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ORIGINAL ARTICLE

A Novel MEF2C Loss-of-Function Mutation Associated with Congenital Double Outlet Right Ventricle

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Abstract

Congenital heart defect (CHD) represents the most prevalent birth defect, and accounts for substantial morbidity and mortality in humans. Aggregating evidence demonstrates the genetic basis for CHD. However, CHD is a heterogeneous disease, and the genetic determinants underlying CHD in most patients remain unknown. In the present study, a cohort of 186 unrelated cases with CHD and 300 unrelated control individuals were recruited. The coding exons and flanking introns of the *MEF2C* gene, which encodes a transcription factor crucial for proper cardiovascular development, were sequenced in all study participants. The functional effect of an identified *MEF2C* mutation was characterized using a dual-luciferase reporter assay system. As a result, a novel heterozygous MEF2C mutation, p.R15C, was detected in an index patient with congenital double outlet right ventricle (DORV) as well as ventricular septal defect. Analysis of the proband's pedigree showed that the mutation co-segregated with CHD with complete penetrance. The missense mutation, which changed the evolutionarily conserved amino acid, was absent in 300 control individuals. Functional deciphers revealed that the mutant MEF2C protein had a significantly decreased transcriptional activity. Furthermore, the mutation significantly reduced the synergistic activation between MEF2C and GATA4, another transcription factor linked to CHD. This study firstly associates *MEF2C* loss-of-function mutation with DORV in humans, which provides novel insight into the molecular pathogenesis of CHD, suggesting potential implications for genetic counseling and personalized treatment of CHD patients.

Keywords Congenital heart disease · Double outlet right ventricle · Genetics · Transcription factor · MEF2C · Reporter gene assay

Introduction

Congenital heart defect (CHD), a structural malformation caused by abnormal development of the heart or cardiothoracic major blood vessels, represents the most prevalent form of birth defect in humans, accounting for about one-third of all major developmental deformities [1, 2]. Each year there are approximately 1.35 million neonates who are born with CHD worldwide, with an annual incidence of roughly 1% in

Cai-Xia lu, Wei Wang, and Qian Wang have contributed equally to the work.

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live births [1, 2]. As a cardiovascular developmental abnormality, CHD is usually classified into 25 distinct clinical types, of which 21 designate specific anatomic or hemodynamic lesions, including ventricular septal defect (VSD), atrial septal defect, patent ductus arteriosus, pulmonary stenosis, tetralogy of Fallot, double outlet right ventricle (DORV), transposition of the great arteries, aortic stenosis, truncus arteriosus, coarctation of the aorta, pulmonary atresia, and endocardial cushion defect [1]. Although minor cardiovascular defects can resolve spontaneously [1], major anomalies may require timely surgical treatment and can lead to poor quality of life [3, 4], reduced exercise capacity [5], delayed cerebral development [6, 7], brain injury [8], arterial thromboembolism [9], infective endocarditis [10, 11], pulmonary hypertension [12–15], congestive heart failure [16–19], arrhythmias [20–24], and sudden cardiac death [25–29]. As such, CHD is still the most frequent cause of infant deaths attributable to birth defects, with nearly 24% of infants who died of a birth defect having a heart malformation [1]. Although great advancement made in pediatric care during recent decades has allowed more than 90% of neonates with CHD to survive into adulthood, it results in an increasing number of adults living with CHD, and moreover, the morbidity and mortality in adult CHD patients are much higher compared with the general population [30–32]. Despite their significant clinical importance, the etiologies underlying CHD remain largely unclear.

