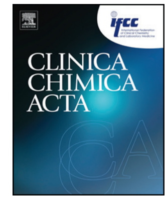
Clinica Chimica Acta 446 (2015) 132–140

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim



Targeted next-generation sequencing (NGS) of nine candidate genes with custom AmpliSeq in patients and a cardiomyopathy risk group

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ARTICLE INFO

Article history:
Received 27 November 2014
Received in revised form 23 March 2015
Accepted 8 April 2015
Available online 17 April 2015

Keywords:
Mutations
SNP
Cardiomyopathy
NGS
Group risk
Students

ABSTRACT

Background: Hypertrophic cardiomyopathy is a common genetic cardiac disease. Prevention and early diagnosis of this disease are very important. Because of the large number of causative genes and the high rate of mutations involved in the pathogenesis of this disease, traditional methods of early diagnosis are ineffective.
Methods: We developed a custom AmpliSeq panel for NGS sequencing of the coding sequences of *ACTC1*, *MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNI3*, *TNNT2*, *TPM1*, and *CASQ2*. A genetic analysis of student cohorts (with and without cardiomyopathy risk in their medical histories) and patients with cardiomyopathies was performed. For the statistical and bioinformatics analysis, Polyphen2, SIFT, SnpSift and PLINK software were used. To select genetic markers in the patients with cardiomyopathy and in the students of the high risk group, four additive models were applied.
Results: Our AmpliSeq custom panel allowed us to efficiently explore targeted sequences. Based on the score analysis, we detected three substitutions in the *MYBPC3* and *CASQ2* genes and six combinations between loci in the *MYBPC3*, *MYH7* and *CASQ2* genes that were responsible for cardiomyopathy risk in our cohorts. We also detected substitutions in the *TNNT2* gene that can be considered as protective against cardiomyopathy.
Conclusion: We used NGS with AmpliSeq libraries and Ion PGM sequencing to develop improved predictive information for patients at risk of cardiomyopathy.

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1. Introduction

Cardiomyopathy is a disease of the heart muscle. It affects people of all ages and is mostly inherited. It is not curable but can usually be treated successfully, with most of those affected going on to lead long and full lives.

Hypertrophic cardiomyopathy (HCM) is the most common type of disease characterized by asymmetric hypertrophy of the left and/or right ventricular myocardium. The prevalence of HCM in the general population is 0.2% or 1/500. Clinical manifestations of hypertrophic cardiomyopathy are greatly heterogeneous and range from asymptomatic left ventricular hypertrophy to serious arrhythmias, progressive heart failure and sudden cardiac death. HCM is a significant cause of sudden unexpected death at any age, and it can also cause exercise disability at almost any age. Annual mortality for HCM patients is 3–4% in adults and over 6% in children [4].

HCM is a genetically heterogeneous disease that is mainly inherited via autosomal dominance. It is caused by one or more mutations in the genes encoding proteins of the sarcomere and mutations in some other genes. Approximately 90% of these pathogenic mutations are missense mutations [12]. Currently, there are more than 18 known genes that can cause HCM when mutated [3]. The most important genes encode the protein components of the cardiac sarcomere, which perform contractile, structural and regulatory functions. These include thick

Abbreviations: CM, cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; NGS, next-generation sequencing; indels, insertion and/or deletion; SNP, single nucleotide polymorphism; MNP, multiple nucleotide polymorphism.

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http://dx.doi.org/10.1016/j.cca.2015.04.014
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filament proteins (*MYH7*, *MYL2*, and *MYL3*), thin filament proteins (*TNNT2*, *TNNI3*, *TNNC1*, *TPM1*, and *ACTC*), intermediate filament proteins (*MYBPC3*), and Z-disc proteins (*ACTN2*, *MYOZ2*), which adjoin the sarcomere. Mutations in the myosin heavy chain (*MYH7*) and myosin-binding protein C (*MYBPC3*) are the most common and account for roughly 80% of sarcomeric HCM [12].

Direct capillary sequencing is mainly used to screen cardiomyopathy patients for genetic mutations [1]. Despite the development of scanning methods, such as high-resolution melting (HRM) analysis and other techniques [5], targeted Sanger sequencing panels using traditional individual exon-by-exon sequencing remain expensive and time consuming. Thus, massively parallel next-generation sequencing (NGS) approaches are beginning to supplant Sanger sequencing [4,5]. The use of NGS has the potential to be substantially more effective than Sanger sequencing [7]. To date, the six main NGS platforms are MiSeq and HiSeq (Illumina), GS-FLX Titanium (Roche), Ion PGM™ and Solid 4 Systems (Life Technologies), and Complete Genomics platforms [3,6, 8–11]. The advantages of NGS for cardiomyopathy causative gene analysis have been repeatedly demonstrated [8,10]. However, those studies had small sample sizes; thus, the clinical sensitivity of NGS can be improved by performing additional studies.

