

Next-generation sequencing identifies pathogenic and modifier mutations in a consanguineous Chinese family with hypertrophic cardiomyopathy

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Abstract

Hypertrophic cardiomyopathy (HCM) is a highly heterogeneous disease displaying considerable interfamilial and intrafamilial phenotypic variation, including disease severity, age of onset, and disease progression. This poorly understood variance raises the possibility of genetic modifier effects, particularly in *MYBPC3*-associated HCM.

In a large consanguineous Chinese HCM family, we identified 8 members harboring the *MYBPC3* c.3624delC (p.Lys1209Serfs) disease-causing mutation, but with very disparate phenotypes. Genotyping ruled out the modifying effect of previously described variants in renin-angiotensin-aldosterone system. Afterwards, we screened for modifying variants in all known causing genes and closely related genes for cardiomyopathy and channelopathy by performing targeted next-generation sequencing. For first time, we showed that a c.1598C>T (p.Ser533Leu) mutation in voltage-dependent L-type calcium channel subunit beta-2 (*CACNB2*) was present in all severely affected HCM patients, but not in those moderately affected or genotype-positive phenotype-negative patients. This *CACNB2* p.Ser533Leu mutation is extremely conserved in evolution, and was not found in 550 healthy controls.

Our results suggest that *CACNB2* is a possible candidate genetic modifier of *MYBPC3*-associated familial HCM, but more genetic evidence and functional experiments are needed to confirm.

Abbreviations: HCM = hypertrophic cardiomyopathy, LVH = left ventricular hypertrophy, MYBPC3 = myosin-binding protein C, MYH7 = β -myosin heavy chain, NGS = next-generation sequencing, RAAS = renin-angiotensin-aldosterone system.

Keywords: *cacnb2*, genetic modifier, hypertrophic cardiomyopathy, *mybpc3*, next-generation sequencing

1. Introduction

Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disease, present in 1 in 500 of the general population. More than 1500 mutations in ≥ 11 causative genes have been identified in HCM, most of which are unique to individual families.^[1] It is estimated that about 70% of patients who are positive for genetic tests carry mutations in β -myosin heavy chain (*MYH7*) and myosin-binding protein C (*MYBPC3*) genes; these 2 genes account for up to 50% of all clinically

diagnosed cases of HCM, whereas other genes account for <10% of cases.^[2] Clinically, HCM is a highly heterogeneous disease with not only considerable interfamilial, but also intrafamilial variability in disease phenotypes, which include disease severity, age of onset, and disease progression.^[1,3] The extensive phenotypic variability cannot be fully explained by the causal mutations because even siblings sharing the same pathological mutation still show huge discordant phenotypes. This unexplained variance raises the possibility of contribution from other genetic factors, the so-called modifier genes.

It is of great importance to identify these genetic modifiers in light of genetic-based diagnosis, risk stratification, and disease treatment. Pharmacologic interventions of the modifier genes involved in HCM-specific pathways might provide new therapeutic opportunities. However, identification of such modifier genes remains challenging and systemic exploration is absent in HCM. Among these few modifiers investigated in HCM, the most studied was genetic variations in the renin-angiotensin-aldosterone system (RAAS) genes. It was first proposed in a HCM family with a causal *MYBPC3* mutation that patients with the pro-left ventricular hypertrophy (LVH) genotypes in RAAS genes manifested cardiac hypertrophy, whereas those without pro-LVH genotypes did not.^[4] However, this observation was not confirmed in a recent study involving a large population of 368 sporadic HCM patients who carried founder mutations in *MYBPC3* gene,^[5] indicating that there are other currently unknown modifiers to be discovered.

Family study is important in modifier gene investigation because the population is homogeneous and more readily available than a large sample of unrelated patients, which is always a genetically heterogeneous population. In a Chinese HCM family, we recently identified 8 members harboring a

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MYBPC3 disease-causing mutation but with very disparate phenotypes. It is most likely that other functional mutations in relevant genes provide synergistic effects with *MYBPC3* mutation and lead to an early-onset and severe phenotype. We aimed to identify these possible genetic variants using the next-generation sequencing (NGS) technology to analyze all known causing genes and closely related genes for cardiomyopathy and channelopathy, given ion cycling deficiency and sarcomeric structure impairment were the major pathological mechanisms of HCM.^[6]

2. METHODS

2.1. Subjects

This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki, and approved by the local ethics committee at the Affiliated Drum Tower Hospital, Nanjing University School of Medicine. Written informed consent was obtained from all subjects.