It has been demonstrated that CHD is a complex multifactorial disorder with both environmental and genetic risk factors involved in the pathogenesis of CHD [33–36]. An expanding list of well-recognized environmental risk factors for CHD include maternal exposures to toxic chemicals, drugs, tobacco smoke, or ionizing radiation during the first trimester of pregnancy and maternal conditions such as viral infection, autoimmune, diabetes, and hypercholesterolemia as well as maternal old age and obesity [35, 36]. However, increasing evidence highlights the genetic determinants for familial CHD, which is predominantly transmitted in an autosomal dominant fashion in the family, though familial transmission of CHD also occurs in other inheritance patterns, encompassing autosomal recessive and X-linked modes [33–36]. Irrespective of chromosomal abnormalities such as trisomy of chromosome 21 and chromosome 22q11 deletion syndrome [34], an increasing number of mutations in over 60 genes, including those coding for cardiac core transcription factors, cardiac sarcomeric proteins, signaling molecules, and chromatin modifiers, have been associated with syndromic or non-syndromic CHD in humans [33–73]. Among these well-established CHD-causing genes, the overwhelming majority encode cardiac transcription factors, including the homeodomain-containing protein NKX2-5, zinc finger proteins GATA4, GATA5, and GATA6, basic helix–loop–helix transcription factors HAND1 and HAND2, and T-box transcription factors TBX1, TBX5, and TBX20 [48, 74]. Theses transcription factors display partially overlapping expression patterns and share cross-talk functions during cardiovascular morphogenesis, indicating that they comprise a key regulatory network essential for proper heart development [74]. Nevertheless, CHD is of remarkable genetic heterogeneity, and the genetic basis underpinning CHD in most cases remains poorly understood.

As a member of the myocyte enhancer factor-2 (MEF2) family of MADS (MCM1, agamous, deficiens, serum response factor)-box transcription factors that are expressed at high levels in various cells, MEF2C has been implicated in transcriptional regulation of all three muscle lineages, including cardiogenic precursor cells and differentiated cardiomyocytes during embryogenesis [75]. In the mouse, the *Mef2c* gene is required for normal cardiogenesis, and targeted deletion of the *Mef2c* gene in mice results in embryonic death due to loss of the right ventricle of the heart, failure of the heart to undergo rightward looping morphogenesis, and diminished expression of several key cardiac-specific genes [75]. Moreover, in mice inactivation of the *Mef2c* gene in the anterior second heart field, a late differentiating population of cardiac progenitors, leads to a spectrum of outflow tract alignment defects ranging from overriding aorta to DORV and dextro-transposition of the great arteries [76]. These observational results make it justifiable to scan *MEF2C* as a prime candidate gene for CHD in patients.

Materials and Methods

Ethical Statement

This research was performed in conformity with the ethical principles outlined in the Declaration of Helsinki. The research protocol was reviewed and approved by the local institutional ethical committee of Tongji Hospital, Tongji University, Shanghai, China [Approval No. LL(H)-09-07]. Written informed consent was obtained from the guardians of the CHD patients and the control subjects prior to commencement of the investigation.

Study Population

In all, 186 unrelated patients affected with CHD were enrolled from the Chinese Han population. Among them, there were 101 males and 85 females with a mean age of 5.3 ± 3.8 years, ranging from 0 to 16 years of age. The available close relatives of the index patient carrying an identified *MEF2C* mutation were also recruited. Phenotypic characteristics of the affected individuals and their family members were derived from detailed clinical records, and medical evaluations based on echocardiography, cardiac

catheterization, and/or surgical findings. Probands with known chromosomal abnormalities, other recognized syndromes, or known maternal exposure to significant toxicants during the first trimester of pregnancy were excluded from the present study. A total of 300 healthy ethnically matched volunteers with no history of CHD were enlisted as controls, of whom there were 160 males and 140 females at an average age of 5.1 ± 3.5 years, ranging from 1 to 16 years of age. Cardiac phenotypes of Chinese control volunteers were determined mainly by echocardiography.

