated patients with CHDs, and a *de novo* heterozygous mutation, p.K132X, was identified in a patient with double outlet right ventricle (DORV), as well as ventricular septal defect. The nonsense mutation, which was predicted to produce a truncated HAND1 protein lacking 84 carboxyl-terminal amino acids, was absent in 600 control chromosomes. Functional analyses revealed that the HAND1 K132X mutant had no transcriptional activity. Furthermore, the mutation disrupted the synergistic activation between HAND1 and GATA binding protein 4 (GATA4), another cardiac core transcription factor causally linked to CHDs. To the best of our knowledge, this is the first report on the association of *HAND1* loss-of-function mutation with an enhanced susceptibility to DORV in humans. These findings expand the phenotypic spectrum linked to *HAND1* mutations, suggesting potential implications for the development of novelo prophylactic and therapeutic strategies for DORV.

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Introduction

Congenital heart defects (CHDs), a series of developmental anomalies in the structures of the heart and the great endothoracic blood vessels, encompassing ventricular septal defect (VSD), atrial septal defect, tetralogy of Fallot, double outlet right ventricle (DORV), transposition of the great arteries, pulmonary atresia and persistent truncus ateriosus, are the most common form of birth defects in humans, with an estimated prevalence of 1% in live births worldwide (1). It was reported that in 2013, there were >34 million individuals living with CHDs worldwide (2). Severe CHDs may result in reduced exercise performance and quality of life (3-6), fetal brain injury and neurodevelopmental delay (7,8), pulmonary hypertension and Eisenmenger syndrome (9), cardiac enlargement and heart failure (10,11), cardiac arrhythmias and sudden cardiac death (12-14). In fact, CHDs are responsible for approximately 30% of all birth defect-related deaths (1). Globally in 2010, CHDs led to approximately 223,000 deaths (15). Although great advances in cardiac surgical techniques and intensive care have allowed the overwhelming majority of children with CHDs to survive into adulthood, unfortunately, the late morbidity and mortality rates are still high in the survivors (16-18). Therefore, CHDs have laid a heavy economic

burden on patients and healthcare systems, and this burden is anticipated to be even heavier in the future due to an increasing number of CHDs in adults (16-18). Despite the important clinical significance, the causes of CHDs among most patients remain largely unclear.

It has been previously reported that non-inherited modifiable factors, including maternal illnesses, nutritional deficiencies, and exposure to drugs, toxicants or polluted air during the first trimester of pregnancy may confer an increased vulnerability to CHDs (19). However, a growing body of evidence strongly suggests that genetic defects are the predominant cause of CHDs, and mutations in a great number of genes, particularly those coding for transcription factors essential for cardiovascular morphogenesis, including NK2 homeobox (NKX2)-5, NKX2-6, GATA binding protein (GATA)4, GATA5, GATA6, T-Bo (TBX)1, TBX5, TBX20, paired like homeodomain 2 (PITX2) and heart and neural crest derivatives expressed transcript (HAND)2, have been associated with various CHDs (20-57). Nevertheless, CHDs are a genetically heterogeneous disorder, and the genetic basis underlying CHDs in an overwhelming majority of cases remains to be elucidated.

