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ORIGINAL ARTICLE

ISL1 loss-of-function mutation contributes to congenital heart defects

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Abstract

Congenital heart defect (CHD) is the most common form of birth deformity and is responsible for substantial morbidity and mortality in humans. Increasing evidence has convincingly demonstrated that genetic defects play a pivotal role in the pathogenesis of CHD. However, CHD is a genetically heterogeneous disorder and the genetic basis underpinning CHD in the vast majority of cases remains elusive. This study was sought to identify the pathogenic mutation in the *ISL1* gene contributing to CHD. A cohort of 210 unrelated patients with CHD and a total of 256 unrelated healthy individuals used as controls were registered. The coding exons and splicing boundaries of *ISL1* were sequenced in all study subjects. The functional effect of an identified *ISL1* mutation was evaluated using a dual-luciferase reporter assay system. A novel heterozygous *ISL1* mutation, c.409G > T or p.E137X, was identified in an index patient with congenital patent ductus arteriosus and ventricular septal defect. Analysis of the proband's pedigree revealed that the mutation co-segregated with CHD, which was transmitted in the family in an autosomal dominant pattern with complete penetrance. The nonsense mutation was absent in 512 control chromosomes. Functional analysis unveiled that the mutant ISL1 protein failed to transactivate the promoter of *MEF2C*, alone or in synergy with TBX20. This study firstly implicates *ISL1* loss-of-function mutation with CHD in humans, which provides novel insight into the molecular mechanism of CHD, implying potential implications for genetic counseling and individually tailored treatment of CHD patients.

Keywords Congenital heart defect · Patent ductus arteriosus · Molecular genetics · Transcription factor · ISL1 · Reporter gene assay

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Introduction

Congenital heart defect (CHD) is a cardiovascular structural deformity that arises from aberrant development of the heart or cardiothoracic great blood vessels during embryogenesis [1]. It is the most common form of birth defect in humans, occurring in approximately 1% of all live newborns, and accounting for almost one-third of all major developmental malformations [1, 2]. Each year about 1.35 million neonates are born with CHD worldwide [1, 2]. Clinically, CHD has been categorized into 25 different types, of which 21 designate specific anatomic or hemodynamic lesions, including patent ductus arteriosus (PDA), ventricular septal defect (VSD), tetralogy of Fallot, atrial septal defect, double outlet right ventricle, transposition of the great arteries, persistent truncus arteriosus, aortic stenosis, coarctation of the aorta, pulmonary stenosis, pulmonary atresia, anomalous pulmonary venous connection and endocardial cushion defect [2]. Although minor cardiovascular defects may resolve spontaneously [2], major abnormalities require timely surgical or catheter-based treatment and otherwise may lead to degraded quality of life [3], decreased exercise performance [4], retarded cerebral development or brain injury [5, 6], pulmonary hypertension [7–10], thromboembolic or hemorrhagic stroke [11–13], metabolic disorder [14], infective endocarditis [15–18], myocardial fibrosis [19, 20], cardiac dysfunction or heart failure [21–25], arrhythmias [26–29], and death [30–32]. Hence, CHD remains the most prevalent cause of infant defect-related demises, with nearly 24% of infants who died of a birth defect having a cardiovascular defect [2]. Although great progress in surgical treatment of pediatric CHD has allowed more than 90% of livebirths with CHD to survive into adulthood, it brings about an ever-increasing population of adults living with CHD and presently there are more adults with CHD than children with CHD [1]. Moreover, the morbidity and mortality in adult CHD patients are much higher than those in the general population [33–42]. Despite significant clinical importance, the underlying etiologies of CHD remain largely elusive.

Cardiac morphogenesis is a complex biological process and both genetic and environmental risk factors may interrupt the process, leading to CHD [1, 43–46]. The well-known environmental risk factors for CHD encompass maternal conditions such as viral infection, diabetes mellitus and autoimmune disorder, and maternal exposures to drugs, tobacco smoke, toxic chemicals or ionizing radiation during the first trimester of pregnancy [46]. However, aggregating evidence underscores the genetic components for CHD, especially for familial CHD, which is transmitted predominantly in an autosomal dominant pattern in the family, though familial transmission of CHD is also observed in other inheritance modes, including autosomal recessive and X-linked modes [1, 43–46]. Regardless of chromosomal abnormalities such as chromosome 22q11 deletion and trisomy of chromosome 21, mutations in more than 60 genes, including those encoding cardiac transcription factors, signaling molecules, cardiac sarcomeric proteins and chromatin modifiers, have been causally linked to CHD in humans [1, 43–72]. Among these well-established CHD-related genes, most code for cardiac transcription factors, including GATA4, GATA5, GATA6, NKX2-5, CASZ1, HAND1, HAND2, NR2F2, MEF2C, TBX1, TBX5 and TBX20 [1, 43–72]. Nevertheless, CHD is a genetically heterogeneous malady, and the genetic determinants underlying CHD in most patients remain unclear.

