

Research Paper

MEF2C loss-of-function mutation contributes to congenital heart defects

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Abstract

Congenital heart disease (CHD) is the most common type of developmental abnormality in humans, and is a leading cause for substantially increased morbidity and mortality in affected individuals. Increasing studies demonstrates a pivotal role of genetic defects in the pathogenesis of CHD, and presently mutations in more than 60 genes have been associated with CHD. Nevertheless, CHD is of pronounced genetic heterogeneity, and the genetic basis underpinning CHD in a large proportion of patients remains unclear. In the present study, the whole coding exons and splicing donors/acceptors of the *MEF2C* gene, which codes for a transcription factor essential for normal cardiovascular development, were sequenced in 200 unrelated patients affected with CHD, and a novel heterozygous missense mutation, p.L38P, was identified in an index patient with patent ductus arteriosus (PDA) and ventricular septal defect (VSD). Genetic scan of the mutation carrier's family members available showed that the mutation was present in all affected family members but absent in unaffected family members. Analysis of the proband's pedigree revealed that the mutation co-segregated with PDA, which was transmitted as an autosomal dominant trait with complete penetrance. The mutation changed the amino acid that was completely conserved evolutionarily, and did not exist in 300 unrelated, ethnically-matched healthy individuals used as controls. Functional deciphers by using a dual-luciferase reporter assay system unveiled that the mutant *MEF2C* protein had a significantly reduced transcriptional activity. Furthermore, the mutation significantly diminished the synergistic activation between *MEF2C* and *GATA4*, another cardiac core transcription factor that has been causally linked to CHD. In conclusion, this is the first report on the association of a *MEF2C* loss-of-function mutation with an increased vulnerability to CHD in humans, which provides novel insight into the molecular mechanisms underlying CHD, implying potential implications for early diagnosis and timely prophylaxis of CHD.

Key words: Congenital heart disease; Genetics; Transcription factor; *MEF2C*; Reporter gene assay

Introduction

Congenital heart disease (CHD) is the most common type of developmental defect in humans, occurring in almost 3% of neonates when including bicuspid aortic valve, and accounting for

approximately one-third of all major birth defects [1-3]. Each year about 1.35 million newborns are born with CHD worldwide, with an estimated incidence of 1% in live births and as high as 10% in still births [1,2]. As a group of structural abnormalities of the heart, CHD is usually classified into 25 different clinical types, including ventricular septal defect (VSD), atrial septal defect, atrioventricular canal defect, patent ductus arteriosus (PDA), tetralogy of Fallot, double outlet right ventricle, transposition of the great arteries, interrupted aortic arch, aortic stenosis, truncus arteriosus, coarctation of the aorta, tricuspid atresia, pulmonary stenosis (PS), pulmonary atresia, hypoplastic left heart, single ventricle, and abnormal pulmonary venous connection [1]. Although mild cardiovascular anomalies can resolve spontaneously [1], major defects may require timely surgical treatment and otherwise can result in degraded health-related quality of life [4], reduced exercise performance [5], retarded central neural development and brain injury [6-9], cerebral and pulmonary thromboembolism [10,11], infective endocarditis [12-16], pulmonary arterial hypertension [17-22], chronic heart failure [23-26], supraventricular and ventricular arrhythmias [27-31] and sudden cardiac death [32-37]. Therefore, CHD represents the most prevalent cause of infant birth defect-related demises, with roughly 24% of infants who died of a birth defect having a cardiovascular deformity [1]. Due to vast advances in medical care and surgical management of CHD during the last 50 years, today 95 % of CHD patients survive to adulthood, which has resulted in a growing population of adults living with CHD, and now there are more adults living with CHD than children [38-41]. However, the morbidity and mortality rates in adult CHD patients are much higher compared with the general population [38-41]. Despite important clinical significance, the causes responsible for CHD in the majority of patients remain unknown.

