RESEARCH ARTICLE

Finding the candidate sequence variants for diagnosis of hypertrophic cardiomyopathy in East Slovak patients

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Background: Hypertrophic cardiomyopathy is a heterogeneous myocardial disease. Mutations appearing in several genes might be a potential cause of the disease. The aim of the study was to analyze selected exons of the sarcomeric and non-sarcomeric genes, with the purpose to identify potential candidate genetic variants and to understand etiopathogenetic mechanisms of hypertrophic cardiomyopathy in East Slovak patients.

Methods: This study recruited 23 unrelated patients with hypertrophic cardiomyopathy, namely, 13 men and 10 women (mean age of 58.09±15.82 years) and 25 healthy controls in order to determine the candidate sequence variants, in the selected exons of six cardiomyopathy genes (*MYBPC3*, *MYH7*, *NEBL*, *SCN5A*, *TNNI3*, *TNNT2*), by conventional capillary-based Sanger sequencing method and standard protocols.

Results: Molecular genetic results confirmed the presence of 43 sequence variants in the selected exons of six cardiomyopathy genes, 58.14% of detected variants were novel. The majority of detected sequence variants were confirmed within exon 23 of *MYH7* gene. Only 11 genetic alterations were predicted to be potentially pathogenic.

Conclusions: In our study, we identified known and novel sequence variants in 23 unrelated patients with hypertrophic cardiomyopathy, but we did not observe any strong mutation hotspot. The results of our study assumed that exon 23 of *MYH7* gene can be in potential affinity to hypertrophic cardiomyopathy in our cohort of patients. The sequence variants identified in this study may be further investigated in order to determine their functions in disease pathogenesis and improve management, diagnosis, and treatment in Slovak patients.

KEYWORDS

DNA sequencing, East Slovak patients, hypertrophic cardiomyopathy, pathogenic variants

1 | INTRODUCTION

Hypertrophic cardiomyopathy is a complex genetic cardiovascular disease with the pathological hallmark of unexplained left ventricular hypertrophy. This pathological entity is considered to be the most common familial heart disease with reported cases in all races and both sexes with estimated prevalence data 1:500 in general populations.¹

The extrapolation of the latest data confirms that there are approximately 10 000 patients with a diagnosis of hypertrophic cardiomyopathy in Slovakia,² but the real number is still unknown, what is the consequence of incomplete penetrance, late onset of disease symptoms or asymptomatic occurrence.³

A lot of patients are asymptomatic and the targeted genetic testing is complicated because of the disease multifactorial

etiology. Hypertrophic cardiomyopathy is associated with the extensive genetic heterogeneity (>1400 mutations in 11 or more genes) and with the high frequency of private mutations.^{1,4} Mutations in several sarcomeric and non-sarcomeric genes which may affect the morphology and functions of the heart^{5,6} are considered to be the main causes of disease. The set of the candidate pathogenic cardiomyopathy genes has been successfully identified, but the knowledge of the full genetic background and the etiopathology of primary hypertrophic cardiomyopathy remains incomplete.⁷

The most affected are genes encoding cardiac myosin binding protein C (*MYBPC3*), cardiac β -myosin heavy chain (*MYH7*), and cardiac troponin isoforms I (*TNNI3*) and T (*TNNT2*).⁸ The role of other sarcomeric and non-sarcomeric genes is reevaluated.⁹ In fact, pathogenic sequence variants are not identified in all sporadic and familial cases. On the other hand, some patients with hypertrophic cardiomyopathy carry more than one causative mutation in the same gene or in different genes which may be a potential factor of hypertrophic cardiomyopathy severity.¹⁰

A precise definition of the genetic causes of hypertrophic cardiomyopathy is necessary, whereas it can improve the diagnosis and clinical management of patients. The detection of pathogenic sequence variants may provide certainty in diagnosis and eliminate ambiguities associated with phenotypic heterogeneity. In this study, we tried to identify candidate disease sequence variants within six cardiomyopathy genes, namely, in selected exons of *MYBPC3*, *MYH7*, *NEBL*, *SCN5A*, *TNNI3*, *TNNT2* gene in East Slovak hypertrophic cardiomyopathy patients.

2 | PATIENTS AND METHODS

2.1 | Demographic and clinical characteristics of patients

The study population consisted of 23 hypertrophic cardiomyopathy patients (13 men and 10 women), with a mean age of 58.09 ± 15.82 years. The total number of controls enrolled in our study was 25 healthy probands of similar age and gender, all recruited from the Cardiocentre of Faculty Hospital J.A. Reiman, Presov (Slovak republic) and cardiology clinics. The clinical diagnosis of hypertrophic cardiomyopathy, established by experts in cardiology, was based on the European Society of Cardiology (ESC) Guidelines on diagnosis and management of hypertrophic cardiomyopathy and World Health Organization criteria.^{3,11}

Demographic, anthropological and clinical characteristics of the patients with hypertrophic cardiomyopathy, including personal and family history, clinical symptoms, 12-lead electrocardiography (ECG) data, echocardiography results and indicated treatment information, were collected. Written, informed consent was obtained from all probands. All procedures performed in this study were in accordance with the ethical standards of the research committee at the University of Presov and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.2 | DNA samples

Genomic DNA was extracted from peripheral blood leukocytes by RepliaPrep™ Blood gDNA isolation kit (Promega, Madison, WI, USA) and standard protocols.