As a member of the homeodomain-containing family of transcription factors, ISL1 is highly expressed in the fetal heart and is essential for proper cardiovascular development [73–75]. In the mouse, targeted disruption of the *Isl1* gene results in embryonic death due to severe developmental defects of the heart, including loss of the outflow tract, right ventricle, and much of the atria [76]. In humans, several single nucleotide polymorphisms of *ISL1* have been associated with increased risk of CHD [77, 78]. These observational findings make it rational to screen *ISL1* as a prime candidate gene for CHD in patients.

Materials and methods

Study participants

In this study, 210 unrelated patients with CHD were recruited from the Chinese Han population. Among them, there were 118 male and 92 female cases with an average age of 7.1 ± 5.6 years, ranging from 1 to 32 years of age. The available family members of the index patient carrying an identified *ISL1* mutation were also enrolled. Two-hundred and fifty-six ethnically-matched individuals, who had no CHD or positive family history of CHD, were enlisted as controls, of whom there were 143 males and 113 females with an average age of 6.8 ± 5.5 years, ranging from 1 to 30 years of age. All study subjects underwent comprehensive clinical appraisal, including detailed medical history, physical examination, electrocardiogram and echocardiogram, as well as cardiac catheterization and/or surgical proceedings for patients. Probands with known chromosomal abnormalities or syndromic CHD, such Holt–Oram syndrome, Turner syndrome and Marfan syndrome, were excluded from the current study. This investigation was conducted in conformity with the ethical principles of the Declaration of Helsinki and was approved by the Research Ethics Committees of the

First Affiliated Hospital of Soochow University and Shanghai Chest Hospital, Shanghai Jiao Tong University. Written informed consent was obtained from the guardians of the CHD patients and the control subjects prior to the study.

Genetic analysis of *ISL1*

Peripheral venous blood specimens were collected from all the study subjects. Genomic DNA was purified from blood leukocytes with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The genomic DNA sequence of the human *ISL1* gene (accession no. NG_023040.1) was derived from the Nucleotide database at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/nuccore/NG_023040.1?from=5001&to=16607&report=genbank). With the help of the online program Primer-BLAT (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?ORGANISM=9606&INPUT_SEQUENCE=NG_023040.1&LINK_LOC=nuccore&PRIMER5_START=5001&PRIMER3_END=16607), the primers to amplify the coding regions and splicing boundaries of *ISL1* by polymerase chain reaction (PCR) were designed as given in Table 1. Genomic DNA of interest was performed by PCR using HotStar Taq DNA Polymerase (Qiagen) on a Veriti Thermal Cycler (Life Technologies, Carlsbad, CA, USA) under recommended reagent concentrations and reaction conditions. PCR sequencing of the purified amplicons was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies) on an ABI PRISM 3130 XL DNA Analyzer (Life Technologies). The position of an exonic sequence variation was numbered in accordance with the reference sequence of the *ISL1* mRNA transcript at the Nucleotide database (https://www.ncbi.nlm.nih.gov/nuccore/NM_002202.2), with an accession number of NM_002202.2. Additionally, the Single Nucleotide Polymorphism database (https://www.ncbi.nlm.nih.gov/snp/?term=ISL1), the Exome Variant Server database (http://evs.gs.washington.edu/EVS), the 1000 Genomes Project database (http://www.internationalgenome.org/), and the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/all.php) were queried to verify the novelty of an identified *ISL1* sequence variance.