Previous studies have demonstrated that the etiology of CHD is complex and is associated with both environmental and genetic causes [2,3,41-44]. The well-established environmental risk factors contributing to CHD encompass maternal exposures to toxic chemicals, drugs or ionizing radiation during the first trimester of pregnancy and maternal conditions such as viral infection, immune disorder and diabetes mellitus [41,42]. However, epidemiologic studies strongly suggest genetic defects as the predominant cause of CHD, which is predominantly transmitted 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 fashions

[41-44]. Genetically, regardless of chromosomal duplications and deletions including trisomy 21, trisomy 18 and 22q11.2 microdeletion [45], more than 60 genes, including those encoding cardiac transcription factors, cardiac structural proteins, signaling transducer molecules and chromatin modifiers, have been causally linked to CHD in humans [41-89]. Of these CHD-associated genes, most code for cardiac transcription factors, including the homeobox-containing protein NKX2-5, zinc-finger proteins GATA4, GATA5 and GATA6, and T-box transcription factors TBX1, TBX5 and TBX20 [89,90]. These transcription factors show partially overlapping expression profiles and functional characteristics during embryonic cardiogenesis, suggesting that they constitute a regulatory network crucial for normal cardiovascular development [90]. Nevertheless, CHD is a very heterogeneous disease, and the genetic determinants for CHD in the majority of patients remain unclear.

As a member of the MADS (MCM1, agamous, *deficiens*, serum response factor)-box containing transcription factor family, the myocyte enhancer factor 2C (MEF2C) is highly expressed in various cells, and plays an important role in transcriptional regulation of all three muscle lineages, including cardiac precursor cells and differentiated cardiomyocytes during embryogenesis [91]. In mice, targeted disruption of the *Mef2c* gene led to embryonic death due to loss of the right ventricle of the heart, failure of the heart to undergo rightward looping morphogenesis [91]. Additionally, in fetal mice ablation of the *Mef2c* gene in the anterior second heart field, a late differentiating population of cardiac progenitors, caused a spectrum of outflow tract malformations ranging from overriding aorta to double outlet right ventricle and transposition of the great arteries [92]. These findings make it reasonable to screen *MEF2C* as a preferred candidate gene for CHD in different cohorts of patients.

Materials and methods

Ethics

This study was conducted in compliance with the ethical principles of the Declaration of Helsinki. The study protocol was approved by the Medical Ethics Committee of Tongji Hospital, Tongji University, Shanghai, China [approval no. LL(H)-09-07]. Prior to the study, written informed consent was obtained from the guardians of the CHD patients and control individuals.

Study participants

In this study, a consecutive cohort of 200 unrelated patients suffered from CHD was recruited

from the Chinese Han population from January 2014 to December 2016, including 110 males and 90 females with an average age of 4.2 ± 3.5 years, ranging from 0 to 15 years of age. The available family members of the index patient harboring an identified *MEF2C* mutation were also enrolled. A total of 300 healthy, ethnicity- and geographic-matched individuals were registered as controls, including 162 males and 138 females at a mean age of 4.1 ± 3.3 years, ranging from 1 to 14 years of age. Study participants underwent comprehensive clinical evaluation including individual and familial histories, medical records, electrocardiogram and echocardiography. Magnetic resonance imaging, cardiac angiography or cardiac surgery was performed when indicated. Diagnosis of CHD was made according to the echocardiography, magnetic resonance imaging, angiography or direct view during cardiac surgery. The patients with known chromosomal abnormalities or syndromic CHD, such as DiGeorge syndrome, Turner syndrome, Marfan syndrome, Holt–Oram syndrome and Down syndrome, were excluded from the study.

Genetic screening of the human *MEF2C* gene

Peripheral venous whole-blood samples were collected from all the study participants and genomic DNA was isolated from blood leukocytes using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions for DNA isolation. The coding exons and flanking exon–intron boundaries of the *MEF2C* gene (transcript variant 1) were sequenced in 200 unrelated patients with CHD and in 300 ethnicity- and geographic-matched control individuals. 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). The primers utilized to amplify the coding regions and splice junction sites of *MEF2C* by polymerase chain reaction (PCR) were designed as shown in Table 1, with the online Primer-BLAST program (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>). PCR was performed in a 25 μ L mixture containing 20–40 ng of genomic DNA, 1 \times Buffer (Qiagen, Hilden, Germany), 0.5 μ M of each primer, 0.2 mM dNTPs and 0.02 U/ μ L of HotStar Taq DNA Polymerase (Qiagen). PCR cycling was carried out on Veriti Thermal Cycler (Applied Biosystems, Waltham, MA, USA) using a pre-denaturation cycle at 95°C for 15 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing of primers at 62°C for 30 s and extension at 72°C for 1 min as well as a final extension

