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ORIGINAL ARTICLE

Identification and genetic analysis of rare variants in myosin family genes in 412 Han Chinese congenital heart disease patients

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Abstract

Background: Myosin family genes, including those encoding myosin heavy chain 6, myosin heavy chain 7, myosin light chain 3, and myosin light chain 2 (MYL2), are important genetic factors in congenital heart disease (CHD). However, how these genes contribute to CHD in the Han Chinese population remains unclear.

Methods: We sequenced myosin family genes in a Han Chinese cohort comprising 412 CHD patients and 213 matched controls in the present study. A zebrafish model was used to evaluate the pathogenicity of rare mutations in MYL2.

Results: We identified 30 known mutations and 12 novel mutations. Furthermore, the contributions of two novel mutations, MYL2 p.Ile158Thr and p.Val146Met, to CHD were analyzed. The p.Ile158Thr mutation increased MYL2 expression. In zebrafish embryos, injection of *myl2b*-targeting morpholinos led to aberrant cardiac structures, an effect that was reversed by expression of wild-type MYL2 but not MYL2 p.Ile158Thr and pVal146Met.

Conclusions: Overall, our findings suggest that MYL2 p.Ile158Thr and p.Val146Met contribute to the etiology of CHD. The results also indicate the importance of MYL2 in heart formation.

KEYWORDS
congenital heart disease, *MYL2*, myosin family, rare variants, zebrafish

1 | INTRODUCTION

Congenital heart disease (CHD) is a common congenital disorder that affects eight out of every thousand live births worldwide and is often associated with fetal loss (Blue et al., 2012; van der Bom et al., 2011). CHD ranked first among all birth defects from 2005 to 2019, with increasing incidence in China (Department of Women and Children Health, 2021). The epidemiology of CHD suggests the importance of genetic contributions (Zaidi & Brueckner, 2017). Exploring the genetic etiology of CHD will help improve its clinical diagnosis (Dong et al., 2022; Gong et al., 2022; Williams et al., 2019). However, which genes are involved and how they contribute to CHD remains unknown in the Han Chinese population.

Cardiac myosin is the molecular motor that powers heart contraction and plays an important role in heart development (Barrick & Greenberg, 2021). Cardiac myosin is a heterohexameric protein consisting of two myosin heavy chains (MHCs), two regulatory light chains (RLCs), and two essential light chains (ELCs) (Winkelmann et al., 2015). There are two kinds of cardiac myosin isoforms in the ventricle and atrium, composed of different MHCs, ELCs, and RLCs. The primary ventricular isoforms are myosin heavy chain 7 (*MYH7*, OMIM accession number: 160760), myosin light chain 3 (*MYL3*, OMIM accession number: 160790), and myosin light chain 2 (*MYL2*, OMIM accession number: 160781), whereas the primary atrial isoforms are myosin heavy chain 6 (*MYH6*, OMIM accession number: 160710), myosin light chain 4 (*MYL4*, OMIM accession number: 160770), and myosin light chain 7 (*MYL7*, OMIM accession number: 613993) (England & Loughna, 2013). Previous studies have identified dysregulated myosin family genes, suggesting that cardiac myosin subunits are associated with CHD or severe heart diseases. For example, mutation within the segment of the *MYH6* gene encoding the motor domain affected myofibril formation (Granados-Riveron et al., 2010). The MYH6 stop codon mutation Arg1279X, which results in the elimination of the 660 amino acids, was found to be associated with CHD (Razmara & Garshasbi, 2018). The mutation Arg403Gln in MYH7 has been associated with hypertrophic cardiomyopathy (Dausse et al., 1993; Erdmann et al., 2003; Moolman et al., 1993). Met149Val and Arg154His mutations in MYL3 have been associated with cardiomyopathy involving mid-left ventricular chamber thickening (Poetter et al., 1996). The results of these studies indicate that cardiac myosin is essential for heart function.

MYL2 is an 18.8 kDa protein in the myosin neck/tail region with an exposed binding site for interacting proteins. MYL2 phosphorylation by myosin light chain kinase (MLCK) is essential for the activation of myosin during cardiac contraction (Barrick & Greenberg, 2021). MYL2 Ser15 phosphorylation by MLCK causes the myosin heads to move closer to the thin filament, modulating the kinetics of cardiac contraction (Levine et al., 1996; Montezano et al., 2018; Szczesna et al., 2001). The results of a previous study revealed that the MYL2 Arg58Gln mutation in hypertrophic cardiomyopathy could dramatically inhibit MYL2 Ser15 phosphorylation (Szczesna et al., 2001).

We sequenced cardiac myosin genes, including MYH6, MYH7, MYL2 and MYL3, from 412 Han Chinese CHD patients to determine which genes contribute to CHD in this population. We identified 30 known and 12 novel variants, including synonymous and missense variants. Four of these MYL2 variants were novel, two of which were missense. The potential pathogenicity of two rare variants (MYL2 p. Ile158Thr and p. Val146Met) was investigated. MYL2 p. Ile158Thr significantly increased MYL2 protein expression. Both variants increased the aberration rate in zebrafish embryos.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

The studies described in the paper were conducted in accordance with the tenets of the 1975 Declaration of Helsinki, and the protocols were previously reviewed and approved by the Ethics Committee of the School of Life Sciences, Fudan University. Written informed consent from the parents or guardians of the children was obtained.

2.2 | Patients and samples

Blood samples from 412 CHD patients (mean age 2.9 ± 2.7 years, 55.6% male) were collected from the Cardiovascular Disease Institute of Jinan Military Command (Jinan, Shandong, China) between March 2008 and 2013 (Qiao et al., 2016). Sporadic CHD cases were diagnosed based on echocardiography, with some diagnoses further confirmed surgically. Patients who had clinical features of developmental anomalies were excluded. Additionally, patients were excluded if they had a positive family history of CHD in a first-degree relative, if the mother had maternal diabetes mellitus, and if the mother was exposed to known teratogens or any therapeutic drugs during pregnancy. All CHD cases were classified according to previously described methodology. Simultaneously, 213 ethnically and gender-matched control volunteers (mean age 7.1 ± 3.7 years, 49.8% male) from the same geographical area were enrolled. Control individuals with any

congenital anomalies or cardiac diseases were excluded (Table S1). Approximately 2 ml of peripheral blood was collected from each test case. Genomic DNA was isolated from peripheral blood using conventional reagents and quantified using a NanoDrop2000 (Thermo Scientific).

2.3 | Exome sequencing and sanger sequencing

The genomic structures of human myosin family genes were determined using NCBI GenBank (MYH6: NM_002471.4, MYH7: NM_000257.4, MYL2: NM_000432.4, MYL3: NM_000258.3).