As a component of screening individuals at risk for cardiomyopathy, genetic testing [10] to determine individuals' predispositions for cardiomyopathies has been proposed. Patients at risk for cardiomyopathies are defined not only as individuals who have a close family member with established cardiomyopathy but also as individuals with a poor cardiac medical history. The results of genetic testing may influence the management of at-risk individuals, which may in turn lead to improved outcomes. Thus, the aim of our study was to use NGS sequencing to determine the common genetic profile of patients with cardiomyopathies and to compare it with the genetic profile of an at-risk group.

2. Material and methods

2.1. Patients

Students at Saint-Petersburg State University ($N = 45$) and patients with cardiomyopathies from Russia ($N = 25$) and Belarus ($N = 13$) were included in this study. The study was cleared by the Saint-Petersburg State University Ethics Review Board for human studies (No. 40 from 07.03.2012), and all patients signed an informed consent.

All groups were subdivided into three cohorts: patients with cardiomyopathies (32 females and 6 males), students with cardiomyopathy risk in their medical history (14 females and 10 males) and students without cardiomyopathy risk in their medical history (15 females and 6 males). Students that composed the at-risk group included those with chest pain (at rest and during physical exertion), episodes of tachycardia (without cause), cardiac arrhythmia, syncope (without cause), arterial hypertension, a positive family history of cardiovascular disease, or a positive family history of cardiac arrhythmia. No ultrasonography or cardio MRI markers have been found in the both groups of students. A short list of the characteristics of the groups is given in Table 1 (a, b).

2.2. AmpliSeq panel genes

The nine genes that have been most clearly demonstrated to be associated with HCM were selected for this study [1]. The design of the AmpliSeq panel was performed by Ion AmpliSeq™ Designer (Life Technologies, USA). The design allowed for the analysis of 133 exons (padding: ± 50 bp) by the targeted resequencing of 242 amplicons (global size: 24.6 kb/patient). The submitted custom AmpliSeq loci for *ACTC1*, *MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNI3*, *TNNT2*, *TPM1*, and *CASQ2* are given in Supplemental 1. The coverage of the submitted loci was more than 99.9%, with a read depth above 30 reads (30X) for each targeted nucleotide. One sample with low coverage (2–6 reads for locus) was excluded from the study.

2.3. DNA isolation

DNA samples from the blood of all patients were isolated by phenol-chloroform extraction as described previously [2]. DNA concentration was determined using Qubit™ software (Invitrogen, USA) with Qubit™ DNA HS Assay Kits according to the manufacturer's instructions.

2.4. Library preparation

Libraries were constructed using the Ion AmpliSeq Library Kit v2.0 (Life Technologies, USA) according to the manufacturer's instructions. Validation and quantification of the libraries were performed on the 2200 TapeStation Instrument using the High Sensitivity D1K Reagents and High Sensitivity D1K ScreenTape (Agilent technologies, USA).

Table 1
Demographics and clinical characteristics.

a

Group	Anamnesis		
	Number of patients	Gender (male/female)	Age at diagnosis
Students (healthy)	21	6/15	18.3 ± 0.8
Students (at-risk group)	24	10/14	18.8 ± 1.2
Patients with HCM (Belarus)	13	6/7	26.9 ± 6.0
Patients with HCM (Russian)	25	0/25	50.1 ± 15.4

b

Group	Medical history					
	Chest pain (at rest and during physical exertion)	Arterial hypertension	Cardiac arrhythmia	Syncope	Positive family history of HCM	Positive family history of cardiovascular disease
Students (healthy)	0/21 (0%)	0/21 (0%)	0/21 (0%)	0/21 (0%)	0/21 (0%)	0/21 (0%)
Students (at-risk group)	0/24 (0%)	6/24 (25.0%)	8/24 (33.3%)	4/24 (16.7%)	0/24 (0%)	13/24 (54.2%)
Patients with HCM (Belarus)	9/13 (69.2%)	2/13 (15.4%)	8/13 (61.5%)	5/13 (38.5%)	7/13 (53.8%)	8/13 (61.5%)
Patients with HCM (Russian)	21/25 (84.0%)	21/25 (84.0%)	11/25 (44.0%)	13/25 (52.0%)	1/25 (4.0%)	No data

2.5. Ion Torrent PGM sequencing

Amplified libraries were submitted to emulsion PCR using the Ion OneTouch™ 2 system with the Ion PGM™ Template OT2 200 Kit (Life Technologies, USA) according to the manufacturer's instructions. Ion sphere particles (ISP) were enriched using the E/S module. ISPs were loaded and sequenced on an Ion 314™ Chip or Ion 316™ Chip by PGM (Life Technologies, USA).