Genetic Analysis of MEF2C

Peripheral venous blood samples were drawn from all the study participants and genomic DNA was extracted from blood leukocytes with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. The referential genomic DNA sequence of the human *MEF2C* gene (Accession No. NC_000005.10) was derived from the Nucleotide database at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/nuccore/NC_000005.10?from=88718241&to=88904105&report=genbank&strand=true). With the aid of the online program Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?ORGANISM=9606&INPUT_SEQUENCE=NC_000005.10&LINK_LOC=nuccore&PRIMER5_START=88718241&PRIMER3_END=88904105), the primer pairs to amplify the coding exons and flanking introns of *MEF2C* by polymerase chain reaction (PCR) were designed as shown in Table 1. The primers for amplification of the 5′-untranslated region (UTR) and 3′-UTR of *MEF2C* (transcript variant 1) by PCR were designed as shown in Table S1. Amplification of genomic DNA was carried out by PCR using HotStar Taq DNA Polymerase (Qiagen) on a Veriti Thermal Cycler (Applied Biosystems, Waltham, MA, USA) under recommended reagent concentrations and standard reaction conditions. Amplified DNA fragments were fractionated by electrophoresis on a 1.5% agarose gel and isolated with the QIAquick Gel Extraction Kit (Qiagen). Direct PCR sequencing of a purified amplicon was conducted with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3130XL DNA Analyzer (Applied Biosystems). For a detected *MEF2C* sequence variation, a second independent PCR-sequencing analysis was performed to verify it. The position of an exonic sequence variation was numbered according to the reference sequence of the *MEF2C* mRNA transcript variant 1 at the Nucleotide database (Accession No. NM_002397.4). In addition, such public databases for human sequence variations as the single nucleotide polymorphism (http://www.ncbi.nlm.nih.gov/SNP), human gene mutation (http://www.hgmd.org), 1000 Genomes (http://www.1000genomes.org/), and Exome Variant Server (http://evs.gs.washington.edu/EVS) databases were consulted to confirm the novelty of an identified *MEF2C* sequence variance.

Multiple Alignments of MEF2C Protein Sequences Across Species

The MEF2C protein of human was aligned with those of chimpanzee, monkey, dog, cattle, mouse, rat, fowl, zebrafish, fruit fly, mosquito, and frog using the online MUSCLE software (https://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&dopt=MultipleAlignment&list_uids=31087).

Prediction of the Pathogenic Potential of a Novel MEF2C Variation

The causative potential of a novel *MEF2C* variation was predicted by MutationTaster (http://www.mutationtaster.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), PROVEAN (http://provean.jcvi.org/index.php), and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html).

Table 1 Primer pairs to amplify the coding exons and flanking introns of the *MEF2C* gene

Coding exon	Forward primer (5′→3′)	Backward primer (5′→3′)	Product size (bp)
1	TCAGCAGGAACGAATGCAGGA	TGCGTGCAATGTAGCAATGT	521
2	ACCAGTTCCCTGGTCACCTTC	AGGTCCAAACTCCCCTGCTT	529
3, 4, 5	CCCCTGAATGTCTCTTACAGCCT	TAAATGAGCTGCCCGTGGGA	542
6	CTTGCAGTCATTTCTGGCACC	AAGCAGTGTTGGCTTTGCCG	592
7	CGTTTGAGCACAGCATGGCAC	ACTCCTGCTTCAGAAAAGTGCT	382
8	TTGGGTTACTTGTCCCATGGAGG	AAGCAAGGCTCTGTCAATGGC	472
9	CCTGGGCTGAAAACCGGGTA	TCACCTGTGAGTGATGCCAGA	405
10	GCAACAGCTGGTCTCTTGAAGG	CGGCGCAGGCCCTAAATAAAG	428
11	TCGCTGATGCTTTATCTCCCCT	CAGAGCTCGCTGTCCTCTTA	493
12, 13	GCATGCTCTGGTGTCTATGCG	CCCCTTCCCCATTAAGGTAT	505

Plasmids and Site-Targeted Mutagenesis

Human heart cDNAs were prepared as described previously [42]. The wild-type full-length open read frame of the human *MEF2C* gene (transcript variant 1; Accession No. NM_002397.4) was amplified by PCR using the pfuUltra high-fidelity DNA polymerase (Stratagene, Santa Clara, CA, USA) and a pair of primers (forward primer: 5′-TGG GCTAGCAGAGAGAGAAGAAAAACGGG-3′; reverse primer: 5′-CCAGCGGCCGCACTAGTAAGTAATAAT CTGA-3′). The amplicons were doubly digested by restriction enzymes *Nhe*I and *Not*I (TaKaRa, Dalian, Liaoning, China). The digested product with a length of 1493 bp was separated by 1.5% agarose gel electrophoresis, purified with the QIAquick Gel Extraction Kit (Qiagen), and then inserted into the *Nhe*I–*Not*I sites of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to generate a recombinant expression plasmid MEF2C-pcDNA3.1. The non-synonymous variant found in CHD patients in the coding region of *MEF2C* was introduced into the wild-type MEF2C-pcDNA3.1 plasmid by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) with a complementary pair of primers according to the manufacturer's instructions, and was verified by sequencing. The recombinant expression plasmid GATA4-pSSRa and the reporter plasmid ANF-luciferase (ANF-luc), which contains the 2600-bp 5′-untranslated region of the *ANF* gene and expresses firefly luciferase, were kind gifts provided by Dr. Ichiro Shiojima from Chiba University School of Medicine, Japan.

