DOI: 10.4149/BLL_2019_007

Bratisl Med J 2019; 120 (1)

46 – 51

EXPERIMENTAL STUDY

Targeted next-generation sequencing in Slovak cardiomyopathy patients

Nagyova E[1,2,6], Radvanszky J[4,5], Hyblova M[1,6], Simovicova V[7], Goncalvesova E[7], Asselbergs FW[2,3,8], Kadasi L[1,5], Szemes T[1,4,6], Minarik G[1,4,6]

Department of Molecular Biology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovakia.
E.Nagyova@umcutrecht.nl

ABSTRACT

OBJECTIVES: For the first time we used targeted next-generation sequencing to detect candidate pathogenic variants in Slovak cardiomyopathy patients.

BACKGROUND: Targeted next-generation sequencing is considered to be the best practice in genetic diagnostics of cardiomyopathies. However, in Slovakia, with high cardiomyopathies prevalence of 1/440, the current diagnostic tests are still based on Sanger sequencing of a few genes. Consequently, little is known about the exact contribution of pathogenic variants in known cardiomyopathy genes in Slovak patients.

METHODS: We used a panel of 46 known cardiomyopathy-associated genes to detect genetic variants in 16 Slovak cardiomyopathy patients (6 dilated, 8 hypertrophic, 2 non-compaction subtypes).

RESULTS: We identified candidate pathogenic variants in 11 of 16 patients (69 %). Genes with higher count of candidate pathogenic variants were *MYBPC3, MYH* and *TTN,* each with 3 different variants. Seven variants *ACTC1* (c.329C>T), *ANKRD1* (c.683G>T), *MYH7* (c.1025C>T), *PKP2* (c.2003delA), *TTN* (c.51655C>T, c.84841G>T, c.101874_101881delAGAATTTG) have been detected for the first time and might represent Slovak-specific genetic cause.

CONCLUSIONS: We have performed genetic testing of previously untested Slovak cardiomyopathy patients using next-generation sequencing cardiomyopathy gene panel. Given the high percentage of candidate pathogenic variants it should be recommended to implement this method into routine genetic diagnostic practice in Slovakia *(Tab. 4, Ref. 39).* Text in PDF *www.elis.sk.*

KEY WORDS: cardiomyopathy, Slovak patients, next-generation sequencing, gene panel, genetic testing.

Abbreviations: ENA – European Nucleotide Archive, CMs – Cardiomyopathies, NGS – Next-generation sequencing, DCM – Dilated cardiomyopathy, HCM – Hypertrophic cardiomyopathy, NNCM – Non-compaction cardiomyopathy, MSR – MiSeq Reporter, BWA – Burrows-Wheeler Aligner, GATK – Genome Analysis Toolkit, ExAC – The Exome Aggregation Consortium, AoCGV – Atlas of Cardiac Genetic Variation, ACMG – American College of Medical Genetics and Genomics, AMP – Association for Molecular Pathology.

[1]Department of Molecular Biology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovakia, [2]Department of Cardiology, Division Heart & Lungs, UMC Utrecht, University of Utrecht, Utrecht, Netherlands, [3]Durrer Center for Cardiovascular Research, Netherlands Heart Institute, Amsterdam, Netherlands, [4]Geneton Ltd., Bratislava, Bratislava, Slovakia, [5]Institute for Clinical and Translational Research, Biomedical Research Centre, Slovak Academy of Sciences, Bratislava, Slovakia, [6]Medirex Inc., Bratislava, Slovakia, [7]Heart Failure and Transplant Department, National Cardiovascular Institute, Bratislava, Slovakia, and [8]Institute of Cardiovascular Science, Faculty of Population Health Sciences, University College London, London, United Kingdom

Address for correspondence: E. Nagyova, MSc, Department of Molecular Biology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovicova 6, SK-842 15 Bratislava 4, Slovakia.
Phone: +31.8875.57381

Acknowledgements: E. Nagyova was supported by The National Scholarship Programme of Slovak Republic for the Support of Mobility of Students, PhD Students, University Teachers and Researchers. Folkert W. Asselbergs is supported by a Dekker scholarship-Junior Staff Member 2014T001 – Netherlands Heart Foundation and UCL Hospitals NIHR Biomedical Research Centre. This work was supported as part of the project REVOGENE – Research center for molecular genetics (ITMS 26240220067) supported by the Research & Development Operational Programme funded by the ERDF.

