

A novel HAND2 loss-of-function mutation responsible for tetralogy of Fallot

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Abstract. Congenital heart disease (CHD), the most common type of developmental abnormality, is associated with substantial morbidity and mortality in humans worldwide. The basic helix-loop-helix transcription factor, heart and neural crest derivatives expressed 2 (HAND2), has been demonstrated to be crucial for normal cardiovascular development in animal models. However, whether a genetically defective HAND2 contributes to congenital heart disease (CHD) in humans remains to be explored. In this study, the entire coding region and splicing boundaries of the *HAND2* gene were sequenced in a cohort of 145 unrelated patients with CHD. A total of 200 unrelated, ethnically-matched healthy individuals used as controls were also genotyped for *HAND2*. The functional effect of the mutant HAND2 was characterized in contrast to its wild-type counterpart by using a dual-luciferase reporter assay system. As a result, a novel heterozygous HAND2 mutation, p.L47P, was identified in a patient with tetralogy of Fallot (TOF). The misense mutation, which altered the amino acid conserved evolutionarily among species, was absent in 400 control chromosomes. Functional analyses unveiled that the mutant HAND2 had a significantly decreased transcriptional activity. Furthermore, the mutation markedly reduced

the synergistic activation between HAND2 and GATA4 or NKX2.5, other two cardiac key transcription factors involved in the pathogenesis of CHD. To the best of our knowledge, this study is the first to report the association of a HAND2 loss-of-function mutation with an increased vulnerability to TOF in humans, which provides novel insight into the molecular mechanism underpinning CHD, suggesting potential implications for the genetic counseling of families with CHD.

Introduction

Congenital heart disease (CHD) represents the most common form of birth defect, accounting for approximately one-third of all major congenital abnormalities, and each year, approximately 1.35 million infants are born with CHD worldwide (1). The estimated prevalence of CHD is 1% in live births, and up to 10% in stillbirths (2-4). In terms of specific anatomic or hemodynamic lesions, various CHDs are clinically classified into at least 21 distinct categories, including ventricular septal defect, atrial septal defect, endocardial cushion defect, tetralogy of Fallot (TOF), Ebstein's anomaly, double outlet of right ventricle, transposition of the great arteries, patent ductus arteriosus, persistent truncus arteriosus, coarctation of the aorta, aortic stenosis, pulmonary atresia, tricuspid atresia, interrupted aortic arch, total anomalous pulmonary venous connection and hypoplastic left heart syndrome, of which TOF is the most common type of cyanotic CHD, accounting for approximately 10% of all CHD cases (4). Severe CHD may give rise to a diminished quality of life, decreased exercise performance, retarded fetal brain development, depression, infective endocarditis, thromboembolism, pulmonary arterial hypertension, Eisenmenger's syndrome, heart failure, arrhythmias and even death (4-11). Hence, CHD is responsible for substantial morbidity and mortality, which lays a heavy economic burden on patients and health care systems (4). Despite important clinical significance, the etiologies of CHD remain largely unknown.

Cardiogenesis from the early embryo to the formation of a fully functional four-chambered heart is a complex and dynamic process that necessitates a harmonious concerto of transcription factors, adhesion molecules, ion channels,

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signaling molecules and structural proteins, and both environmental and genetic risk factors may disrupt this biological process of heart development, resulting in a wide variety of CHDs (12). Although environmental exposures are also relevant, a growing number of studies have demonstrated that genetic defects are the leading cause of CHD, and thus far, mutations in >60 genes have been causally linked to CHD (13-25). Among these CHD-causative genes, those encoding cardiac transcription factors, including homeodomain-containing protein, NK2 homeobox 5 (NKX2.5), GATA-binding protein 4 (GATA4) and T-box transcription factor 5 (TBX5), are the most commonly involved genes in the pathogenesis of CHD, underscoring the pivotal roles of cardiac transcription factors in cardiovascular development and disease (26).