Luciferase Assays

HeLa cells were incubated in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum as well as 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C and in an incubator with an atmosphere of 5% CO_2. Cells were seeded in 12-well plates 24 h before being transfected with various plasmids using the Lipofectamine 2000® reagent (Invitrogen) according to the manufacturer's protocol. Additionally, the pGL4.75 (Promega, Madison, WI, USA) vector expressing a renilla luciferase was co-transfected into the cells as an internal control for transfection efficiency. For transient transfection experiments, HeLa cells were transfected with 1.0 μg of wild-type MEF2C-pcDNA3.1, 1.0 μg of R15C-mutant MEF2C-pcDNA3.1, 0.5 μg of wild-type MEF2C-pcDNA3.1, or 0.5 μg of wild-type MEF2C-pcDNA3.1 together with 0.5 μg of R15C-mutant MEF2C-pcDNA3.1 was used, in combination with 1.0 μg of ANF-luc and 0.04 μg of pGL4.75 (Promega). In order to evaluate the ability of the mutant MEF2C to transcriptionally activate the *ANF* promoter in synergy with GATA4, the same amount (0.4 μg) of expression plasmid DNA (empty pcDNA3.1, wild-type MEF2C-pcDNA3.1, R15C-mutant MEF2C-pcDNA3.1 or GATA4-pSSRa) was used alone or together, in the presence of 1.0 μg of ANF-luc and 0.04 μg of pGL4.75. The empty plasmid pcDNA3.1 was used as a negative control. Cells were cultured at 37 °C and harvested 36 h after transfection. Luciferase activity of the lysates was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Firefly luciferase data were normalized to the transfection control (renilla luciferase readings). For each construct, a minimum of three independent experiments were each performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

Statistical Analysis

Statistical analyses were performed using the SPSS software package for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± SD. Categorical variables were given as a number and percentage. Comparison of continuous variables between two groups was made using Student's unpaired *t* test; whereas categorical variables were compared with Pearson's χ^2 test or Fisher's exact test, when appropriate. A two-sided $p < 0.05$ indicated significant difference.

Results

Clinical Features of the Study Participants

In the current study, 186 unrelated CHD patients were clinically evaluated in contrast to 300 unrelated healthy control individuals. The patients and controls were matched in ethnicity, gender, and age. All the cases had echocardiogram-documented CHD, of whom approximately 19% had a positive family history of CHD. The control subjects were healthy with a negative family history of CHD, and their echocardiograms showed normal cardiovascular images with no evidence of structural heart defects. The baseline clinical characteristics of the patients with CHD are summarized in Table 2.

Identification of a Novel MEF2C Mutation

By sequence analysis of the *MEF2C* gene in 186 unrelated patients affected with CHD, a missense mutation was detected in a female patient who was 1 year old, with a mutational prevalence nearly 0.54%. Specifically, a substitution of thymine for cytosine at the first nucleotide of codon 15 (c.43C>T), predicting the transition of arginine at amino acid position 15 to cysteine (p.R15C), was discovered in a girl with DORV and VSD, who had a positive family history of CHD. The DNA sequencing electropherograms

Table 2 Baseline clinical characteristics of the patients with congenital heart defects ($n = 186$)

Parameter	*n* or mean	% or range
Gender		
Male	101	54
Female	85	46
Age (years)	5	0–16
Positive family history of CHD	35	19
Distribution of different forms of CHD		
Isolated CHD	95	51
VSD	32	17
ASD	23	12
PDA	12	6
DORV	8	4
TA	4	2
TGA	4	2
CoA	3	2
AS	3	2
APVC	2	1
PS	2	1
PA	1	1
ECD	1	1
Complex CHD	91	49
TOF	28	15
VSD + PDA	16	9
DORV + VSD	15	8
VSD + ASD	13	7
ASD + PDA	8	4
TGA + VSD	6	3
TA + VSD	3	2
TOF + ASD	2	1
Incidence of arrhythmias		
Atrioventricular block	7	4
Atrial fibrillation	3	2
Treatment		
Surgical repair	103	55
Percutaneous closure	67	36
Follow-up	16	9