A large consanguineous Chinese HCM family with considerable phenotype heterogeneity was clinically examined (Fig. 1). An ethnically matched group of 550 control subjects with normal echocardiograms was also included. Venous blood samples were collected for DNA analysis.

2.2. Diagnosis

Clinical diagnosis of HCM in adult patients is based on the presence of left ventricular end-diastolic wall thickness ≥ 15 mm in ≥ 1 left ventricular myocardial segments by 2-dimensional echocardiography after excluding other cardiac or systemic diseases capable of producing a similar degree of hypertrophy.^[7-9] For the diagnosis of HCM in first-degree relatives of patients with unequivocal HCM, the cut-off of left ventricular wall thickness was 13 mm.^[8] Genetic testing is also used in the identification of affected relatives in families known to

have HCM; those relatives who carry HCM-definitive mutation but without cardiac hypertrophy are called genotype-positive phenotype-negative HCM patients.^[7,9]

2.3. Echocardiography

Echocardiography was performed by an experienced operator blinded of both genotype and control or study subject status. M-mode, 2-dimensional images, and Doppler recordings were obtained using a Philips Sonos 5500 ultrasound system. Left ventricular ejection fraction (LVEF) was assessed by using Simpson biplane method. Left ventricular end-diastolic diameter (LVDD) and left ventricular septal and posterior wall thickness were assessed from 2-dimensional images. Peak early (E) and late (A) transmitral velocities, E/A ratio, and E-wave deceleration time were measured from spectral Doppler images. Tissue Doppler e' velocity was measured at the septal and lateral corners of the mitral annulus. Subjects with normal echocardiograms were classified as unaffected members or members with unknown status.

2.4. Sanger sequencing for mutations in *MYBPC3* and *MYH7*

Genomic DNA was extracted from human peripheral blood samples using the DNeasy kit (Qiagen). DNA concentration and purity were measured with a Nanodrop 2000 spectrophotometer (Thermo scientific), and calibrated to 50 ng/ μ L for further use. The polymerase chain reaction (PCR) was performed using primers that cover all exons and exon-intron boundaries of *MYBPC3* and *MYH7*.^[10-11] PCR products were purified with the Gel Extraction kit (Omega) and sequenced on both strands using the BigDye Terminator v3.1 kit (Applied Biosystems). Sequence alignments were carried out with the ABI 3100 Genetic Analyzer (Applied Biosystems).

2.5. Genotyping the 5 polymorphisms in RAAS

Five polymorphisms previously investigated in RAAS were selected for genotyping. That includes rs4646994 in the angiotensin converting enzyme gene (*ACE*), rs5186 in the angiotensin II receptor type 1 gene (*AGTR1*), rs1800875 in the cardiac chymase A gene (*CMA1*), rs699 in the gene encoding angiotensinogen (*AGT*), and rs1799998 in the aldolase synthase gene (*CYP11B2*). The deletion/insertion (D/I) polymorphism (rs4646994) in *ACE* was genotyped as previously described^[5] and genotyping of other polymorphisms was determined by Sanger sequencing.

2.6. Targeted ngs

Targeted NGS (including library construction, capture, and sequencing) was carried out at MyGenostics Gene Technologies (Beijing, China). The Cardiomyopathy panel (MyGenostics) was designed to cover 104 genes, which were known causes or related genes for cardiomyopathy and channelopathy (Table S1, <http://links.lww.com/MD/B745>). Targeted gene enrichment was performed with the GenCap Custom Enrichment Kit (MyGenostics) according to the GenCap protocol, as described previously.^[12-13] Captured DNA libraries were sequenced with the Illumina HiSeq 2000 instrument (Illumina, San Diego, CA) for 100-bp paired reads.

