RESEARCH ARTICLE

Analysis of selected genes associated with cardiomyopathy by next-generation sequencing

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Background: As the leading cause of congestive heart failure, cardiomyopathy represents a heterogeneous group of heart muscle disorders. Despite considerable progress being made in the genetic diagnosis of cardiomyopathy by detection of the mutations in the most prevalent cardiomyopathy genes, the cause remains unsolved in many patients. High-throughput mutation screening in the disease genes for cardiomyopathy is now possible because of using target enrichment followed by next-generation sequencing. The aim of the study was to analyze a panel of genes associated with dilated or hypertrophic cardiomyopathy based on previously published results in order to identify the subjects at risk.

Methods: The method of next-generation sequencing by IlluminaHiSeq 2500 platform was used to detect sequence variants in 16 individuals diagnosed with dilated or hypertrophic cardiomyopathy. Detected variants were filtered and the functional impact of amino acid changes was predicted by computational programs.

Results: DNA samples of the 16 patients were analyzed by whole exome sequencing. We identified six nonsynonymous variants that were shown to be pathogenic in all used prediction softwares: *rs3744998* (*EPG5*), *rs11551768* (*MGME1*), *rs148374985* (*MURC*), *rs78461695* (*PLEC*), *rs17158558* (*RET*) and *rs2295190* (*SYNE1*). Two of the analyzed sequence variants had minor allele frequency (MAF) < 0.01: *rs148374985* (*MURC*), *rs34580776* (*MYBPC3*).

Conclusion: Our data support the potential role of the detected variants in pathogenesis of dilated or hypertrophic cardiomyopathy; however, the possibility that these variants might not be true disease-causing variants but are susceptibility alleles that require additional mutations or injury to cause the clinical phenotype of disease must be considered.

KEYWORDS

bioinformatic analysis, cardiomyopathies, next-generation sequencing, panel of genes, pathogenic variants

1 | INTRODUCTION

Cardiomyopathies (CMP) are one of the major causes of sudden death and/or progressive heart failure. Cardiomyopathies are a small group of

related but clinically distinguishable primary diseases of the heart muscle, such as hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM), and left ventricular non-compaction cardiomyopathy (LVNC). Primary cardiomyopathies

are described as diseases in which the heart is the sole or predominantly involved organ in contrast with secondary cardiomyopathies which are described as diseases in which myocardial dysfunction is a part of a systemic disorder. However, the difficulty to distinguish primary and secondary disorders in this way is illustrated by the fact that many of the diseases classified as primary cardiomyopathies can be associated with major extra-cardiac manifestations; conversely, the pathology in many of the diseases classified as secondary cardiomyopathies can predominantly involve the heart.¹

Hypertrophic cardiomyopathy is defined by the presence of increased ventricular wall thickness or mass in the absence of loading conditions (hypertension, valve disease) sufficient to cause the observed abnormality. HCM occurs in approximately 1:500 of the general population.² Many individuals have familial disease with an autosomal dominant pattern of inheritance caused by mutations in genes that encode different proteins of the cardiac sarcomere. In approximately 80% of HCM patients carry mutations in the *MYH7* and *MYBPC3* genes, which encode the heavy chain of β -myosin and C-binding protein of myosin.³ Mutations in the troponin complex genes: *TNNI2*, *TNNI3* and *TPM1* are also relatively common (10%-15% of cases with genetic defects) in HCM.⁴

Dilated cardiomyopathy is defined by the presence of left ventricular dilatation and left ventricular systolic dysfunction in the absence of abnormal loading conditions (hypertension, valve disease) or coronary artery disease sufficient to cause global systolic impairment. The prevalence of DCM in the general population is unknown, but it clearly varies with age and geography. At least 25% of patients in Western populations have evidence for familial disease with predominantly autosomal dominant inheritance.⁵ Genetics appears to play a role in 20%-50% of idiopathic DCM cases, with a heterogeneous inheritance model, both Mendelian (autosomal dominant, autosomal recessive, X-linked) and mitochondrial inheritance, with incomplete penetrance, variable expression, and sometimes digenic inheritance.⁶ So far, >40 genes have been implicated in DCM⁷ encoding cytoskeletal (desmin, tafazzin, δ -sarcoglycan, dystrophin, metavinculin) as well as nuclear proteins (emerin and lamin A/C).⁸