The HAND subset of basic helix-loop-helix (bHLH) transcription factors is composed of two members, HAND1 and HAND2, which are required for the normal cardiovascular development in fish, chicks, rodents and humans (58). The HAND1 protein has a functionally important structural domain termed bHLH, which consists of a short stretch of basic amino acids followed by an amphipathic α helix, a loop and an additional α helix, and is required for binding to target gene DNA and protein-protein combinatorial interactions (59). A previous study demonstrated that HAND1 can directly activate the cardiac *ANF* promoter, alone or in synergy with transcriptionally cooperative partners, including GATA4, myocyte enhancer factor 2 (MEF2) and HAND2 (60). In chicks, HAND1 and HAND2 are both expressed in the bilateral heart primordia and subsequently throughout the primitive tubular heart, as well as its derivatives during embryonic genesis, and the treatment of chick embryos with *HAND1* and *HAND2* antisense oligonucleotides has revealed that either oligonucleotide alone has no effect on embryonic development, whereas together they arrest development at the looping heart tube stage (61). In mice, HAND1 is highly expressed in distinct regions of the linear heart tube during embryogenesis and, after looping, becomes localized to both primary heart fields, specifically in the outer curvature of the presumptive left ventricle and the developing outflow tract, and also at a lower level in the outer curvature of the right ventricle (62). Mice lacking *Hand1* suffer from defective cardiac looping, failed chamber septation, anomalous ventricular myocardial differentiation and early embryonic lethality resulting from cardiac failure (63,64). Mouse embryos homozygous for the cardiac-specific *Hand1*-null allele present diverse cardiac deformations, including membranous VSD, overriding aorta and hyperplastic atrioventricular valves, and DORV (65). In humans, HAND1 is expressed in cardiac tissues within both ventricles (66), and mutations in *HAND1* have been causally linked to hypoplastic hearts and cardiac septal defects (67-69). However, the prevalence and spectrum of *HAND1* mutations in other cohorts of patients with various CHDs remain to be investigated.

Thus, in this study, the coding exons and flanking introns of the *HAND1* gene, which encodes a basic helix-loop-helix transcription factor crucial for cardiovascular development, were sequenced in 158 unrelated patients with CHDs. We identified a *de novo* heterozygous mutation, p.K132X in a patient with DORV. Thus, this mutation may be associated with an enhanced susceptibility to DORV. Our findings may provide new insight into the pathogenesis of DORV and CHDs at the genetic level.

Materials and methods

Ethics. This study was conducted in conformity with the ethical principles for medical research outlined in the Declaration of Helsinki. The study protocol was approved by the local institutional Ethics Committee of Tongji Hospital, Tongji University, Shanghai, China [approval no. LL(H)-09-07], and written informed consent was obtained from the patients or their guardians prior to the study.

Study population. A cohort of 158 unrelated patients (87 males and 71 females, with an average age of 3.3 years) with non-syndromic CHDs was recruited from the Chinese Han population. The available relatives of the CHD cases were also enlisted. All patients underwent a comprehensive clinical evaluation, including medical history, complete physical examination, 12-lead electrocardiogram and two-dimensional transthoracic echocardiography with color flow Doppler. Cardiac catheterization, angiography and cardiac magnetic resonance imaging were carried out only when indicated. The cardiac phenotype was confirmed by echocardiography in all patients with CHDs. Patients with known chromosomal abnormalities or other recognized syndromic CHDs were excluded from the study. A total of 300 healthy subjects (162 males and 138 females, with an average age of 3.2 years), who were matched to the patietns with CHD in age, gender and ethnicity, were enrolled as controls. All the control individuals underwent transthoracic echocardiography and their cardiac morphologic structures were shown to be normal.

Mutational analysis of HAND1. Peripheral venous blood samples were drawn from the study participants and the genomic DNA was isolated from leukocytes using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA) according to the manual of procedure. The referential genomic DNA sequence of *HAND1* was derived from GenBank (GenBank ID: NC_000005.10), an online nucleotide database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/nucleotide/). With the aid of the online Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?ORGANISM=9606&INPUT_SEQUENCE=NC_000005.10&LINK_LOC=nuccore&PRIMER5_START=154474972&PRIMER3_END=154478264), the primers used to amplify the coding exons and splicing junction sites of *HAND1* by polymerase chain reaction (PCR) were designed as shown in Table I. PCR was carried out using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a Veriti Thermal Cycler (Applied Biosystems, Foster, CA, USA). The amplicons were fractionated by electrophoresis on a 2% agarose gel and then purified with the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The purified amplicons were

Table I. Primers used for the amplification of the coding regions and splicing junction sites of the *HAND1* gene.