2.7. Data analysis

Mapping of the sequencing reads to the human genome reference sequence (hg19) was performed with the burrows-wheeler

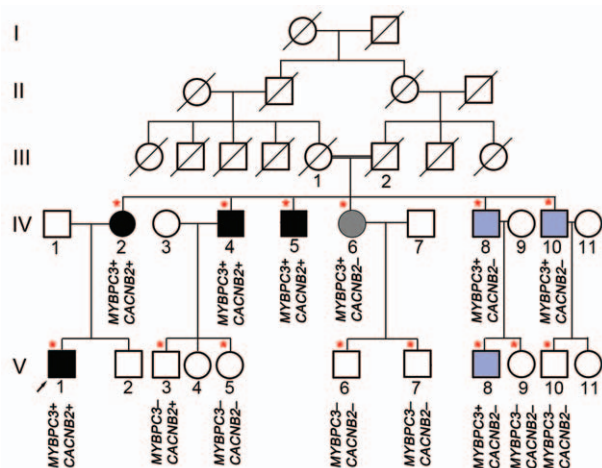


Figure 1. Pedigree of a consanguineous Chinese family with hereditary hypertrophic cardiomyopathy. Squares denote male family members, circles female family members, black solid squares and circles severely affected HCM members, gray solid circle moderately affected HCM member, blue solid circles genotype-positive phenotype-negative, open squares and circles healthy members with wild-type *MYBPC3*. A slash across the symbols denotes deceased individual, and asterisks denotes family members whose samples were sequenced. Genotypes for *MYBPC3* and *CACNB2* were shown below the symbol for each individual with a plus (+) indicating mutant and a minus (-) indicating wild type for that genotype.

alignment tool.^[14] The Short Oligonucleotide Analysis Package (SOAPSnp) and the Genome Analysis Toolkit were used to discover single-nucleotide polymorphism and insertion-deletion, respectively.^[15–16] Gene related annotation was mainly done with ANNOVAR.^[17] The pathogenicity of a variant was determined based on frequency in the population and *in silico* prediction. We excluded common variants (frequency > 1%) present in the National Heart, Lung, and Blood Institute ESP (Exome Sequencing Project), the 1000 Genomes database, and exomes generated in house from 550 unaffected Chinese control samples. Variants annotated in the Human Gene Mutation Database (HGMD) were regarded as disease-causing. All nonsynonymous variants were subjected to *in silico* analysis, which included functional annotation algorithms such as SIFT, PolyPhen2, GERP++, and MutationTaster. All variants identified by NGS and deemed as pathogenic were verified by Sanger sequencing, using primers designed from Primer 3 online software (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm>).

3. Results

3.1. Description of the pedigree

The index patient (V-1) was presented to our institution at age 28 with exertional dyspnea and chest discomfort for 1 month. His electrocardiogram showed prominent LVH. Transthoracic echocardiography confirmed a severe form of HCM with a marked hypertrophic septum (27mm) and a left-ventricular outflow obstruction (LVOTO) of 32mmHg. Exercise echocardiography revealed that LVOTO increased to 60mmHg. He received metoprolol treatment (190mg/day), but refused to receive an implantable cardioverter defibrillator (ICD), percutaneous transluminal septal myocardial ablation (PTSDMA), or septal myectomy because he remained fully active after drug therapy with only mild functional limitation (functional class New York Heart Association class II). He unfortunately suffered sudden death 1 year later when he was 29 years' old. His mother (IV-2) suffered from exertional angina for 10 years and was diagnosed with HCM showing atypical hypertrophy (23mm). Her symptoms improved on treatment with metoprolol, and her cardiac function remained stable from her recent echocardiography.

Two uncles of the index patient (IV-4 and IV-5) showed very similar clinical pictures: they both suffered from exertional dyspnea, had permanent atrial fibrillation, and hypertrophic interventricular septum (19mm). Rest echocardiogram did not show evidence of LVOTO. IV-4 received pharmacological therapy of beta-blockers and low-dose loop diuretics, but unfortunately encountered sudden death at age 52. IV-5 did not receive any treatment and his NYHA functional class remained II to III. IV-6 was diagnosed with HCM at the age 64 as part of clinical screening of the family. Her echocardiogram showed moderate asymmetrical hypertrophy with a maximum wall thickness (MWT) of 15mm and normal LV dimensions and systolic function. IV-8 was diagnosed with hypertension (grade 2) at age 38 and did not receive any antihypertensive agents for 7 years. Ambulatory blood pressure monitoring at this screening confirmed uncontrolled hypertension with an average blood pressure of 155/99mmHg. Auxiliary examinations ruled out secondary hypertension. He is asymptomatic and his echocardiogram showed a symmetric LV hypertrophy and a mildly increased LV chamber dilation and reduced systolic function. It was unlikely the burned-out phenotype of hypertrophic cardiomyopathy.