step at 72°C for 6 min. Amplicons were fractionated by electrophoresis on a 1.5% agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen). Both strands of each PCR product were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems) according to the protocols. The identified *MEF2C* variant was confirmed by sequencing two independent PCR-generated amplicons from the same patient. 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). Additionally, the identified sequence variation was queried in the single nucleotide polymorphism database (<http://www.ncbi.nlm.nih.gov/SNP>), the human gene mutation database (<http://www.hgmd.org>), the 1000 Genome Project database (<http://www.1000genomes.org>), and the Exome Aggregation Consortium (<http://exac.broadinstitute.org>) to confirm its novelty.

Table 1. Primers to amplify the coding and adjacent non-coding sequences of the *MEF2C* gene.

Coding exon	Forward primer (5'→3')	Backward primer (5'→3')	Amplicon (bp)
1	ACGAATGCAGGAATTT GGGAACGTG	CTTTGTTTGATGATGCG TGCAATGT	525
2	AGGGCTCTGACAACTA CACACC	TCCAAACTCCCCTGCTT GCC	371
3	CCCTGAATGTCTCTTAC AGCCTT	CTAAATGAGCTGCCCGT GGGA	542
4	CTGAACGTCTTGAGAGC TCITCTT	GACCTAGCTCGGGGT GGAT	483
5	TGTGGGCCITTAGCCTT TCICG	TTGTGTCCAAGGATGGT ATCCAG	387
6	TTGGGTACTGTGCCA TGGAGG	AAGCAAGGCTCTGTCA ATGGC	472
7	GCTACAGCCAAGCAGT GTGG	CATGCCATTGAGGGA AGCG	510
8	GTTACTATGGCAACAGC TGGICT	AATGCTGCAGTGCTGTG GACC	456
9	TGCGACTATGCTACTT CAGAG	ATTATTACAGAGCTCGC TGCC	577
10	TGCTCTGGTGTCTATGC GAGTC	ACGGCACATATAATGC ATATCG	527

Sequence alignment of *MEF2C* across species

Conservation of the amino acid altered by a missense allelic variant was evaluated by aligning the protein of human *MEF2C* to 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/homologs?cmd=Retrieve&dopt=MultipleAlignment&list_uids=31087).

Prediction of the causative potential of a new MEF2C variation in silico

The disease-causing potential of a new *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).

Plasmids and site-targeted mutagenesis

Human heart cDNAs were prepared as described previously [57]. With the human heart cDNAs as templates, the full-length coding sequences for human *MEF2C* gene (transcript variant 1; accession no. NM_002397.4) were amplified by PCR using the pfuUltra high-fidelity DNA polymerase (Stratagene, Santa Clara, CA, USA) and a pair of primers (forward primer: 5'-TGGGCTAGCAGAG-AGAGAAGAAAACGGG-3'; reverse primer: 5'-CCAGCGGCCGCACTAGTAAGTAATAATCTGA-3'). The amplified products were doubly digested by restriction enzymes *NheI* and *NotI* (TaKaRa, Dalian, Liaoning, China). The resultant product with a length of 1493 base pairs was separated by 1.5% agarose gel electrophoresis, extracted with the QIAquick Gel Extraction Kit (Qiagen), and then inserted into the *NheI-NotI* sites of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to produce a recombinant expression plasmid MEF2C-pcDNA3.1. The identified mutation was introduced into the wild-type MEF2C-pcDNA3.1 plasmid by site-directed mutagenesis using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) with a complementary pair of primers (Forward: 5'-ATGAGCTGAGCGTGCCGTGTGACTGTGAGAT-3'; Reverse: 5'-ATCTCACAGTCACACGGCAGC-CTCAGTCTCAT-3') according to the manufacturer's protocols, and was validated 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 kindly provided by Dr. Ichiro Shiojima from Chiba University School of Medicine, Japan.