Coding exon	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)
1	GAGCGGCGTTAATAGGGCTG	TTCGACTACCTGCATGGCCT	666
2	GGAACTCCGCGCATAAAGGC	CGTGCGATCCAAGTGTGTGG	478

bp, base pairs.

PCR-sequenced under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems) with BigDye® Terminator v3.1 Cycle Sequencing kits (Applied Biosystems). The DNA sequences were analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). For an identified sequence variance, the Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/), the 1000 Genomes Project (1000GP; http://www.1000genomes.org/data) database, the NCBI's single nucleotide polymorphism (SNP; http://www.ncbi.nlm.nih.gov/snp) database and PubMed (http://www.ncbi.nlm.nih.gov/pubmed) database were consulted to confirm its novelty.

Alignment of multiple HAND1 protein sequences among species. The amino acid sequences of the HAND1 protein in humans were aligned with those in the chimpanzee, monkey, cattle, mouse, rat, fowl, fruitfly and frog using MUSCLE, an online program (http://www.ncbi.nlm.nih.gov/homologene), in order to show the evolutionary conservation for an altered amino acid.

Expression plasmids and site-directed mutagenesis. The recombinant expression plasmid, HAND1-pcDNA3.1, which contains the full-length cDNA of human *HAND1*, was constructed as previously described (69). The identified mutation was introduced into the wild-type HAND1-pcDNA3.1 plasmid by site-directed mutagenesis using a QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and a complementary pair of primers, and was verified by sequencing. The expression plasmid GATA4-pSSRa and the ANF-luciferase reporter (ANF-luc) plasmid, which contains the 2600-bp 5'-flanking region of the *ANF* gene and expresses the Firefly luciferase, were kind gifts from Dr Ichiro Shiojima at Chiba University School of Medicine (Chiba, Japan).

Cell culture, DNA transfection and luciferase assays. HeLa and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and plated at a density of $1x10^5$ cells per well on 24-well plates 24 h prior to transfection. Transfection was performed using Lipofectamine® 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The internal control vector pGL4.75 (Promega Corp.), which expresses the *Renilla* luciferase, was co-transfected in transfection assays to normalize transfection efficiency. For the transfection of HeLa cells, 1.0 μg of empty pcDNA3.1 vector, 1.0 μg of wild-type HAND1-pcDNA3.1, 1.0 μg of mutant HAND1-pcDNA3.1, 0.5 μg of wild-type HAND1-pcDNA3.1, or 0.5 μg of wild-type HAND1-pcDNA3.1 plus 0.5 μg of mutant HAND1-pcDNA3.1 were used in combination with

Table II. Clinical characteristics of the patients with congenital heart defects.

Parameters	No. or mean	% or range
Male/female	87/71	55/45
Age, years	3	0-12
Positive family history of CHDs	11	7
Distribution of distinct forms of CHDs		
Isolated CHDs	95	60
VSD	31	20
ASD	19	12
PDA	13	8
PS	10	6
DORV	5	3
TGA	4	3
PTA	3	2
HLV	3	2
PA	2	1
TAPVC	2	1
CoA	2	1
CAC	1	1
Complex CHDs	63	40
TOF	25	16
VSD + ASD	15	9
DORV + VSD	13	8
VSD + PDA	8	5
PTA + VSD	1	1
TGA + VSD	1	1
Incidence of arrhythmias		
Atrioventricular block	8	5
Atrial fibrillation	5	3
Treatment		
Surgical repair	92	58
Percutaneous closure	32	22
Follow-up	31	20

ASD, atrial septal defect; CAC, common atrioventricular canal; CHDs, congenital heart defects; CoA, coarctation of the aorta; DORV, double outlet right ventricle; HLV, hypoplastic left ventricle; PA, pulmonary atresia; PDA, patent ductus arteriosus; PS, pulmonary stenosis; PTA, persistent truncus arteriosus; TAPVC, total abnormal pulmonary venous connection; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