The basic helix loop helix family of transcription factors, including heart and neural crest derivatives expressed (HAND)1 and HAND2, the only two members identified up to now, has been substantiated to be essential for normal cardiovascular development, with either *Hand1*- or *Hand2*-deficient mice not surviving due to cardiovascular developmental abnormalities (27). In humans, gain- or loss-of-function mutations in *HAND1* have been associated with various CHDs, encompassing hypoplastic left heart syndrome, ventricular septal defect, atrial septal defect and atrioventricular septal defect (28-30). Considering that the expression profiles and functional roles of *HAND2* overlap at least in proportion to those of *HAND1* (27,31-35), we hypothesized that genetically compromised *HAND2* may contribute to the development of CHD in a subset of patients.

Materials and methods

Study subjects. A total of 145 unrelated patients with CHD were enrolled in this study. The available family members of the index patient who carried an identified *HAND* mutation were also included. A total of 200 unrelated individuals without CHD, who were matched to the CHD patients in ethnicity and gender, were recruited as the controls. All the study subjects were from the Han Chinese population. They underwent a comprehensive clinical evaluation, including medical history, physical examination, transthoracic echocardiography, standard 12-lead electrocardiogram and chest X-ray radiography. The clinical types of CHD were defined with two-dimensional continuous wave Doppler and color Doppler techniques on transthoracic echocardiography. When indicated, transesophageal echocardiography, cardiac catheterization and angiography were performed to further clarify the cardiovascular anatomic malformations. Cardiac surgery was carried out in some of the patients with CHD. The patients who suffered from chromosomal abnormalities or syndromic cardiovascular anomalies, such as Axenfeld-Rieger syndrome, DiGeorge syndrome, Alagille syndrome and Holt-Oram syndrome, were excluded from the current study. This study is in conformity with the principles of the Declaration of Helsinki. 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). Written informed consent was obtained from the participants or their guardians prior to the commencement of the study.

Genetic analysis of *HAND2*. Whole blood samples from the patients with CHD and the control individuals were collected. Genomic DNA was isolated from blood leukocytes using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA), according to the manufacture's instructions. With the aid of online Primer 3 (<http://primer3.ut.ee>), the primers used for the amplification of the coding exons and flanking introns of *HAND2* by polymerase chain reaction (PCR) were designed as shown in Table I. The referential genomic DNA sequence of *HAND2* was from GenBank (accession no. NC_000004). PCR was conducted using a standard procedure on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Basically, a PCR mixture consisted of 1X PCR buffer, 1X Q solution, 5 pmol of each primer pairs, 0.2 mM dNTPs, 50 ng of genomic DNA and 1 unit of HotStar TaqDNA polymerase (Qiagen, Hilden, Germany), to a volume of 25 μ l with double distilled water. A typical PCR program was an initial activation of the polymerase (Qiagen) at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 1 min, and elongation at 72°C for 1 min, with a final extension at 72°C for 6 min. The PCR-amplified fragments were purified and sequenced with *HAND2*-specific primers using the BigDye[®] Terminator v3.1 Cycle Sequencing kit on an ABI PRISM 3130 XL DNA Analyzer (both from Applied Biosystems). For an identified mutation in the coding region of *HAND2*, the numbering of it started with the nucleotide A of the initial translation codon ATG (accession no. NM_021973.2). To confirm the novelty of an identified sequence variation, the single nucleotide polymorphism (SNP; <http://www.ncbi.nlm.nih.gov/SNP>) database, the human genome mutation database (HGMD; <http://www.hgmd.org/>), the 1000 genomes project database (1000 Genomes; <http://www.1000genomes.org>) and the exome variant server (EVS; <http://evs.gs.washington.edu/EVS>) database were queried.

Alignment of multiple amino acids of *HAND2* proteins across species. The amino acids of *HAND2* proteins from various species were aligned with the online MUSCLE program (http://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&dopt=MultipleAlignment&list_uids=32092).

In silico analysis of *HAND2* mutation. The functional consequence of an identified sequence variation on *HAND2* protein was predicted by MutationTaster (<http://www.mutationtaster.org/>), PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and SIFT (<http://sift.jcvi.org>).