Restrictive cardiomyopathy is characterized by a pattern of ventricular filling in which increased stiffness of the myocardium causes ventricular pressure to rise precipitously with only small increases in volume. The exact prevalence of RCM is unknown but it is probably the least common type of cardiomyopathy. RCM may be idiopathic or familial, or can result from various systemic disorders, in particular amyloidosis, sarcoidosis, carcinoid heart disease, scleroderma, and anthracycline toxicity.⁹

Classification of cardiomyopathies has some clinical problems, which include the occurrence of different cardiomyopathies caused by the same genetic mutation (in unrelated and related individuals), the same cardiomyopathy resulting from many different mutations, and the evolution of one disease phenotype into another over time. Over the last few years, genetic association studies have taken advantage of the remarkable improvements in high density genotyping and significantly contributed to the discovery of novel loci for cardiomyopathies including HCM and DCM. Since the 1970s, Sanger sequencing has remained

the gold standard for detecting sequence variants in patients. Despite its high reliability and partial automation, it is expensive and relatively slow method. Next-generation sequencing (NGS) represents the dawn of a new diagnostic era, by providing fast and reliable testing for multiple genes at a relatively low cost.¹⁰ Accordingly, high-throughput mutation screening in disease genes for DCM, HCM, and other cardiomyopathies is now possible using target enrichment followed by next-generation sequencing (NGS), yielding precise detection of sequence variants and mutations in multiple disease-relevant genetic loci in parallel, whilst reducing the manual handling steps needed in traditional sequencing assays.¹¹

2 | MATERIALS AND METHODS

2.1 | Study population

Sixteen individuals with established cardiomyopathy recruited from Cardiocenter at J.A Reiman Faculty Hospital in Presov (Slovakia) were studied. Four females and 12 males with mean age of 48 $\pm$ 16 years (range from 21 to 73 years) were included. The DCM (N=11) and HCM (N=5) patients were diagnosed by experts in cardiology according to criteria provided by the World Health Organization and European Society of Cardiology guidelines. Written, informed consent was obtained from all probands and all institutional ethics requirements were met.

Peripheral whole-blood samples of patients were collected during examination and DNA was extracted using RepliaPrep™ BloodgDNA isolation kit (Promega, Madison, WI, USA) following the manufacturer's instructions.

2.2 | Next-generation sequencing

An amount of at least 2 μ g of genomic DNA at a minimum concentration of 50 ng/ μ L extracted from patients was used for whole exome sequencing (WES) by IlluminaHiSeq 2500 platform based on bridging amplification after library preparation and reversible dye terminator for sequencing purposes. The whole exome sequencing on Illumina HiSeq 2500 achieved an average output of 2 $\times$ 40701366 mapped reads, 90.26% of reads was covered at least 30 $\times$. The mean coverage was 98.03%. Criteria for variant calling were a read coverage higher than 30 $\times$.

Sample libraries were prepared using the IlluminaTruSeq DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Exome capture was performed with the Nimblegen Sequence Capture EZ Library v2.0 (Roche, Madison, WI, USA). Samples were sequenced on an IlluminaHiSeq 2500 in pair end mode, generating 100 bp paired end reads. Specific reaction conditions are available on request.