DNA-fragment libraries were generated, and target enrichment was performed using a probe specific for myosin family genes with the Agilent Sure Select XT Custom enrichment system with some modifications at GBP Biotechnology. In brief, 1 μg of genomic DNA was subjected to ultrasonic energy fragmentation with a Covaris S2 instrument. The average size of the genomic DNA fragments was ~250 bp. The ligated libraries were gel purified and, in turn, PCR amplified (Phusion, Thermo Scientific). The quality and quantity of the prepared libraries were assessed using an Agilent 2100 Bioanalyzer. Sequencing was performed on an Illumina HiSeq2000 DNA sequencer (version 3). The annotation process was performed using the Variant Effect Predictor based on the hg19/GRCh37 database.

The MYL2 variants were confirmed by Sanger sequencing (primers, MYL2-genome-F: CCGGGAATTCA TGGCACCTAAGAAAGCAAAG, MYL2-genome-R: CTGC CCCAGAAAGGGCATGAC). All confirmed case-specific MYL2 variants were cross-referenced with the Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org). All missense variants were evaluated by PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al., 2010) and PROVEAN (Protein Variation Effect Analyzer, http://provean.jcvi.org/index.php) (Choi & Chan, 2015).

2.4 | Vector construction and site-directed mutagenesis

MYL2 cDNA was amplified using total RNA extracted from the AC16 cell line and cloned into the pCS2-3Flag vector between EcoRI and XhoI. The MYL2 variants were generated by PCR with specific primers and then digested using DpnI for 4 hours. The products were transformed into *Escherichia coli* DH5α, and a single clone was selected the next day. All plasmids used in this study were confirmed by Sanger sequencing. The primers are listed in Table S2.

2.5 | Cell culture and transfection

Human HEK293T cells and AC16 cells (purchased from ATCC, HTX2571) were cultured in DMEM (Gibco, 11995-065) supplemented with 10% fetal bovine serum (Gibco, 10099-141-FBS). All cells were maintained at 37°C and 5% CO_2 and passaged by using 0.05% trypsin (Gibco, 25300062).

The cells were seeded in new cell plates and incubated until they reached 70–80% confluency before the transfection assay. Lipofectamine 3000 (Invitrogen, L3000015) and Lipofectamine 2000 (Invitrogen, 11668019) were used for the transfection according to the manufacturer's instructions.

2.6 | RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Life Technologies, 15,596,026) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA with HiScript III RT SuperMix (+gDNA wiper, Vazyme, R323-01).

2.7 | Western blot analysis

Cells were washed with cold PBS and lysed in cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1% Triton, complete mini tablet-protease inhibitor cocktail tablets [Roche, 04693132001]). The proteins were separated by 10% SDS–PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk in TBS with Tween-20 (TBST), the membrane was incubated

TABLE 1 The summary of all mutations detected in cardiac myosin family

Protein	Type	Mutations	Novel mutations
MYH6	Missense variant	16	5
	Synonymous variant	7	2
MYH7	Missense variant	6	2
	Synonymous variant	8	2
MYL2	Missense variant	2	1
	Synonymous variant	2	0
MYL3	Synonymous variant	1	0
Total		42	12

Notes: Refseq: NM_002471.4, NP_002462.2 for MYH6. Refseq: NM_000257.4, NP_000248.2 for MYH7. Refseq: NM_000432.4, NP_000423.2 for MYL2. Refseq: NM_000258.3, NP_000249.1 for MYL3.

TABLE 2 The information of missense variants and corresponding patients

Protein	Chromosome position	Amino acid substitution	Reference	Alternate	gnomAD allele counts	Number of patients
MYH6	chr14:23853678	E1846D	C	G	/	1
MYH6	chr14:23853695	G1841R	C	G	/	1
MYH6	chr14:23853806	Q1804K	G	T	98/282876	1
MYH6	chr14:23854154	E1754K	C	T	4/282888	1
MYH6	chr14:23855229	R1691C	G	A	3/251298	1
MYH6	chr14:23857492	A1411T	C	T	8/282872	1
MYH6	chr14:23858195	E1350K	C	T	3/251194	1
MYH6	chr14:23859619	E1127K	C	T	1/246544	1
MYH6	chr14:23861816	E1099D	C	G	/	1
MYH6	chr14:23862194	D1060N	C	T	19/251496	1
MYH6	chr14:23865524	R800C	G	A	7/282774	2
MYH6	chr14:23866254	R696C	G	A	6/251434	1
MYH6	chr14:23870076	V418M	C	T	62/282872	1
MYH6	chr14:23871726	M363T	A	G	5/282632	1
MYH6	chr14:23872953	G257E	C	T	/	2
MYH6	chr14:23873948	G205V	C	A	/	1
MYH7	chr14:23883054	E1902Q	C	G	28/282882	4
MYH7	chr14:23884263	A1834T	C	T	14/250566	1
MYH7	chr14:23884377	R1796W	G	A	1/251482	1
MYH7	chr14:23886464	E1473K	C	T	/	1
MYH7	chr14:23888744	Q1267H	C	G	15/282846	3

Sample ID	Sex	Age	Phenotype	PROVEAN score	PROVEAN prediction	PolyPhen-2 score	PolyPhen-2 prediction
159952	M	25D	VSD	−2.011	Neutral	0.99	PROBABLY DAMAGING
159101	F	7Y	PS	−4.916	Deleterious	0.987	PROBABLY DAMAGING
157881	F	7Y	PS	−2.432	Neutral	0.996	PROBABLY DAMAGING
158415	F	1Y1M	TOF	−2.753	Deleterious	1	PROBABLY DAMAGING
158927	M	4M	CoA, VSD, MI	−5.242	Deleterious	1	PROBABLY DAMAGING
158379	F	7Y	ASD	−2.297	Neutral	0.999	PROBABLY DAMAGING
158341	F	1Y6M	TOF	−2.998	Deleterious	1	PROBABLY DAMAGING
158557	M	2Y5M	DORV, PH	−3.044	Deleterious	1	PROBABLY DAMAGING
158480	F	3Y8M	SV, SA, TGA, PS	−2.416	Neutral	0.06	BENIGN
159300	F	7Y	TOF	−3.899	Deleterious	1	PROBABLY DAMAGING
157977	F	5Y	F3	−6.459	Deleterious	1	PROBABLY DAMAGING
159001	F	3Y4M	TECD, PH				
159666	M	1Y6M	DTGA	−6.92	Deleterious	1	PROBABLY DAMAGING
159621	F	1Y1M	ASD	−2.469	Neutral	1	PROBABLY DAMAGING
159872	M	2Y4M	SV, TGA, PS	−4.986	Deleterious	0.277	BENIGN
158909	M	1Y	TOF	−6.211	Deleterious	0.96	PROBABLY DAMAGING
159230	M	3Y	TOF				
158681	F	6M	AS	−1.334	Neutral	0.011	BENIGN
159012	F	1Y11M	PDA	−1.89	Neutral	0.741	PROBABLY DAMAGING
159901	F	5Y	VSD, ASD, PDA, PH				
158415	F	1Y1M	TOF				
158483	F	14Y	TOF, LVHS				
159608	F	5M	F3	−1.104	Neutral	0.007	BENIGN
158245	M	10M	VSD	−5.473	Deleterious	1	PROBABLY DAMAGING
159771	M	3Y8M	PDA	−3.044	Deleterious	0.798	PROBABLY DAMAGING
157938	M	6Y	DORV	−2.872	Deleterious	0.999	PROBABLY DAMAGING
159111	F	3Y	VSD, PDA, PH				
159948	F	3Y	VSD, ASD				

TABLE 2 (Continued)

Protein	Chromosome position	Amino acid substitution	Reference	Alternate	gnomAD allele counts	Number of patients
MYH7	chr14:23897786	Y501D	A	C	/	1
MYL2	chr12:111348909	I158T	A	G	/	1
MYL2	chr12:111348946	V146M	C	T	10/280354	1

Notes: Reference: Human GRCh37/hg19. Refseq: NM_002471.4, NP_002462.2 for MYH6. Refseq: NM_000257.4, NP_000248.2 for MYH7. Refseq: NM_000432.4, NP_000423.2 for MYL2. Refseq: NM_000258.3, NP_000249.1 for MYL3.