Expression plasmids and site-directed mutagenesis. The human cardiac full-length cDNA was prepared as previously described (22,23,36-38). Human *HAND2* harboring the whole coding region was generated by PCR with the human heart cDNA as a template, cut with the restriction enzymes, *EcoRI* and *NotI*, and then subcloned at the *EcoRI-NotI* sites of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). The identified mutation was introduced into the wild-type *HAND2*-pcDNA3.1 construct by site-directed mutagenesis using a complementary pair of primers and the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA), and verified by direct sequencing. The recombinant expression plasmids GATA4-pSSRa and NKX2.5-pEFSA, and the ANF-luciferase

Table I. Primers to amplify the coding exons and flanking introns of the *HAND2* gene.

Coding exon	Forward primer	Reverse primer	Amplicon size (bp)
1-a	5'-cgagaggattctgcctccgc-3'	5'-acagggccatgctgtagtcg-3'	550
1-b	5'-ggtaggtggtttccccacca-3'	5'-gcccaattggaagaggccg-3'	624
2	5'-ggttcactgtctcctccggc-3'	5'-cgggatcccttaccacacgg-3'	483

bp, base pairs.

Table II. Baseline clinical characteristics of the study patients with CHD (n=145).

Variables	Statistics
Age (years)	3.68±1.52
Male (%)	78 (54)
Positive family history of CHD (%)	6 (4)
Distribution of different types of CHD	
Isolated CHD (%)	93 (64)
VSD (%)	32 (22)
ASD (%)	26 (18)
PDA (%)	12 (8)
DORV (%)	5 (3)
ECD (%)	4 (3)
PTA (%)	4 (3)
Other isolated CHD (%)	10 (7)
Complex CHD (%)	52 (36)
TOF (%)	25 (17)
ASD + VSD (%)	8 (6)
VSD + DORV (%)	5 (3)
VSD + AS (%)	4 (3)
VSD + TGA (%)	3 (2)
Other complex CHD (%)	7 (5)
Arrhythmias	
Cardiac conduction block (%)	6 (4)
Atrial fibrillation (%)	3 (2)
Treatment	
Cardiac surgery (%)	67 (46)
Catheter-based repair (%)	46 (32)
Follow-up (%)	32 (22)

Data are expressed as the means ± standard deviation, number or percentage. CHD, congenital heart disease; VSD, ventricular septal defect; ASD, atrial septal defect; PDA, patent ductus arteriosus; DORV, double outlet right ventricle; ECD, endocardial cushion defect; PTA, persistent truncus arteriosus; TOF, tetralogy of Fallot; AS, aortic stenosis; TGA, transposition of the great arteries.

(ANF-luc) reporter plasmid, which contains 2,600 base pairs upstream of the transcriptional start site of the *ANF* gene and expresses Firefly luciferase, were generously provided by Dr Ichiro Shiojima from Chiba University School of Medicine, Chiba, Japan.

Cell culture and luciferase reporter assays. HeLa cells (from a cell bank of our cardiovascular research laboratory) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 µg/ml of penicillin and 100 µg/ml of streptomycin in an atmosphere of 5% CO₂ at 37°C. Cell transfection was carried out in 6-well plates using Lipofectamine® 2000 reagent (Invitrogen) 24 h after plating. The internal control plasmid, pGL4.75 (hRluc/CMV; Promega), which expresses *Renilla* luciferase, was used in the transfection assays to normalize the transfection efficiency. In the transient transfection of HeLa cells, the same amount (0.6 µg) of plasmid DNA (wild-type *HAND2*-pcDNA3.1, mutant *HAND2*-pcDNA3.1, GATA4-pSSRa or NKX2.5-pEFSA) was used alone or in combination, in the presence of 1.0 µg of ANF-luc and 0.04 µg of pGL4.75. The cells were lysed 48 h after transfection, and the Firefly and *Renilla* luciferase activities were measured using the Dual-Glo luciferase assay system (Promega) according to the manufacturer's instructions. The activity of the *ANF* promoter was expressed as the fold activation of Firefly luciferase relative to *Renilla* luciferase. Three independent experiments were performed in triplicate for each cell transfection, and each value presented was the average of triplicate samples.

Statistical analysis. Continuous data are expressed as the means ± standard deviation (SD). Differences in continuous variables between 2 groups were compared using the Student's unpaired t-test. Differences in categorical variables between 2 groups were compared using the χ^2 test or Fisher's exact test, as indicated. The significance level was set at a two-tailed P-value of <0.05. All statistical analyses were performed with SPSS version 18.0 (SPSS IBM, New York, NY, USA).

