

Identification of novel mutations including a double mutation in patients with inherited cardiomyopathy by a targeted sequencing approach using the Ion Torrent PGM system

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Abstract. Inherited cardiomyopathy is the major cause of sudden cardiac death (SCD) and heart failure (HF). The disease is associated with extensive genetic heterogeneity; pathogenic mutations in cardiac sarcomere protein genes, cytoskeletal protein genes and nuclear envelope protein genes have been linked to its etiology. Early diagnosis is conducive to clinical monitoring and allows for presymptomatic interventions as needed. In the present study, the entire coding sequences and flanking regions of 12 major disease (cardiomyopathy)-related genes [namely myosin, heavy chain 7, cardiac muscle, β (*MYH7*); myosin binding protein C, cardiac (*MYBPC3*); lamin A/C (*LMNA*); troponin I type 3 (cardiac) (*TNNI3*); troponin T type 2 (cardiac) (*TNNT2*); actin, α , cardiac muscle 1 (*ACTC1*); tropomyosin 1 (α) (*TPMI*); sodium channel, voltage gated, type V alpha subunit (*SCN5A*); myosin, light chain 2, regulatory, cardiac, slow (*MYL2*); myosin, heavy chain 6, cardiac muscle, α (*MYH6*); myosin, light chain 3, alkali, ventricular, skeletal, slow (*MYL3*); and protein kinase, AMP-activated, gamma 2 non-catalytic subunit (*PRKAG2*)] in 8 patients with dilated cardiomyopathy (DCM) and in 8 patients with hypertrophic cardiomyopathy (HCM) were amplified and then sequenced using the Ion Torrent Personal Genome Machine (PGM) system. As a result, a novel heterozygous mutation (*MYH7*, p.Asn885Thr) and a variant of uncertain significance (*TNNT2*, p.Arg296His) were identified in 2 patients with HCM. These 2 missense mutations, which were absent in the samples obtained from the 200 healthy control subjects, altered the amino acid that was evolutionarily conserved among a number of vertebrate species; this illus-

trates that these 2 non-synonymous mutations play a role in the pathogenesis of HCM. Moreover, a double heterozygous mutation (*PRKAG2*, p.Gly100Ser plus *MYH7*, p.Arg719Trp) was identified in a patient with severe familial HCM, for the first time to the best of our knowledge. This patient provided us with more information regarding the genotype-phenotype correlation between mutations of *MYH7* and *PRKAG2*. Taken together, these findings provide insight into the molecular mechanisms underlying inherited cardiomyopathy. The mutations identified in this study may be further investigated in the future in order to improve the diagnosis and treatment of patients with inherited cardiomyopathy. Furthermore, our findings indicated that sequencing using the Ion Torrent PGM system is a useful approach for the identification of pathogenic mutations associated with inherited cardiomyopathy, and it may be used for the risk evaluation of individuals with a possible susceptibility to inherited cardiomyopathy.

Introduction

Inherited cardiomyopathies are divided into the following 4 categories according to alterations in ventricular morphology and function: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic right ventricular cardiomyopathy (ARVC) and restrictive cardiomyopathy (RCM) (1,2). It is associated with extensive genetic heterogeneity; inherited cardiomyopathy-linked mutations have been found in over 100 disease-causing genes, including mutations in the genes encoding the following: cardiac sarcomere proteins, cytoskeletal proteins and nuclear envelope proteins (3-5), cardiac development and structural remodeling proteins (6-8), cardiac transcription factors (9-14), Tax-1-binding protein (15) and the RAS-mitogen-activated protein kinase pathway (16). As the leading cause of sudden cardiac death (SCD) in adolescents and young athletes, HCM is the most common type of inherited cardiomyopathy with a morbidity rate of approximately 1 in 500 individuals worldwide, which is characterized by unexplained left ventricular hypertrophy (17,18). DCM is characterized by left ventricular dilatation and systolic dysfunction [with a left ventricular ejection fraction (LVEF)

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of <50%], and affects at least 1/2,500 individuals in the general population (3); it is primarily caused by pathogenic gene mutations inherited in a Mendelian autosomal dominant pattern (19). Molecular genetic testing is crucial for selecting the correct therapy and management strategies for the disease, as well as to evaluate the prognosis of patients with inherited cardiomyopathy and of their family members.

Currently, conventional capillary-based sequencing is the gold standard approach for detecting mutations associated with this disease. However, this method has drawbacks, as not all types of genetic variation are detectable and it is a time-consuming and costly technique. In a striking technological development, the Ion Torrent Personal Genome Machine (PGM) system was launched in 2011 by Life Technologies, and it has been demonstrated to be a more rapid, more sensitive and less costly system, and it also allows the scalable sequencing of samples (20).

In the present study, we enrolled 16 Chinese patients diagnosed with inherited cardiomyopathy (either HCM or DCM) and performed bioinformatics and molecular genetic analyses of the entire coding sequence and flanking regions of 12 major disease (cardiomyopathy)-related genes using the Ion Torrent PGM system. As a result, we identified a novel [myosin, heavy chain 7, cardiac muscle, β (MYH7), p.Asn885Thr] mutation, a variant of uncertain significance [troponin T type 2 (cardiac) (TNNT2), p.Arg296His] and a double heterozygous [protein kinase, AMP-activated, gamma 2 non-catalytic subunit (PRKAG2), p.Gly100Ser plus MYH7, p.Arg719Trp] mutation. The findings of our study expand the mutational spectrum of MYH7 and TNNT2 which are associated with HCM, and enhance our understanding of the molecular mechanisms underlying inherited cardiomyopathy. Furthermore, we present a useful approach for the genetic testing of patients with inherited cardiomyopathy.