2.3 | Variant filtering and classification

The pair-end reads were quality trimmed and aligned with the human genome reference sequence (UCSC Genome build hg19). The analysis was carried out with WEP (Whole Exome sequencing Pipeline web tool), which aims to analyze WES data produced by Illumina

platforms.¹² It is also capable to analyze a user-selected set of the exome samples generating tables reporting variant information and their functional annotation. The analysis pipeline includes 11 modules and perform quality statistics, filtering and trimming of sequence reads, alignment to a reference genome, post alignment analysis with the calculation of mapping rate, statistics and annotation of the detected variants.¹² Values of the variant filters were: coverage>30, ambiguously mapped reads per variant <5 Phred scaled consensus quality>50 and variant confidence/consensus quality>1.5. Data from all patients were filtered only for 110 genes (Table 1) associated with DCM or HCM based on previously published results.¹³ All variants in analysed genes were annotated with the online software. Synonymous and noncoding region variants with the exception of potential splice site variants were excluded, and the remaining coding region variations were considered putative pathogenic mutations. All variants were annotated with the online software Variant Effect Predictor/VEP (<http://www.ensembl.org/info/docs/tools/vep/index.html>).

The putative pathogenic mutations were analyzed computationally using the PolyPhen-2, SIFT, and MutationTaster algorithms.¹⁴⁻¹⁶ These algorithms can distinguish mutations with functional effects from neutral mutations. The putative novel pathogenic variants that were reported neither in the 1000 Genome project database nor in the NCBI dbSNP database and the ExAC database were further confirmed.

3 | RESULTS

Sixteen unrelated patients with DCM or HCM diagnosis were studied. From total screened patients, 31.25% (5/16) were patients with HCM and 68.75% (11/16) were patients with DCM. The subgroup of males was larger because of supposed cardiomyopathy male predominance.¹⁷ The mean age for all patients was 48±16 years, 65±8 years for females (4/16) and 42±14 years for males (12/16). NYHA (New York Heart Association) functional classification system of heart failure was used, which places patients into one of four categories (I-IV) based on how much they are limited during physical activity (www.heart.org). The positive family history was confirmed only in one patient with dilated cardiomyopathy. Other patients were not aware

of the occurrence of the disease in the family, but all of them were diagnosed as idiopathic cardiomyopathy. The cause may be variable expressivity (the clinical phenotype can vary among family members who all have the same mutation) or variable penetrance (sometimes, family members will carry a gene mutation but have completely normal cardiac function) and also age-related penetrance. The detailed characteristics of cardiomyopathy patients are shown in Table 2. In all patients, the genetic pathogenesis of cardiomyopathy was previously unknown.

All successfully mapped sequence reads were analyzed in order to detect sequence variants. After filtering of data we finally work with 18 nonsynonymous variants, which were predicted by SIFT tool as damaging. Summary of nonsynonymous variants is shown in Table 3.

These variants were detected in 16 analysed genes which can be implicated in etiopathogenesis of CMP (*ALMS1*, *EPG5*, *GBE*, *MGME1*, *MURC*, *MYBPC3*, *NEBL*, *PGM1*, *PHLDB3*, *PLEC*, *POMPT1*, *RBM20*, *RET*, *SYNE1*, *SYNE2*, *TTN*). Comparing our data with NCBI dbSNP we identified two variants from a total number of 18 sequence variants (11.11% of selected sequence variants), which were previously associated with CMP: *rs34580776* (*MYBPC3*) and *rs942077* (*RBM20*). Using bioinformatics analysis of the sequencing results, we identified six nonsynonymous variants (33.33% of selected sequence variants) shown to be pathogenic in all prediction software (PolyPhen-2, SIFT and MutationTaster): *rs3744998* (*EPG5*), *rs11551768* (*MGME1*), *rs148374985* (*MURC*), *rs78461695* (*PLEC*), *rs17158558* (*RET*) and *rs2295190* (*SYNE1*). Variants were selected from the synonymous or non-synonymous variants and compared with the reported data from NCBI dbSNP (MAF<0.01). We identified two variants (11.11% of selected sequence variants) with minor allele frequency (MAF)<0.01: *rs148374985* (*MURC*), *rs34580776* (*MYBPC3*), after the comparison of detected variants with ExAC database information (<http://exac.broadinstitute.org/>).