Plasmid constructs and site-directed mutagenesis

RNA isolation and cDNA preparation from human heart tissue were prepared as previously described [79]. The wild-type full-length open read frame of the human *ISL1* gene (accession no. NM_002202.2) was amplified by PCR using the pfuUltra high-fidelity DNA polymerase (Stratagene, Santa Clara, CA, USA) and a specific pair of primers (forward primer: 5′-TGGGCTAGCAACCACCATTTCACTGTGG-3′; reverse primer: 5′-TGGGCGGCCGCAAAATACAGAATGAATGTTC-3′). The amplified product was doubly digested by restriction enzymes *Nhe*I and *Not*I (TaKaRa, Dalian, Liaoning, China). The digested product with a length of 1118 base pairs was separated by 1.0% agarose gel electrophoresis, isolated using the GeneJET™ gel extraction kit (Life Technologies), and then inserted into the *Nhe*I-*Not*I sites of the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) to generate a recombinant expression plasmid ISL1-pcDNA3.1. The nonsense mutation detected in CHD patients was introduced into the wild-type ISL1-pcDNA3.1 plasmid by site-targeted mutagenesis using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) with a complementary pair of primers following the manufacturer's descriptions and was checked by sequencing. For generation of the reporter plasmid MEF2C-luciferase (MEF2C-luc), which expresses firefly luciferase, a 1387-bp promoter region of the *MEF2C* gene (nucleotides 2584–3970; accession No. AY324098) was subcloned into the promoterless pGL3-Basic vector (Promega, Madison, WI, USA) as described previously [80]. The expression plasmid TBX20-pcDNA3.1 was constructed as described previously [58].

Cell transfection and luciferase assay

CHO and 10T1/2 cells were cultured in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum as well as 100 U/ml penicillin and 100 µg/ml streptomycin in an incubator with an atmosphere of 5% CO_2 at 37 °C. Cells were seeded at a density of 1×10^5 cells per well in 12-well plates. After 48 h, transfection was performed using the Lipofectamine 2000® reagent

Table 1 Primers to amplify the coding exons and splicing junctions of the *ISL1* gene

Coding exon	Forward primer (5′→3′)	Backward primer (5′→3′)	Product size (bp)
1	GCGTCAGACCAATGGCGATG	CAGTAAGCATGCAGGCGTGG	499
2	TCCCAGAGTACGCCCTATAAGAG	AACAAGCCTCAATACCCCGGAA	527
3	TGCTGTTTACTTGGGGCGTC	TGTGCTCGGGGAATCAAGGG	590
4	TCCCTTTCACCCTCTTCGCC	TGAAAACCGTGCGATCCTGC	590
5	TTGGGCTGAGCTGTGAAGGT	GCACCCTCCCCACTGAATCT	547
6	GACTGAGAGCTCACCTACTCCC	CTTCGTGCATTTCATGGAGCATT	629

(Invitrogen) following the manufacturer's manual. For transient transfection experiments, CHO cells were transfected with 1.0 μg of wild-type ISL1-pcDNA3.1, 1.0 μg of E137X-mutant ISL1-pcDNA3.1, 1.0 μg of wild-type ISL1-pcDNA3.1 plus 1.0 μg of E137X-mutant ISL1-pcDNA3.1, 0.5 μg of wild-type ISL1-pcDNA3.1, or 0.5 μg of wild-type ISL1-pcDNA3.1 in combination with 0.5 μg of E137X-mutant ISL1-pcDNA3.1, in the presence of 1.0 μg of MEF2C-luc and 0.04 μg of pGL4.75 (Promega). The pGL4.75 vector expressing a renilla luciferase was co-transfected into the cells as an internal control for transfection efficiency. For the negative control, the empty plasmid pcDNA3.1 was used. In order to explore the synergistic effect between ISL1 and TBX20 on the *MEF2C* promoter, 10T1/2 cells were co-transfected with 0.25 μg of wild-type ISL1-pcDNA3.1, or 0.25 μg of E137X-mutant ISL1-pcDNA3.1, or 0.25 μg of wild-type TBX20-pcDNA3.1, or 0.25 μg of wild-type ISL1-pcDNA3.1 plus 0.25 μg of wild-type TBX20-pcDNA3.1, or 0.25 μg of E137X-mutant ISL1-pcDNA3.1 plus 0.25 μg of wild-type TBX20-pcDNA3.1, in the presence of 1.0 μg of MEF2C-luc and 0.04 μg of pGL4.75 (Promega). Cells were incubated at 37 °C and harvested 48 h after transfection. Luciferase activity of the cell lysates was determined in a luminometer with the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. The results were expressed as the ratios of the activities of firefly luciferase (pGL3-Basic) to renilla luciferase (pGL4.75). For each expression plasmid, three independent experiments were each performed in triplicate and the *MEF2C* promoter activity was expressed as mean ± standard deviation (SD).