Abbreviations: AS, aortic stenosis; ASD, atrial septal defect; CAA, coronary artery anomalies; CoA, coarctation of the aorta; DORV, double outlet right ventricle; DTGA, d-transposition of the great arteries; Ebstein, Ebstein anomaly; F3, trilogy of Fallot; ID, isolated dextrocardia; LVHS, left ventricular hypertrophy; MI, mitral insufficiency; PDA, patent ductus arteriosus; PH, pulmonary arterial hypertension; PS, pulmonic stenosis; SA, single atrium; SV, single ventricle; TGA, transposition of the great arteries; TECD, total endocardial cushion defect; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

overnight at 4°C with one of the following primary antibodies: GAPDH (Yeason, 30201ES60) and FLAG (Yeason, 30503ES80). The next day, the membrane was washed with TBST three times and incubated with corresponding anti-mouse (Proteintech, SA00001-1) secondary antibody at a 1:10,000 dilution at room temperature for 1 h.

2.8 | Zebrafish microinjection and microscopic observation

The zebrafish (AB strain and transgenic line [Cmlc2: mCherry] in which cardiomyocytes are specifically labeled with red dye) were maintained in standard conditions at 28.5°C. The antisense MO (5′-AGCAGAGATT ATTAGAGTCCACCTA-3′) targeting the splice site was designed and synthesized by Gene Tools (Philomath, OR). The MO can cleanly skip myl2b-201 & myl2b-202 exon 3, comprising 76 bases, causing a frameshift in the downstream sequence. This alteration brings a premature termination codon in-frame and might trigger nonsense-mediated decay of the transcript (Figure S1).

The plasmids used for the zebrafish experiments were all extracted using an endotoxin-free process. The working concentration of MO was 0.9 nM, and 2.3 nl MO solution was injected into each zebrafish embryo. In the rescue experiments, plasmids at a working concentration of 40 ng/μl or 50 ng/μl were coinjected into 1-cell-stage zebrafish embryos. Embryos were observed and photographed using a Leica MZ95 microscope system at 3 days postfertilization. The embryos were divided into three categories according to the severity of morphology. The zebrafish injections and phenotyping procedures were conducted in a blind manner. The distribution of each group was compared by χ^2 analysis in GraphPad Prism.

All animal procedures were approved by the Ethics Committee of the School of Life Sciences, Fudan University. All animals received humane care and that study protocols comply with the Ethics Committee of the School of Life Sciences's guidelines.

2.9 | Secondary structure prediction of wild type, p. Ile158Thr and p. Val146Met MYL2

Secondary structure prediction was performed using I-TASSER (http://zhanglab.ccmb.med.umich.edu/ITASSER/). The amino acid sequences of p. Ile158Thr and p. Val146Met were compared against template proteins selected from a library of similar structures. The prediction quality was proportional to the value of the C-score (Rock et al., 1994; Roy et al., 2010). The predicted structures were then modeled using the PyMOL (www.pymol.org) molecular visualization system to allow the determination of the C-α distances (Å) between amino acid residues.

2.10 | Statistical analysis

GraphPad Prism was used for statistical analyses and graph generation. All experiments were repeated at least three times. The *t*-test, assuming a two-tailed distribution, was used for comparisons between two groups. Data are expressed as the mean ± *SD* with dots to show the actual data points. Significance is defined as #$p > .05$, *$p < .05$, **$p < .01$, ***$p < .001$ and ****$p < .0001$.

3 | RESULTS

3.1 | Identification of cardiac myosin variants in CHD cases

As previously described, a total of 412 sporadic CHD patients and 213 matched controls were recruited from

Sample ID	Sex	Age	Phenotype	PROVEAN score	PROVEAN prediction	PolyPhen-2 score	PolyPhen-2 prediction
160098	M	1Y	VSD	−8.772	Deleterious	1	PROBABLY DAMAGING
159731	F	5M	PDA, ASD, VSD	−3.054	Deleterious	0.653	PROBABLY DAMAGING
158927	M	4M	CoA, VSD, MI	−1.497	Neutral	0.413	BENIGN

2008 to 2013. Through targeted sequencing of the *MYH6, MYH7, MYL2 and MYL3* genes, we identified 42 rare variants (<0.001% in the gnomAD database) in myosin family genes, including MYH6, MYH7, MYL2 and MYL3 (Table 1). PROVEAN and PolyPhen-2 were used to predict whether the resulting amino acid substitutions impacted the biological functions of the corresponding proteins. The variant information, patient information and functional prediction results are listed in supplement material (Tables 2 and 3), and the patients were divided based on whether they had synonymous or missense variants.

3.2 | Two case-specific missense *MYL2* variants were identified

We focused on the pathogenic evaluation of two missense variants in *MYL2* (Table 4) because of the pivotal regulatory role of this gene in myosin function, as mentioned above. The MYL2 p. Ile158Thr (**NC_000012.11: chr12:111348909A ≥ G**) variant was absent from gnomAD (Karczewski et al., 2020), and the MYL2 p. Val146Met (**NC_000012.11: chr12: 111348946C ≥ T**) variant was rare, with a frequency of 10/280345 in gnomAD. These variants have been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). The accession numbers are **VCV001202626** for MYL2 p. Ile158Thr and **VCV000520474** for MYL2 p. Val146Met.

The MYL2 p. Ile158Thr variant was identified in a 5-month-old female infant with patent ductus arteriosus, atrial septal defect and ventricular septal defect (Table 1). Ile158Thr is located in the EF-hand 3 domain of MYL2. This variant affected a highly conserved residue (Figure 1A) and was predicted to be a pathogenic variant by both SIFT and PolyPhen2.

The p. Val146Met variant was discovered in a 4-month-old male infant with coarctation of the aorta, ventricular septal defect and mitral insufficiency (Table 1). The valine at codon 146 was a highly conserved residue between species (Figure 1A). Both SIFT and PolyPhen2 suggested that this variant was neutral. These two substitutions were absent in our control individuals. Sanger sequencing confirmed the authenticity of the variants in CHD cases (Figure 1B).