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

displaying the heterozygous *MEF2C* mutation of c.43C>T as well as its wild-type control sequence are shown in Fig. 1a. A schematic diagram of the MEF2C protein indicating the key structural domains and location of the mutation identified in this study is shown in Fig. 1b. The missense mutation was neither detected in the 300 control subjects nor reported in the single nucleotide polymorphism, human gene mutation, 1000 Genomes, or Exome Variant Server database (queried again in October 22 2017). Genetic screen of the mutation carrier's family members available showed that the mutation was present in all the affected family members, but absent in unaffected family members. Analysis of the index patient's pedigree revealed that the mutation co-segregated with CHD, which was transmitted in an autosomal dominant pattern in the family with complete penetrance. The pedigree structure of the proband's family is shown in Fig. 1c. The phenotypic characteristics of the proband's living affected family members are shown in Table 3.

MEF2C Sequence Variation Predicted to Be Conserved and Disease-Causing

Alignment of multiple MEF2C protein sequences from various species exhibited that the altered arginine residue at amino acid position 15 was completely conserved evolutionarily (Fig. 2). Additionally, the identified MEF2C sequence variation was predicted to be pathogenic with a *p* value of 1.000 by MutationTaster, probably damaging with a score of 0.999 (sensitivity: 0.09; specificity: 0.99) by PolyPhen-2, deleterious with a PROVEAN score of −7.203 by PROVEAN, and damaging with a SIFT score of 0 and a median information content of 3.4 by SIFT.

Diminished Transcriptional Activity of MEF2C Caused by the Mutation

Previous experiments have demonstrated that MEF2C transcriptionally activates the *ANF* promoter alone or in synergy with GATA4 in HeLa cells [77]. As shown in Fig. 3, the same amount (1.0 μg) of wild-type and R15C-mutant MEF2C-pcDNA3.1 plasmids transcriptionally activated the *ANF* promoter by ~ 16- and ~ 3 -folds, respectively. When 0.5 μg of wild-type MEF2C-pcDNA3.1 was used alone or together with 0.5 μg of R15C-mutant MEF2C-pcDNA3.1, the induced transcriptional activation of the *ANF* promoter was ~ 7 or ~ 8 folds. These data indicate that the R15C-mutant MEF2C has a significantly decreased transcriptional activity.

Reduced Synergistic Transcriptional Activity Between MEF2C Mutant and GATA4

As shown in Fig. 4, in the presence of 0.4 μg of wild-type GATA4, the same amount (0.4 μg) of wild-type and mutant *MEF2C* activated the *ANF* promoter by ~ 52- and ~ 17-folds, respectively, indicating that the MEF2C mutant has a significantly decreased synergistic transcriptional activity with GATA4.