In order to test whether the mutant ISL1 protein is properly expressed by the reconstructed E137X-mutant ISL1-pcDNA3.1 plasmid, 1.0 μg of E243X-mutant ISL1-pcDNA3.1 or 1.0 μg of wild-type ISL1-pcDNA3.1 was transfected into the cultured CHO cells with the Lipofectamine 2000® reagent (Invitrogen). Cells were collected 36 h after transient transfection and total mRNAs were extracted using TRIzol Reagent (Invitrogen). Reverse transcription (RT) of mRNA coding for ISL1 protein was carried out with SuperScript™ IV First-Strand Synthesis System (Invitrogen) and the *ISL1*-specific primer (5′-CACCATGGGAGTTCCTGTCA-3′). Amplification of cDNA by PCR was performed with HotStar Taq DNA Polymerase (Qiagen) and a pair of primers specific to *ISL1* (forward primer: 5′-CATCGAGTGTTTCCG CTGTG-3′; backward primer: 5′-CACCATGGGAGTTCC TGTCA-3′; product size: 496 bp). The amplicons were quantified by electrophoresis analysis on 1.2% agarose gel stained with ethidium bromide.

Statistical analysis

For statistical analyses, the SPSS software package for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA) was used. Continuous variables between two groups were compared with Student's unpaired t test, whereas categorical variables between two groups were compared with Pearson's χ^2 test or Fisher's exact test, when appropriate. A two-sided $P < 0.05$ was considered statistically different.

Results

Clinical characteristics of the study population

In the present study, 210 unrelated CHD patients were clinically investigated in contrast to 256 unrelated control individuals without CHD. Patients were matched with controls for age, gender and ethnicity. All the patients had echocardiogram-documented CHD, of whom about 20% had a positive family history of CHD. The control individuals had no family history of CHD and their echocardiograms showed normal cardiac images without evidence of structural cardiac defects. The demographic and baseline clinical features of the CHD patients are summarized in Table 2.

Identification of a novel *ISL1* mutation

The entire coding exons and exon–intron boundaries as well as partial 3′- and 5′-untranslated regions of the *ISL1* gene were analyzed by direct sequencing in a cohort of 210 patients with CHD and a nonsense mutation was found in an index patient who was five years old. Specifically, a substitution of thymine for guanine at the first nucleotide of codon 137 (c.409G > T), predicting the transition of the codon encoding glutamic acid at amino acid position 137 to a premature termination codon (p.E137X), was detected in a girl with PDA and VSD, who had a positive family history of CHD. The sequence electropherograms of the heterozygous *ISL1* mutation of c.409G > T and its wild-type control are displayed in Fig. 1A. The schematic diagrams of the E137X-mutant and wild-type ISL1 proteins indicating the key structural domains and location of the mutation identified in this study are exhibited in Fig. 1B. The nonsense mutation was neither detected in the 256 control individuals nor reported in the Single Nucleotide Polymorphism, Exome Variant Server, 1000 Genomes Project, and Human Gene Mutation databases (queried again in June 29, 2018). Genetic scan of the mutation carrier′s family members available revealed that the mutation was present in all the affected family members, but absent in unaffected family members. Analysis of the proband′s pedigree unveiled that the mutation co-segregated with CHD, which was transmitted as an autosomal dominant trait in the family with complete penetrance.

Table 2 Demographic and clinical features of the patients with congenital heart defects ($n = 210$)

Parameter	*n* or mean	% or range
Gender		
Male	118	56
Female	92	44
Age (years)	7.1 ± 5.6	1–32
Positive family history of CHD	43	20
Distribution of different forms of CHD		
Isolated CHD	101	48
VSD	34	16
ASD	28	13
PDA	17	8
DORV	6	3
PS	4	2
TA	4	2
TGA	2	1
AS	2	1
PA	2	1
CoA	2	1
Complex CHD	109	52
TOF	34	16
VSD + PDA	28	13
VSD + ASD	14	7
ASD + PDA	10	5
VSD + ASD + PDA	6	3
DORV + VSD	6	3
TGA + VSD	5	2
TA + VSD	4	2
TOF + ASD	2	1
Incidence of arrhythmias		
Atrioventricular block	11	5
Atrial fibrillation	7	3
Treatment		
Surgical repair	102	49
Catheter-based closure	95	45
Follow-up	13	6