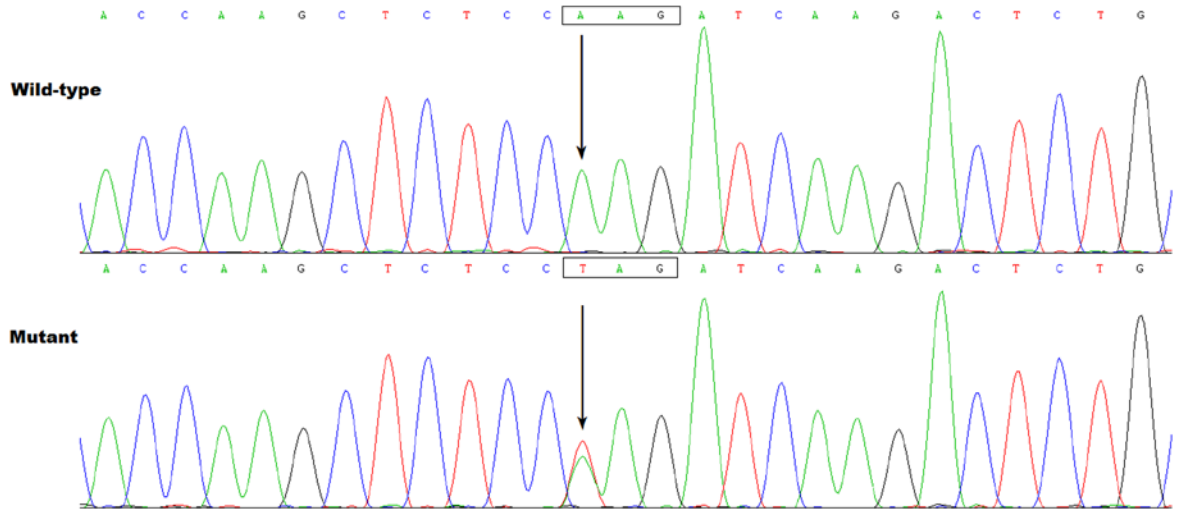

Figure 1. Sequence electropherograms exhibiting the *HAND1* mutation and its wild-type. The arrows indicate the heterozygous nucleotides of T/A in the mutation carrier (mutant) or the homozygous nucleotides of A/A in the corresponding control individual (wild-type). The rectangle marks the nucleotides comprising a codon of *HAND1*.

1.0 μg of ANF-luc and 0.04 μg of pGL4.75. For the transfection of NIH3T3 cells, the same amount (0.5 μg) of plasmid DNA (empty pcDNA3.1 vector, wild-type HAND1-pcDNA3.1, GATA4-pSSRa or mutant HAND1-pcDNA3.1) was used alone or in combination, in the presence of 1.0 μg of ANF-luc and 0.04 μg of pGL4.75. The transfected cells were incubated for 48 h at 37˚C with 5% CO_2, then washed and lysed using 1X passive lysis buffer provided by the Dual-Glo luciferase reporter assay kit (Promega Corp.). The Firefly and *Renilla* luciferase activities were measured using the Dual-Glo luciferase reporter assay kit (Promega Corp.) according to the manufacture's instructions using a GloMax® 96 Luminometer (Promega Corp.). The activity of the *ANF* promoter was expressed as the fold activation of the Firefly luciferase value relative to the *Renilla* luciferase value. At least 3 independent transfection experiments, all of which were conducted in triplicate, were performed to calculate average values and standard deviations.