3.3 | MYL2 p. Ile158Thr significantly influenced the expression pattern of MYL2 protein

Then, I-TASSER was used to analyze the lowest-energy secondary structure of wild-type (WT), p. Ile158Thr and p. Val146Met MYL2 (Figure 1C). As MYL2 Ser15 is a pivotal site for phosphorylation and regulates MYL2 function, the site of MYL2 Ser15 is indicated in the figure. Meanwhile, the distances between the WT residues or mutant residues and S15 are shown. The p. Ile158Thr variant increased the apparent distance between the 158th residue and Ser15 (27.5 Å < 30.0 Å). Also, the p. Val146Met variant increased this distance (40.4 Å < 43.1 Å).

We examined whether MYL2 p. Ile158Thr and p. Val146Met influence protein expression. We constructed MYL2 WT and mutant plasmids and then transfected them into HEK293T cells. The Western blot results showed that the expression level of MYL2 p. Ile158Thr was significantly upregulated (Figure 1D). This finding indicates that the MYL2 p. Ile158Thr substitution strongly influenced the expression of MYL2 protein compared with WT. However, the MYL2 p. Val146Met substitution resulted in no notable change in protein expression levels.

TABLE 3 The information of synonymous variants and corresponding patients

Protein	Chromosome position	Amino acid substitution	Reference	Alternate	gnomAD allele counts	Number of patients	Sample ID	Sex	Age	Phenotype
MYH6	chr14:23852437	E1886E	C	T	/	1	157853	M	10Y	F3, PS
MYH6	chr14:23852443	A1884A	G	A	288/282808	2	158798	F	5Y	Ebstein, ARVD
							160049	M	4 M	TECD
MYH6	chr14:23852455	Y1880Y	G	A	/	1	158516	M	5Y6 M	DORV, PS, CAA
MYH6	chr14:23868079	Y583Y	G	A	26/282854	1	158528	M	3Y3M	Dextrocardia, TECD
MYH6	chr14:23868130	K566K	C	T	1/31386	1	159833	M	1Y8 M	ASD
MYH6	chr14:23869918	D470D	G	A	60/282848	1	158543	M	8 M	DORV, PH
MYH6	chr14:23876244	T63T	G	A	5/251432	1	158681	F	5 M	AS
MYH7	chr14:23888440	L1306L	G	A	178/282850	2	158329	M	6 M	TOF
							158527	M	3Y5 M	TOF
MYH7	chr14:23891486	R1050R	G	T	4/251484	1	159691	M	16Y	DORV, PS
MYH7	chr14:23893269	N923N	G	A	199/282884	1	159925	F	5 M	VSD, ASD, MI, TI
MYH7	chr14:23897011	L557L	C	A	4/251484	1	159691	M	16Y	DORV, PS
MYH7	chr14:23898447	N416N	A	G	5/251492	1	158242	F	7Y3M	VSD, ASD, PDA
MYH7	chr14:23899027	K365K	C	T	32,399/282762	2	157959	F	6Y	PS
							159925	F	5 M	VSD, ASD, MI, TI
MYH7	chr14:23900640	A261A	T	C	/	1	159857	F	2Y5 M	TOF, PDA, MG
MYH7	chr14:23902876	E22E	C	T	/	1	159582	M	1Y2M	ID, SA, SV, PS
MYL2	chr12:111348926	Y152Y	G	A	32/280648	3	158900	F	1 M	VSD, ASD
							159063	M	5Y7M	VSD, PFO
							160124	M	25D	PA, TS, TI, PDA
MYL2	chr12:111352090	R58R	T	C	1/251464	1	159664	F	3Y	TGA, PH
MYL3	chr3:46904776	V35V	G	A	5/250210	2	157998	F	5Y	TOF
							159550	M	3Y	DORV, PS

Notes: Reference: Human GRCh37/hg19 Refseq: NM_002471.4, NP_002462.2 for MYH6. Refseq: NM_000257.4, NP_000248.2 for MYH7. Refseq: NM_000432.4, NP_000423.2 for MYL2. Refseq: NM_000258.3, NP_000249.1 for MYL3.

Abbreviations: AS, aortic stenosis; ASD, atrial septal defect; CAA, coronary artery anomalies; CoA, coarctation of the aorta; DORV, double outlet right ventricle; DTGA, d-transposition of the great arteries; Ebstein, Ebstein anomaly; F3, trilogy of Fallot; ID, isolated dextrocardia; LVHS, left ventricular hypertrophy; MI, mitral insufficiency; PDA, patent ductus arteriosus; PH, pulmonary arterial hypertension; PS, pulmonic stenosis; TECD, total endocardial cushion defect; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; SA, single atrium; SV, single ventricle; VSD, ventricular septal defect.

TABLE 4 Rare missense mutations within the MYL2 coding region detected in this CHD cohort

Type	Varients[a]	Nucleotide change[b]	GnomAD allele counts (frequency)	PROVEAN[c]	PloyPhen-2 prediction[d]	Sample ID (Sex; Age)	Phenotype
Missense variant	p.Ile158Thr	c.473 T > C	\	Deleterious	Probably damaging	159731 (F;5 Month)	PDA, ASD, VSD
Missense variant	p.Val146Met	c.436G > A	10/280354 (3.57e-05)	Neutral	Benign	158927 (M;4 Month)	CoA, VSD, MI

Abbreviations: ASD, atrial septal defect; CoA, coarctation of the aorta; F, female; M, male; MI, mitral insufficiency; PDA, patent ductus arteriosus; VSD, ventricular septal defect.

[a]Reference sequence NP_000423.2.

[b]Reference sequence NM_000432.4, MYL2_v001.

[c]PROVEAN (http://provean.jcvi.org/index.php) Prediction cutoff = −2.5. Prediction outcome can be one of deleterious or neutral.

[d]PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). Prediction outcome can be one of probably damaging, possibly damaging, or benign.

3.4 | MYL2 p. Ile158Thr and p. Val146Met mutants could not rescue the zebrafish developmental defects caused by *myl2b*-MO knockdown

To investigate the effects and functions of MYL2 p. Ile158Thr and p. Val146Met variants in vivo, we used morpholinos (MO) to knock down *myl2b* (the homologous gene of human *MYL2*) in zebrafish. Three days postfertilization, injected zebrafish embryos were divided in three categories according to severity (Figure 2A). Statistical analysis showed that injection with *myl2b*-MO led to cardiac malformation in approximately 60% of embryos, while the control group had only 15% abnormal embryos (Figure 2B).

To investigate the role of the p. Ile158Thr and p. Val146Met variants in zebrafish embryonic development, we injected plasmids carrying WT, p. Ile158Thr and p. Val146Met MYL2 as well as the empty vector (negative control) into zebrafish embryos. WT MYL2 noticeably reduced the aberrations caused by *myl2b*-MO, whereas p. Ile158Thr and p. Val146Met MYL2 demonstrated lower rescue efficacy (Figure 2B and Table S3). This phenomenon suggested that these variants, including p. Ile158Thr or p. Val146Met, compromised the function of MYL2 in zebrafish embryonic cardiac development.