Subjects and methods

Patients and healthy controls. A total of 16 patients with inherited cardiomyopathy were recruited, namely 8 patients with DCM and 8 patients with HCM, who were traditionally diagnosed according to the criteria specified in the American College of Cardiology Foundation/American Heart Association (ACCF/AHA) guideline for the diagnosis and treatment of hypertrophic cardiomyopathy (21) and the European guidelines for the study of familial dilated cardiomyopathies (22). Of the 16 patients, 2 (patients A12 and A13) were considered to have familial HCM (Fig. 1). The demographic and clinical characteristics of the patients, including family history, clinical symptoms, echocardiography results, and 12-lead electrocardiography (ECG) records, were collected. In addition, 100 healthy individuals without any symptoms of cardiovascular disease were enrolled into this study as healthy control subjects. All of the subjects provided written informed consent prior to participating voluntarily in this study. The study protocol was approved by the Ethics Committee of The Affiliated Hospital of Kunming University of Science and Technology (Kunming, China) and complied with the principles of the Declaration of Helsinki.

DNA extraction, genomic library construction and template preparation/amplification. Peripheral whole blood

samples (~2 ml) from each subject were collected in Vacutainer tubes coated with EDTA (BD Biosciences, Franklin Lakes, NJ, USA) and stored at 4°C until DNA extraction. Genomic DNA was extracted from anticoagulated whole blood of each sample using a commercial Blood Genomic DNA Miniprep kit (Axygen, Union City, CA, USA). The majority of the primers were designed using the freely available Lasergene PrimerSelect software (DNASTAR, Madison, WI, USA) and Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA), and then 116 pairs of primer sets were synthesized and we amplified 226 coding exons of the following genes: MYH7; myosin binding protein C, cardiac (MYBPC3); TNNT2; troponin I type 3 (cardiac) (TNNT3); myosin, light chain 2, regulatory, cardiac, slow (MYL2); lamin A/C (LMNA); myosin, light chain 3, alkali, ventricular, skeletal, slow (MYL3); PRKAG2; sodium channel, voltage gated, type V alpha subunit (SCN5A); myosin, heavy chain 6, cardiac muscle, α (MYH6); actin, α , cardiac muscle 1 (ACTC1); and tropomyosin 1 (α) (TPMI); (data available upon request). From this panel, approximately 15 pairs of primer sets having similar annealing temperatures and of similar amplicon size were combined in one reaction pool in order to reduce the reaction times and reagent costs. Polymerase chain reaction (PCR) amplification was performed using PrimeSTAR GXL DNA polymerase (Takara, Otsu, Japan) under the following conditions: DNA denaturation at 98°C for 3 min, followed by 30 cycles of denaturing at 98°C for 10 sec, annealing at 54 or 57°C for 15 sec and extension at 68°C for 1-3 min, and finalized with one extension cycle of 68°C for 5 min (data available upon request). Finally, the multiplex PCR products from each sample were mixed in microtubes at equal concentrations which were determined using the SequalPrep Normalization kit (Invitrogen, Carlsbad, CA, USA). Genomic library construction was performed using the manual (Publication no. 4471989, Revision N) and DNA template preparation was conducted using an Ion OneTouch 2 instrument and an Ion OneTouch enrichment system (ES) (both from Life Technologies, Carlsbad, CA, USA).

Ion Torrent PGM sequencing and bioinformatics analysis. DNA high-throughput sequencing was performed using reagents from the Ion PGM Sequencing 400 kit (obtained from Life Technologies). The prepared samples of Ion Sphere Particles (ISP) were loaded onto an Ion 314 sequencing chip (Life Technologies), and DNA sequencing was performed in the Ion PGM instrument using the Ion PGM 400 sequencing kit set at 640 flows for 160 runs. Raw data from the PGM runs were processed using the Ion Torrent platform-specific pipeline software Torrent Suite v4.0.2 (Life Technologies) to generate sequence reads. The FastQC (v3.4.1.1) plug-in software was used in order to perform the analysis of the mean read depth and alignment quality. The short reads alignment was rapidly and accurately achieved using the Burrows Wheeler Aligner (BWA) Multi-Vision software package. Coverage Analysis (v4.0-r77897) plug-in software was used to assess the number of mapped bases, the percentage of coverage on the target gene.

The sequence variants in the 12 genes in each sample were identified using the Torrent Suite Variant Caller (TSVC; v4.0-r76860) plug-in and browser extensible data (BED) files (chromosome coordinates) that specify the coding regions of

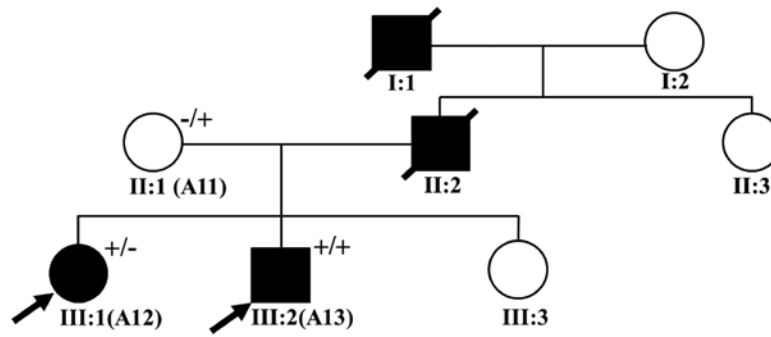


Figure 1. Pedigree of the family with hypertrophic cardiomyopathy (HCM). Male family members are indicated by squares; female family members are indicated by circles, deceased individuals are indicated by symbols with a strikethrough, the unaffected individuals are represented by open symbols, and the solid symbols represent affected individuals. In addition, the probands are marked with a black arrow. The presence of a mutation is indicated by a (+) sign and the absence of mutations is indicated by a (-) sign. III:1 and III:2 are the probands, I:1 and II:2 died of sudden cardiac death, and clinical data were unavailable for the other members; II:1 possesses the mutation but the individual is clinically unaffected, and clinical data were unavailable for the other members.

the target genes within the human reference genome (hg19) retrieved from the NCBI database (build 37) as a reference. The TSVC plug-in generated files in variant caller format (VCF) were further annotated using online Ion Reporter software (<https://ionreporter.lifetechnologies.com/ir/secure/home.html>), and Integrated Genomic Viewer (IGV) software (23) was then used to complete the visualization and to eliminate false-positive variants.