2.6. Computational analysis

Torrent Mapping Alignment Program (Torrent Suite v.4.0), Torrent Variant Caller v.3.6–4.0, samtools/bcftools v.0.1.18 [17], VCFtools v.0.1.7 [18], PLINK v.1.07 [19], SnpSift v.3.6c [16], ANNOVAR rev. 527 [13], Polyphen2 v.2.2.2 [15] and SIFT v.4.0.3 [14] software were used to perform the computational analyses, including sequence alignment, variant calling and variant analysis.

For each patient, reads were aligned against the hg19 reference genome with Torrent Mapping Alignment Program (TMAP), using the default alignment settings. After alignment, the variant calling was performed using Torrent Variant Caller (TVC). Variant calling for each patient was run independently. This resulted in 30 to 50 variants in nine target genes per patient. SnpSift was used to annotate these variants.

Another method used for variant calling was samtools mpileup followed by filtering. It was run as a multi-sample variant caller on all samples simultaneously. Then, mutations were tested for association using Fisher's exact test, carried out by SnpSift. In this test, only diseased and healthy patient groups were taken into account as case and control groups, correspondingly.

A single table was made with the variants determined by Torrent Variant Caller and the results obtained from samtools and SnpSift. This was done using R, Java and Python.

The obtained variants were annotated to determine overlapping information with the genetic database ANNOVAR [13]. Alternative allele frequency values were taken from the 1000 Genomes project (dbSNP build id 138).

The impact of non-synonymous amino acid substitution was assessed in silico, using Polyphen 2 and SIFT programs.

3. Results

An NGS workflow with custom AmpliSeq panel was used for the genotyping of 82 DNA samples from patients with cardiomyopathies and students. Four separate runs, containing 19 to 24 patient samples each, were performed (three runs with a 314-chip and one run with a 316-chip). The coverage was comparable between runs. Each 314-chip generated an average of 350–490 thousand reads resulting in an average of 1.8–2.1 million Q20 bases per sample sequenced (Q20 = 95.1–98.9% chance of the correct base being identified). The mean read length was approximately 91–102 bp. The 316-chips generated an average of 3.1 million reads from 19 of our samples, resulting in an average of 6.1 million Q20 bases per sample sequenced (Q20 = 98.7% chance of the correct base being identified). The mean read length was approximately 110–130 bp. According to previous studies, each targeted nucleotide of the exons was sequenced at 30X coverage. The mutations in exon 13 and exon 22 of the *MYH7* gene, as well as the mutation in exon 17 of the *MYBPC3* were verified by automated Sanger sequencing (see Supplemental 2).

Table 2
Main markers for HCM identified in patients and in the at-risk group compared to the control group (under dominant model).

Gene	Start position	Nucleotide change	Sequence change	Type	rsID	Diseased risk healthy in %	*Score*	*Score2*	*p*-Value (CC_DOM model, snpSift)	Polyphen 2	SIFT	Clinical verification
MYH7	23892910	c.2945T>C (NM_000257.2)	G	SNP	rs145532615	5/0/0	20	−100	0.41	Probably damaging	Damaging	Andreasen et al. [7]
TNNT2	201335977	c.222T>G (NM_000364.3)	G	SNP	–	5/0/0	20	−100	–	Probably damaging	Damaging	–
MYBPC3	47367871	c.977G>A (NM_000256.3)	T	SNP	rs34580776	5/4/0	19	−99	0.41	Benign	Damaging	Jääskeläinen et al. (2014)
MYBPC3	47357487	c.2678G>T (NM_000256.3)	T	SNP	–	5/17/0	16	−96	–	Probably damaging	Damaging	–
MYBPC3	47364248	c.1505C>T (NM_000256.3)	T	SNP	rs397515907	3/0/0	10	−50	0.64	Probably damaging	Damaging	–
MYH7	23884861	c.5134C>T (NM_000257.2)	A	SNP	rs121913650	3/0/0	10	−50	0.64	Probably damaging	Damaging	Hougs et al. (2005)
MYH7	23884860	c.5135G>A (NM_000257.2)	T	SNP	rs193922390	3/0/0	10	−50	0.64	–	Damaging	Gruner et al. [25]
MYBPC3	47353797	c.3640T>C (NM_000256.3)	G	SNP	–	3/0/0	10	−50	0.64	Probably damaging	Damaging	Christiaans et al. (2010)
MYL2	111352091	c.173G>A (NM_000432.3)	T	SNP	rs104894369	3/0/0	10	−50	0.64	Probably damaging	Tolerated	–
TPM1	63351791	c.404delA (NM_000366.5)	Del	DEL	–	21/0/0	80	−400	4.73E−04	–	–	–
CASQ2	116245530	c.1014 + 12delG (NM_001232.3)	Del	DEL	–	13/4/0	49	−249	8.62E−05	–	–	–
MYBPC3	47370130	c.655-38delG (NM_000256.3)	Del	DEL	–	11/0/0	40	−200	8.24E−05	–	–	–
MYBPC3	47360055	c.2308 + 16delC (NM_000256.3)	Del	DEL	–	3/0/0	10	−50	4.92E−05	–	–	–
CASQ2	116311173	c.-11delT (NM_001232.3)	Del	DEL	–	3/0/0	10	−50	1.72E−03	–	–	–
MYBPC3	47369105	c.852-75insGA (NM_000256.3)	GA	DEL, INS	–	0/4/0	−1	1	3.25E−04	–	–	–
TNNT2	201338793	c.97 + 151delC (NM_000364.3)	Del	DEL	–	0/0/10	−100	20	1.80E−05	–	–	–
TNNT2	201335874	c.223 + 92G>C (NM_000364.3)	C	SNP	–	0/0/29	−300	60	1.902E−07	–	–	–
TNNT2	201335873	c.223 + 93C>G (NM_000364.3)	G	SNP	–	0/0/33	−350	70	2.535E−04	–	–	–