Cell transfection and luciferase assays

HeLa cells were cultured in Dulbecco's modified Eagle's media containing 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin in an incubator with 5% CO₂ at 37°C. Cells were seeded in 24-well plates 24 h before transfection with various plasmids using the Lipofectamine 3000[®] reagent (Invitrogen) according to the manufacturer's instructions. The pGL4.75 (Promega) vector expressing a renilla luciferase was co-transfected into

the cells as an internal control to normalize transfection efficiency. For transient transfection experiments, HeLa cells were transfected with 1.0 µg of wild-type MEF2C-pcDNA3.1, 1.0 µg of L38P-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 L38P-mutant MEF2C-pcDNA3.1, in combination with 1.0 µg of ANF-luc and 0.04 µg of pGL4.75 (Promega). To evaluate the synergistic transcriptional activation, the same amount (0.4 µg) of expression plasmid DNA (empty pcDNA3.1, wild-type MEF2C-pcDNA3.1, L38P-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. Transfections were conducted in three independent experiments in triplicate. Cells were harvested and lysed 36 h after transfection. Luciferase activity of the lysates was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's manual. Firefly luciferase data was normalized to the renilla luciferase readings, and the results were expressed as mean ± standard deviations (SD).

Statistical analysis

The data was statistically analyzed 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 expressed as a number and percentage. Comparison of continuous variables between two groups was performed 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 2-tailed probability value of $P < 0.05$ was considered to be significantly different.

Results

Clinical features of the study participants

In the present study, 200 unrelated CHD patients was clinically investigated in contrast to 300 unrelated healthy control individuals. The patients and controls were matched in ethnicity, gender, age, and geography. All the patients 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 were normal with no evidence of cardiovascular structural deformities. The demographic and clinical characteristics of the cohort of CHD patients that participated in the study are summarized in Table 2.

Table 2. Demographic and clinical features of the patients with congenital heart disease (n = 200).

Parameter	n or mean	% or range
Gender		
Male	110	55
Female	90	45
Age (years)	4	0–15
Positive family history of CHD	38	19
Distribution of different forms of CHD		
Isolated CHD	108	54
VSD	32	16
ASD	25	12.5
PDA	16	8
AS	8	4
PS	6	3
DORV	5	2.5
TGA	5	2.5
TA	4	2
CoA	4	2
PA	2	1
SV	1	0.5
Complex CHD	92	46
TOF	27	13.5
VSD + ASD	22	11
VSD + PDA	21	10.5
DORV + VSD	7	3.5
ASD + PDA	5	2.5
TGA + VSD	5	2.5
TA + VSD	3	1.5
VSD + ASD + PDA	2	1
Incidence of arrhythmias		
Atrioventricular block	7	3.5
Atrial fibrillation	4	2
Treatment		
Surgical treatment	106	53
Catheter-based repair	69	34.5
Follow-up	25	12.5

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

Identification of a novel MEF2C mutation

Sequence analysis of the *MEF2C* gene in our patient's cohort led to identification of a non-synonymous heterozygous variant in a male patient who was one year old, with a mutational prevalence of 0.5%. Specifically, a substitution of cytosine for thymine at the second nucleotide of codon 38 (c.113T>C), which was predicted to result in the change of leucine at amino acid position 38 into proline (p.L38P), was detected in a boy with PDA and VSD, who had positive family history of CHD. The DNA sequence chromatograms that exhibited the heterozygous allelic mutation of c.113T>C and its homozygous wild-type control base are shown in Figure 1A. A schematic drawing of the MEF2C protein displaying the functionally important structural domains with approximated localization of the mutation found in the current study is illustrated in Figure 1B. The missense mutation was neither discovered in the 300 control subjects nor reported in

the single nucleotide polymorphism, human gene mutation, 1000 Genomes Project and Exome Aggregation Consortium database (queried again in June 6, 2017). 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 PDA, which was transmitted as an autosomal dominant trait in the family with complete penetrance. Additionally, the proband's father (II-5) and uncle (II-1) had also VSD and PS, respectively, while his grandfather (I-1) had also VSD and PS and died of heart failure at the age of 58 years. Besides, the affected adult family members had also stereotypic movements, mental retardation, and paroxysmal epilepsy. The pedigree structure of the proband's family is represented in Figure 1C. The clinical characteristics and molecular findings of the proband's affected family members are listed in Table 3.

Table 3. Phenotypic characteristics and genotypes for MEF2C of the affected family members.