Molecular genetic analysis. We analyzed mutations that have been reported to be associated with the disease phenotype in the PubMed Database (<http://www.ncbi.nlm.nih.gov/pubmed/>) or by the Human Gene Mutation Database (HGMD; <http://www.hgmd.cf.ac.uk>). In addition to the above, the mutations meeting the following criteria were putatively considered pathogenic (24): i) if the mutation had a minor allele frequency (MAF) of <10% in the NCBI dbSNP137 (<http://www.ncbi.nlm.nih.gov/projects/SNP/>), the 1000 Genomes Project (<http://www.1000genomes.org/>) and NHLBI Grand Opportunity Exome Sequencing Project (ESP; <https://esp.gs.washington.edu/drupal/>) databases; ii) pathogenicity prediction programs that assess the functional significance of amino acid substitutions were used, including PolyPhen-2 (25), SIFT (26) or MutationTaster algorithms (27), which labeled the mutation as pathogenic; iii) the novel mutations were absent from the 100 unrelated healthy control subjects in order to rule out the possibility of the polymorphisms existing in the normal population; iv) evolutionary conservation analysis of the novel mutation was performed in a number of vertebrate species (namely, *Macaca mulatta*, *Felis catus*, *Mus musculus*, *Gallus gallus*, *Danio rerio* and *Xenopus tropicalis*). The Clustal W alignment program (<http://www.genome.jp/tools/clustalw/>) was used to align orthologs from eukaryotes, and the weblogo (<http://weblogo.berkeley.edu/logo.cgi>) format was used for aligning eukaryotic species. All of the putative pathogenic mutations were verified by PCR and conventional capillary-based sequencing analysis using an ABI 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Results

Demographic and clinical characteristics of the patients with DCM and HCM. The recorded demographic and clinical

characteristics of all 16 patients (8 patients diagnosed with DCM and 8 patients diagnosed with HCM) are presented in Table I. The patients with DCM had a median age at diagnosis of 44 years (ranging from 16 to 70 years and SD of 17.2 years) and those with HCM had a median age at diagnosis of 37.1 years (ranging from 10 to 57 years and SD of 19.2 years). Echocardiography revealed a mildly dilated left ventricular end-systolic diameter (LVESD, an average of 57.8 mm) and left ventricular end-diastolic diameter (LVED, an average of 68.0 mm) and LVEF (an average of 32.6%) of <50% in the patients with DCM. Echocardiography also revealed that the patients with HCM experienced significant concentric left ventricular hypertrophy (LVH) with severe alterations in interventricular septum thickness (IVST, average of 19.2 mm) and left ventricular posterior wall thickness (LVPWT; average of 11.5 mm).

Among the patients with familial HCM (Fig. 1), the proband of this family (A13, III:2) was a young child, who at the age of 10 years was diagnosed with a malignant HCM phenotype that manifested as a greater LVPWT with evident septal asymmetry, an enlarged left atrium (LA), decreased left ventricular systolic and diastolic function (IVST, 21 mm; LVPWT, 12 mm; LA, 40 mm; and LVEF, 48%); at the time of the initial diagnosis the child was experiencing chest pain and syncope. The family history revealed that the grandfather of the proband had died from heart disease at age 42 and that his father had succumbed to sudden cardiac death at age 40. In the absence of clinical data, the child's mother (A11, II:1) was assumed to be free of clinical symptoms. Patient A12 (III:1), who is the proband's elder sister, was diagnosed at the age of 21 with a severe HCM phenotype (IVST, 25.9 mm; LVPWT, 9.4 mm; and LA, 26.9 mm).

Analysis of raw data collected by performing multiplex PCR amplification followed by Ion Torrent PGM sequencing. To rapidly detect HCM- and DCM-related mutations of the 12 target genes, 116 primer pairs were designed for multiplex PCR in order to amplify a total of 154,537 base pairs (bp): the PCR fragment size ranged from 245 to 2906 bp (data available upon request). To distinguish the sequence data of each individual sample, we linked an Ion Xpress Barcode Adapter sequence to each fragment in the library. Following the optimization of PCR

Table I. Available demographic and clinical characteristics of patients with inherited cardiomyopathy.

Subject no.	Gender	Age (years)	Disease	Family History	Echocardiogram					
					IVST (mm)	LVED (mm)	LVESD (mm)	LVPWT (mm)	LA (mm)	LVEF (%)
19	Male	70	DCM	Negative	N/A	N/A	N/A	N/A	N/A	N/A
A7	Male	16	DCM	Negative	8	61	N/A	8.8	35	45.0
A63	Male	41	DCM	Positive	9.3	60.6	48	8.6	40	38.0
A64	Female	56	DCM	Positive	9.9	67.4	59.6	8.5	58.2	24.0
38	Male	40	DCM	Negative	9.2	90.1	81.6	8.5	61.8	19.0
A51	Male	59	DCM	Negative	N/A	N/A	N/A	N/A	N/A	N/A
A88	Male	30	DCM	Negative	7.0	N/A	N/A	8.0	40	22.0
A37	Female	40	DCM	Positive	7.9	61.3	42	6.8	32.6	48.0
Mean ± SD	N/A	44.0±17.2	N/A	N/A	8.5±1.0	68.0±12.6	57.8±17.5	8.2±0.7	44.6±12.3	32.6±12.5
A36	Female	49	HCM	Positive	22	44.2	31.7	10.9	32	54.0
A15	Male	14	HCM	Positive	18.8	37.2	24.0	16.0	46.0	65.0
51	Female	57	HCM	Negative	11.7	37.4	26.1	11.5	29.1	58.0
A94	Male	57	HCM	Positive	16.3	49.6	26.2	13.5	52.5	78.0
A12	Female	21	HCM	Positive	25.9	32.0	18.4	9.4	26.9	75.0
A13	Male	10	HCM	Positive	21.0	N/A	N/A	12.0	40.0	48.0
64	Male	42	HCM	Negative	18.1	51.7	34.4	9.8	44.0	61.0
A86	Male	47	HCM	Positive	20.0	49.2	32.3	8.8	32.0	63.0
Mean ± SD	N/A	37.1±19.2	N/A	N/A	19.2±4.2	43.0±7.6	27.6±5.6	11.5±2.4	37.8±9.2	62.6±10.1

HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; IVST, interventricular septum thickness; LVED, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVPWT, left ventricular posterior wall thickness; LA, left atrium; LVEF, left ventricular ejection fraction; N/A, not applicable.

amplification conditions, we used only 6 microtubes containing multiplexed PCR pools in order to perform 116 PCR reactions on each sample (data available upon request).

In general, an Ion 314 semiconductor chip can generate an amount of 300 kb data on the microporous surface, and as it only utilizes 50% of the microporous, we assumed that the average reads length was 200 bp, and each of the nucleotide sequencing has a depth of 30x (Q30=99.9% certainty that the correct base was called). Therefore, a pool of library DNA from 5 to 6 patient samples was amplified using an Ion OneTouch 2 system and then loaded onto an Ion 314 semiconductor chip. Finally, runs of all 16 samples were performed in 3 independent replicates. The average 314 semiconductor chip loading obtained was 77.6% (ranging from 75 to 81%), and an average PGM run generated raw data of approximately 49.9 Mb on the 314 semiconductor chip (ranging from 49.1 to 50.5 Mb) (data available upon request) and a mean read length of 160 bp (ranging from 147 to 181 bp) (data available upon request). The sequencing data quality was assessed using the FastQC plug-in software, which revealed that the average Q value was approximately 30 (Q30=99.9% certainty that the correct base was called) (data available upon request).

Finally, upon completion of the analysis, we obtained a total of 783,648 raw reads, including 112,378,736 raw bases, the average for 7,023,671 bases/sample (Table II). This resulted in an average of 89% of bases per sample sequenced, indicative of a quality of >Q20 (where Q20=99% certainty that the

correct base was called). Total reads were mapped uniquely to the human reference genome (hg19) retrieved from the NCBI database (build 37) using the Burrows Wheeler Aligner (BWA) Multi-Vision software package. Approximately 96.6% of the bases were matched to hg19 (Table II), and approximately 90% of the bases were matched to coding regions of the target gene(s) using the Coverage Analysis plug-in. It was found that the average depth of coverage of all the exons was at least 28-fold across all 16 samples (Table II). According to previous research, the majority of the raw sequencing data were considered as qualified (28,29).

Identification of pathogenic mutations in order to improve diagnosis. Sequence analysis using the Ion Torrent Variant Caller (v4.0-r76860) plug-in revealed a total of 1,399 nucleotide variations in the 16 patient samples. These variations were positioned in the 5'-UTR, 3'-UTR and in both introns and exons, with an average of 87 variants per patient (Table II). The identified variations were annotated using online Ion Reporter software: 1,368 (97.8%) of them were predicted to be non-coding or synonymous, whereas 31 (2.2%) were non-synonymous and insertion or deletion variants that lead to alterations in 1 or more amino acids (Table II). Non-synonymous and frame shift variations were described (data available upon request).

The entire potential non-synonymous and frameshift variations sites were filtered (24). Among these variants, 5 known heterozygous mutations (MYH7, p.Arg719Trp, p.Ala26Val

Table II. Ion Torrent PGM run statistics and potential disease mutations in patients with inherited cardiomyopathy.

Subject no.	Total bases	≥Q20 bases	Reads	Mapped reads	Mean depth	Variants	Synonymous	Insertions and deletions	Non-synonymous
19	14,418,988	13,103,929	107,304	103,943	50.06	90	5	1	1
A15	7,791,816	7,066,013	58,703	56,546	29.74	36	7	0	3
A36	5,324,605	4,823,023	39,257	37,978	14.74	16	5	0	1
A37	5,831,466	5,307,572	40,876	39,480	21.54	37	8	0	2
A7	4,812,050	4,088,627	43,384	41,417	21.88	98	4	0	4
A13	6,089,346	5,237,958	46,431	44,261	23.60	89	5	0	2
A63	7,376,806	6,320,407	53,364	51,338	32.39	84	5	1	0
A64	8,285,404	7,132,742	61,327	58,623	37.20	116	6	1	1
A94	4,111,436	3,522,751	32,388	30,602	15.55	89	7	1	1
A12	5,626,106	5,101,170	39,302	38,165	26.36	91	6	0	3
38	7,807,093	7,039,285	48,538	47,368	33.25	128	7	0	1
51	7,785,380	7,012,358	46,493	45,430	27.94	124	5	0	3
64	7,546,099	6,803,657	46,726	45,536	27.51	127	8	0	2
A51	6,792,213	6,086,301	40,887	39,770	31.94	93	5	0	1
A86	5,261,059	4,714,393	32,785	31,808	22.73	76	6	0	1
A88	7,518,870	6,751,598	45,884	44,622	31.58	105	8	0	1
Mean	7,023,671	6,256,987	48,978	47,305	28	87	-	-	-

Table III. PCR primers used for the validation of the gene sequence variants.