The main goal of this study was to identify genetic markers in patients with cardiomyopathies and students in an at-risk group that were not detected in the student control group and to identify markers present only in the control group. Two additive models were considered. We made the assumption that clinical effect depends on the presence of alternative alleles or their combinations (dominant models) and alternative genotypes or their combinations between detected variants (recessive models). These assumptions resulted in four different models. For each model a single table was generated.

The resulting tables include the following columns: chromosome (chr), start position (start pos.), end position (end pos.), gene name, reference nucleotide (ref), observed nucleotide (nuc.), mutation type, mutation rsID (rsID), number of patients with this mutation (polymorphism) within each group (diseased, risk or healthy), *Score* (based on the number of patients with this mutation within each group), *Score2*, alternative allele frequency (alt allele freq, taken from 1000 Genomes project), *p*-value (from Fisher's exact test), adjusted *p*-value (*p*-value adjusted with Bonferroni method), polyphen 2 and SIFT prediction characteristics.

We also used two score functions (*Score* and *Score2* values) and *p*-value for the analysis. The first score was calculated by the following formula: $Score = 10 * Number_in_Diseased - 50 * Number_in_Healthy - Number_in_Risk$, where $Number_in_X$ is the number of patients with this mutation in the *X* patient group. This score was used to select mutations specific to the diseased group. The score formula can be explained as follows: for every patient in the diseased group with a mutation, that mutation was given 10 points; for every patient in the healthy group with a mutation, that mutation lost 50 points; for every patient in the at-risk group with a mutation, that mutation lost one point (we assumed the probability of the patient becoming diseased was small). To select mutations specific to the control group a similar function, *Score2*, was used: $Score2 = 10 * Number_in_Healthy - 50 * Number_in_Diseased + Number_in_Risk$.

In the first dominant model (Supplemental 3.1), we identified 449 substitutions in the selected samples: 107 indels, 303 SNPs, 12 MNPs and 27 complex mutations in the target regions and nearby regions. The main markers were selected using the following features: highest possible *Score*, highest possible *Score2*, lowest possible *p*-value, most important by SIFT or Polyphen2 analysis, and markers known to be associated with HCM (described in OMIM). The majority of these markers (with $Score > 0$, "damaging" function/clinical function or with p-value <0.01 and without low covered patients) are shown in Table 2.

Eighteen different variants are included in Table 2: 11 SNPs and 7 ins/del. Interestingly, all ins/del variants were detected as homozygous. Nine of them represented variants with $Score > 10$ and disruption of protein function. There were substitutions in *MYH7* (c.2945T>C, c.5134C>T, and c.5135G>A), *TNNT2* (c.222T>G), *MYBPC3* (c.977G>A, c.2678G>T, c.1505C>T, and c.3640T>C), and *MYL2* (c.173C>T). The mutations in *MYH7* (c.2945T>C, c.5134C>T, and c.5135G>A) and *MYBPC3* (c.977G>A and c.3640T>C) are associated with HCM, as was shown in previous studies [7,25,43,54,57]. Nine substitutions were statistically significant (p-value < 0.01). They were localized in *TPM1* (c.404delA), *CASQ2* (c.1014 + 12delG and c.-11delT), *MYBPC3* (c.655-38delG, c.2308 + 16delC, and c.852-75insGA), and *TNNT2* (c.97 + 151delC, c.223 + 92G>C, and c.223 + 93C>G).