Individual	Gender	Age (years)	Cardiac phenotype	MEF2C mutation
Family A				
I-1	M	58*	PDA, VSD, PS	NA
II-1	M	32	PDA, PS	+/-
II-5	M	26	PDA, VSD	+/-
III-1	F	5	PDA	+/-
III-4	M	1	PDA, VSD	+/-

M, male; F, female; PDA, patent ductus arteriosus; VSD, ventricular septal defect; PS, pulmonary stenosis; NA, not available; +/-, heterozygote.

*Age at death.

Evolutionary conservation of the altered amino acid

As shown in Figure 2, multiple alignments of the MEF2C protein sequences across species exhibited that the altered leucine at amino acid 38 was completely conserved evolutionarily.

A novel MEF2C variation predicted to be pathogenic

The identified MEF2C variation c.113T>C was predicted to be disease-causing 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 -6.438 by PROVEAN, and damaging with a SIFT score of 0 and a median information content of 3.41 by SIFT.

Reduced transcriptional activity of MEF2C caused by the mutation

As shown in Figure 3, the same amount (1.0 µg) of wild-type and L38P-mutant MEF2C-pcDNA3.1

plasmids transcriptionally activated the *ANF* promoter by ~ 14 folds and ~ 2 folds, respectively. When 0.5 μg of wild-type MEF2C-pcDNA3.1 was used alone or together with 0.5 μg of L38P-mutant MEF2C-pcDNA3.1, the induced transcriptional activation of the *ANF* promoter was ~ 7 -fold or ~ 6 -fold.

Diminished synergistic activation between MEF2C and GATA4 resulted from the mutation

As shown in Figure 4, in the presence of 0.4 μg of wild-type GATA4, the same amount (0.4 μg) of wild-type and L38P-mutant MEF2C activated the *ANF* promoter by ~ 39 folds and ~ 14 folds, respectively; while in the absence of wild-type GATA4, the same

amount (0.4 μg) of wild-type and L38P-mutant MEF2C activated the *ANF* promoter by ~ 4 folds and ~ 2 folds, respectively.

Discussion

In the present study, a novel heterozygous mutation (c.113T>C, equivalent to p.L38P) in the *MEF2C* gene was discovered in a family with PDA as well as VSD and PS. The missense mutation, which was absent in the 600 control chromosomes, co-segregated with CHD in the family with complete penetrance. Functional analyses demonstrated that the L38P-mutant MEF2C protein had a significantly diminished transcriptional activity. Furthermore, the mutation significantly decreased the synergistic transcriptional activation between MEF2C and GATA4, another cardiac core transcription factor that has been associated with CHD. Hence, it is very likely that genetically compromised MEF2C contributes to CHD in this family.

To date, there are four members of the MEF2 family found in vertebrates, encompassing MEF2A, MEF2B, MEF2C and MEF2D, of which MEF2B and MEF2C are firstly activated in the heart mesoderm at approximately embryonic day 7.5; while MEF2A and MEF2D are expressed after birth for 24 hours [93]. MEF2C is widely expressed in many types of cells in vivo to regulate tissue-specific gene expression during embryonic period of eukaryote organisms, including cardiac muscle, skeletal muscle, neural, chondroid, immune and endothelial cells [93]. In humans, *MEF2C* maps on chromosome 5q14.3, which codes for several isoforms of proteins, including isoform 1 with 473 amino acids. The human MEF2C protein contains five key structural domains, including MADS, MEF2, transcriptional activation domain 1 (TAD1), transcriptional activation domain 2 (TAD2), and nuclear localization signal (NLS) [93]. The highly conserved MADS domain at the amino-terminus of MEF2C consists of 56 amino acids, and its