Statistical analysis. Statistical analyses were performed using the SPSS version 17.0 software package (SPSS, Inc., Chicago, IL, USA). Data are expressed as the means and standard deviation, unless otherwise indicated. The numeric variables were compared between 2 groups using the Student's unpaired t-test. A comparison of the categorical variables between 2 groups was 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

Baseline clinical features of the study subjects. In this study, 158 unrelated patients with isolated CHDs (87 males and 71 females, with an average age of 3.3 years) were clinically evaluated in contrast with 300 ethnically-matched, unrelated healthy individuals (162 males and 138 females, with an average age of 3.2 years). All the patients had echocardiographically documented CHDs. Of the 158 patients with CHD, 11 (approximately 7%) had a positive family history of CHDs. The control individuals were physically and mentally healthy with no structural cardiac defects confirmed by echocardiogram, and they had a negative family history of CHDs. There were no significant differences between the patient and control groups as regards demographic characteristics, including age, gender and ethnicity. The baseline clinical characteristics of the 158 unrelated patients with CHDs are presented in Table II.

Discovery of a de novo HAND1 mutation. By PCR sequencing, a heterozygous mutation in *HAND1* was identified in one of the 158 unrelated patients with isolated CHDs, with a mutational prevalence of approximately 0.63%. Specifically, a substitution of thymine (T) for adenine (A) in the first nucleotide of codon 132 (c.394A>T), predicting the conversion of the codon coding for lysine (K) into a stop codon (X) at amino acid position 132 (p.K132X), was discovered in a 5-month-old boy affected with congenital DORV, as well as VSD, who had no family history of CHDs. The nonsense mutation was neither found in the mutation carrier's healthy parents, nor detected in the 300 unrelated control individuals, indicating it is a *de novo* mutation. The sequence chromatograms showing the heterozygous *HAND1* mutation of c.394A>T, as well as its control sequence are shown in Fig. 1. A schematic diagram of HAND1 showing the bHLH structural domain and the location of the identified mutation is shown in Fig. 2. The identified *HAND1* mutation c.394A>T has not been reported in the HGMD, 1000GP, SNP and PubMed databases (accessed on September 12, 2016), suggesting that it is a novel mutation.

Alignment of multiple HAND1 protein sequences across species. Multiple alignments of the HAND1 protein sequences among various species displayed that the altered lysine at amino acid position 132 (p.K132) was completely conserved evolutionarily (Fig. 3).

No transcriptional activity of the mutant HAND1 protein. As shown in Fig. 4, dual-luciferase assays in the cultured HeLa cells revealed that the same amount (1.0 μg) of wild-type and

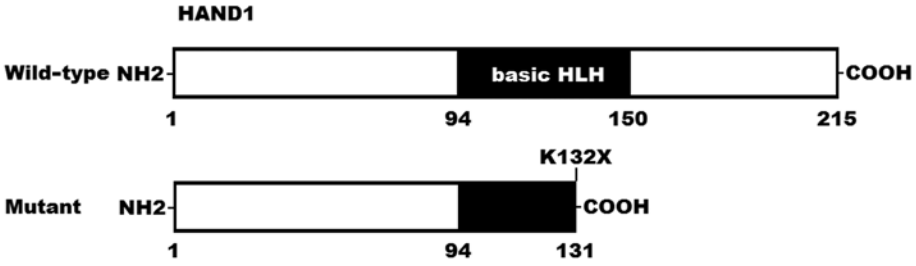

Figure 2. Schematic diagram depicting the HAND1 protein structures with the identified mutation indicated. The mutation identified in a patient with double outlet right ventricle as well as ventricular septal defect is shown above the structural domain. NH2, amino-terminus; bHLH, basic helix loop helix; COOH, carboxyl-terminus.