The following mutations were found only in the patients with cardiomyopathy and in the at-risk group of students: *MYBPC3* (c.977G>A and c.2678G>T) and *CASQ2* (c.1014 + 12delG). A variant of *MYBPC3* (c.977G>A) was found in two patients and one at-risk student. A *MYBPC3* marker (c.2678G>T) was identified in two patients and four at-risk students, A *CASQ2* marker (c.1014 + 12delG) was identified in five patients and one at-risk student. According to the SNPSIFT analysis, the most significant mutations were substitutions in the *TNNT2* gene. Some of them (c.97 + 151delC, c.223 + 92G>C, and c.223 + 93C>G) occurred only in the student control group. These variants can be considered as protective against cardiomyopathy.

In the second (recessive) model (Supplemental 3.2), we identified 127 homozygous mutations in the selected samples: 41 indels, 72 SNPs, six MNPs and eight complex mutations in target regions and nearby regions. The main identified markers are shown in Table 3.

Four variants were included in Table 3. Among those mutations, c.3288G>A of *MYBPC3* was only observed in affected patients and at-risk students. However, it was not "Damaging" (by Polyphen2) and had no clinical effect [6].

In the third (dominant) combination model (Supplemental 3.3), we identified more than 2800 two-gene variants combinations in the selected samples, and only 10 of them were selected as main markers. As shown in Table 4, they included the following associated mutations: *MYBPC3* (c.706A>G)–*MYH7* (c.3973-30A>G), *MYBPC3* (c.3288G>A)–*MYH7* (c.1095G>A), *MYBPC3* (c.3815-66C>T)–*MYH7* (c.1128C>T), *MYBPC3* (c.706A>G)–*MYH7* (c.3853 + 27T>A), *MYBPC3* (c.706A>G)–*CASQ2* (c.939 + 23C>T), *MYH7* (c.1128C>T)–*ACTC1* (c.808 + 76G>C), *MYH7* (c.1128C>T)–*CASQ2* (c.1185C>T), *MYBPC3* (c.1223 + 29G>A)–*MYH7* (c.1095G>A), *MYH7* (c.1408-117C>T)–*MYH7* (c.1128C>T), and *MYBPC3* (c.786C>T)–*TPM1* (c.375-75A>G). Only the mutation in *MYBPC3* (c.706A>G) was associated with HCM, as was shown in previous studies [6,13,44].

Among the mutations that occurred only in patients with cardiomyopathy and at-risk students, there were six coupled mutations: *MYBPC3* (c.706A>G)–*MYH7* (c.3973-30A>G), *MYBPC3* (c.3288G>A)–*MYH7* (c.1095G>A), *MYBPC3* (c.3815-66C>T)–*MYH7* (c.1128C>T), *MYBPC3* (c.706A>G)–*MYH7* (c.3853 + 27 T>A), *MYBPC3* (c.706A>G)–*CASQ2* (c.939 + 23C>T), and *MYBPC3* (c.1223 + 29G>A)–*MYH7* (c.1095G>A).

In the fourth (recessive) combination model (Supplemental 3.4), we identified 313 two gene homozygous variant combinations, but no significant markers were found.

We also compared the mutations identified here with three sets of previously known mutations: HCM-associated variants listed in the OMIM database (MIM 160760, 160710, 102540, 600958, 160781, 160790, 191044, 191045, 191010, 114251); previously identified cardiomyopathy markers; and SIFT and Polyphen2 markers with score >10 (according to the terms of Table 2). After filtering, we detected 22 variants (3, 11 and 8 correspondingly). Most variants were found in *MYBPC3* and *MYH7*. Only the following eight variants were among mutations of our interest: *MYH7* (c.5134C>T, c.5135G>A), *MYBPC3* (c.977G>A, c.2678G>T, c.1505C>T, c.3640T>C), *MYL2* (c.173C>T), *TNNT2* (c.222T>G) (see Table 2).

Key mutations were identified in 20 of 38 patients. Two patients had more than three mutations. One Belarus male had four key mutations, and one Russian female had three key mutations. These three mutations were also present in the Belarus male, and they

Table 3
Main markers for HCM identified in patients and in the at-risk group compared to the control group (under recessive model).