Introduction

Cardiomyopathies (CMs) are a heterogeneous group of disorders of cardiac muscle with high morbidity and mortality, leading to chronic end-stage heart failure or sudden cardiac death (1). In cardiomyopathies, mutations have been detected in more than 40 genes that encode the crucial elements of cardiomyocytes, such as sarcomeric filaments, calcium-metabolizing proteins, desmosomes, and mitochondrial enzymes (2, 3).

Mutation detection based on next-generation sequencing (NGS) of gene panels of known CM genes is currently considered the best practice for genetic analysis in CMs (4). Knowledge about genetic cause of patient's CM can help to predict disease prognosis, to indicate treatment, such as the use of cardiac devices, or to recognize family members at risk even before the disease has clinically manifested (5, 6).

Tab. 1. Clinical characterization of the Slovak patient cohort.

Sample	Candidate pathogenic variants (No)	Sex	CM subtype	Age presentation/ last evaluation	Transplantation (age)/ Death (age)	LV ejection fraction (%)	Affected family members
1	1	M	DCM	46/53	Y(50)/N	21[1]	N/A
2	1	M	DCM	26/38	WL/Y(N/A)	15	N/A
3	2	M	DCM	40/46	N/N	25	N
4	1	M	DCM	52/59	Y(58)/N	20[1]	Y
5	0	M	DCM	46/52	N/N	20	Y
6	1	F	DCM	48/48	N/N	45	N
7	0	F	HCM	74/74	N/N	64	Y
8	2	F	HCM	12/38	N/N	normal	Y
9	0	M	HCM	27/55	N/N	35	N/A
10	1	M	HCM	25/37	WL/N	65	Y
11	2	M	HCM	59/59	N/N	50	N/A
12	0	F	HCM	62/66	N/N	70	N/A
13	1	M	HCM	38/42	N/N	60	Y
14	2	M	HCM	23/23	N/N	65	Y
15	1	M	NNCM	20/23	N/N	45	N/A
16	0	M	NNCM	42/42	N/N	17	N/A

M – male; F – female; CM – cardiomyopathy; NNCM – non-compaction cardiomyopathy ; DCM – dilated cardiomyopathy; HCM – hypertrophic cardiomyopathy; LV – left ventricle; N/A – not available; N – no; Y – yes; WL – waiting list; [1] – before transplantation

In Slovakia, data on genetic etiology of cardiomyopathies in general is largely missing. In the 2013 report of the Slovak national center of health information, there were 12,317 patients with known cardiomyopathy (7,680 men and 4,637 women) which represents prevalence of 1/440 (National Health Information Center (7). During years 2010 and 1998, 1000 patients with heart failure referred by cardiologists to a single tertiary center in order to assess the indication for heart transplantation were hospitalized and monitored (86.8 % men and 13.2 % women, mean age 49.0 ± 10.9 years). The majority of patients (80.8 %) had severe left ventricular systolic dysfunction with ejection fraction < 30 %; 1.8 % of patients had preserved left ventricular ejection fraction ≥ 50 % (8). Despite of the number of CM patients and the availability of NGS methods, the current diagnostic tests in Slovakia are still based on Sanger sequencing of a few genes (9).

Here, we have performed genetic testing of previously untested Slovak cardiomyopathy patients using standard NGS cardiomyopathy gene panel in order to demonstrate the beneficial effect of implementing modern methods in diagnostic practice.

Material and methods

Patient information

All patients signed an informed consent to participate in the study. The genomic DNA samples from 6 dilated (DCM), 8 hypertrophic (HCM) and 2 non-compaction (NNCM) cardiomyopathy patients (Tab. 1) were obtained from the Heart Failure and Transplant Department, National Cardiovascular Institute, Bratislava, Slovakia.

Next-generation sequencing

Genomic DNA was extracted from whole blood according to standard procedures and samples were purified by ZYMO DNA Clean & Concentrator™-5 preps (Zymo Reasearch, USA) according to instructions of the manufacturer. Further, 50ng of DNA from each sample was processed into adapter-tagged DNA library according to the TruSight Rapid Capture (Illumina, San Diego, CA, USA) guide including tagmentation, purification, first amplification, pre-enrichment pooling, 2 steps of enrichment and final amplification of the library. In enrichment steps, coding exons of 46 cardiomyopathy genes (*ABCC9, ACTC1, ACTN2, ANKRD1, CASQ2, CAV3, CRYAB, CSRP3, CTF1, DES, DSC2, DSG2, DSP, DTNA, EMD, FHL2, GLA, JUP, LAMA4, LAMP2,*

Tab. 2. Amount of variants after filtering.