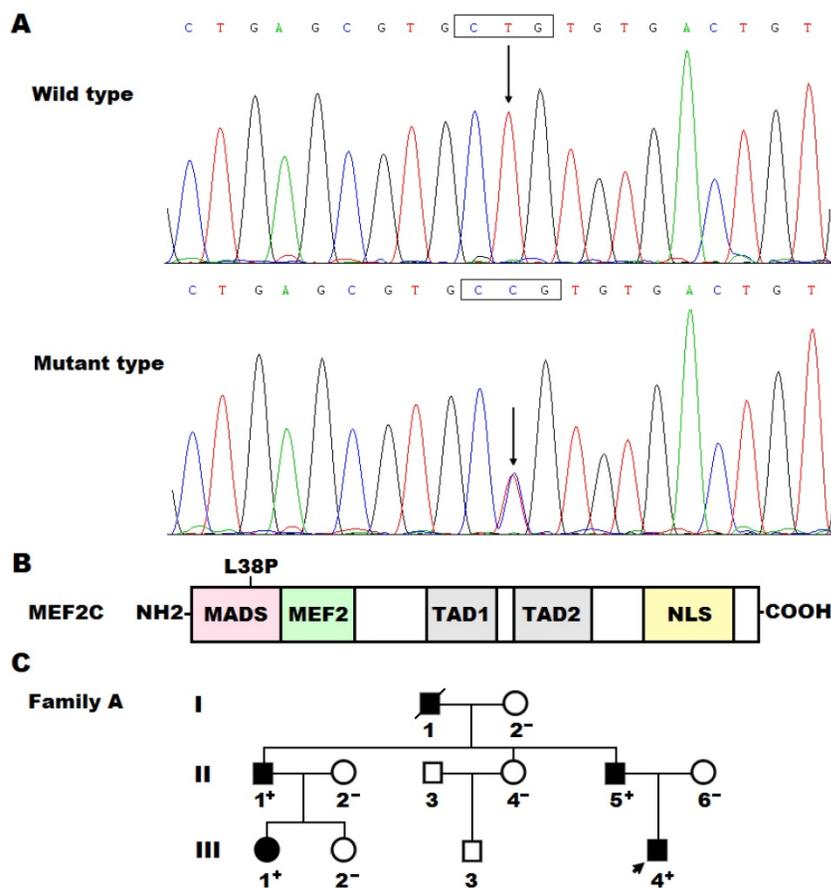


Figure 1. A novel *MEF2C* mutation associated with congenital heart disease. (A) DNA sequence chromatograms displaying the heterozygous *MEF2C* mutation and its corresponding wild-type control. The arrow points to the heterozygous nucleotides of T/C in the proband (mutant type) or the homozygous nucleotides of T/T in a control individual (wild type). The rectangle marks the nucleotides comprising codon 38 of *MEF2C*. (B) Schematic diagram delineating the structural domains of the MEF2C protein and the location of the mutation involved in congenital heart disease. The mutation identified in patients with congenital heart disease is shown above the structural domains. NH2, amino terminus; MADS, MCM1, agamous, deficiencies, serum response factor; MEF2, myocyte enhancer factor 2; TAD1, transcriptional activation domain 1; TAD2, transcriptional activation domain 2; NLS, nuclear localization signal; COOH, carboxyl terminus. (C) Pedigree structure of the family with congenital heart disease. The family was designated as family A. Family members are recognized 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.

main role is to mediate DNA binding, dimerization, and interactions with co-factors. The MEF2 domain is adjoining the MADS domain and comprises 30 highly conserved amino acids, starting from amino acid 57 to amino acid 86. In combination with the MADS domain, the MEF2 domain plays an important role in mediating dimerization and DNA binding. The TAD1 and TAD2 domains function as the transcriptional activators; while the NLS domain is located at the Carboxyl-terminus of MEF2C, which is responsible for nuclear translocation of the protein [93]. In the current study, the mutation detected in CHD patients was located at the MADS domain of MEF2C, and thus was anticipated to impair the transcriptional activity of MEF2C mainly by interfering with its binding to the promoters of target genes, including the *ANF* gene highly expressed in the heart during embryogenesis [94]. Functional deciphers demonstrated that the L38P-mutant MEF2C protein had significantly reduced transcriptional activation of the *ANF* promoter alone or in synergy with GATA4. These results suggest that haploinsufficiency for *MEF2C* is potentially an alternative pathological mechanism underlying CHD.