CHD congenital heart defect, *VSD* ventricular septal defect, *ASD* atrial septal defect, *PDA* patent ductus arteriosus, *DORV* double outlet right ventricle, *PS* pulmonary stenosis, *TA* truncus arteriosus, *TGA* transposition of the great arteries, *AS* aortic stenosis, *PA* pulmonary atresia, *CoA* coarctation of the aorta, *TOF* tetralogy of Fallot

The pedigree structure of the proband's family is shown in Fig. 1C. The phenotypic features of the proband's affected family members are shown in Table 3.

Failure to transactivate the promoter of *MEF2C* by the mutant ISL1 protein

Previous studies have validated that ISL1 transcriptionally activates the *MEF2C* promoter in vivo and in vitro, alone or in synergy with TBX20 [80–82]. As shown in Fig. 2, the same amount (1.0 μg) of wild-type and E137X-mutant ISL1-pcDNA3.1 plasmids transcriptionally activated the *MEF2C* promoter in CHO cells by ~ 10 folds and ~ onefolds, respectively. When 1.0 μg of wild-type ISL1-pcDNA3.1 and 1.0 μg of E137X-mutant ISL1-pcDNA3.1 were co-transfected, the induced transcriptional activation of the *MEF2C* promoter was ~ ninefold. Additionally, when 0.5 μg of wild-type ISL1-pcDNA3.1 together with 0.5 μg of empty ISL1-pcDNA3.1 or 0.5 μg of E137X-mutant ISL1-pcDNA3.1 was used, the induced transcriptional activation of the *MEF2C* promoter was ~ sixfold or ~ fivefold. These data suggest that the E137X-mutant ISL1 has neither transcriptional activation of target genes nor dominant-negative effect on its wild-type counterpart.

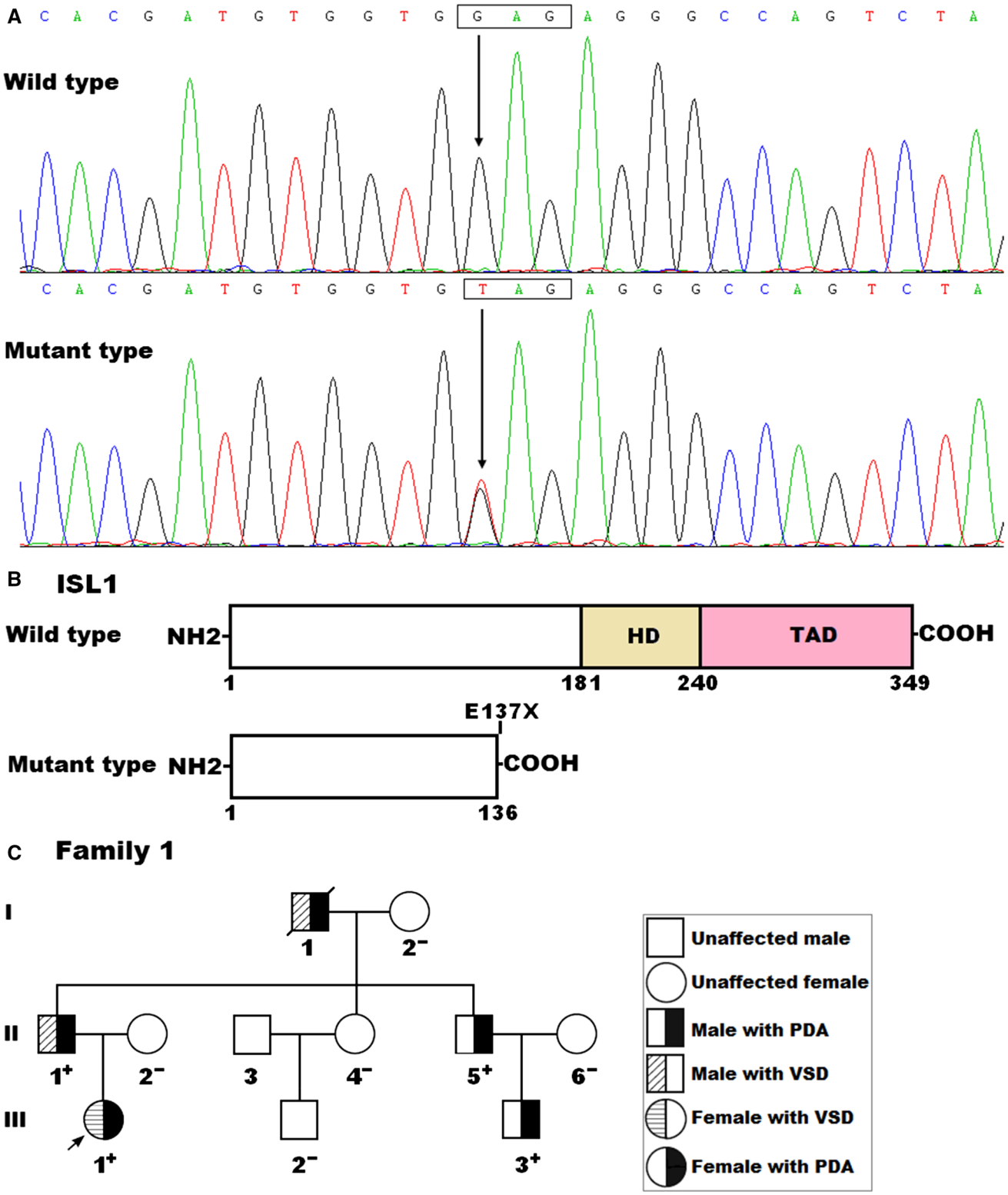
As shown in Fig. 3, the same amount (0.25 μg) of wild-type and E137X-mutant ISL1-pcDNA3.1 plasmids transcriptionally activated the *MEF2C* promoter in 10T1/2 cells by ~ eightfolds and ~ onefold, respectively. In the presence of 0.25 μg of TBX20-pcDNA3.1, the induced synergistic transcriptional activity by the same amount (0.25 μg) of wild-type or E137X-mutant ISL1-pcDNA3.1 plasmid was ~ 28-fold or ~ tenfold. These data indicate that the E137X mutation disrupts the synergistic transcriptional activation between ISL1 and TBX20.