The clinical characteristics of the family were shown in Table 1. All family members were examined, and no member in the family showed phenotypes of Brugada syndrome.

3.2. Identification of the mutation in MYBPC3 by Sanger sequencing

It is well accepted that variants in *MYBPC3* and *MYH7* account for >70% of HCM cases in which a mutation has been defined. We sequenced *MYBPC3* and *MYH7* genes in the 4 affected HCM patients and revealed no disease-causing mutation in *MYH7*, but the presence in all affected individuals of a heterozygous c.3624delC mutation within *MYBPC3* (11p11.2), leading to a frameshift and a premature termination codon (p.Lys1209Serfs) and thus causing the loss of 49 amino acids in the protein (Fig. 2). This mutation has been reported previously in several HCM patients^[18–19], but was not found in the 1000 Genomes Project or the Exome Sequencing Project, nor in the 550 ethnically matched healthy control individuals in our study. In this family, we identified 8 members harboring this disease-causing mutation. All these 8 members were regarded as HCM patients, either as

Table 1

Clinical characteristics of family members.

Patient No.	Sex	Age, y	ASO, y	LVDD, cm	IVSTD, cm	LVPWTD, cm	AoD, cm	LAD, cm	EF (%)	E, m/s	A, m/s	E/A	e', cm/s	HR, bpm	Cardiac rhythm
IV-2*	F	51	40	5.3	1 (2.3*)	1	3.1	3.4	63	0.4	0.6	0.67	8.5	66	Sinus
IV-4	M	52	47	5.5	1.9	1.18	3.4	6.8	47	0.5	0.6	0.83	9.4	123	AF
IV-5	M	66	45	5.7	1.9	1.2	2.9	6.5	44	0.4	0.6	0.67	10.0	90	AF
IV-6	F	64	NA	4.7	1.5	0.9	3.1	3.6	61	0.4	0.9	0.44	10.5	75	Sinus
IV-8	M	45	NA	5.7	1.1	1.1	3.3	4.3	51	0.8	0.6	1.33	12.7	86	Sinus
IV-10	F	43	NA	5.1	0.9	0.9	3	3.75	61	0.5	0.7	0.71	12.0	75	Sinus
V-1	M	29	27	4.3	2.7	1.0	2.6	3.9	55	0.5	0.65	0.77	8.9	76	Sinus
V-3	M	17	NA	5.4	0.8	0.8	2.5	3.6	60	1.0	0.6	1.67	13.1	66	Sinus
V-5	F	22	NA	5.1	0.75	0.75	2.6	3.6	67	0.8	0.6	1.33	14.5	75	Sinus
V-6	M	45	NA	4.9	0.8	0.8	2.9	3.5	65	0.8	0.7	1.14	12.0	78	Sinus
V-7	M	41	NA	5.15	0.8	0.8	3	3.45	60	0.7	0.65	1.08	13.1	93	Sinus
V-8	M	18	NA	4.3	0.9	0.9	2.3	2.8	61	0.6	0.5	1.2	14.3	88	Sinus
V-9	F	12	NA	4	0.5	0.5	2.1	2.35	61	0.95	0.65	1.46	15.2	76	Sinus
V-10	M	11	NA	3.9	0.55	0.55	2.1	2.3	62	0.95	0.8	1.19	14.8	88	Sinus

AF = atrial fibrillation, ASO = age of symptom onset, EF = ejection fraction; F = female, HR = heart rate, IVSTD = interventricular septal end-diastolic thickness, LAD = left atrial diameter, LVDD = left ventricular diastolic diameter; LVPWTD = left ventricular posterior wall thickness at end-diastole, M = male; NA = not applicable.