Gene symbol	Nucleotide changes	Primers (5'→3')	Fragment size (bp)
MYH7	c.2155C>T	Sense: GCTAATCAGTGACAAAGCCAGGATC Antisense: AGGGTGGGAAGAGCCAACAGTAGC	1,434
PRKAG2	c.298G>A	Sense: CAGTCCTGTGTGGTCAGAACTTGG Antisense: GGACCAGAAGGATTACGCTTTGAT	907
MYH7	c.77C>T	Sense: AGCCAGCTTCTGCTCACTCCAG Antisense: GCCACTTGTAAGGGTTGACGGT	1,013
MYBPC3	c.706A>G	Sense: CACCATACTTGGCTAATTTTCGT Antisense: GGATGACTGTTGACGGGACATAATGT	1,542
MYH7	c.2654A>C	Sense: GCTAATCAGTGACAAAGCCAGGATC Antisense: AGGGTGGGAAGAGCCAACAGTAGC	1,434
MYH6	c.5410C>A	Sense: TGATGGAGGAGGGAAAGGTGATT Antisense: GGGTGCCAGGTGAACGGTTAA	2,286
MYH7	c.1987C>T	Sense: GCAGAATCCATGTCCACCTGT Antisense: TGTCTAGGAGGTCCTGTTCC	1,248
TNNT2	c.887G>A	Sense: AGGGTGATTGTGAGGGTTACAG Antisense: GAGGGTCAAGAGAATGTGTCGT	2,007

and p.Arg663Cys; PRKAG2, p.Gly100Ser and MYBPC3, p.Ser236Gly) are already known to be associated with inherited cardiomyopathy, and one variant was of uncertain significance (TNNT2, p.Arg296His). Of note, we identified a novel A>C mutation located at nucleotide position c.2654 (according to the cDNA reference sequence, GenBank accession number NM_000257.3) within exon 22 of *MYH7*, which resulted in the replacement of asparagine at the 885th amino

acid with threonine (p.Asn885Thr), as shown in Table IV. These HCM- and DCM-related pathogenic mutations were validated by conventional capillary-based sequencing (Figs. 2A and B and 3), and the PCR primers for the first generation sequencing are listed in Table III. All of the putative mutations were verified by Sanger sequencing (Figs. 1-3), demonstrating that Ion Torrent PGM sequencing achieves a high degree of accuracy with regard to detecting rare mutations.

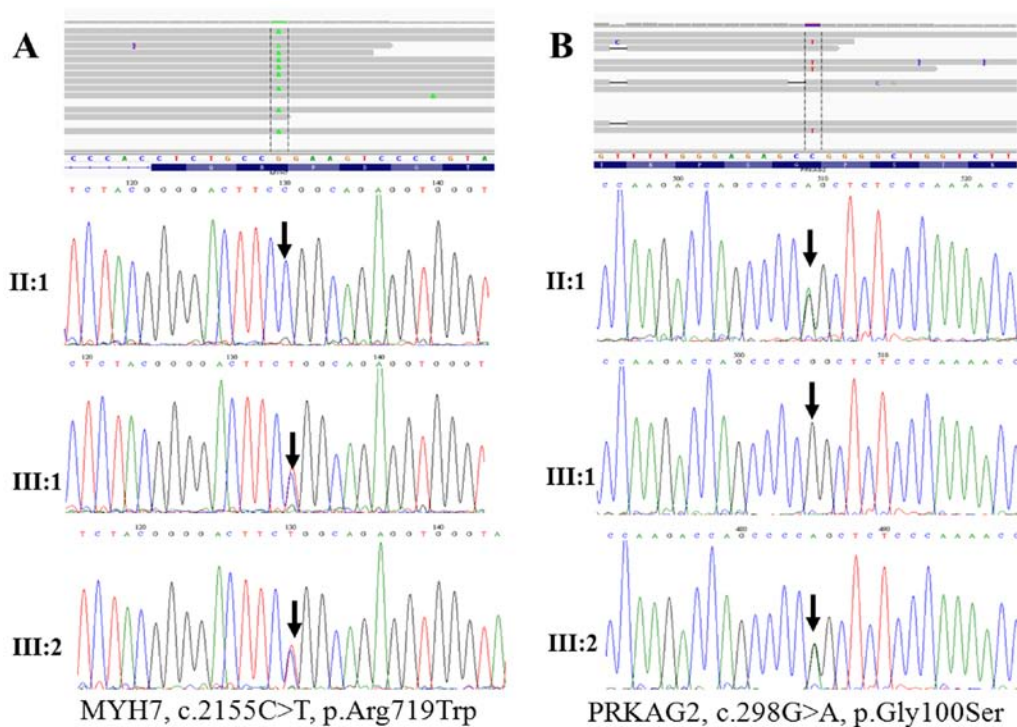


Figure 2. Pedigree of the family with hypertrophic cardiomyopathy (HCM) by Sanger sequencing analysis. (A) Sanger sequencing showing the results for the MYH7, p.Arg719Trp mutation in family members; the results were positive in III:1, III:2, and negative in II:1. (B) Sanger sequencing showing the results for the PRKAG2, p.Gly100Ser mutation in family members; the results were positive in II:1, III:2 and negative in III:1.