Besides, quantitative RT-PCR was performed and the results demonstrated that both wild-type ISL1-pcDNA3.1 and E137X-mutant ISL1-pcDNA3.1 were equally efficient in transcribing *ISL1* mRNAs, indicating that wild-type ISL1-pcDNA3.1 and E137X-mutant ISL1-pcDNA3.1 produce the same amounts of ISL1 proteins (data not shown).

Discussion

In this research, a novel heterozygous mutation (c.409G > T or p.E137X) in the *ISL1* gene was identified in a family with PDA and VSD. The nonsense mutation, which was absent in the 512 control chromosomes, co-segregated with CHD in the family with complete penetrance. Functional analysis demonstrated that the E137X-mutant ISL1 protein had no transcriptional activity. Therefore, it is very likely that genetically compromised *ISL1* contributes to PDA and VSD in this family.

In humans, *ISL1* maps on chromosome 5q11.1, encoding a protein with 349 amino acids. The encoded ISL1 protein, as a member of the homeodomain family of transcription factors, binds to the enhancer region of the target genes, playing a key role in regulating expression of target genes, which are central to the heart development [73–76]. The human ISL1 protein has two functionally important structural domains, including homeodomain (HD) and transcriptional activation domain (TAD) [82, 83]. The highly



conserved HD domain comprises 60 amino acids (amino acids 181–240) and its main functional role is to bind to consensus DNA sequence in the promoter of a target gene. Adjacent to the HD domain is the TAD domain which consists of 109 highly conserved amino acids, starting from amino acid 241 to amino acid 349, and is responsible for activating transcription [82, 83]. In the present study, the mutation identified in CHD patients was predicted to generate a truncated protein with only 136 amino-terminal amino acids left, lacking the DNA-binding and transcriptional