Results

Clinical characteristics of the study participants. In this study, 145 unrelated patients with CHD were clinically investigated in contrast to 200 unrelated control individuals without CHD. All the patients with CHD had congenital cardiac defects confirmed by an echocardiogram or further by cardiac surgery. Based on the medical histories and echocardiographic records, the control individuals had neither CHD nor a positive family history of CHD. There were no differences in ethnicity, gender and age between the patient and control groups. The baseline clinical characteristics of the study patients with CHD are presented in Table II.

Identification of a novel mutation in *HAND2*. By direct PCR-sequencing of the *HAND2* gene in the 145 unrelated

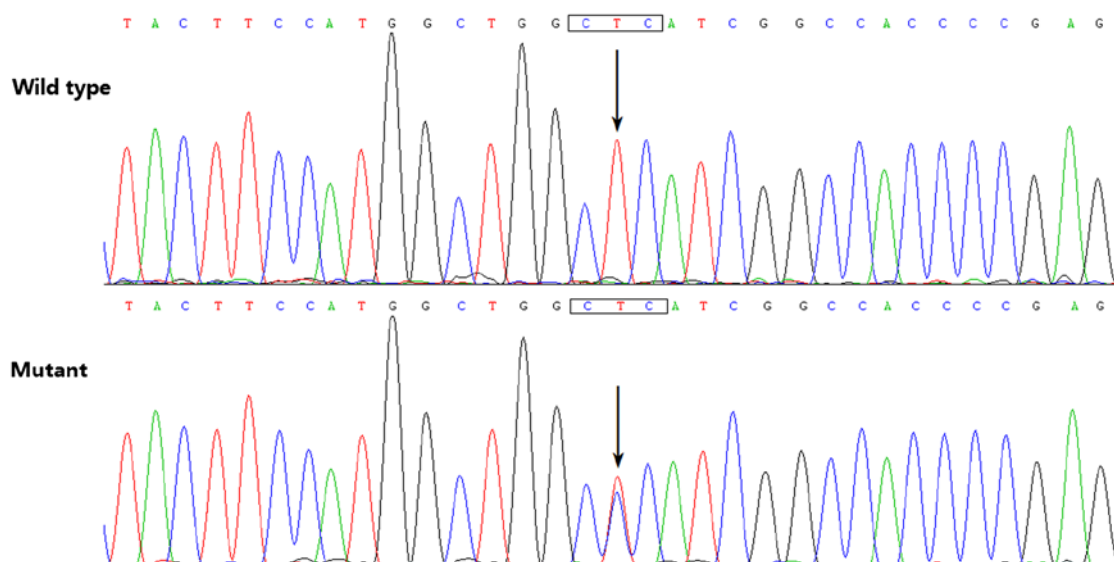


Figure 1. Sequence electropherograms displaying the heterozygous *HAND2* mutation and its wild-type control. The arrow indicates the heterozygous nucleotides of T/C in the patient with tetralogy of Fallot (mutant) or the homozygous nucleotides of T/T in the corresponding control subject (wild-type). The rectangle marks the nucleotides that comprise a codon of *HAND2*.

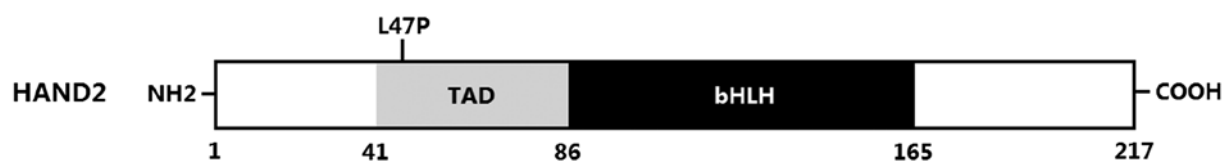


Figure 2. Schematic diagram depicting the structural domains of *HAND2*. The mutation identified in the patient with tetralogy of Fallot is shown above the structural domains. NH2, amino-terminus; TAD, transcriptional activation domain; bHLH, basic helix-loop-helix; COOH, carboxyl-terminus.