4 | DISCUSSION

The first gene mutation identified to be causing heart disease was reported in 1993¹⁸; since then, more than 1400 mutations that

TABLE 1 List of selected genes associated with cardiomyopathies¹³

ABCC9	BOLA3	DPM3	FHL2	HFE	LAMP2	NEBL	POU1F1	SGCB	TMEM70
ACAD8	BRCC3	DSC2	FKRP	HFE2	LDB3	NEXN	PRDM16	SGCD	TMPO
ACTA1	CAV3	DSG2	FKTN	HSPB6	LMNA	NKX2-5	PSEN1	SKI	TNNC1
ACTC1	CPT2	DSP	FOXD4	CHKB	MGME1	PDLIM3	PSEN2	SLC22A5	TNNI3
ACTN2	CRYAB	DYSF	GATA4	CHRM2	MTCP1	PGM1	RBM20	SYNE1	TNNT2
ADCY5	CSRP3	EMD	GATAD1	ILK	MURC	PKP2	RET	SYNE2	TPM1
ALG6	DES	EPG5	GBE1	ISL1	MYBPC3	PLEC	RYR	TAZ	TTN
ALMS1	DMD	ERBB3	GLB1	ITGB1BP2	MYH6	PLN	SCARB2	TBX20	TXNRD2
ANKRD1	DMPK	EYA4	GNPTAB	JUP	MYH7	PNPLA2	SCN5A	TCAP	UBR1
ANO5	DNAJC19	FBN1	HADHA	KCNH2	MYL2	POLG	SDHA	TERT	VCL
BAG3	DOLK	FHL1	HADHB	LAMA4	MYPN	POMT1	SGCA	TMEM43	XPNPEP3

Patient ID	Type of CMP	Sex	Age	NYHA	LVEF, %	Family history
CMP01	HCM	M	39	I	60	No
CMP02	DCM	M	49	I-II	20-23	No
CMP03	DCM	M	49	III	40	No
CMP04	DCM	M	57	III	28-30	No
CMP05	DCM	M	51	III	29-30	No
CMP06	DCM	M	43	III	33	No
CMP07	DCM	F	73	III	30	Yes
CMP08	HCM	M	33	I	68-70	No
CMP09	DCM	M	59	II	50	No
CMP10	DCM	M	53	II	48-50	No
CMP11	DCM	M	25	I-II	65	No
CMP12	DCM	F	71	III	48-50	No
CMP13	DCM	F	63	III	30	No
CMP14	HCM	M	21	II	50	No
CMP15	HCM	M	21	II	40	No
CMP16	HCM	F	55	II	65-68	No

DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; M, male; F, female; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction.

TABLE 2 Overview of the patients in the study

encode sarcomere proteins have been detected in at least 20 genes,¹⁹ calcium-handling proteins, Z-disc proteins, etc. In this study, the whole exome sequencing was used to find cardiomyopathy associated genes and mutations in patients with DCM and HCM. Coverage is one of the most important factors in calling a variant. The variant calls with low coverage might not indicate a true variant in the exome. Nevertheless, a high coverage not necessarily indicates a true variant either.²⁰

The criteria for variant calling were a read coverage higher than 30x. The functional impact of amino acid changes was predicted using three computational programs (PolyPhen2, SIFT and MutationTaster). The usefulness of expanded gene testing is most obvious for overlapping clinical entities, such as HCM and DCM, which are each associated with more than 40 disease genes.²¹ Restricting the genetic analysis to only frequently affected, well-established disease genes would ultimately risk overlooking of variants in genes with a prognostic impact, such as *ANK2*.²²

In our study two variants previously described in the etiology of CMP were identified. We detected a rare variant (MAF=0.0008) *rs34580776* (*MYBPC3*) in 2/16 patients (1 HCM and 1 DCM patient) with a potential functional effect on the protein (R/Q), which was also predicted by SIFT and MutationTaster. *MYBPC3* gene encodes cardiac myosin binding protein C, which is found in cardiac muscle cells and is associated with the sarcomere. In particular, *MYH7* and *MYBPC3* sarcomere proteins mutations are most commonly related to HCM.²³

Mutation *rs34580776* in the *MYBPC3* gene was documented in previous studies on HCM and also in DCM patients.²⁴⁻²⁶