A transgenic zebrafish model (cmlc2: mCherry) was used to further confirm the effect of the two variants on the embryonic heart. Imaging results indicated that p. Ile158Thr and p. Val146Met MYL2 led to structural disorders in zebrafish hearts, while zebrafish with WT MYL2 had an intact heart structure with a distinct atrium and ventricle (Figure 2C). After injecting *myl2b*-MO, the hearts of embryonic zebrafish appeared abnormal, resembling a line or a pipe. This abnormal heart could not beat naturally; however, the phenotype could be reversed by MYL2 WT injection. In contrast, the injection of p. Ile158Thr and p. Val146Met MYL2 into abnormal embryos failed to produce a natural cardiac structure with a distinct atrium or ventricle. These results suggest that MYL2 acts as a vital cardiac regulator during early cardiac formation in zebrafish. This detailed evidence supports our previous conclusions that MYL2 p. Ile158Thr and p. Val146Met variants are harmful to the heart.

4 | DISCUSSION

The present case–control study of 412 CHD patients and 213 matched controls was designed to investigate the associations between dysfunctional myosin family genes and the risk of CHD. To the best of our knowledge, this is the first report of 42 potential risk variants in four members

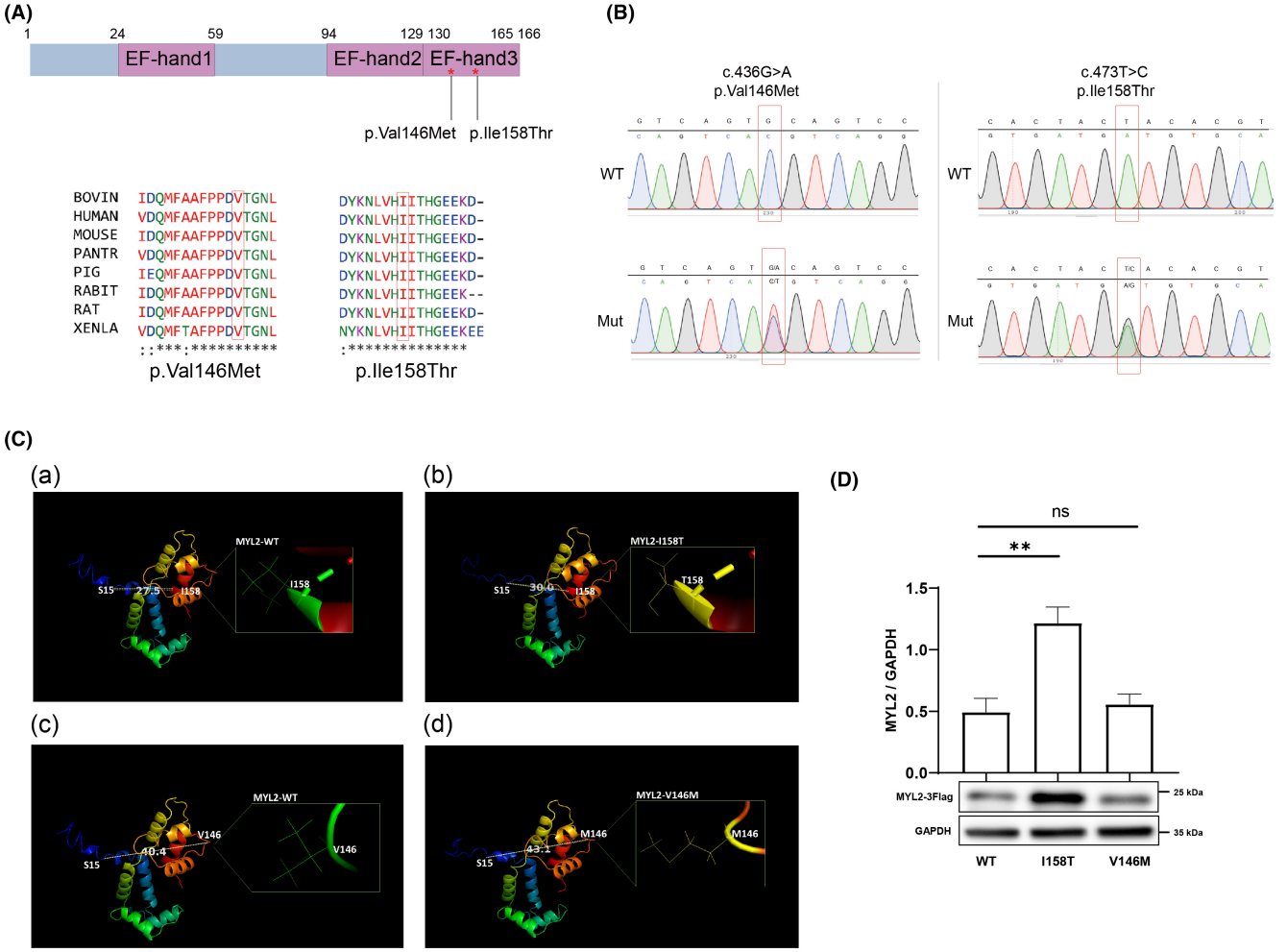
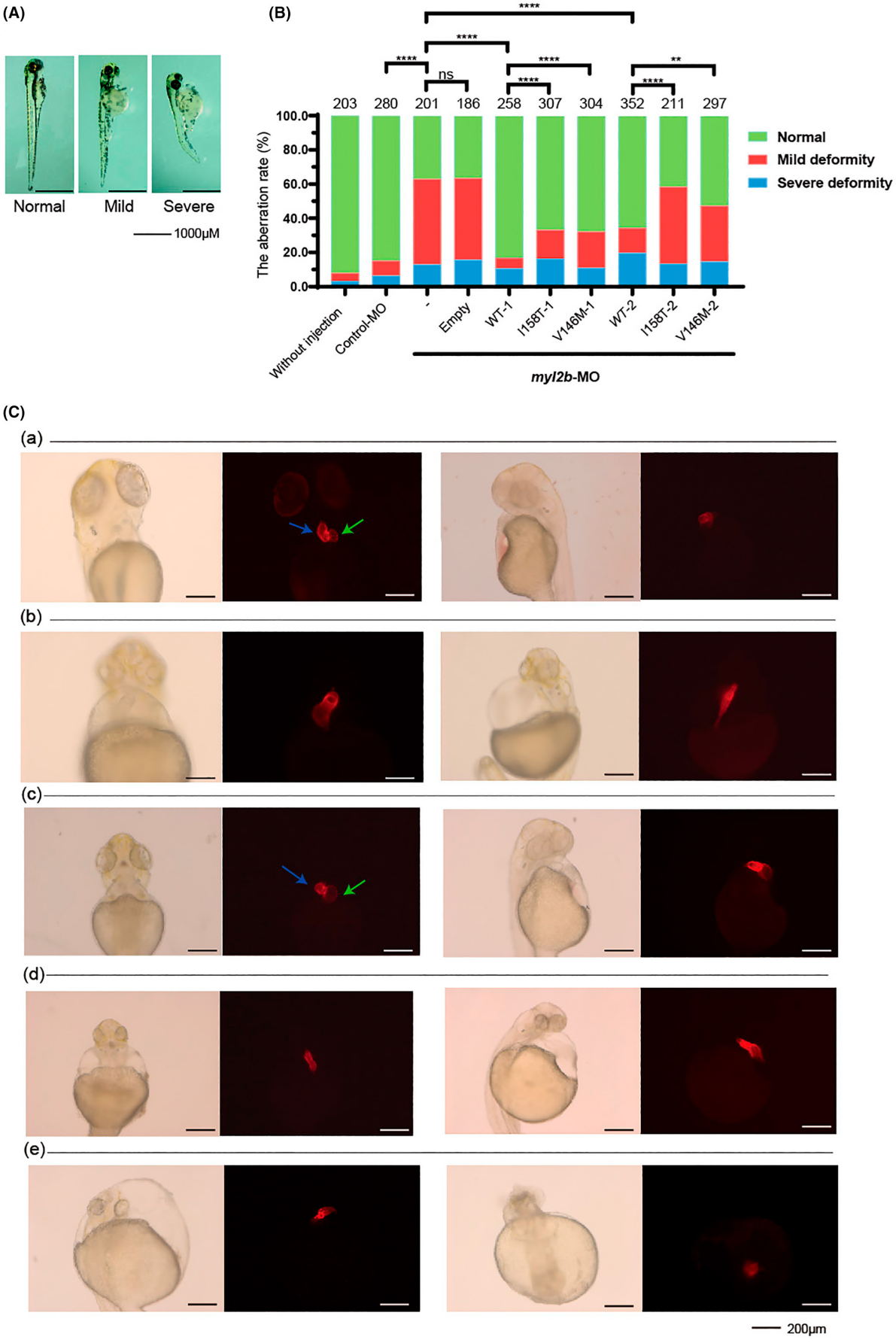