Association of *MEF2C* loss-of-function mutation with enhanced susceptibility to CHD may be partially explained by abnormal cardiovascular development. In fruit fly, there is a single *Mef2* gene that encodes a protein with extensive homology to the MADS domains of the mammalian MEF2 proteins, and

during embryonic development, its expression is initiated at gastrulation within mesodermal precursor cells in the ventral furrow and then becomes restricted to the somatic, cardiac, and visceral muscle lineages [95]. In the embryos of fruit fly, loss-of-function mutation of *Mef2* gives rise to a block in the development of all three types of muscle cells, including cardiac, somatic and visceral muscle cells [95]. In zebrafish, there are two *Mef2c* genes, namely *Mef2ca* and *Mef2cb*, which are expressed similarly in the bilateral heart fields at the primitive heart tube stage, and knockdown of a single *Mef2ca* leads to delayed heart development, but mutants with loss of a single *Mef2cb* have a functional heart [96]. In frog, knocking down either *Mef2c* or *Mef2d* by corresponding antisense morpholino oligonucleotides results in congenital heart defects including morphological anomalies, pericardial edema, and brachycardia [97]. In mice, MEF2C is the first member of the MEF2 family expressed during embryogenesis in cells of the cardiac mesoderm that form the primitive heart tube [98], and targeted deletion of the *Mef2c* gene causes severe cardiac structural abnormalities and embryonic lethality in homozygous mutants due to the failure of heart tube to undergo looping morphogenesis and the absence of the right ventricular region of the heart [91]. Furthermore, in mice ablation of *Mef2c* in the anterior second heart field causes outflow tract defects, such as overriding

aorta, double outlet right ventricle and transposition of the great arteries [92]. In humans, all four MEF2 transcript variants (MEF2A, MEF2B, MEF2C and MEF2D) are expressed in all developmental stages of the heart [99]. More importantly, Kodo and colleagues made a sequence analysis of the *MEF2C* gene in 256 non-syndromic, non-familial patients with cardiac outflow tract defects, and identified a novel sequence variant (p.A103V) in a female patient with pulmonary atresia and VSD [100]. Functional assays using a luciferase reporter showed that the

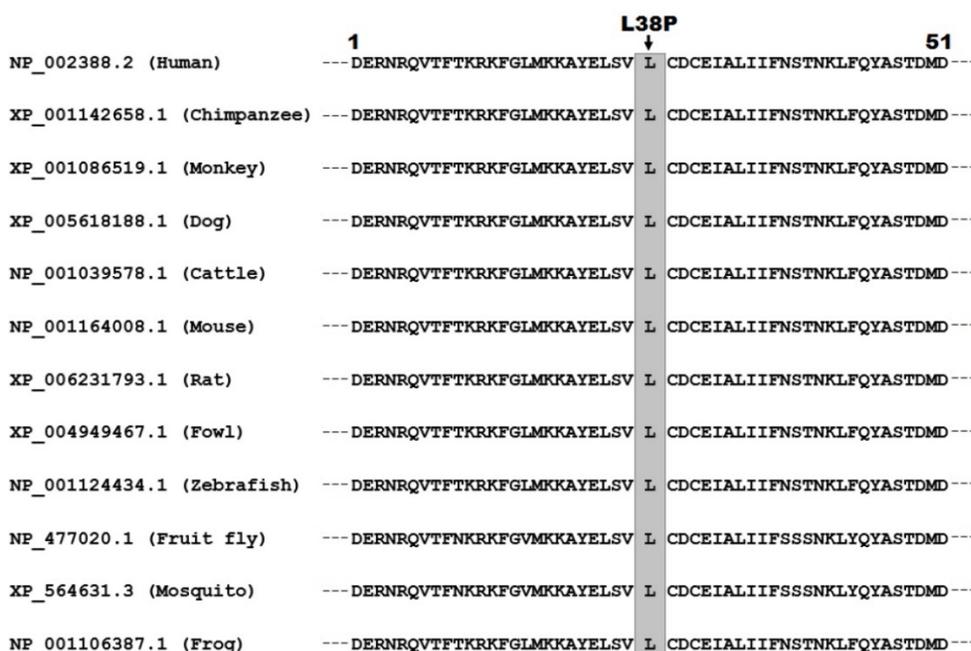


Figure 2. Alignment of multiple MEF2C proteins across species. Multiple alignments of MEF2C proteins among various species exhibited that the altered leucine at amino acid 38 was completely conserved evolutionarily.

A103V-mutant MEF2C protein had a significantly increased transcriptional activity, and furthermore, overexpression of the A103V-mutant MEF2C in a fish model system disturbed early cardiac development [100]. Taken collectively, these findings together with the results of the current study indicate that genetically compromised *MEF2C* predisposes to CHD in humans.