Another variant associated with CMP, but mainly with DCM was found in the gene *RBM20* (RNA binding motif protein 20), which is highly expressed in cardiac tissues and regulates splicing by processing of pre-mRNA.²⁷ Transcript splicing is regarded as one of the primary

mechanisms by which phenotypic variation is generated²⁸ and studies supported the role of *RBM20* as a proximal regulator in a pathway that regulates cardiac morphology and as disease susceptibility locus for DCM.²⁹

Variant *rs942077* in *RBM20* was found in 3% of subjects with DCM, but the association between this variant and transplantation rate and frequency of implantable cardioverter defibrillator therapy has not been proven.³⁰ In our study, the variant *rs942077* was identified in 10/11 DCM patients and 5/5 HCM patients.

From a total number of 18 variants we filtered six, which were predicted by SIFT as damaging, by PolyPhen2 as probably/possibly damaging and by MutationTaster as disease causing. Two of them were rare variants according to MAF value (MAF<0.01), which were not previously described in etiopathogenesis of CMP, but may be potential markers for CMP.

The rare variant *rs148374985* is located in the *MURC* gene (muscle restricted coiled-coil), which encodes Z-line component protein predominantly expressed in the heart, activates *RhoA/ROCK* pathway implicated in heart failure, expression of atrial natriuretic peptide and regulates myofiber organization.³⁰⁻³² Experimental data implicate *MURC* as a biologically plausible gene for human cardiomyopathies and strongly support the causal role of the *MURC* gene variants in DCM.^{32,33}

The second filtered rare variant was *rs78461695* in *PLEC* gene (human plectin gene). It acts as multi-functional linker protein and signaling scaffold that centrally orchestrates the structural and functional organization of filamentous cytoskeletal networks.³⁴

Mutations in the gene *PLEC* causing a variety of rare diseases referred to like plectinomyopathies, but some studies also describe plectin-related cardiac disease comprising mild left ventricular hypertrophy,³⁵ reduced ejection fraction in combination with

TABLE 3 Variants found in the selected established cardiomyopathy genes

Gene	GRCh37 pos.	rs ID	Seq	AA	NO. DCM/ HCM	SIFT	PP2	MT	Clin. sig.	MAF Eur.	MAF
ALMS1	73679956	rs28730854	c.6305C>T	Ser/Leu	1/0	D	Pro	P	NA	0.038 59	0.025 88
EPG5	43497710	rs3744998	c.3173T>C	Val/Ala	10/4	D	Pro	DC	NA	0.5555	0.4909
GBE1	81698130	rs2229519	c.568A>T	Arg/Gly	7/3	D	Ben	P	NA	0.3675	0.3746
MGME1	17950545	rs11551768	c.43A>T	Ser/Cys	3/2	D	Pos	DC	NA	0.1208	0.1041
MURC	103348618	rs148374985	c.980C>A	Pro/His	1/0	D	Pos	DC	NA	0.000 285	0.000 181
MYBPC3	47357479	rs35078470	c.2686A>G	Val/Met	1/0	D	Ben	DC	NA	0.0183	0.012 75
MYBPC3	47367871	rs34580776	c.977G>A	Arg/Gln	1/1	D	Ben	DC	Likely benign- Primary DCM, Primary FHCM	0.007 814	0.005 506
NEBL	21134282	rs41277370	c.1132G>C	Asp/His	2/1	D	Pro	P	Benign/CMP	0.083 24	0.065 19
PGM1	64114301	rs11208257	c.1258T>C	Tyr/His	5/2	D	Ben	P	NA	0.1911	0.224
PHLDB3	44001379	rs11083711	c.716A>G	Gln/Arg	2/1	D	Pro	P	NA	0.059 32	0.077 14
PLEC	144992269	rs78461695	c.12131G>A	Thr/Met	1/0	D	Pro	DC	NA	0.013 05	0.009 26
POMT1	134390870	rs11243406	c.1233C>A	Asp/Glu	2/0	D	Pro	P	Benign allele	0.049 43	0.034 16
RBM20	112595719	rs942077	c.3667G>C	Glu/Gln	10/5	D	Pro	P	Benign/CMP	0.8681	0.7637
RET	43620335	rs17158558	c.2944C>T	Arg/Cys	1/0	D	Pro	DC	Benign, risk factor	0.018 38	0.019 24
SYNE1	152453291	rs35591210	c.26060C>T	Thr/Ile	1/1	D	Ben	DC	Likely benign	0.073 92	0.051 33
SYNE1	152443744	rs2295190	c.26221G>T	Leu/Met	1/2	D	Pro	DC	Likely benign	0.1516	0.1182
SYNE2	64447776	rs9944035	c.1721T>C	Ile/Thr	5/1	D	Pos	P	Benign	0.076 21	0.099 54
TTN	179615931	rs922985	c.11311G>C	Leu/Phe	10/5	D	Ben	P	Benign	0.9997	0.9926