◂**Fig. 1** Novel *ISL1* mutation associated with congenital heart defects. (A) Sequence electropherograms displaying the heterozygous *ISL1* mutation and its homozygous wild-type control. The arrow points to the heterozygous nucleotides of G/T in the proband (mutant type) or the homozygous nucleotides of G/G in a control subject (wild type). The rectangle denotes the nucleotides comprising a codon of *ISL1*. (B) Schematic diagrams showing the structural domains of the wild-type and E137X-mutant ISL1 protein and the location of the mutation causally linked to congenital heart defects. The mutation identified in patients with congenital heart defects is marked above the structural domains. NH2 means amino terminus; HD, homeodomain; TAD, transcriptional activation domain; COOH, carboxyl terminus. (C) Pedigree structure of the family with congenital heart defects. The family was designated as family 1. Family members are identified by generations and numbers. The proband is indicated by an arrow. Squares indicate male family members and circles indicate female members. Phenotypes are indicated as: right half black symbols, patent ductus arteriosus (PDA); left half shaded symbols, ventricular septal defect (VSD); the symbol with a diagonal, the dead member. "+" means a carrier of the heterozygous nonsense mutation; "–", a non-carrier

Table 3 Phenotypic characteristics and status of ISL1 mutation of the family members with congenital heart defects

Individual	Gender	Age (years)	Cardiac phenotype	ISL1 mutation
Family 1				E137X
I-1	M	54[a]	PDA, sub-aortic VSD	NA
II-1	M	31	PDA, sub-aortic VSD	±
II-5	M	26	PDA	±
III-1	F	5	PDA, sub-aortic VSD	±
III-3	M	1	PDA	±

M male, *F* female, *PDA* patent ductus arteriosus, *VSD* ventricular septal defect, *NA* not available, ± heterozygote

[a]Age at death

activation domains and, thus, was anticipated to nullify its transcriptional activation of target genes, including the *MEF2C* gene that has been associated with CHD [59, 65, 84]. Functional deciphers demonstrated that the E137X-mutant ISL1 protein had no transcriptional activation of the *MEF2C* promoter. Furthermore, the mutation disrupted the synergic activation between ISL1 and TBX20, another cardiac core transcriptional factor that has been causally linked to CHD [58]. Importantly, co-immunoprecipitation experiments performed in cultivated HeLa cells demonstrated the physical interactions between ISL1 and TBX20, and reporter gene analyses revealed that TBX20 could potently activate the *MEF2C* and *NKX2-5* promoters alone or synergistically with ISL1 [81]. Besides, as an important transcriptional co-regulator of ISL1, TBX20 was also involved in directly up-regulating the expression of other key cardiac genes, including PITX2, FGF10 and MYH7, singly or in synergy with ISL1, NKX2-5 and GATA4 [81]. These findings indicate that haploinsufficiency resulted from an *ISL1* mutation is likely to be an alternative pathological mechanism of CHD.

Previous investigations have revealed that a premature translation termination codon may cause degradation of mRNA in many types of organisms and cell lines by a mechanism named as nonsense-mediated mRNA decay (NMD), a translation-dependent, multi-step process that monitors and degrades irregular or faulty mRNAs [79, 85]. In the present research, the nonsense mutation in *ISL1* yielded a premature translation termination codon; therefore, the mutant *ISL1* mRNAs were likely to undergo NMD, although not all nonsense mutations triggered NMD [86]. At present, we could not validate NMD in the mutation carriers due to the unavailability of their cardiac tissue specimens, where the mutant ISL1 protein might be expressed. Even if the mutant mRNAs underwent NMD, the overall abundance of ISL1 mRNAs would be decreased by a half, leading to haploinsufficiency, which was in line with our functional results. Notably, downstream intron or pre-mRNA splicing, which causes the deposition of a multi-protein complex, termed as the exon-junction-complex, 20–24 nucleotides upstream of each exon–exon junction, is required for the degradation of mRNA containing a premature translation termination codon by the NMD mechanism. Hence, NMD could not occur in the context of cDNA constructs [79, 85].