* A moderate asymmetrical hypertrophy of 2.3cm localized to apex of the left ventricle.

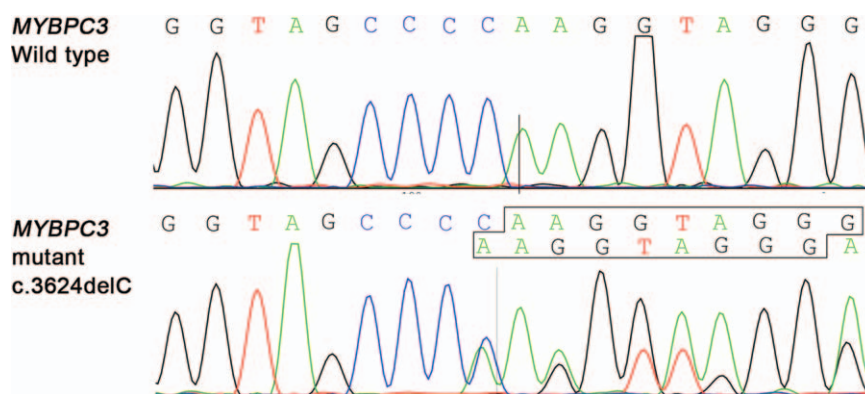


Figure 2. Electropherograms showing the *MYBPC3* c.3624delC (p.Lys1209Serfs) heterozygous mutation and a wild type, control sequence.

genotype-positive phenotype-positive or genotype-positive phenotype-negative patients.

3.3. Targeted NGS for modifying variants

Marked clinical heterogeneity was noted among these patients who shared the same disease-causing *MYBPC3* mutation: 4 (IV-2, IV-4, IV-5, and V-1) were considered severely affected because they had a greater maximal wall thickness (MWT), an earlier onset of HCM phenotype associated with marked symptoms and cardiac events (cardiac death or admission for heart failure or stroke); 1 (IV-6) was considered as moderately affected because she had documented LVH, but less MWT and no evidence of cardiac symptoms or event; the other 3 (IV-8, IV-10, and V-8) were genotype-positive phenotype-negative patients with no evidence of LVH or relevant clinical symptoms. However, V-8 needs follow-up reevaluation because he was only 18 at his last clinical evaluation. This remarkable phenotypic variation indicates that disease modifiers exist.

We first genotyped the five variations in *RAAS* genes and revealed no correlation between HCM phenotypes and these genotypes, which ruled out the modifying effect of these *RAAS* variants. We then performed targeted NGS for the index HCM patient using a panel covering all known causing genes and closely related genes of cardiomyopathy and channelopathy, and the *RAAS* genes. The NGS captured 99.5% of the target region. A mean coverage of 307× was reached and 92.7% of target regions were covered to a depth of at least 20×. In total, we identified 301 genetic variants in the index patient across the target region. After excluding synonymous genetic variants, 135 exonic or splice-site variants remained. Further excluding common variants ($\geq 1.0\%$) reported in Exome Sequencing Project, 1000 Genomes, and exomes generated in house left us with 5 candidate variants (Table 2). Two of these, c.3624delC (p.Lys1209Serfs) within

MYBPC3 and c.1598C>T (p.Ser533Leu) within voltage-dependent L-type calcium channel subunit beta-2 (*CACNB2*; 10p12.31), were predicted to be “damaging” by all algorithms, whereas the other 3 mutations, c.1058C>T (p.Ala353Val) within transforming growth factor beta 1 (*TGFB1*; 19q13.2), c.8134G>T (p.Ala2712Ser) within A-kinase anchoring protein 9 (*AKAP9*, 7q21.2), and c.3923G>A (p.Arg1308Gln) within desmoplakin (*DSP*; 6p24.3), were predicted “benign” according to at least 2 functional annotation algorithms.