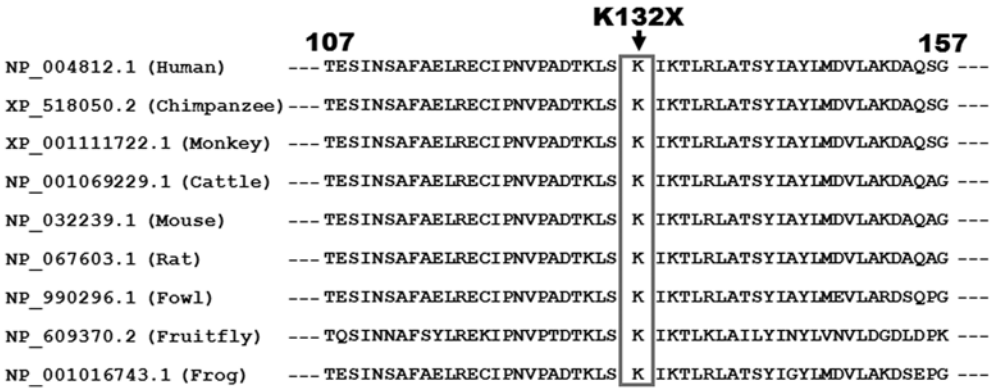

Figure 3. Alignment of multiple HAND1 protein sequences across species. The altered lysine at amino acid 132 (p.K132) is completely conserved evolutionarily among various species.

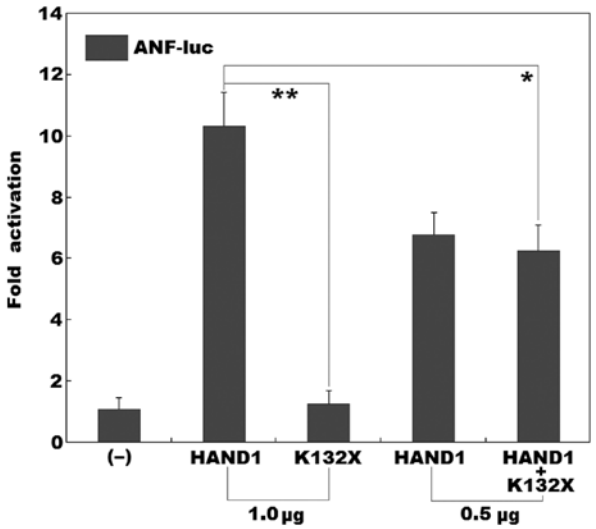

Figure 4. No transcriptional activity of the mutant HAND1 protein. HeLa cell transfection assays measuring activation of the *ANF* promoter revealed that the K132X-mutant HAND1 protein had no transcriptional activity. Experiments were performed in triplicate, and mean and standard deviations are given. **P<0.001, *P<0.01.

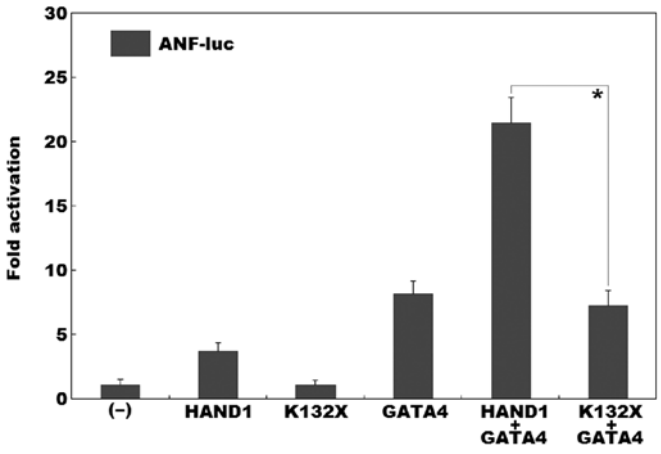

Figure 5. Synergistic transcriptional activation disrupted by the mutation. NIH3T3 cell transfection assays measuring synergistic activation of the *ANF* promoter between HAND1 and GATA4 unveiled disrupted synergistic activation by the K132X mutation. Experiments were performed in triplicate, and mean and standard deviations are given. *P<0.001.

K132X-mutant HAND1-pcDNA3.1 plasmids transcriptionally activated the *ANF* promoter by approximately 10-fold and approximately 1-fold, respectively. When 0.5 μg of wild-type HAND1-pcDNA3.1 was used together with the same amount (0.5 μg) of 132X-mutant HAND1-pcDNA3.1, the induced activation of the *ANF* promoter was approximately 6-fold.