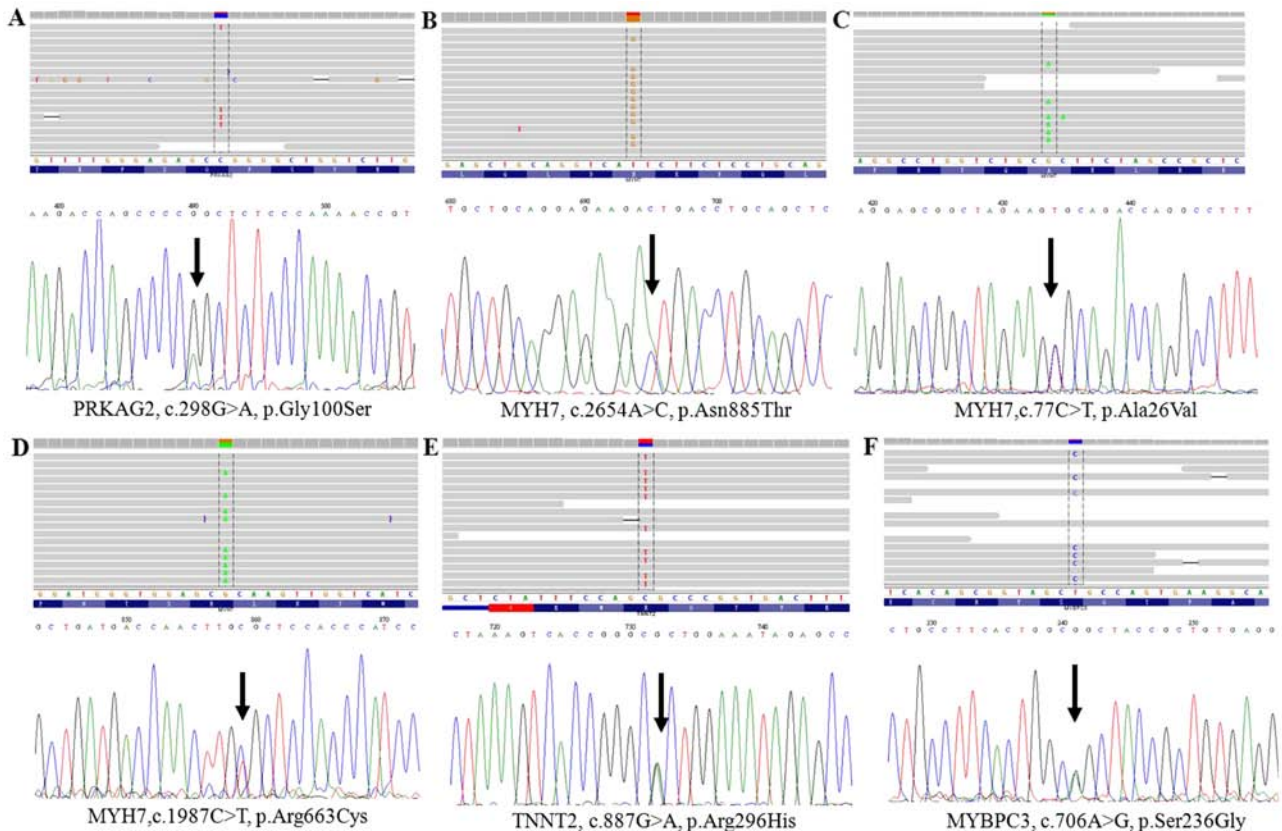


Figure 3. (A-F) Sanger sequencing showing gene mutations observed in this study of cardiomyopathy patients from China. Mutation sites are indicated by arrows.

Notably, the novel MYH7, p.Asn885Thr mutation was consistently predicted to be deleterious by the PolyPhen-2 (25),

SIFT (26), and MutationTaster (27) algorithms which showed that the mutation is probably damaging with scores

Table IV. Pathogenic mutations detected in subjects.

Subject no.	Gene symbol	Ref Chr.	Transcript	Nucleotide changes	Amino acid changes	SIFT	Poly Phen-2	Mutation Taster	MAF in 1000G	MAF in ExAC (EA)	Novel/known (Refs.)
A12	MYH7	chr14:23895180	NM_000257.3	c.2155C>T	p.Arg719Trp	N/A	N/A	N/A	0.000	0.000	Known (46)
A13	MYH7	chr14:23895180	NM_000257.3	c.2155C>T	p.Arg719Trp	N/A	N/A	N/A	0.000	0.000	Known (46)
A11	PRKAG2	chr7:151478406	NM_016203.3	c.298G>A	p.Gly100Ser	N/A	N/A	N/A	0.071	0.035	Known (47)
A13	PRKAG2	chr7:151478406	NM_016203.3	c.298G>A	p.Gly100Ser	N/A	N/A	N/A	0.071	0.035	Known (47)
A15	PRKAG2	chr7:151478406	NM_016203.3	c.298G>A	p.Gly100Ser	N/A	N/A	N/A	0.071	0.035	Known (47)
A37	MYH7	chr14:23902865	NM_000257.3	c.77C>T	p.Ala26Val	N/A	N/A	N/A	0.008	0.006	Known (35)
A94	MYBPC3	chr11:47370041	NM_000256.3	c.706A>G	p.Ser236Gly	N/A	N/A	N/A	0.031	0.030	Known (52)
A36	MYH7	chr14:23894003	NM_000257.3	c.2654A>C	p.Asn885Thr	NT	PD	DC	0.000	0.000	Novel
64	MYH7	chr14:23896043	NM_000257.3	c.1987C>T	p.Arg663Cys	N/A	N/A	N/A	0.000	0.000	Known (53)
A86	TNNT2	chr1:201328348	NM_001276345.1	c.887G>A	p.Arg296His	NT	PD	DC	0.000	0.000	VUS

N/A, not applicable; NT, not tolerated; PD, probably damaging; DC, disease causing; MAF, minor allele frequency; 1000G, 1000 Genomes Project; EA, East Asian; ExAC, Exome Aggregation Consortium; VUS, variant of uncertain significance.

of 0.997 and 0.996 on the HumDiv and HumVar models, respectively (Fig. 4C). Secondly, this mutation was not found in reference alleles from the 100 healthy controls, and was also absent from the HGMD database (www.hgmd.cf.ac.uk), NCBI dbSNP137 (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and 1000 Genomes project (<http://www.1000genomes.org/>). Finally, the mutation, p.Asn885Thr, occurs within a 3 helix bundle with the second helix interrupted and it was highly conserved across a number of species (Fig. 4A). As with the mutation of p.Asn885Thr in MYH7, notably, a variant of uncertain significance (TNNT2, p.Arg296His) was predicted to be probably damaging to amino acids using *in silico* programs, which is relevant for the pathogenicity of HCM (Fig. 4B and D).

Discussion

The identification of pathogenic mutations is critical for understanding the molecular pathogenesis of inherited cardiomyopathy, as this in turn will aid the clinical diagnosis of this disease. In the present study, we established a method for the rapid detection of potentially pathogenic mutations in a panel of 12 major genes closely associated with the occurrence of HCM or DCM using the Ion Torrent PGM system. A novel heterozygous mutation (MYH7, p.Asn885Thr) and a variant of uncertain significance (TNNT2, p.Arg296His) were identified.