3.4. Sanger sequencing confirmation in family members

Next we determined these 5 genotypes in all family members through Sanger sequencing. We found that among the 8 HCM patients carrying the *MYBPC3* mutation, those severely affected (IV-2, IV-4, IV-5, and V-1) also carried the *CACNB2* c.1598C>T (p.Ser533Leu) mutation (Fig. 3), whereas the patient moderately affected (IV-6) and those 3 genotype-positive phenotype-negative patients (IV-8, IV-10, and V-8) did not carry this mutation in *CACNB2*. However, segregation analysis of the other 3 mutations in the family confirmed no association between these mutations and the phenotypes (Table 3). The good segregation of HCM phenotype with *CACNB2* genotype suggests that *CACNB2* might serve as a functional modifier in *MYBPC3*-related HCM. To support this, the affected amino acid of this mutation in *CACNB2* is extremely conserved in the evolution (Fig. 4).

4. Discussion

4.1. *MYBPC3*-Lys1209Serfs causes HCM in humans

The *MYBPC3*-Lys1209Serfs mutation has been previously reported in several patients with HCM in 2 studies (including 1 familial HCM), both of which were conducted in Chinese population.¹⁹ This variant was not reported in public databases,

Table 2

Genetic variants identified after targeted next-generation sequencing followed by adequate filtering.

Chr	Position	Gene	Transcript	Exon	Nucleotide change	Amino acid change	Pathogenicity*	Control	Associated diseases
11	47354120	<i>MYBPC3</i>	NM_000256	32	c.3624delC	p.P1208fs	NA	0/550	HCM, DCM, LVNC
10	47367774	<i>CACNB2</i>	NM_201597	14	c.1598C>T	p.S533L	Probably damaging	0/550	Brugada syndrome
19	41837072	<i>TGFB1</i>	NM_000660	7	c.1058C>T	p.A353V	Benign	0/550	
7	91711950	<i>AKAP9</i>	NM_005751	32	c.8134G>T	p.A2712S	Benign	0/550	Long QT Syndrome
6	7580346	<i>DSP</i>	NM_004415	23	c.3923G>A	p.R1308Q	Possibly damaging	2/550	ARVC

ARVC = arrhythmogenic right ventricular cardiomyopathy, DCM = dilated cardiomyopathy, HCM = hypertrophic cardiomyopathy, LVNC = left ventricular non compaction, NA = not applicable.

* Predictions are based on PolyPhen_2 program.

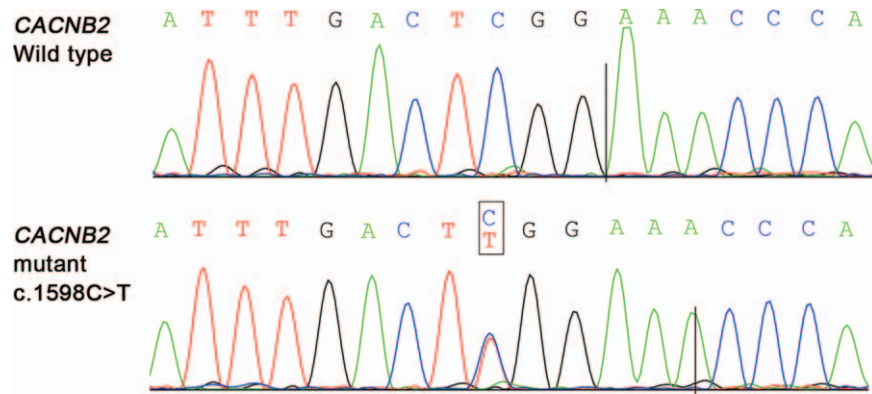


Figure 3. Electropherograms showing the *CACNB2* c.1598C>T (p.Ser533Leu) heterozygous mutation and a wild type, control sequence.

and was predicted to cause loss of 49 amino acids. The *MYBPC3* truncation mutation might result in haploinsufficiency because truncated proteins were prone to be degraded through nonsense-mediated decay.^[20]

4.2. MYBPC3-Lys1209Serfs and other MYBPC3 mutations are associated with incomplete penetrance and mild prognosis

The *MYBPC3*-Lys1209Serfs mutation was first reported in a 3-generation family with HCM.^[19] Onset of cardiac symptoms did not occur until the sixth decade of life in this family, and all HCM patients lived to their 90s without the protection of ICDs. Consistently, other *MYBPC3* mutations were also reported relatively mild in prognosis and were even considered as founder mutations in several general populations.^[21] Our study confirmed that remarkable incomplete penetrance exists in *MYBPC3*-mutation-carrying HCM patients: 3 family members did not have echocardiographic manifestations of HCM at ages 18, 43, and 45 years. However, 4 other HCM patients carrying the identical mutation showed earlier disease onset (3 at their 40s and 1 at 20s), severe echocardiographic manifestations (greater MWT), and much worse prognosis (1 suffered sudden cardiac death at 20s and 1 at 40s) than the 3 genotype-positive phenotype-negative patients and most HCM patients reported in previously published literatures. These discrepancies indicate that a modifier gene may contribute to deteriorate disease phenotype in addition to the *MYBPC3* mutation.