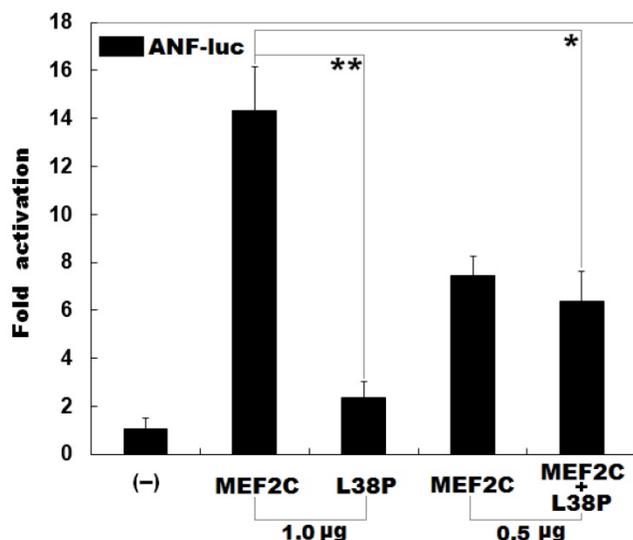


Figure 3. Reduced transcriptional activity of MEF2C caused by the mutation. Activation of the ANF promoter driven luciferase in HeLa cells by wild-type MEF2C or L38P-mutant MEF2C (L38P), alone or together, showed significantly reduced transcriptional activity by the mutant protein. Three independent transfection experiments were conducted in triplicate for each expression plasmid, and mean and standard deviations are shown. ** indicates $t = 10.7379$, $p = 0.00043$; * indicates $t = 6.29383$, $p = 0.00326$, when compared with wild-type MEF2C (1.0 µg).

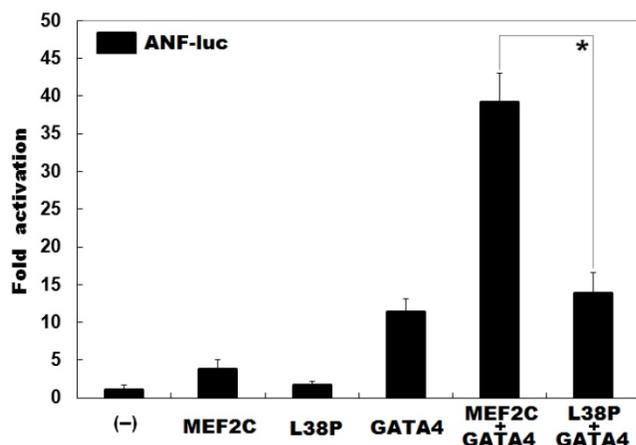


Figure 4. Diminished synergistic transcriptional activation between MEF2C and GATA4 resulted from the mutation. In the presence of GATA4, activation of the ANF promoter driven luciferase reporter in HeLa cells by wild-type MEF2C or L38P-mutant MEF2C (L38P) showed significantly diminished synergistic transcriptional activation between L38P-mutant MEF2C and GATA4. Three independent transfection experiments were done in triplicate for each expression plasmid, and mean and standard deviations are given. * indicates $t = 9.37232$, $p = 0.00072$, when compared with the wild-type counterpart.

Interestingly, in the present study all the affected adult family members harboring the identified MEF2C mutation had also mental retardation, stereotypic movements, and paroxysmal epilepsy. Factually, the *MEF2C* gene plays a crucial role not only in myogenesis but also in neurogenesis, and *MEF2C* haploinsufficiency has been involved in the pathogenesis of central nervous system disease [101]. Clinically, the *MEF2C* haploinsufficiency syndrome has been well recognized as a neurodevelopmental disorder, which is characterized mainly by intellectual disability with inability to speak, limited walking ability, hypotonia, stereotypic movement, epilepsy, and minor brain malformation [101]. Therefore, this study expands the phenotypic spectrum linked to *MEF2C* mutation.

In conclusion, this is the first report on the association of *MEF2C* loss-of-function mutation with increased vulnerability to CHD in humans, which provides novel insight into the molecular mechanism of CHD, suggesting potential implications for an improved strategy in early diagnosis and individualized treatment of CHD.

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Competing Interests

The authors have declared that no competing interest exists.

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