FIGURE 1 Schematic representation of missense *MYL2* variants. (A) MYL2 protein with 166 amino acids showing three EF-hand domains. The red stars indicate positions of MYL2 variants, and their evolutionary conservation across the species is shown below. (B) DNA sequence chromatogram of wild-type and mutant variants. The sequences were confirmed by Sanger sequencing. The red squares mark the positions of variants. WT, wild-type; MUT, mutant. (C) The secondary structure prediction of WT, p. Ile158Thr and p. Val146Met MYL2 was performed using I-TASSER. (a). MYL2-WT structure, which emphasized the Ile158 site. The distance between the C-α of Ser15 and the C-α of Ile158 is 27.5 Å. (b) MYL2-I158T structure, which emphasized the Thr158 site. The distance between the C-α of Ser15 and the C-α of Thr158 is 30.0 Å, which is longer than the relevant distance in MYL2-WT. (c). MYL2-WT structure, which emphasized the Var146 site. The distance between the C-α of Ser15 and the C-α of Var146 is 40.4 Å. (d). MYL2-V146 M structure, which emphasized the Met146 site. The distance between the C-α of Ser15 and the C-α of Met146 is 43.1 Å, which is shorter than the relevant distance in MYL2-WT. (D) Western blot analysis of wild-type and mutant MYL2 proteins in HEK293T cells. GAPDH was used as a loading control for the cell lysate. ns $p > .05$, *$p < .05$, ** $< .01$.

FIGURE 2 *MYL2* variant effects in zebrafish embryos. (A) Typical three-day postfertilization zebrafish morphology in three categories according to severity. We clustered all injected zebrafish embryos into normal/mild deformity/severe deformity groups. The zebrafish were mainly divided by heart abnormalities. "Normal" indicated no apparent malformation in either the heart or body. "Mild" and "severe" indicated the degree of heart failure and malformation. (B) Myl2b-knockdown zebrafish embryos rescued by human WT, p. Ile158Thr, p. Val146Met MYL2 plasmids, respectively. Two different dosage sets were injected into zebrafish embryos. The WT-1, I158T-1 and V146 M-1 groups were injected with 40 ng/μl plasmids. The WT-2, I158T-2 and V146 M-2 groups were injected with 50 ng/μl plasmids. The Y-axis represents the percentages of the three categories according to degrees in Figure 2A. The number above each bar is the total number of embryos examined under each experimental condition. ns $p > .05$, *$p < .05$, ** $< .01$, ***$p < .001$, ****$p < .0001$. (C) Transgenic zebrafish line Tg (Cmlc2: mCherry) embryos in which cardiomyocytes are specifically labeled with red dye were injected with human MYL2, p. Ile158Thr, p. Val146Met plasmids to depict the effects of p. Ile158Thr and p. Val146Met on zebrafish heart. The figure shows the five groups. (a). Wild-type zebrafish. (b). The zebrafish injected with myl2b-MO. (c). The zebrafish injected with myl2b-MO and human MYL2-WT plasmids. (d). The zebrafish injected with myl2b-MO and human MYL2 p. Ile158Thr plasmids. (e). The zebrafish injected with myl2b-MO and human MYL2 p. Val146Met plasmids. Two representative orientations of each experimental condition were presented, and each orientation consisted of a light field and a fluorescence microscope. The blue arrows indicate the heart ventricle, and the green arrows indicate the heart atrium.


(A)
Normal
Mild
Severe
1000μM
(B)
****
****
****
ns
****
****
**
****
203 280 201 186 258 307 304 352 211 297
100.0
80.0
60.0
40.0
20.0
0.0
The aberration rate (%)
Normal
Mild deformity
Severe deformity
Without injection
Control-MO
-
Empty
WT-1
I158T-1
V146M-1
WT-2
I158T-2
V146M-2
myl2b-MO
(C)
(a)
(b)
(c)
(d)
(e)
200μm

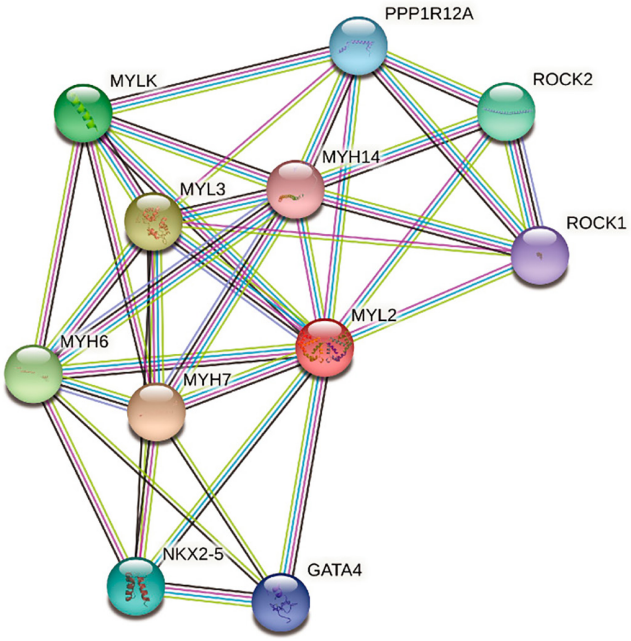


FIGURE 3 Ten potential direct interactors of MYL2 detected by the STRING database (https://string-db.org/)