Sample	VCF all	VCF PASS	VCF PASS+ VCF GQ 99	After filters	Rare variants
1	164	146	146	1	1
2	131	115	115	2	1
3	123	98	98	4	2
4	181	147	128	2	2
5	185	118	103	1	0
6	187	132	113	2	1
7	173	83	66	0	0
8	123	100	87	3	2
9	156	139	139	1	0
10	161	146	145	1	1
11	143	132	132	2	2
12	229	96	75	1	1
13	130	95	95	1	1
14	153	124	118	2	2
15	152	109	106	3	1
16	151	104	101	0	0
Mean	158.9	117.8	110.4	1.6	1.1
STDEV	27.9	20.9	23.8	1.1	0.8

VCF PASS – passed all quality filters; GQ – Genotype Quality; Rare variants – information from Atlas of Cardiac Genetic Variation

Tab. 3. Potentially causal rare variants found in Slovak patient cohort.

Sample	CM subtype	Gene	Transcript	Genomic location	Nucleotide change	Amino acid change	Zygosity
1	DCM	*TTN*	ENST00000589042	2:179474495	c.51655C>T	p.Gln1721Ter	heterozygous
2	DCM	*TTN*	ENST00000589042	2:179399460-179399468	c.101874_101881del AGAATTTG	p.Ile33958MetfsX25	heterozygous
3	DCM	*ANKRD1*	ENST00000371697	10:92675606	c.683G>T	p.Arg228Met	heterozygous DG
3	DCM	*TPM1*	ENST00000288398	15:63353405	c.572G>A	p.Arg191Gln	heterozygous DG
4	DCM	*PKP2*	ENST00000070846	12:32974431-32974432	c.2003delA	p.Lys668ArgfsX16	heterozygous CP
4	DCM	*PKP2*	ENST00000070846	12:32996139	c.1487T>A	p.Val496Asp	heterozygous CP
6	DCM	*TTN*	ENST00000589042	2:179426018	c.84841G>T	p.Gly28281Ter	heterozygous
8	HCM	*MYH7*	ENST00000355349	14:23900677	c.746G>A	p.Arg249Gln	heterozygous DG
8	HCM	*DSP*	ENST00000379802	6:7559520	c.484C>G	p.Arg162Gly	heterozygous DG
10	HCM	*MYBPC3*	ENST00000545968	11:47354445-47354448	c.3407_3409delACT	p.Tyr1136del	heterozygous
11	HCM	*MYBPC3*	ENST00000545968	11:47369211	c.842G>A	p.Arg281Gln	heterozygous DG
11	HCM	*MYH6*	ENST00000356287	14:23874928	c.253G>A	p.Asp85Asn	heterozygous DG
13	HCM	*MYBPC3*	ENST00000545968	11:47372084-47372086	c.373_374delGC	p.Ala125Terfs	heterozygous
14	HCM	*MYH7*	ENST00000355349	14:23899097	c.1025C>T	p.Thr342Ile	heterozygous CP
14	HCM	*MYH7*	ENST00000355349	14: 23896042	c.1988G>A	p.Arg663His	heterozygous CP
15	DCM	*ACTC1*	ENST00000290378	15:35085571	c.329C>T	p.Ala110Val	heterozygous

Data from Variant Effect Predictor (Ensembl GRCh37 release 88 – Mar 2017); DG – digenic; CP – compound; CM – cardiomyopathy; Genomic location – Human Genome Assembly GRCh37

Tab. 4. Classification of potentially causal rare variants found in Slovak patient cohort.

Sample	Gene	Nucleotide change	AoCGV	Clinvar	InterVar	Classification
1	*TTN*	c.51655C>T	rare variant not detected in ExAC	no evidence	pathogenic	likely pathogenic
2	*TTN*	c.101874_101881delAGAATTTG	rare variant not detected in ExAC	no evidence	N/A	unknown significance
3	*ANKRD1*	c.683G>T	rare variant not detected in ExAC	no evidence	uncertain significance	unknown significance
3	*TPM1*	c.572G>A	not in AoCGV	no evidence	uncertain significance	unknown significance
4	*PKP2*	c.2003delA	rare variant not detected in ExAC	no evidence	N/A	unknown significance
4	*PKP2*	c.1487T>A	rare variant	no evidence	likely benign	unknown significance
6	*TTN*	c.84841G>T	rare variant not detected in ExAC	no evidence	pathogenic	likely pathogenic
8	*MYH7*	c.746G>A	rare variant not detected in ExAC	likely pathogenic	likely pathogenic	likely pathogenic
8	*DSP*	c.484C>G	rare variant	no evidence	uncertain significance	unknown significance
10	*MYBPC3*	c.3407_3409delACT	rare variant not detected in ExAC	unknown significance	N/A	unknown significance
11	*MYBPC3*	c.842G>A	rare variant	no evidence	uncertain significance	unknown significance
11	*MYH6*	c.253G>A	rare variant	unknown significance	uncertain significance	unknown significance
13	*MYBPC3*	c.373_374delGC	rare variant not detected in ExAC	likely pathogenic	N/A	likely pathogenic
14	*MYH7*	c.1025C>T	rare variant not detected in ExAC	no evidence	likely pathogenic	likely pathogenic
14	*MYH7*	c.1988G>A	rare variant	likely pathogenic	pathogenic	likely pathogenic
15	*ACTC1*	c.329C>T	rare variant not detected in ExAC	no evidence	likely pathogenic	likely pathogenic