Synergistic transactivational failure caused by the mutation. As shown in Fig. 5, dual-luciferase assays in the cultured NIH3T3 cells revealed that the same amount (0.5 μg) of wild-type and K132X-mutant HAND1 activated the *ANF* promoter by approximately 4-fold and approximately 1-fold, respectively; while in the presence of 0.5 μg of wild-type *GATA4*, the same amount (0.5 μg) of wild-type and K132X-mutant HAND1

activated the *ANF* promoter by approximately 22-fold and approximately 7-fold, respectively.

Discussion

In the current study, a novel heterozygous mutation, p.K132X, was identified in a patient with isolated DORV, as well as VSD. The nonsense mutation, which was absent in the 600 reference chromosomes, altered the amino acid that was completely conserved evolutionarily across species, and was predicted to generate a truncated protein with partial bHLH domain left. Functional tests revealed that the K132-mutant HAND1 protein had no transcriptional activation of the *ANF* promoter. Furthermore, the mutation abrogated the synergistic activation of the *ANF* promoter between HAND1 and GATA4. Therefore, it is likely that the identified *HAND1* mutation predisposes to DORV, as well as VSD.

In humans, the *HAND1* gene, as the *eHAND* gene, is located at chromosome 5q33, coding for a transcription factor protein of 215 amino acids. In this study, the *HAND1* mutation identified in a patient with CHD was predicted to generate a truncated protein losing the partial bHLH domain; thus, it is reasonably anticipated to disable *HAND1*. Functional analyses substantiated that the mutant *HAND1* lost the transcriptional activation of the *ANF* promoter. Furthermore, the mutation disrupted the synergistic activation between HAND1 and GATA4, another cardiac core transcription factor previously associated with CHDs in humans (70). These findings strongly suggest that haploinsufficiency caused by the *HAND1* mutation is probably an alternative pathological mechanism of CHDs.

Somatic or germline mutations in *HAND1* have been previously associated with various CHDs in humans (67-69). By direct PCR sequencing of the *HAND1* gene in human heart tissues derived from 31 unrelated patients with hypoplastic hearts, Reamon-Buettner *et al* (67) identified a common frameshift mutation (p.A126fsX12) in 24 of 31 hypoplastic left or right ventricles. Luciferase assays revealed that the resulting mutant protein was unable to modulate the transcription of reporter genes, suggesting that functionally impaired *HAND1* leads to hypoplastic human hearts. Subsequently, Reamon-Buettner *et al* (67) sequenced *HAND1* in a cohort of 68 malformed hearts affected primarily by septation defects, and detected 32 different nonsynonymous mutations, of which 12 are in the bHLH domain of HAND1. Functional analyses using yeast and mammalian cells have revealed that the transcriptional activity of HAND1 is reduced or abolished by certain mutations, suggesting that genetically compromised *HAND1* may also be responsible for septation defects of the human hearts. Chen *et al* (68) screened the coding regions of *HAND1* in 498 unrelated individuals affected with non-syndromic CHDs, and found 2 novel non-synonymous mutations of p.G73S and p.K152N in 2 patients suffering from VSD, respectively. Yeast two-hybrid and liquid β-galactosidase assays indicated that both mutations increased the transcriptional activity of HAND1, probably by enhancing the capability of HAND1 to form homodimers. In this study, a *de novo* HAND1 mutation of p.K132X was discovered in a patient with DORV and VSD, thus expanding the clinical phenotypic spectrum linked to *HAND1* mutations. Taken collectively, these findings highlight the exquisite sensitivity of the developing cardiovascular system to the function of *HAND1*, suggesting a key role of *HAND1* in human heart development and CHDs.

In conclusion, this study firstly associates *HAND1* loss-of-function mutation with enhanced susceptibility to DORV and VSD, which adds significant insight to the molecular pathogenesis underpinning CHDs, suggesting potential implications for precise diagnosis and genetic counseling of patients with CHD.

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