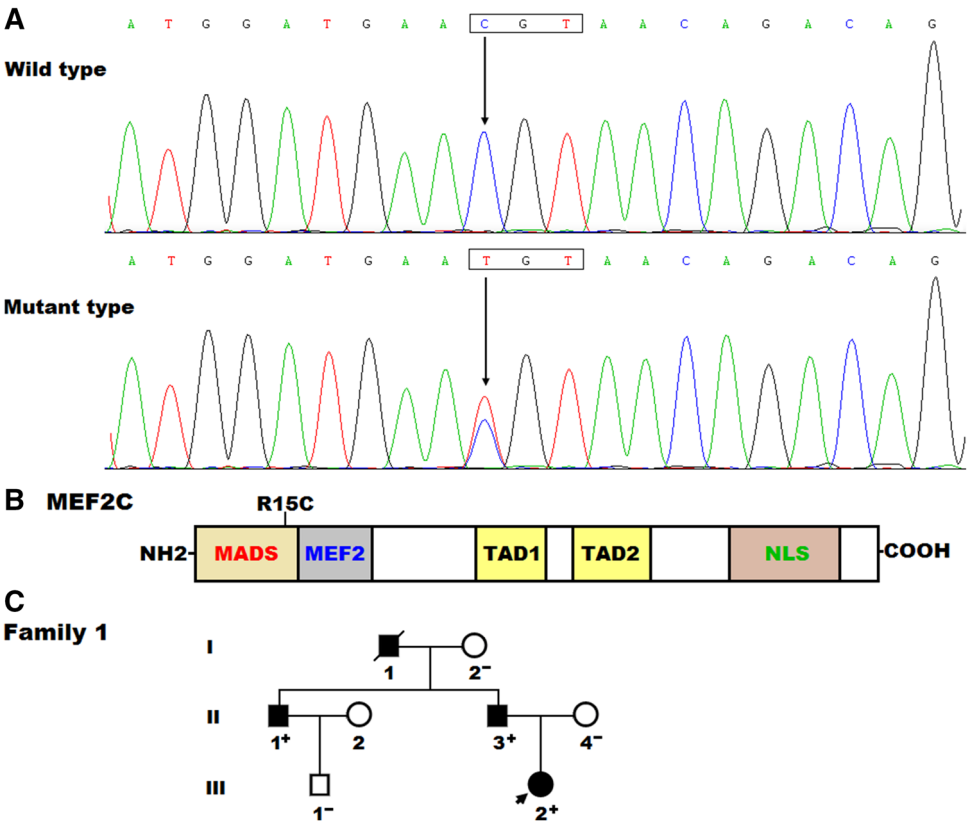

Fig. 1 Novel *MEF2C* mutation associated with congenital heart defects. **a** Sequence electropherograms showing the heterozygous *MEF2C* mutation as well as its wild-type control. The arrow points to the heterozygous nucleotides of C/T in the proband (mutant type) or the homozygous nucleotides of C/C in a control individual (wild type). The rectangle marks the nucleotides comprising a codon of *MEF2C*. **b** Schematic diagram depicting the structural domains of the MEF2C protein and the location of the mutation linked to congenital heart defects. The mutation identified in patients with congenital heart defects is noted above the structural domains. *NH2* amino terminus; MADS, MCM1, agamous, deficiens, serum response factor; *MEF2* myocyte enhancer factor 2; *TAD* transcriptional activation domain; *NLS* nuclear location signal; *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. Square indicates male family member; circle, female member; closed symbol, affected member; open symbol, unaffected member; arrow, proband; "+," carrier of the heterozygous missense mutation; "–," non-carrier

Table 3 Phenotypic characteristics and status of MEF2C mutation of the affected family members

Individual	Gender	Age (years)	Cardiac phenotype	MEF2C mutation
Family 1				R15C
I-1	M	52[a]	DORV, VSD	NA
II-1	M	30	DORV, VSD	±
II-3	M	27	DORV, VSD	±
III-2	F	1	DORV, VSD	±

M male, *F* female, *VSD* ventricular septal defect, *DORV* double outlet right ventricle, *NA* not available, ± heterozygote

[a]Age at death

Discussion

In this study, a novel heterozygous mutation (c.43C>T or p.R15C) in the *MEF2C* gene was identified in a family with DORV and VSD. The missense mutation, which was absent in the 600 control chromosomes, co-segregated with CHD in the family with complete penetrance. Functional data showed that the R15C-mutant MEF2C protein had a significantly reduced transcriptional activity. Therefore, it is very likely that mutated *MEF2C* predisposes to DORV and VSD in this family.

In vertebrates, there are four members of the MEF2 family, including MEF2A, MEF2B, MEF2C, and MEF2D, of which MEF2C and MEF2B are firstly expressed in the cardiac mesoderm at approximately embryonic day 7.5; while MEF2D and MEF2A are expressed after birth for 24 h [78]. MEF2C proteins are expressed ubiquitously