The Ion Torrent PGM technique is based on the PCR amplification of DNA obtained from subjects which is followed by pooling and barcode labeling of the fragments in each sample and high-throughput sequencing (30,31). The multiplex amplification primers were designed to amplify the principal HCM and DCM disease-related genes in a condensed format that required only 6 microtubes. This process avoids the need for multiple amplifications and reduces the reagent cost per patient. Approximately 90% of the obtained sequences were matched to the coding regions of the target genes using the Coverage Analysis plug-in; however, 10% of the coding region was not covered. This may be due to several factors. Firstly, as the primer pairs were mixed in one reaction, multiplex PCR has a low specific amplification. In addition, the sequence stretches of low complexity and as well as GC-rich regions are difficult to capture (30). Such uncovered regions must be completed using conventional capillary-based sequencing, although there is a risk of missing some mutations using this technique. Despite the drawback regarding lower sequence coverage, the Ion Torrent PGM-based approach using multiplex PCR remains a high-throughput, low-cost method for the detection of mutations.

Herein, we constructed a library to sequence the genomic DNA isolated from patients (19, A15, A36, A37 and A7). Similarly, the second (A13, A63, A64, A94, A12 and 38) and third (51, 64, A51, A86 and A88) pool were also used to construct libraries, respectively, and a total of 8 patients were identified as carriers of pathogenic mutations (Table IV). We detected mutations in 12 disease genes in 7 (7/8) patients with HCM and in 2 (2/8) of the 8 patients with DCM. Of these 7 mutations, 5 are known (MYH7, p.Arg719Trp, p.Ala26Val and p.Arg663Cys; PRKAG2, p.Gly100Ser and MYBPC3, p.Ser236Gly), one is a variant of uncertain significance (TNNT2, p.Arg296His) and one is a novel mutation (MYH7, p.Asn885Thr). Our DCM mutation detection rates are consistent with those of a previous study (32), but the HCM detection rate was higher than that

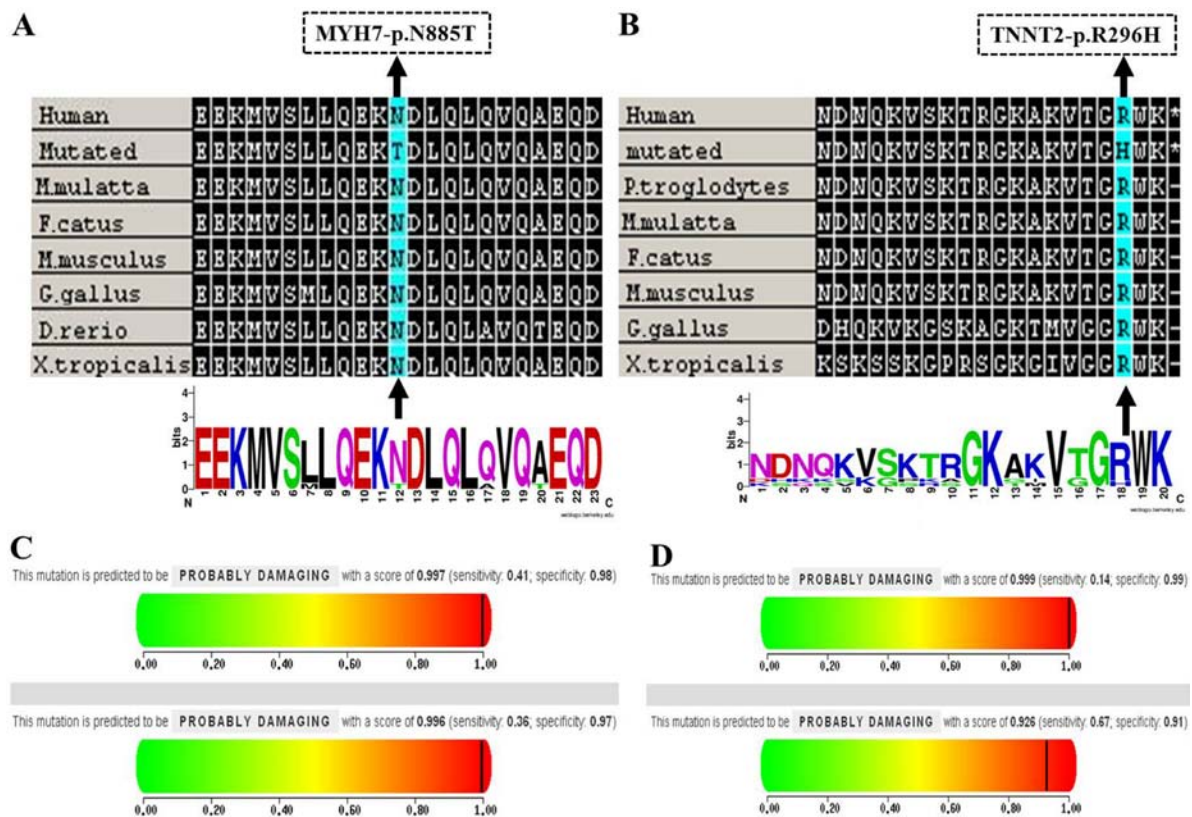


Figure 4. (A and B) Evolutionary conservation analyses of the MYH7, p.Asn885Thr and TNNT2, p.Arg296His mutations were performed, respectively. Clustal W was used to align sequences from *Homo sapiens*, *Macaca mulatta*, *Felis catus*, *Mus musculus*, *Gallus gallus*, *Danio rerio* and *Xenopus tropicalis* and WebLogo was used to generate sequence logos. The MYH7, p.Asn885Thr and TNNT2, p.Arg296His mutations are marked by a black arrow; (C and D) Results of the PolyPhen-2 analysis predicting the pathogenicity of the mutations of MYH7, p.Asn885Thr and TNNT2, p.Arg296His, respectively.