Data from Variant Effect Predictor (Ensembl GRCh37 release 88 – Mar 2017); AoCGV – Atlas of Cardiac Genetic Variation; ExAC – The Exome Aggregation Consortium; N/A – not available; InterVar – Clinical Interpretation of genetic variants by ACMG/AMP 2015 guideline

LDB3, LMNA, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOZ2, NEXN, PKP2, PLN, PRKAG2, RBM20, RYR2, SGCD, TAZ, TCAP, TMEM43, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, VCL) were enriched using the TruSight Cardiomyopathy Sequencing Panel oligos (Illumina, San Diego, CA, USA).

Concentration of libraries was measured using Qubit® dsDNA HS kit (Invitrogen, CA, USA). Template size distribution was verified using Agilent® HighSensitivity DNA Chip and 2100 Bioanalyser (Agilent Technologies, Germany). Finally, DNA templates were normalized to 4nM concentration, denaturated by 0.2N NaOH (SIGMA-ALDRICH, Germany). Bridge PCR amplification and sequencing run of the panel of genes was then performed on the pooled samples of our 16 patients in two separate runs on MiSeq (Illumina, San Diego, CA, USA) using paired-end setting of 2x150bp reads.

Data analysis and interpretation

Sequence data generated from TruSight Cardiomyopathy enriched libraries were analyzed by the on-instrument MiSeq Reporter (MSR) software. The samples were demultiplexed and fastq files were generated. The software used the Burrows-Wheeler Aligner (BWA) (10) to align the reads against the human reference genome GRCh37/hg19 to create bam files, and the Genome Analysis Toolkit (GATK) (11) to perform variant analysis of the target regions specified in the manifest file to produce vcf files. Filtering criteria used in vcf files were coverage of the genetic position at least 20x, minimum genotype quality > 99, variant frequency (percentage of reads supporting the alternate allele) > 0.20, locus genotype quality < 10.0000 or not present, indel repeat length > 8, strand bias > -10, site genotype conflicts with proximal indel call removed, site mapping quality < 0.0000, locus quality score normalized by allele depth < 0.0000.

Ensembl Variant Effect Predictor software (12) was used to annotate variants in filtered vcf files. We filtered out annotated variants with population frequency > 0.5 % according to the data from ExAC database (The Exome Aggregation Consortium) (13), using reference data from non-Finland European population. We also filtered out data with other than missense, frameshift, splice region, in-frame deletions, stop gain and stop loss consequence. Because of the controversial interpretation (14) missense variants in the *TTN* gene were listed separately. For the selection of candidate causative mutations we have considered data from Atlas of Cardiac Genetic Variation (AoCGV) (15) and from ClinVar databases (16) while prioritized variants referred as rare and/or pathogenic. The clinical interpretation of genetic variants by American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) 2015 guidelines was made using online tool InterVar (17). We classified variants referred in ClinVar database or after InverVar classification as "pathogenic" or "likely pathogenic" as likely pathogenic and other variants as variants of unknown significance.

Results

We analyzed genomic DNA samples from 16 cardiomyopathy patients (6 dilated – DCM, 8 hypertrophic – HCM and 2 non-compaction – NNCM) (Tab. 1) using NGS workflow including the TruSight Cardiomyopathy panel of 46 selected genes.

The mean region coverage depth of the sequenced libraries was comparable between the two produced runs: 283x ± 108 and 308x ± 301. Target coverage at the sequencing depth of 20x was 99.6 % ± 0.4 for the first run and 95.8 % ± 4.4 for the second.

On average, 159 ± 28 variants were detected per patient. After filtering we obtained 1.6 ± 1 variants per patient (Tab. 2). In total, 26 variants were considered for further assessment.

We identified potentially causal variants in 11 of 16 patients (69 %) in nine known genes (Tab. 3). All selected variants were classified in AoCGV as 'rare variants' or they were not previously detected in the ExAC, the 1000 Genomes or the Exome Sequencing Project database (> 70 000 samples).