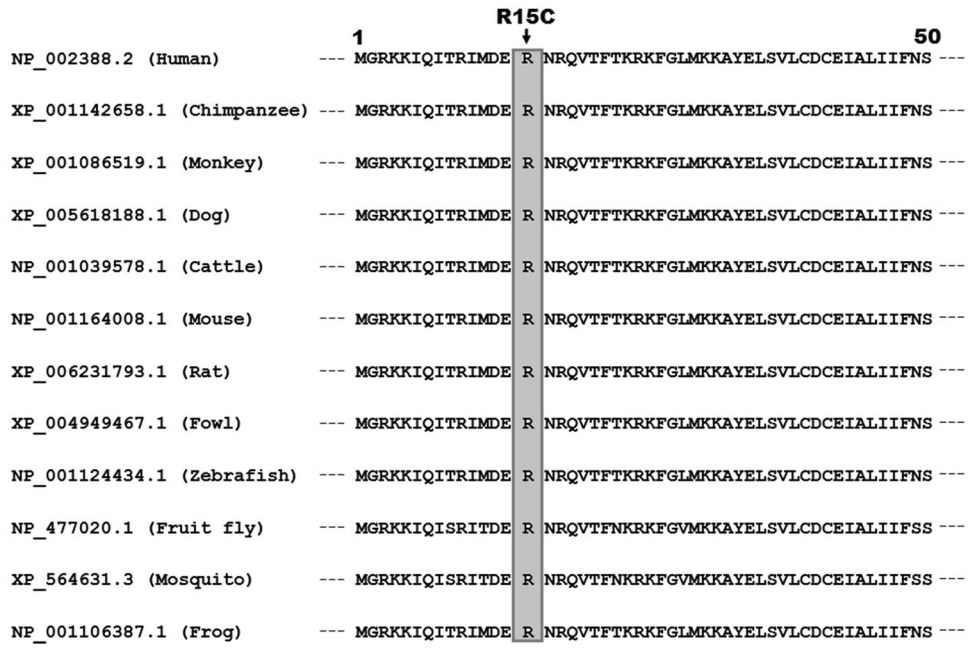

Fig. 2 Multiple alignments of MEF2C proteins from various species. Alignment of multiple MEF2C proteins across species displayed that the altered arginine at amino acid 15 was completely conserved evolutionarily

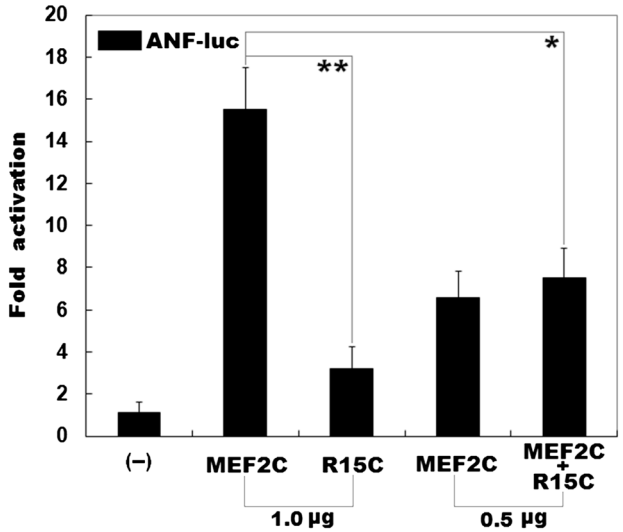

Fig. 3 Diminished transcriptional activity of MEF2C resulted from the mutation. Activation of the *ANF* promoter-driven luciferase in HeLa cells by wild-type MEF2C or R15C-mutant MEF2C (R15C), alone or together, showed significantly decreased transcriptional activation by the mutant protein. Experiments were performed in triplicate, and means with standard deviations are shown. $^{**}t = 9.59826$, $p = 0.00066$; $^{*}t = 4.76792$, $p = 0.00885$, when compared with wild-type MEF2C (1.0 μg)

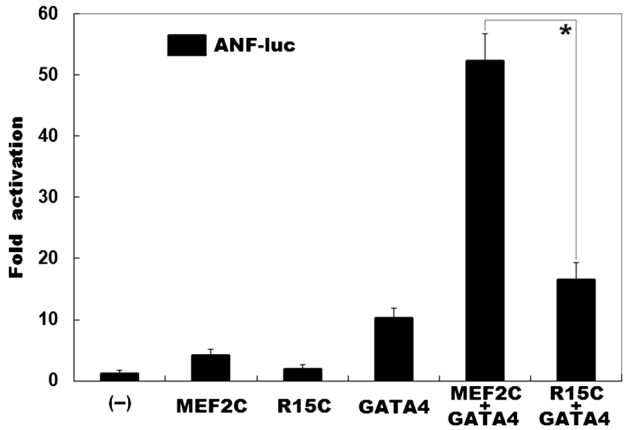

Fig. 4 Decreased synergistic transcriptional activity between R15C-mutant MEF2C and GATA4. In the presence of GATA4, activation of the *ANF* promoter-driven luciferase reporter in HeLa cells by wild-type MEF2C or R15C-mutant MEF2C (R15C) showed significantly reduced synergistic transcriptional activity caused by the mutation. Experiments were carried out in triplicate, and mean and standard deviations are shown. $^{*}t = 11.614$, $p = 0.00031$, when compared with the wild-type counterpart