In addition, recent researches have associated several common polymorphisms of the *ISL1* gene with an increased risk of CHD [77, 78]. Stevens and colleagues [77] in stage 1 made a case–control analysis of 30 polymorphisms mapping to the *ISL1* locus in 300 pediatric patients with complex CHD and 2201 healthy children, and discovered that eight polymorphisms (rs6867206, rs4865656, rs6869844, rs2115322, rs6449600, IVS1 + 17C > T, rs1017, rs6449612) in and flanking *ISL1* were significantly associated with complex CHD. To independently validate their findings, in stage 2 they performed a replication study of these candidate polymorphisms in 1044 new cases and 3934 independent controls and confirmed the association of these polymorphisms within and around *ISL1* with increased risk of non-syndromic CHD [77]. To determine the association of genetic polymorphisms in and near the *ISL1* gene with CHD in the Chinese Han population, Luo and partners [78] analyzed nine polymorphisms (rs6867206, rs6869844, rs3762977, rs1017, rs6449612, rs4865656, rs2115322, rs6449600, rs150104955) of *ISL1* in 233 patients with CHD and 288 healthy subjects, and found that one polymorphism (rs1017) in *ISL1* was significantly associated with simple CHD. These data provide strong evidence that *ISL1* plays an important role in the development of heart and pathogenesis of CHD.

Notably, previous investigations have causally linked over 60 genes, including those coding for cardiac transcription

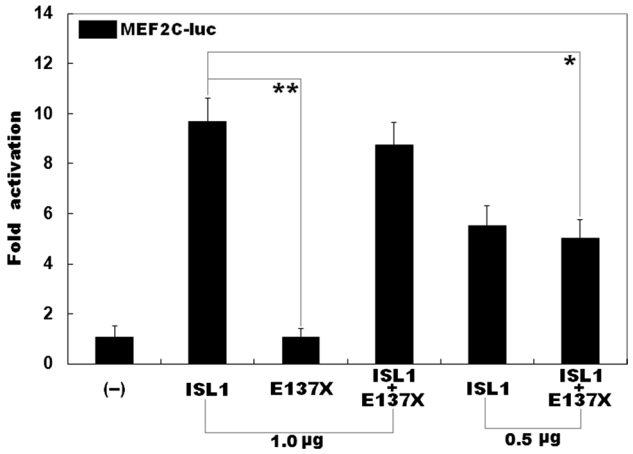


Fig. 2 Inability to transcriptionally activate the *MEF2C* promoter by the mutant ISL1. Activation of the *MEF2C* promoter driven luciferase in CHO cells by wild-type ISL1 or E127X-mutant ISL1 (E137X), alone or together, showed no transcriptional activation by the mutant protein. Three independent transfection experiments were performed in triplicate. The results are represented by means with standard deviations. ** indicates $t=14.7009$, $p=0.00012$; * indicates $t=6.76641$, $p=0.00249$, when compared with wild-type ISL1 (1.0 μg)

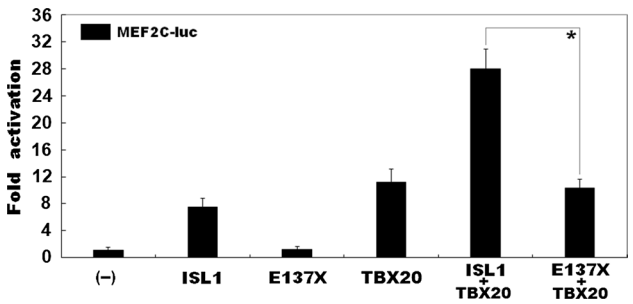


Fig. 3 Synergic transcriptional activation between ISL1 and TBX20 disrupted by the mutation. In the presence of TBX20, activation of the *MEF2C* promoter driven luciferase in 10T1/2 cells by wild-type ISL1 or E127X-mutant ISL1 (E137X) showed significantly reduced transcriptional activity by the mutant protein. Three independent transfection experiments were performed in triplicate. The results are represented by means with standard deviations. * indicates $t=9.52952$, $p=0.000677$, when compared with wild-type counterpart

factors, to CHD in humans [1, 43–46]. In the current study, as previously described, we have analyzed several other cardiac transcription factors in the index patient who carried an identified *ISL1* mutation, including GATA4 [64], GATA5 [87], GATA6 [72], TBX1 [69], TBX5 [88], TBX20 [58], HAND1 [52, 53], HAND2 [89], NKX2-5 [54], MEF2C [59, 65], PITX2 [90], CASZ1 [91], NR2F2 [63] and MESP1 [60], and detected no pathogenic mutations. Nevertheless, we cannot rule out the possibility that other genes may also contribute to the pathogenesis of CHD.

In conclusion, this study firstly associates *ISL1* loss-of-function mutation with CHD in humans, which adds novel insight to the molecular pathogenesis of CHD, suggesting potential implications for genetic counseling and individualized treatment of the patients with CHD.

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Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study or their legal guardians.

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