According to ACMG/AMP 2015 guidelines and data from ClinVar database we classified seven variants as likely pathogenic and nine variants as variants of unknown significance (Tab. 4). The most frequently mutated genes were *MYBPC3*, *MYH,* and *TTN* (three different variants).

Six of eleven patients were carrying one heterozygous variant, 3 patients were carrying two heterozygous variants in two different genes and 2 patients have shown compound heterozygosity (Table 3).

Four 'rare variants' (*DSP* c.484C>G; *MYBPC3* c.842G>A; *PKP2* c.1487T>A; *TPM1* c.572G>A) and seven 'rare variants, previously not detected in the ExAC database' (*ACTC1* c.329C>T; *ANKRD1* c.683G>T; *MYH7* c.1025C>T; *PKP2* c.1459C>T, c.2003delA; *TTN* c.51655C>T, c.84841G>T) that were detected in our cohort, have, to our knowledge, not been reported previously neither as a disease-causing or as a benign polymorphism (Tab. 4).

Discussion

Due to the large variation in clinical manifestations of HCM and DCM, ranging from asymptomatic forms to progressive heart failure and sudden cardiac death, not all individuals can be diagnosed properly relying solely on phenotype characteristics. Genetic testing provides clues to predict disease prognosis, to indicate treatment, such as the use of cardiac devices, and to recognize family members at risk. Here, we used a standard NGS panel of 46 cardiomyopathy genes to determine the genetic yield in 16 Slovak patients for the first time.

Seven rare variants that have been detected in our cohort were previously not detected in ExAC database (*ACTC1* c.329C>T; *ANKRD1* c.683G>T; *MYH7* c.1025C>T; *PKP2* c.1459C>T, c.2003delA; *TTN* c.51655C>T, c.84841G>T) and to our knowledge they have not been reported previously as a disease cause or as a benign polymorphism. However, without population specific genotype databases such as the Dutch GoNL database (18), it is difficult to evaluate if these might represent population-specific variants in Slovakia.

Three variants (*MYH7* c.746G>A; *MYBPC3* c.373_374delGC, c.3407_3409delACT) were previously not detected in the ExAC database but they are mentioned in the ClinVar database.

We classified 7 of 16 candidate pathogenic variants as likely pathogenic (*ACTC1* c.329C>T; *MYBPC3* c.373_374delGC; *MYH7* c.746G>A, c.1988G>A, c.1025C>T; *TTN* c.51655C>T, c.84841G>T) and nine variants as variants of unknown significance (*ANKRD1* c.683G>T; *DSP* c.484C>G; *MYBPC3* c.842G>A; *MYH6* c.253G>A; *PKP2* c.2003delA, c.1487T>A; *TPM1* c.572G>A; *TTN* c.101874_101881delAGAATTTG, c.84841G>T).

In other studies the diagnostic yield of NGS cardiomyopathy panels was at 35.2 % in Finish cohort (145 permanent pacemaker patients) (37), 33.3 % in Russian cohort (38 cardiomyopathy patients) (38), 45 % in Tunisian cohort (11 HCM patients) (39). The higher diagnostic yield in our cohort (69 %) could be influenced by the fact that we have selected patients with the most severe cardiomyopathy phenotype which were not previously genetically tested. In countries where genetic testing in cardiomyopathies is a routine practice the most common genetic causes are already tested in many patients and they are not necessary included in new studies. Furthermore, variants selected in our study are candidate pathogenic variants and we expect the real diagnostic yield to be lower after further analysis, e.g. segregation analysis in patient families, which was not possible in our study.

In Slovakia there is an urgent need to modernize genetic diagnostic practice. Even genes already well known to be associated with cardiomyopathies are not routinely tested in Slovak patients. Slovak population might be largely underdiagnosed and borderline cardiomyopathy patients might be classified incorrectly. Given the high percentage of candidate pathogenic variants in other countries as well as in our preliminary data (69 %) it should be recommended to implement NGS cardiomyopathy gene panels in routine genetic diagnostic practice also in Slovakia. The detection of pathogenic variants is especially important for cascade screening in families and identification of family members in risk which should be regularly checked by cardiologist to get proper treatment on time or even to prevent sudden cardiac death. One of the most important steps to make progress in cardiology routine testing is to increase the knowledge of cardiologist about benefits connected with proper genetic testing for patients and their families and make those tests widely available by involving the insurance companies, as it is common in other countries.

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Receiced September 18, 2018.
Accepted October 22, 2018.