during embryonic period of eukaryote organisms to regulate tissue-specific gene expression in many types of cells, such as cardiac muscle, skeletal muscle, neural, chondroid, immune, and endothelial cells [78]. In humans, *MEF2C* maps on chromosome 5q14.3, encoding multiple isoforms of proteins, including isoform 1 with 473 amino acids.

The human MEF2C protein possesses five functionally important structural domains, including MADS, MEF2, TAD1, TAD2, and NLS [78]. The highly conserved MADS domain at the N-terminus of MEF2C comprises 56 amino acids, and its main role is to mediate DNA binding, dimerization, and co-factor interactions. Adjacent to the MADS domain is the MEF2 domain which consists of 30 highly conserved amino acids, starting from amino acid 57 to amino acid 86, and is important in mediating dimerization and DNA binding. The transcriptional activation domains

TAD1 and TAD2 are responsible for activating transcription, while the nuclear localization signal NLS domain at the C-terminus of MEF2C is required for nuclear translocation of the protein [78]. In the present study, the mutation identified in CHD patients was located at the MADS domain of MEF2C, and thus was anticipated to impair the transactivational function of MEF2C mainly by interfering with its binding to the promoters of target genes, including the *ANF* gene abundantly expressed in the embryonic hearts [77]. Functional assays showed that the R15C-mutant MEF2C protein had significantly diminished transcriptional activation of the *ANF* promoter alone or in synergy with GATA4. These findings imply that haploinsufficiency resulted from a *MEF2C* mutation is potentially an alternative pathological mechanism of CHD.

Notably, previous studies have related mutations in more than 60 genes, including those encoding cardiac transcription factors, to CHD in humans [33–36, 48]. In this study, as previously described, we have screened several other cardiac transcription factors in the index patient who harbored an identified MEF2C mutation, including NKX2-5 [66], NKX2-6 [63], GATA4 [46], GATA5 [56], GATA6 [71], HAND1 [62], HAND2 [51], TBX1 [79], TBX5 [80], TBX20 [41], PITX2 [59], CASZ1 [42], NR2F2 [54] and MESP1 [69], and found no pathogenic mutations. Nevertheless, we can't exclude the possibility that other genes may also contribute to the pathogenesis of CHD.

Additionally, functional data provided are rather robust, but unfortunately with two caveats. Firstly, data were obtained in HeLa cells rather than in primary cultures of cardiomyocyte, and therefore its relevance to cardiac function was minimal. In cultured cardiomyocytes, the inducibility levels of target genes might be smaller but more relevant from the biological point of view. Secondly, it was hard to reconcile that impaired transactivation of *ANF* might be linked to CHD. These functional experiments were proof of concept only. In fact it is highly desirable to repeat similar transcriptional activation experiments using more relevant targets. Similarly, it would be interesting if competition assays with other *MEF2* family members are provided given the fact that the identified mutation might alter dimerization.

It is interesting that *MEF2C* mutations have been found in patients with neurodevelopmental disorder, which is characteristic of intellectual disability with inability to speak, hypotonia, stereotypic movement, and epilepsy [81]. Besides, *MEF2C* mutations have also been involved in dilated cardiomyopathy [82] and other congenital heart defects, including patent ductus arteriosus, VSD, pulmonary atresia, and pulmonary stenosis [53, 83]. In the present research, a novel *MEF2C* mutation was identified in patients with DORV. Therefore, this research expands the phenotypic spectrum linked to MEF2C mutation.

In conclusion, this study firstly associates *MEF2C* loss-of-function mutation with DORV in humans, which provides novel insight into the molecular mechanism underpinning CHD, suggesting potential implications for genetic counseling and personalized management of the patients with MEF2C-related CHD.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interests.

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