of the myosin gene family in Han Chinese CHD patients. Our findings provide a comprehensive perspective on the roles of these genes in this population. The variants are divided into two groups, and we believe both groups represent good resources for further studies. We should note that the missense variants and the synonymous variants may play an important role in regulating the expression of corresponding genes. It is now recognized that some synonymous variants can influence the splicing process. For instance, a synonymous variant in MYL2 (p. Tyr152Tyr, chr12:110911122 c.456C > A), which was found in three patients in the cohort exhibiting similar CHD types, is predicted to affect the splicing process (Type: alteration of auxiliary sequence; Interpretation: significant alteration of ESE/ESS motifs ratio) by Human Splicing Finder (https://www.genomnis.com/access-hsf). The prediction is insufficient to confirm the function of the synonymous variant; thus, further research is still needed.

The *MYL2* gene is associated with many heart diseases. The relationship between MYL2 and hypertrophic cardiomyopathy has been widely studied (Gil et al., 2020) (Manivannan et al., 2020; Yin et al., 2019). However, few studies have demonstrated the relationship between *MYL2* and CHD. We believe that part of the reason for is that it is quite challenging to elucidate the potential functions of MYL2 in heart formation since most studies focus on the structures of MYL2 in mature myocardium.

Ca^{2+} binding and phosphorylation play a key role in regulating the thick filament protein myosin, ultimately leading to cardiac muscle contraction (Irving et al., 1992). MYL2 is a regulatory part of the myosin molecular complex located in the lever arm region that helps stabilize the α-helical neck region of the beta-myosin heavy chain. The EF-hands in MYL2 control Ca^{2+} binding and phosphorylation, modulating cardiac muscle contraction speed and force (Morano, 1999). Some previously reported MYL2 mutations that disrupt the Ca + binding site could perturb MYL2 binding with Ca^{2+} and result in changes beyond the sarcomere. For instance, the MYL2-R58Q pathogenic variant localizes near the Ca^{2+} binding site and has been associated previously with a clinically severe phenotype (Varnava et al., 2001; Yin et al., 2019). In this study, we found two missense variants in the same motif of MYL2, referred to as EF-hand 3. Together with the zebrafish experiment in which two MYL2 variants led to a structural heart disorder, this finding led us to speculate that the two variants have a negative effect on myosin function during heart formation by a loss-of-function effect. Similar to MYL2-R58Q, the variants in the MYL2 EF-hand may impair the interaction with myosin heavy chain by reducing Ca^{2+} transients. We also found that the MYL2 p. Ile158Thr variant resulted in significantly higher MYL2 protein expression levels than WT MYL2. The upregulated expression of MYL2 may lead to the imbalance of myosin family proteins during sarcomere formation.

To further explore the potential involvement of MYL2 in CHD, we examined its protein partners and its participation in signaling pathways. By querying the STRING database, we found that MYL2 interacts directly with 10 proteins (Figure 3). Four proteins (MYH6, MYH7, MYL3 and MYH14) in the myosin family interact with MYL2. These proteins are the basic components of the myosin II complex (GO:0016460) and participate in cardiac muscle contraction (KEGG pathway: hsa04260). Rock1, Rock2 and MYLK are serine/threonine protein kinases. They catalyze specific sites in MYL2 and regulate myosin-light-chain-phosphatase activity (GO:0035507). NKX2-5 and GATA4 are important transcriptional activators during septum secundum development (GO:0003285). More information about the ten proteins can be found in Table 5.

According to the ACMG/AMP guidelines (Richards et al., 2015), our results indicate that the MYL2 c.473T > C (p. Ile158Thr) should be classified as a pathogenic variant (PS3, PS4, PM1, PM2, PP2) and MYL2 c.436G > A (p. Val146Met) should be classified as a likely pathogenic variant (PS4, PM1, PP2).

Although our study revealed some rare myosin family gene variants, it did not directly elucidate the relationships between these genes and CHD. Further molecular experiments are needed to demonstrate the functional risk factors for CHD associated with these variants. For example, one could edit the hESC and induce cardiac cells to test the specific function of mutant MYL2. A mouse

TABLE 5 Potential direct interactors of MYL2 detected by the STRING database (https://string-db.org/)

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
MYH14	MYH7	ENSP00000470298	ENSP00000347507	Myosin heavy chain 9/10/11/14; Myosin-14; Cellular myosin that appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping; Deafness associated genes	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	0.916
MYH6	MYH7	ENSP00000386041	ENSP00000347507	Myosin heavy chain 6/7; Myosin-6; Muscle contraction; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	0.932
MYL2	MYH7	ENSP00000228841	ENSP00000347507	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	0.997
MYL3	MYH7	ENSP00000379210	ENSP00000347507	Myosin light chain 3; Regulatory light chain of myosin. Does not bind calcium; EF-hand domain containing	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	0.997

(Continues)

TABLE 5 (Continued)

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
MYLK	MYH7	ENSP00000353452	ENSP00000347507	Myosin light chain kinase, smooth muscle; Calcium/calmodulin-dependent myosin light chain kinase implicated in smooth muscle contraction via phosphorylation of myosin light chains (MLC). Also regulates Actin-myosin interaction through a non-kinase activity. Phosphorylates PTK2B/PYK2 and myosin light-chains. Involved in the inflammatory response (e.g., apoptosis, vascular permeability, leukocyte diapedesis), cell motility and morphology, airway hyperreactivity and other activities relevant to asthma. Required for tonic airway smooth muscle contraction that is necessary for physiological [...]	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	0.56
NKX2-5	MYH7	ENSP00000327758	ENSP00000347507	Homeobox protein Nkx-2.5; Implicated in commitment to and/or differentiation of the myocardial lineage. Acts as a transcriptional activator of ANF in cooperation with GATA4 (By similarity). Binds to the core DNA motif of NPPA promoter. It is transcriptionally controlled by PBX1 and acts as a transcriptional repressor of CDKN2B (By similarity). It is required for spleen development; NKL subclass homeoboxes and pseudogenes	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	0.729
GATA4	MYL2	ENSP00000334458	ENSP00000228841	Transcription factor GATA-4; Transcriptional activator that binds to the consensus sequence 5′-AGATAG-3′ and plays a key role in cardiac development and function. In cooperation with TBX5, it binds to cardiac super-enhancers and promotes cardiomyocyte gene expression, while it downregulates endocardial and endothelial gene expression. Involved in bone morphogenetic protein (BMP)-mediated induction of cardiac-specific gene expression. Binds to BMP response element (BMPRE) DNA sequences within cardiac activating regions (By similarity). Acts as a transcriptional activator of ANF in coop [...]	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.963

TABLE 5 (Continued)