in earlier studies (33,34). As shown in Table IV, 9 subjects [a total of 7 subjects with HCM, one subject (A37) with DCM, and one (A11) subject who was clinically asymptomatic] carried mutations; subject A13 carried a double heterozygous mutation (PRKAG2, p.Gly100Ser plus MYH7, p.Arg719Trp). Mutations were distributed mostly in MYH7 (50%, 5/10) and PRKAG2 (30%, 3/10), followed by MYBPC3 (10%, 1/10) and TNNT2 (10%, 1/10). Mutations were not found in the LMNA, MYH6, MYL2, TPM1, MYL3, SCN5A, ACTC1 and TNNI3 genes. No pathogenic mutations were detected in 87.5% (7/8) of the patients with DCM. This may be due to the fact that there are fewer known DCM-related genes compared with the number of HCM-related genes (3,35); thus, the likelihood of detecting a mutation using our limited gene panel is low. Alternatively, lifestyle and environmental factors may play a more important role in the etiology of DCM which is not taken into account when performing genetic screening alone (32).

To the best of our knowledge, this is the first study to describe the novel MYH7 mutation (p.Asn885Thr) in patients with HCM. Sequence conservation analysis revealed that this residue is highly conserved across a number of species (Fig. 4A), thereby impairing its contractile function. Our results suggest that this novel mutation may be functionally deleterious and thus, play an important role in the pathogenesis of HCM, and it expands the mutational spectrum of the MYH7 gene. Moreover, to the best of our knowledge we are the first to report a variant of uncertain significance, p.Arg296His in TNNT2 which is associated with

HCM; this illustrated that the status of the variant TNNT2, p.Arg296His may be upgraded to pathogenic. Determining the pathogenicity of a mutation remains a major clinical challenge (36); further independent studies with *in vitro* or animal models (37,38) are essential in order to validate our results.

His558Arg polymorphism of the SCN5A gene is associated with dilated cardiomyopathy (39), atrial fibrillation (40), idiopathic cardiac conduction disorders (41) and Brugada syndrome (42). His558Arg is a common polymorphism that interacts with the other mutation, eventually modifying or modulating the disease phenotypes (39,42). We identified the common polymorphism of His558Arg in the SCN5A gene in 4 patients with DCM (subject nos. 19, A51, A7 and A37) and validated these findings using Sanger sequencing (data available upon request). Of note, the mutation MYH7, p.Ala26Val plus the common polymorphism of SCN5A, His558Arg were detected in one patient (A37) with DCM. Potentially, a polymorphism of His558Arg modifies or modulates the variant MYH7, p.Ala26Val which causes DCM, and may affect the age of onset, the severity and rate of progression of DCM.

Some patients with inherited cardiomyopathy may have more than one disease-causing mutation, which can occur in either the same gene (compound heterozygotes) or in different genes (double heterozygotes) (43,44). As a consequence of these complex mutations, the individual usually has a malignant phenotype of inherited cardiomyopathy (45). Notably, we have identified, for the first time to the best of our

knowledge, a double heterozygous (MYH7, p.Arg719Trp plus PRKAG2, p.Gly100Ser) mutation in a proband (III:2) with familial HCM. He exhibited a malignant phenotype of HCM that manifested with increased interventricular septum thickness (Table I and Fig. 2A and B). His elder sister (III:1) was also diagnosed with HCM and carried MYH7, p.Arg719Trp, but not PRKAG2, p.Gly100Ser. Family screening revealed that the proband's 45-year-old mother (II:1), who was asymptomatic, was also affected, and carried the PRKAG2, p.Gly100Ser mutation (Fig. 2A and B). These results suggest that the pathogenic MYH7, Arg719Trp mutation probably originated in the proband's father and grandfather. The MYH7, p.Arg719Trp (46) and PRKAG2, p.Gly100Ser (47) mutations have previously been shown to be associated with malignant familial HCM and sporadic HCM, respectively. We suggest that PRKAG2, p.Gly100Ser exacerbates the clinical severity of HCM in individuals with the MYH7 p.Arg719Trp mutation and thus, have a 'double dose' gene mutation effect (48,49). This is associated with a poor prognosis, and explains why the proband exhibited an early onset malignant phenotype of HCM (50). As a mutation carrier, the proband's mother (A11) is a family member at risk who was clinically asymptomatic (51); long-term follow-up is therefore essential in this subject. However, her children are at high risk of developing HCM, and thus genetic testing may be particularly helpful in this group. Indeed, we have recommended genetic testing for all first-degree relatives of the proband, since it may facilitate clinical decisions that impact diagnosis and treatment strategies.

In conclusion, Ion Torrent PGM targeted sequencing is a rapid and cost-effective method for the clinical genetic screening of patients with inherited cardiomyopathy. Correct recognition of the responsible gene mutations is essential for providing optimal presymptomatic intervention and genetic counseling for probands and their family members. The gene panel testing of 12 major disease-related genes in patients with inherited cardiomyopathy reported in this study has the potential to assist in revealing the etiology of genetically heterogeneous HCM, and it is a highly reliable and effective method for the screening of pathogenic mutations in candidate genes. However, the coverage of these targeted regions must be further improved. Furthermore, we detected a novel (MYH7, p.Asn885Thr) mutation and a variant of uncertain significance (TNNT2, p.Arg296His) that we suggest be upgraded to pathogenic status; these add new data to the spectrum of mutations in HCM. Moreover, a double heterozygous (PRKAG2, p.Gly100Ser plus MYH7, p.Arg719Trp) mutation was found in a proband with familial HCM. This data has the potential to allow us to better facilitate risk stratification and guide familial management of the disease. Finally, we note that genes beyond this initial 12-gene panel should be included in future tests as their relevance to DCM becomes clear.

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