AA, amino acid change; Ben, benign; Clin. significance, clinical significance by dbSNP NCBI; CMP, cardiomyopathy; DC, disease causing; DCM, dilated cardiomyopathy; FHCM, familial hypertrophic cardiomyopathy; HCM, hypertrophic cardiomyopathy; MAF, total minor allele frequency (ExAC); MAF Eur., European (Non-Finnish) minor allele frequency (ExAC); MT, Mutation Taster; NA, not analysed; NO, number of patients with variant, dilated cardiomyopathy/hypertrophic cardiomyopathy; P, polymorphism; Pos, possible damaging; PP2, PolyPhen2; Pro, probably damaging; rsID, variant ID; Seq, sequence change; Start pos, start position.

arterial fibrillation,³⁶ dilated cardiomyopathy³⁷ and left ventricular non-compaction cardiomyopathy.³⁸ Considering these findings, *PLEC* gene might be a candidate gene for involvement in CMP.

Unique sequence variants detected only in one DCM or HCM patient were confirmed in seven (38.89%) cases. MAF values of two these variants were predicted to be <0.01 according ExAC for European (non-Finnish) population. All these prediction software confirmed potential damaging effect only one unique sequence variant with MAF<0.01 detected in *MURC* gene (*rs148374985*). Sequence variants of *EPG5* gene (*rs3744998*), *RBM20* gene (*rs942077*), and *TTN* gene (*rs922985*) were detected almost in all screened cardiomyopathy patients. We hypothesized that these variants are single nucleotide polymorphism with high frequency in Europe population based on ExAC database MAF values. The *TTN* gene sequence variant MAF value is 99.97% for Europe (non-Finnish) population and this variant was not confirmed only in one DCM patient. Variant *rs3744998* (*EPG* gene) was predicted by online software (SIFT, PolyPhen2, and MutationTaster) like potential damaging, but MAF value was 55.55% in Europe (without non-Finnish population) and 49% in general population.

Multiple variants were confirmed in all patients. We detected ten sequence variants in patient CMP4 and only three sequence variants were identified in two cases (patients CMP07 and CMP12). The effect of this variant in pathogenesis of diseases, including cardiomyopathy must be supported by other precise studies.

Accordingly, several lines of genetic and functional data have to be incorporated in discerning clinical significance of the DNA sequence variants in sporadic cases or small families. Although our data support the potential role of the detected variants in DCM or HCM patients, these variants may not be true disease-causing variants but are susceptibility alleles that require additional mutations or injury to cause the clinical phenotype. There is also a possibility that these variants are functional variants that do not play significant roles in susceptibility to cardiomyopathy. There is also a support that CMP might be oligogenic and the growing number of publications contribute this idea.³⁹

4.1 | Study limitation

In this study, the number of recruited participants was limited, and the obtained clinical data were not consistent for all subjects. In addition, we used the accepted normal range of each clinical parameter in this study. The main limitation was that none of the detected variants in our study was finally clearly pathogenic in absence of familial and functional studies. A larger study of patients with cardiomyopathy and Sanger resequencing will be essential to further confirm the cardiomyopathy genetic profile of patients analysed in this study.

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ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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