Gene	Start position	Nucleotide change	Sequence change	Type	rsID	Diseased risk healthy in %	*Score*	*Score2*	*p*-Value (CC_REC model, snpSift)	Polyphen 2	SIFT	Clinical verification
MYBPC3	47354787	c.3288G>A (NM_000256.3)	T	SNP	rs1052373	16/12/0	57	−297	0.06	Benign	Tolerated	Li et al. [6]
MYBPC3	47370130	c.655-38delG (NM_000256.3)	Del	DEL	–	11/0/0	40	−200	1.98E−03	–	–	–
CASQ2	116311173	c.-11delT (NM_001232.3)	Del	DEL	–	3/0/0	10	−50	1.72E−03	–	–	–
MYBPC3	47355061	c.3190 + 47delC (NM_000256.3)	Del	DEL	–	0/0/5	−50	10	1.69E−03	–	–	–

Table 4
Main two gene variant combinations markers for HCM identified in patients and in the at-risk group compared to the control group (under dominant model).

Gene 1	Start position 1	Nucleotide change 1	rsID 1	Polyphen 2 1	SIFT 1	Gene 2	Start position 2	Nucleotide change 2	rsID 2	Polyphen 2 2	SIFT 2	Diseased risk healthy in %	*Score*	*Score2*	Clinical verification
MYBPC3	47370041	c.706A>G (NM_000256.3)	rs3729989	Benign	Tolerated	*MYH7*	23887645	c.3973-30A>G (NM_000257.2)	rs7159367	Possibly damaging	–	18/8/0	68	−348	Jääskeläinen et al. (2002); [6,13]
MYBPC3	47354787	c.3288G>A (NM_000256.3)	rs1052373	–	Tolerated	*MYH7*	23899027	c.1095G>A (NM_000257.2)	rs735711	Possibly damaging	–	18/17/0	66	−346	–
MYBPC3	47353498	c.3815-66C>T (NM_000256.3)	rs2290146	–	–	*MYH7*	23898994	c.1128C>T (NM_000257.2)	rs2231126	Possibly damaging	Tolerated	16/4/0	59	−299	–
MYBPC3	47370041	c.706A>G (NM_000256.3)	rs3729989	Benign	Tolerated	*MYH7*	23888665	c.3853 + 27 T>A (NM_000257.2)	rs2277475	–	–	16/4/0	59	−299	Jääskeläinen et al. (2002); [6,13]
MYBPC3	47370041	c.706A>G (NM_000256.3)	rs3729989	Benign	Tolerated	*CASQ2*	116247790	c.939 + 23C>T (NM_001232.3)	rs3811003	–	–	16/8/0	58	−298	Jääskeläinen et al. (2002); [6,13]
MYH7	23898994	c.1128C>T (NM_000257.2)	rs2231126	Possibly damaging	Tolerated	*ACTC1*	35084215	c.808 + 76G>C (NM_005159.4)	rs3729755	–	–	13/0/0	50	−250	–
MYH7	23898994	c.1128C>T (NM_000257.2)	rs2231126	Possibly damaging	Tolerated	*CASQ2*	116243877	c.1185C>T (NM_001232.3)	rs7413162	–	Tolerated	13/0/0	50	−250	–
MYBPC3	47365014	c.1223 + 29G>A (NM_000256.3)	rs11570078	–	–	*MYH7*	23899027	c.1095G>A (NM_000257.2)	rs735711	Possibly damaging	–	13/4/0	49	−249	–
MYH7	23897996	c.1408-117C>T (NM_000257.2)	rs3729814	–	–	*MYH7*	23898994	c.1128C>T (NM_000257.2)	rs2231126	Possibly damaging	Tolerated	18/4/5	19	−339	–
MYBPC3	47369443	c.786C>T (NM_000256.3)	rs11570058	–	–	*TPM1*	63351687	c.375-75A>G (NM_001018004)	rs4775614	Possibly damaging	–	16/17/5	6	−286	–

were in the *MYBPC3* gene. Known OMIM variants were present in three patients from Belarus only.

4. Discussion

Today, next-generation sequencing is considered a high throughput mutation detection method for genes for which large cohorts of patients have to be investigated, such as patients with cardiomyopathies. NGS technology allows for the analysis of substantially larger genomic regions at a lower cost than other methodologies [4]. At the same time, the number of variants of unknown (unexplored) significance has also increased with next-generation sequencing. Additionally, the percent of individuals who have more than one mutation that is thought to be pathogenic is increasing. A study in 2013 reported that 9.5% (19/200) of patients from China with HCM had multiple pathogenic mutations and that the number of mutations correlated with disease severity [20]. In our study, nine of 38 patients had multiple pathogenic mutations (23.7%). Two patients with arrhythmia had more than three identical mutations (see Table 5). Interestingly, two of those identical mutations in the two patients with arrhythmia (c.977G>A and c.2547C>T) are associated with cardiac arrhythmias and not cardiomyopathy. Both of those mutations were also detected in one at-risk student (he also had arrhythmia in his medical history and his mother had the same pathology). A previous study by Roncarati et al. showed that a non-synonymous change, rs34580776-c.977G>A, carried by c.3697C>T had a potential functional effect on the protein [26]. Thus, these mutations may provoke both arrhythmia and cardiomyopathy and also potentiate the severity of cardiomyopathy, making them risk factors for HCM.