4.3. Evidences support channelopathy gene mutations could modify HCM phenotype

It is generally accepted that other disease modifiers exist beyond the specific disease-causing genetic mutations. HCM is a disease with complex pathogenesis, among which the most well-known were cycling deficiency and sarcomeric structure impairment.^[6] Adrenergic system are also involved in the process of LVH or heart failure,^[22-23] and reports showed possible modifying effect of variations in their related genes.^[4-5] In our study, however, we did not find associations between these variations in adrenergic system and HCM phenotypes.

Accumulating evidences support that HCM patients carrying >1 sarcomere gene mutation were associated with more remarkable phenotypes and more adverse outcomes than those with a single mutation.^[19,24] However, this is not the case in our study because we did not find patients carrying multiple sarcomere mutations. Instead, for the first time, we showed that *CACNB2* is a possible genetic modifier of *MYBPC3*-associated familial HCM. Our findings were supported by a recently published study, in which Lopes et al^[25] used targeted high-throughput sequencing to investigate the genotype–phenotype associations in HCM, and demonstrated that in patients harboring sarcomere protein gene mutations, those who also carried Ankyrin-B gene (*ANK2*) variants had greater maximum wall thickness. It is notable that mutations in both *ANK2* and *CACNB2* could lead to channelopathies.^[26] In this regard, it is reasonable to postulate that variants in non-sarcomere genes, particularly in candidate genes for channelopathies, are potential genetic modifiers of *MYBPC3*-related HCM.

Table 3
Detailed information of the 5 identified genetic variants in 8 HCM patients.

	MYBPC3	CACNB2	TGFB1	AKAP9	DSP
IV-2	+	+	-	-	+
IV-4	+	+	-	-	+
IV-5	+	+	-	-	-
IV-6	+	-	-	-	-
IV-8	+	-	-	-	+
IV-10	+	-	-	-	-
V-1	+	+	+	+	+
V-8	+	-	-	-	+

Genotypes were shown for each individual with a plus (+) indicating mutant and a minus (-) indicating wild type for that genotype. AKAP = A-kinase anchoring protein 9, *CACNB2* = calcium channel subunit beta-2, DSP = desmoplakin, HCM = hypertrophic cardiomyopathy, *MYBPC3* = myosin-binding protein C, TGFB1 = transforming growth factor beta 1.

Mutant	RGLSRQETFD L ETQESRDSAY
Human	RGLSRQETFD S ETQESRDSAY
Chimpanzee	RGLSRQETFD S ETQESRDSAY
Pig	RGLSRQETLD S ETQESRDSAY
Cow	RGLSRQETLD S ETQESRDSAY
Dog	RGLSRQETFD S ETQESRDSAY
Mouse	RGLSRQETFD S ETQESRDSAY
Rat	RGLSRQETFD S ETQESRDSAY
Rabbit	RGLSRQETFD S ETQESRDSAY
Chicken	RGLPRQETFD S ETQDSRDSAY
Microbat	RGLSRQETFD S ETQDSRDSAY

Figure 4. Alignment of homologous *CACNB2* protein sequences that flank the amino acid substitutions.