patients with CHD, a transition of thymine to cytosine in the second nucleotide of codon 47 (c.140T>C), predicting the substitution of proline at amino acid position 47 for leucine (p. L47P), was identified in a male patient with TOF, who was half a year old without a positive family history of CHD. Additionally, sequence analysis of *HAND2* in the parents of the mutation carriers revealed no mutation, indicating that the identified mutation was a *de novo* mutation. The DNA sequencing electropherograms showing the identified heterozygous *HAND2* mutation of c.140T>C in comparison with its control sequence are shown in Fig. 1. A schematic diagram of *HAND2* depicting the functionally important structural domains and the location of the mutation detected in this study is presented in Fig. 2. The missense mutation was neither observed in the 200 control individuals nor found in the SNP, HGMD, 1000 Genomes and EVS databases.

Alignment of multiple amino acids of *HAND2* proteins from various species. Alignment of the amino acids of the human *HAND2* protein with those of chimpanzee, monkey, dog, cattle, mouse, rat, zebrafish and frog exhibited that the altered leucine at amino acid residue 47 of human *HAND2* was completely conserved evolutionarily (Fig. 3).

Causative potential of the identified *HAND2* sequence variation. The *HAND2* sequence variation of c.140T>C was predicted to be disease-causing by MutationTaster, with a

P-value of 1.0000, probably damaging by PolyPhen-2, with a score of 0.999 (sensitivity 0.14; specificity 0.99) and intolerated by SIFT, with a score of 0.02.

Functional impairment of the *HAND2* protein caused by the mutation. As shown in Fig. 4, the same amount (0.6 μ g) of wild-type and L47P-mutant *HAND2* transcriptionally activated the ANF promoter by ~8- and 2-fold, respectively (wild-type vs. mutant, $t=5.6462$, $P=0.0048$). In the presence of 0.6 μ g of wild-type *GATA4*, the same amount (0.6 μ g) of wild-type and L47P-mutant *HAND2* activated the ANF promoter by ~35- and 14-fold, respectively (wild-type vs. mutant, $t=10.3947$, $P=0.0005$); while in the presence of 0.6 μ g of wild-type *NKX2.5*, the same amount (0.6 μ g) of wild-type and L47P-mutant *HAND2* activated the ANF promoter by ~24- and 11-fold, respectively (wild-type vs. mutant, $t=8.5137$, $P=0.0010$). These results reveal that the L47P-mutant *HAND2* has a significantly reduced transcriptional activity, and furthermore, the mutation markedly diminishes the synergistic activation between *HAND2* and *GATA4* or between *HAND2* and *NKX2.5*.

Discussion

TOF, characterized by four distinct anatomic features, including pulmonary outflow tract obstruction, overriding aortic root, ventricular septal defect and right ventricular hypertrophy, constitutes approximately 7-10% of all CHD cases, corre-

	24	L47P	72
NP_068808.1 (Human)	--- AAAAAAAAAA--SRC SHEENPYFHGW	L	IGHPEMSPPDYSMALSYSP EYASGA ---
XP_003310653.1 (Chimpanzee)	--- AAAAAAAAAA--SRC SHEENPYFHGW	L	IGHPEMSPPDYSMALSYSP EYASGA ---
XP_001085842.1 (Monkey)	--- AAAAAAAAAA--SRC SHEENPYFHGW	L	IGHPEMSPPDYSMALSYSP EYASGA ---
XP_005635942.1 (Dog)	--- GAAAAAAAAAGPPARC SHEEEPLFYGW	L	IGHPEMSPLDYSMALSYSP EYASGA ---
XP_003586406.1 (Cattle)	--- ---AAAAAA--SRC SHEENPYFHGW	L	IGHPEMSPPDYSMALSYSP EYASGA ---
NP_034532.3 (Mouse)	--- AAAAAAAAAA--SRC SHEENPYFHGW	L	IGHPEMSPPDYSMALSYSP EYASGA ---
NP_073187.1 (Rat)	--- AAAAAAAAAA--SRC SHEENPYFHGW	L	IGHPEMSPPDYSMALSYSP EYASGA ---
NP_571701.2 (Zebrafish)	--- AAA-----SRC-HEEPPYFHGW	L	ISHPEMSPPDYTMAPSYSPEYSTGA ---
NP_001093695.1 (Frog)	--- AAAAA-----SRC-HEENPYFHGW	L	ISHPEMSPPDYSMAPSYSPEYANGA ---

Figure 3. Multiple alignments of the amino acids of HAND2 proteins across various species. Alignment of the amino acids of human HAND2 protein with those of chimpanzee, monkey, dog, cattle, mouse, rat, zebrafish and frog displayed that the altered leucine at amino acid 47 of human HAND2 was completely conserved evolutionarily.