The complexities of the cardiomyopathy diagnosis in some cases are due to de novo mutations. Only genetic testing can reliably determine the presence or absence of the same mutation in a close relative. The absence of a mutation means that the individual has not inherited the familial predisposition to HCM and, thus, has a similar risk of developing HCM as the general population [20].

Another difficulty in cardiomyopathy diagnostics is the number of pathogenic mutations in the population. These mutations appear to have incomplete penetrance [21]. Their presence in both patients and the normal population significantly complicate diagnostic screening for cardiomyopathies. In our study, we tried to assess the NGS methodology in three target groups: patients with cardiomyopathies, an at-risk students group and a control group.

To evaluate the mutations, we used multiple traditional scores, such as *p*-value, Polyphen-2 and SIFT prediction scores, as well as the simple statistical measures *Score* and *Score2* based on the number of patients with a mutation in the selected groups. *p*-Value and prediction scores have been widely used in similar studies for mutation significance measuring (for example, [10,24]). *Score* and *Score2* were used to rank mutations in the present study (more patients with a mutations generated a higher score). A mutation was treated as a main marker if its significance was confirmed (p-value < 0.01, disruption of protein function or an association with CM shown previously) or its *Score* (or *Score2*) was high enough to be statistically meaningful.

Two recessive and two dominant models were used to compare the results of our study. Our recessive models did not show any significance for determining the at-risk group. However, our dominant models were more informative for these purposes. The at-risk groups in these models, as shown previously [4], corresponded to an autosomal dominant pattern of cardiomyopathy inheritance. However, the results provided by the dominant models were different. Two mutations in the *MYBPC3* gene (c.977G>A and c.2678G>T) and another one in the *CASQ2* gene (c.1014 + 12delG) were found to be significant for cardiomyopathy testing in the at-risk group. We assumed that *MYBPC3* gene (c.977G>A) genetic counselling should be offered for individuals and their family members with those variants. Meanwhile, the analysis of the complex mutations in other genes, including *MYBPC3*

Table 5
Key mutations in patients with cardiomyopathy.

Gene	Start position	rsID/nucleotide change	Clinical verification	Diseased risk healthy in %	Score	Bel 01	Bel 02	Bel 03	Bel 04	Bel 05	Bel 08	Bel 09	Bel 43	Bel 44	Bel 45	Bel 63	Bel 64	Rus 10	Rus 01	Rus 02	Rus 03
OMIM																					
MYH7	23884861	rs121913650/c.5134C>T	[27]	3/0/0	10			+													
MYL2	111352091	rs104894369/c.173G>A	[28–32]	3/0/0	10						+										
MYH7	23898488	rs3218714/c.1207C>T	[32–35]	3/0/0	10		+														
Clinical verification																					
MYBPC3	47358997	rs3729953/c.2547C>T	[26]	13/8/0	48									+		+					
MYBPC3	47353740	rs397516037/c.3697C>T	[36–38]	5/0/0	20											+					
MYH7	23892910	rs145532615/c.2945 T>C	[7,39–41]	5/0/0	20																
MYBPC3	47367871	rs34580776/c.977G>A	[42–45]	5/4/0	19											+					
CASQ2	116243875	rs397516641/c.1185_1187delCGA	[46]	3/0/0	10		+														
MYBPC3	47364248	rs397515907/c.1505G>A	[47–52]	3/0/0	10			+													
MYH7	23894116	rs397516155/c.2539_2541delAAG	[24,53]	3/0/0	10				+												
MYBPC3	47353797	c.3640T>C	[54]	3/0/0	10										+						
MYL3	46902303	rs139794067/c.170C>G	[55]	3/0/0	10											+					
MYH7	23884860	rs193922390/c.5135G>A	[25]	3/0/0	10																
MYH7	23894049	rs138049878/c.2608C>T	[7,56]	3/0/0	10																
SIFT/Polyphen 2																					
TNNT2	201335977	c.222T>G	–	5/0/0	20												+				
MYBPC3	47357487	c.2678G>T	–	5/17/0	16										+						
MYH7	23894024		–	3/0/0	10																
MYH7	23896043	rs397516127/c.1987C>T	–	3/0/0	10																
MYH7	23896053		–	3/0/0	10																
MYH7	23898165		–	3/0/0	10							+									
MYBPC3	47357493		–	3/0/0	10																
MYL3	46901027		–	3/0/0	10																