The proposed concept that channelopathy gene mutations may contribute to HCM phenotype was further advanced by a study screening for mutations in 7 Ca^{2+} regulatory genes (i.e., *SRI*, *FKBP1B*, *CASQ2*, *PLN*, *SLN*, *CALR3*, and *CALM*) in a cohort of 252 unrelated familial HCM patients.^[27] Four variants with pathogenic significance were detected in these Ca^{2+} regulatory genes, among which 2 patients also carried the *MYBPC3* sarcomere mutations.^[27] The modifying effect of these variants was not fully illustrated because the family pedigrees were small and screening for other relatives was unavailable. In light of our observations and the data reviewed above, it is likely that most of the evidences concerning modifier genes in HCM are obtained on the basis of *MYBPC3* mutations. This may be because of the “benign” nature of most *MYBPC3* mutations,^[31] as *MYBPC3* mutations are usually associated with a mild hypertrophy, late-onset, incomplete penetrance, and a better prognosis. Besides, *MYBPC3* gene is the only gene reported in HCM with founder mutations, for example, 2% to 8% of all major Indian populations carry a *MYBPC3* deletion, and 0.4% of Iceland population carries a *MYBPC3* missense mutation.^[21] It would be of great interest to investigate genetic modifiers in these populations with founder mutations.^[21]

4.4. Possible mechanisms of the *CACNB2* mutation modifying disease phenotype

CACNB2 encodes for an auxiliary voltage-dependent L-type calcium-channel (LTCC) $\text{Ca}_v\beta_2$ subunit and is predominantly expressed in the heart. The $\text{Ca}_v\beta_2$ subunit improves the calcium current by promoting the voltage-dependent opening of Ca^{2+} channel and enhancing channel surface expression, probably through direct interaction with actin. Mice lacking $\text{Ca}_v\beta_2$ have functionally compromised heart and die at embryonic day 10.5.^[28] Diminished L-type Ca^{2+} currents were observed in these $\text{Ca}_v\beta_2^{-/-}$ mice, as well as in patients with Brugada syndrome harboring loss-of-function mutations in *CACNB2*.^[28] Based on algorithmic prediction, it is likely that the *CACNB2* mutation identified in our study could attenuate L-type calcium current in cardiomyocytes, and then inhibits the activation of calcium/calmodulin (Ca^{2+} /CaM)-dependent protein kinase II (CaMKII), a key regulator of cardiac physiology and pathology.^[29] Subsequently, on the one hand, CaMKII-mediated phosphorylation of cMyBP-C, which is critical in maintaining sarcomeric structure and function, is markedly decreased. Dephosphorylated cMyBP-C is prone to degradation,^[30] and thus leads to dramatic insufficiency of cMyBP-C. This situation is worsened given that the *MYBPC3* truncation mutation itself causes haploinsufficiency. It is very likely that the serious cMyBP-C deficiency significantly exacerbates the development of HCM in those severely affected patients because a lack of functional full-length cMyBP-C has been demonstrated to cause HCM phenotypes.^[31] On the other hand, CaMKII directly binds to the C terminus of $\text{Ca}_v\beta_2$ subunit and facilitates LTCC calcium currents through phosphorylation of $\text{Ca}_v\beta_2$. In *CACNB2* mutation carriers, this process is inhibited and Ca^{2+} currents are further reduced. Considerable Ca^{2+} is stored in the sarcomere, but mutant sarcomeres may trap more Ca^{2+} than normal sarcomeres, causing a reduction in cytoplasmic Ca^{2+} .^[32] Therefore, Ca^{2+} current dysfunction is extremely evident in those severely affected HCM patients who carry both the *MYBPC3* and *CACNB2* mutations, which could explain the high rate of sudden cardiac death of these patients.

4.5. Study limitations

Several limitations should be acknowledged in our study. First, we used targeted NGS technology instead of a hypothesis-free approach such as exome or genome sequencing. However, our sequencing panel included all known causing genes and closely related genes of cardiomyopathy and channelopathy, which very likely cover the modifier gene if it exists. Second, the mechanism of how *MYBPC3* interacts with *CACNB2* remains undetermined in our study; we were unable to obtain the left ventricular tissue of HCM patients for functional analyses because consent of myocardial biopsy was not provided and none of these patients received heart transplantation or postmortem examination. More genetic evidence and functional experiments are needed to confirm our observations.

In this study, for the first time we showed that *CACNB2* is a possible candidate hypertrophy-modifying gene contributing to disease variability of *MYBPC3*-associated familial HCM. We provided a new possible mechanism of HCM pathogenesis, that is, a combination effect of sarcomere gene and channelopathy gene mutations, which however needs confirmation with more genetic evidence and functional experiments.

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