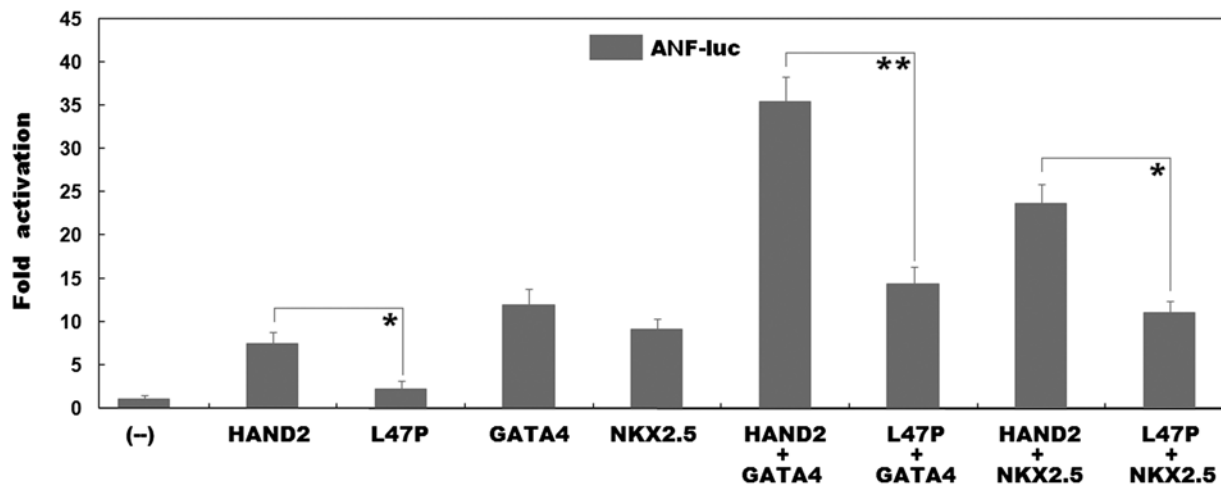


Figure 4. Functional defects of HAND2 caused by the mutation. Activation of the *ANF* promoter driven luciferase in HeLa cells by wild-type or L47P-mutant HAND2 (L47P), alone or in combination with GATA4 or NKX2.5, revealed significantly decreased activation or synergistic activation by the mutant protein. Experiments were performed in triplicate, and the means \pm standard deviations are shown. * $p < 0.005$ and ** $p < 0.001$ compared with the wild-type counterparts.

sponding to 3 of every 10,000 live births, with males being affected slightly more often than females (39). If not treated surgically, 25% of cases with severe obstruction succumb to the disease within the first year, 40% succumb by the age of 3 years, 70% by the age of 10 years, and 95% by the age of 40 years (39). Therefore, it is of pronounced clinical significance to ascertain the molecular basis of TOF. In the present study, a novel heterozygous mutation, p.L47P, in HAND2 was identified in a child with TOF. The missense mutation was absent in the 400 control chromosomes from a control population matched for ethnicity and gender. The mutation, which altered the amino acid conserved evolutionally across species, was predicted to be pathogenic by MutationTaster, PolyPhen-2 and SIFT. Reporter gene assays unveiled that the L47P-mutant HAND2 possessed a significantly reduced transcriptional activity. Furthermore, the L47P mutation markedly decreased the synergistic activation between HAND2 and GATA4 or HAND2 and NKX2.5. Therefore, it is possible that functionally compromised HAND2 predisposes to CHD in a subset of patients.