Gene	Rus 04	Rus 05	Rus 06	Rus 07	Rus 38	Rus 39	Rus 40	Rus 41	Rus 42	Rus 50	Rus 51	Rus 52	Rus 53	Rus 54	Rus 55	Rus 56	Rus 57	Rus 58	Rus 59	Rus 60	Rus 61	Rus 62
OMIM																						
MYH7																						
MYL2																						
MYH7																						
Clinical verification																						
MYBPC3			+			+						+										
MYBPC3												+										
MYH7																			+			+
MYBPC3												+										
CASQ2																						
MYBPC3																						
MYH7																						
MYBPC3																						
MYL3																						
MYH7		+																				
MYH7	+																					
SIFT/Polyphen 2																						
TNNT2										+												
MYBPC3								+														
MYH7						+																
MYH7								+														
MYH7															+							
MYH7																						
MYBPC3									+													
MYL3										+												

(c.706A>G)–*MYH7* (c.3973-30A>G), *MYBPC3* (c.3288G>A)–*MYH7* (c.1095G>A), *MYBPC3* (c.3815-66C>T)–MYH7 (c.1128C>T), MYBPC3 (c.706A>G)–MYH7 (c.3853 + 27T>A), *MYBPC3* (c.706A>G)–*CASQ2* (c.939 + 23C>T), and *MYBPC3* (c.1223 + 29G>A)–*MYH7* (c.1095G>A) (see Table 4), may also be useful, especially because the number of mutations in an individual may influence disease severity [20]. However, information about these mutations cannot be used as a verification of the diagnosis.

In our study, the following mutations were identified for the first time: *MYBPC3* (c.2678G>T) and *CASQ2* (c.1014 + 12delG) and the combinations *MYBPC3* (c.3288G>A)–*MYH7* (c.1095G>A), *MYBPC3* (c.3815-66C>T)–*MYH7* (c.1128C>T), and *MYBPC3* (c.1223 + 29G>A)–*MYH7* (c.1095G>A). It is important to note that with the exception of mutations in *CASQ2*, the rest of the mutations were identified based on score analysis using polyphen 2 and SIFT prediction. Thus, score analysis with polyphen 2/SIFT prediction can be more robust and informative than scoring with SnpSift analysis.

In our study, we detected pathogenic mutations (see Tables 2, 4, 5) and protective cardiomyopathy risk variants that had not been identified previously. All of the protective variants were confined to the *TNNT2* gene. Why these variants are protective and the molecular mechanism of their protection is not yet clear.

Although our patient groups were different in terms of demographics, we assumed this difference was not important for our comparisons. The found mutations cannot be associated with patients' sex because the target genes were autosomal. The groups of patients from two countries included in the study (Russians and Belarusians) had no obvious population differences [22].

To further show the homogeneity of our data we conducted two additional experiments. Principal component analysis (PCA) of the found variants in the nine target genes for all our samples (similar to a study in 1000 Genomes project) [23] detected no population bias in our groups. Then, *p*-value distribution and Q–Q plot analyses were performed (see Supplemental 4). These analyses showed no significant difference between Russian and Belarusian diseased patients.

We speculate that HCM is more likely a complex rather than a single-gene disease. Thus, it is difficult to use partial genetic information to stratify the risk of patients. Novel genetic and environmental causes of HCM should be revealed in order to fully determine the pathogenic mechanisms of HCM. Considering that the majority of HCM genetic markers are associated with a predicted disruption of protein functions in our HCM cohort and others, it is highly likely that the score analysis used in this study will fit other populations as well.

5. Conclusion

NGS is a fast and cost-effective method for the clinical genetic screening of patients with cardiomyopathies. To facilitate a possible diagnostic application of NGS, unknown variants and the determination of additive effects of multiple variants should be addressed in future studies. This will provide improved prognostic and/or predictive information and personalize the medical care of patients. The development of multiple databases of target gene sequences will also assist in the understanding of different genetic variants. These data will progressively improve the knowledge of novel genetic variants and will further indicate the benefit of clinical diagnostic sequencing for patients with cardiomyopathies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2015.04.014.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgements

In this research, we used equipment from the SPbGU resource centre “Development of molecular and cellular technology” and “SPbGU Biobank”. This work was financially supported by the Russian Scientific Foundation, grant No. 14-50-00069 (research) and by the Government of Russian Federation, grant 074-U01 (bioinformatics).

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