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
MYH14	MYL2	ENSP00000470298	ENSP00000228841	Myosin heavy chain 9/10/11/14; Myosin-14; Cellular myosin that appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping; Deafness associated genes	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.963
MYH6	MYL2	ENSP00000386041	ENSP00000228841	Myosin heavy chain 6/7; Myosin-6; Muscle contraction; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.985
MYH7	MYL2	ENSP00000347507	ENSP00000228841	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.997

(Continues)

TABLE 5 (Continued)

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
MYL3	MYL2	ENSP00000379210	ENSP00000228841	Myosin light chain 3; Regulatory light chain of myosin. Does not bind calcium; EF-hand domain containing	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.993
MYLK	MYL2	ENSP00000353452	ENSP00000228841	Myosin light chain kinase, smooth muscle; Calcium/calmodulin-dependent myosin light chain kinase implicated in smooth muscle contraction via phosphorylation of myosin light chains (MLC). Also regulates Actin-myosin interaction through a non-kinase activity. Phosphorylates PTK2B/PYK2 and myosin light-chains. Involved in the inflammatory response (e.g., apoptosis, vascular permeability, leukocyte diapedesis), cell motility and morphology, airway hyperreactivity and other activities relevant to asthma. Required for tonic airway smooth muscle contraction that is necessary for physiological [...]	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.979
NKX2-5	MYL2	ENSP00000327758	ENSP00000228841	Homeobox protein Nkx-2.5; Implicated in commitment to and/or differentiation of the myocardial lineage. Acts as a transcriptional activator of ANF in cooperation with GATA4 (By similarity). Binds to the core DNA motif of NPPA promoter. It is transcriptionally controlled by PBX1 and acts as a transcriptional repressor of CDKN2B (By similarity). It is required for spleen development; NKL subclass homeoboxes and pseudogenes	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.978

TABLE 5 (Continued)

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
PPP1R12A	MYL2	ENSP00000389168	ENSP00000228841	Protein phosphatase 1 regulatory subunit 12A; Key regulator of protein phosphatase 1C (PPP1C). Mediates binding to myosin. As part of the PPP1C complex, involved in dephosphorylation of PLK1. Capable of inhibiting HIF1AN-dependent suppression of HIF1A activity; Ankyrin repeat domain containing	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.969
ROCK1	MYL2	ENSP00000382697	ENSP00000228841	Rho-associated protein kinase 1; Protein kinase which is a key regulator of Actin cytoskeleton and cell polarity. Involved in regulation of smooth muscle contraction, Actin cytoskeleton organization, stress fiber and focal adhesion formation, neurite retraction, cell adhesion and motility via phosphorylation of DAPK3, GFAP, LIMK1, LIMK2, MYL9/MLC2, PFN1 and PPP1R12A. Phosphorylates FHOD1 and acts synergistically with it to promote SRC-dependent non-apoptotic plasma membrane blebbing. Phosphorylates JIP3 and regulates the recruitment of JNK to JIP3 upon UVB-induced stress. Acts as a sup [...]	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.963

(Continues)

TABLE 5 (Continued)

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
ROCK2	MYL2	ENSP00000317985	ENSP00000228841	Rho-associated protein kinase 2; Protein kinase which is a key regulator of Actin cytoskeleton and cell polarity. Involved in regulation of smooth muscle contraction, Actin cytoskeleton organization, stress fiber and focal adhesion formation, neurite retraction, cell adhesion and motility via phosphorylation of ADD1, BRCA2, CNN1, EZR, DPYSL2, EP300, MSN, MYL9/MLC2, NPM1, RDX, PPP1R12A and VIM. Phosphorylates SORL1 and IRF4. Acts as a negative regulator of VEGF-induced angiogenic endothelial cell activation. Positively regulates the activation of p42/MAPK1-p44/MAPK3 and of p90RSK/RPS6KA [...]	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	0.978
MYH14	MYL3	ENSP00000470298	ENSP00000379210	Myosin heavy chain 9/10/11/14; Myosin-14; Cellular myosin that appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping; Deafness associated genes	Myosin light chain 3; Regulatory light chain of myosin. Does not bind calcium; EF-hand domain containing	0.948
MYH6	MYL3	ENSP00000386041	ENSP00000379210	Myosin heavy chain 6/7; Myosin-6; Muscle contraction; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	Myosin light chain 3; Regulatory light chain of myosin. Does not bind calcium; EF-hand domain containing	0.99
MYH7	MYL3	ENSP00000347507	ENSP00000379210	Myosin heavy chain 6/7; Myosin-7; Myosins are Actin-based motor molecules with ATPase activity essential for muscle contraction. Forms regular bipolar thick filaments that, together with Actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle; Belongs to the TRAFAC class myosin-kinesin ATPase superfamily. Myosin family	Myosin light chain 3; Regulatory light chain of myosin. Does not bind calcium; EF-hand domain containing	0.997

TABLE 5 (Continued)

Node1	Node2	Node1 accession	Node2 accession	Node1 annotation	Node2 annotation	Score
MYL2	MYL3	ENSP00000228841	ENSP00000379210	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform; Contractile protein that plays a role in heart development and function (By similarity). Following phosphorylation, plays a role in cross-bridge cycling kinetics and cardiac muscle contraction by increasing myosin lever arm stiffness and promoting myosin head diffusion; as a consequence of the increase in maximum contraction force and calcium sensitivity of contraction force. These events altogether slow down myosin kinetics and prolong duty cycle resulting in accumulated myosins being cooperatively recruited to Actin [...]	Myosin light chain 3; Regulatory light chain of myosin. Does not bind calcium; EF-hand domain containing	0.993

experiment is needed to determine whether the novel mutations cause CHD and to investigate the underlying mechanisms.

5 | CONCLUSIONS

In summary, we identified 42 rare variants in Han Chinese CHD patients that could be explored for pathogenicity in the future. MYL2 p.Ile158Thr were validated to affect protein expression in HEK293T. In zebrafish, the injection of MYL2 p. Ile158Thr and p. Val146Met into embryos failed to rescue abnormal phenotypes produced by *myl2b*-MO. Our findings suggest that MYL2 p.Ile158Thr and p.Val146Met contribute to the etiology of CHD. The results also indicate the importance of MYL2 in heart formation.

AUTHOR CONTRIBUTION

The authors' responsibilities were as follows: Y.Z. and H.W. conceived and designed the experiments. Y.Z. performed the experiments on cells and zebrafishes. R.P. collected CHD samples and performed sequencing analysis. Y.Z. analyzed the data and wrote the manuscript. Y.Z. and H.W. edited the manuscript. H.W. provided financial support. All authors reviewed/edited the manuscript and approved the final version.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests that are directly or indirectly related to the work. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

ETHICS STATEMENT

This study was conducted in accordance with the Declaration of Helsinki. Written informed consent from the parents or guardians of the children was obtained. All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Fudan University. The

protocols were previously reviewed and approved by the Ethics Committee of the School of Life Sciences, Fudan University.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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