In humans, *HAND2* is located on chromosome 4q33, with a transcript of 2.3 kb in length encoding a protein of 217 amino acids, and is strongly expressed in the human heart (40). The HAND2 protein harbors two functionally important structural domains, a transcriptional activation domain and a basic helix-loop-helix domain. The former is required for the transcriptional activation of downstream genes, and the latter is responsible for the binding to target DNAs and the interactions with transcriptionally cooperative partners (41). Previous studies have demonstrated that HAND2 transcriptionally activates multiple target genes highly expressed in the heart during embryogenesis, including *ANF*, alone or in synergy with such cooperative partners as GATA4, NKX2.5 and myocyte enhancer factor 2C (MEF2C) (42-45). In the present study, the mutation identified in a patient with CHD was located in the transcriptional activation domain of the HAND2 protein, and biological assays revealed that the mutation significantly diminished the transcriptional activation of the *ANF* promoter driven by HAND2, and furthermore, the mutation markedly decreased the synergistic activation between HAND2 and

GATA4 or HAND2 and NKX2.5, two other cardiac core transcription factors that are most commonly linked to CHD in humans (13). These findings suggest that haploinsufficiency caused by HAND2 mutation is likely an alternative mechanism underlying CHD.

The association of genetically defective *Hand2* with increased vulnerability to CHD has been substantiated in animal models. In zebrafish, *Hand2*-mutant embryos have been shown to have defects in myocardial development from an early stage, with a reduced number of myocardial precursors and an improperly patterned myocardial tissue, which were preceded by the aberrant morphogenesis of the cardiogenic regions of the lateral plate mesoderm (46). Additionally, gene expression profiles in *Hand2*-mutant embryos revealed an essential role of *Hand2* in the establishment of a favorable environment for cardiac fusion through the negative regulation of fibronectin (47). In chicks, treatment of stage 8 chick embryos with *Hand2* and *Hand1* antisense oligonucleotides demonstrated that either oligonucleotide alone did not disrupt embryogenesis, whereas in combination, they inhibited cardiac development at the looping heart tube stage (33). In mice, the targeted disruption of *Hand2* has been shown to give rise to embryonic lethality on embryonic day 10.5, mainly due to right ventricular hypoplasia and vascular deformities (34). In rescued mouse embryos by activating adrenergic receptors, the deletion of *Hand2* has been shown to lead to the misalignment of the outflow tract and aortic arch arteries, and ventricular septal defect, double outlet right ventricle, interrupted aortic artery, pulmonary stenosis, as well as retroesophageal right subclavian artery (48). Moreover, the conditional ablation of *Hand2* alleles in specific cardiac cell populations at defined developmental points recapitulated the complete *Hand2*-null phenotype. Specifically, the loss of *Hand2* at later stages of development and in restricted areas of the second heart field has been shown to cause various cardiovascular abnormalities, including hypoplastic right ventricle, tricuspid atresia, truncus arteriosus and ventricular septal defect (49). Besides, the endocardial nullification of *Hand2* contributes to the abnormal development of tricuspid valve, intraventricular septum and ventricles (50). By contrast, mice with an increased copy number of *Hand2*, which were generated by transgene with a bacterial artificial chromosome containing *Hand2*, also presented with congenital heart defects (51). Taken collectively, these results suggest that *Hand2* plays pivotal roles in cardiovascular morphogenesis, and the imbalanced dosage of HAND2 confers an increased predisposition to CHD.

In humans, previous studies have demonstrated that patients with chromosomal deletion or duplication that involved chromosome 4q33, the locus of *HAND2*, are liable to CHD, including pulmonary atresia, ventricular septal defect, coarctation of the aorta and TOF (40). In addition, *HAND2* sequence variations were also discovered in patients with CHD, encompassing TOF, patent ductus arteriosus, pulmonary atresia, atrial septal defect, atrioventricular septal defect, pulmonary stenosis and double outlet right ventricle. However, the functional roles of these CHD-related mutations remain to be characterized (52).

In conclusion, to the best of our knowledge, this is the first study on the association of HAND2 loss-of-function mutation with an enhanced susceptibility to TOF in humans, providing novel insight into the molecular mechanisms responsible for

the development of CHD, and implying potential implications for the xgenetic counseling of families with CHD.

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