An amount of at least 1.5 μ L of genomic DNA at a minimum concentration of 50 ng/ μ L extracted from blood was used for detection of sequence variants in the selected exons 12, 24, 25 of *MYBPC3*, exon 23 of *MYH7*, exon 12 *NEBL*, exons 12, 20, 21 of *SCN5A*, exon 7 of *TNNI3*, and exon 9 of *TNNT2* gene by Sanger sequencing method.

The candidate genes and the exons appropriate for Sanger sequencing analyses in the East Slovak patients with hypertrophic cardiomyopathy were selected according to our previous next-generation sequencing (NGS) data and data obtained from the databases PubMed Database (<http://www.ncbi.nlm.nih.gov/pubmed/>) and Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk>).

Table 1 summarizes genes included in our study, as well as their function, associated disorder(s), and chromosomal position.

2.3 | Data analysis

Sequencing was performed by using 24 capillaries 3500xL Genetic Analyzer (Life Technologies, Foster City, CA, USA), terminator kit BigDye, version v3.1 (Thermo Fisher Scientific, Waltham, MA USA), and standard sequencing protocols. The sequencing primers were designed by software Primer3 (v0.4.0; Madison, WI, USA). Sequences of primers and specific reaction conditions are available on request.

The sequence variants identified in selected exons of genes in each sample were analyzed by the SeqScape v.2.7 and Sequencing Analysis v5.4 (Life Technologies) software and compared with the data obtained from Ensembl (www.ensembl.org) and NCBI database (<https://www.ncbi.nlm.nih.gov/gene/>).

Several free online tools (Mutation Taster, PhD-SNP, Polyphen2, PredictSNP, SIFT, and SNAP2) were applied in order to assess sequence- and structure-based features.

3 | RESULTS

The selected exons were amplified and sequenced as candidates for hypertrophic cardiomyopathy by using DNA samples from 23 patients diagnosed with hypertrophic cardiomyopathy and 25 healthy controls from Eastern Slovakia. The investigation presents the results of the Sanger sequencing analysis of the sarcomeric and non-sarcomeric gene exons.

Within analyzed cardiomyopathy exons (exons 12, 24, 25 of *MYBPC3* gene, exon 23 of *MYH7* gene, exon 12 of *NEBL* gene, exons 12, 20, 21 of *SCN5A* gene, exon 7 of *TNNI3* gene, and exon 9 of *TNNT2* gene), 43 sequence variants, due known and novel, were detected, Table 2. To be more precise, we detected only single nucleotide changes.

Four of identified sequence variants were predicted to be synonymous, while 38 sequence variants were missense alterations disrupting

TABLE 1 Genes included in the study of patients with hypertrophic cardiomyopathy

Gene	Analyzed exons	Location	Transcript ID	Protein Access	Protein class	Function	Disease conditions ^a
MYBPC3	e.12 e.24 e.25	11p11.2	ENST00000545968.5	Q14896	Sarcomere	Contraction	DCM, HCM, LVNC, RCM, SCD Atrial standstill Familial hypercholesterolemia Diastolic heart failure
MYH7	e.23	14q11.2	ENST00000355349.3	P12883	Sarcomere	Force generation; Contraction	DCM, HCM, RCM Myopathies Muscular dystrophy Wolff-Parkinson-white syndrome Glycogen storage disease
NEBL	e.12	10p12.31	ENST00000377122.8	O76041	Z-disk	Cardiac myofibril assembly; Contraction	DCM Endocardial fibroelastosis
SCN5A	e.12 e.20 e.21	3p22.2	ENST00000413689.5	Q14524	Sodium channel	Sodium ion transport	BS, DCM, LQT Progressive heart block Familial progressive cardiac conduction defect
TNNI3	e.7	19q13.42	ENST00000344887.9	P19429	Sarcomere	Contraction	DCM, HCM, RCM Rheumatic myocarditis Intermediate coronary syndrome Congestive heart failure Fabry disease Duchenne muscular dystrophy Congenital heart disease
TNNT2	e.9	1q32.1	ENST00000509001.5	P45379	Sarcomere	Contraction	DCM, HCM, RCM, LVNC Progressive familial heart block Intermediate coronary syndrome Myocardial infarction

BS, Brugada syndrome; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; LVNC, left ventricular noncompaction cardiomyopathy; LQT, Long QT syndrome; RCM, restrictive cardiomyopathy; SCD sudden cardiac death.

^aThe most frequent disease conditions associated with selected genes. Table summarized data from GeneCards (www.genecards.org/), Ensembl (www.ensembl.org/), and UniProt (www.uniprot.org/) databases.

TABLE 2 Detected sequence variants in the cohort of East Slovak patients with hypertrophic cardiomyopathy

Gene, exon	All variants (N=43)	Ms (N=38)	Syn (N=4)	Ns (N=1)	Novel (N=25)
MYH7 e. 23	12	12	0	0	10
MYBPC3 e. 12	4	3	1	0	4
MYBPC3 e. 24	1	1	0	0	1
MYBPC3 e. 25	5	4	1	0	2
NEBL e. 12	3	3	0	0	1
SCN5A e. 12	4	4	0	0	0
SCN5A e. 20	1	0	1	0	0
SCN5A e. 21	3	3	0	0	0
TNNT2 e. 9	8	7	0	1	6
TNNI3 e. 7	2	1	1	0	1

Ms, missense variants; N, number of cases; Ns, nonsense variants; Syn, synonymous variants; Novel, number of novel variants detected in each analyzed exon.

protein functions. We identified one novel nonsense variant within the coding region of *TNNT2* gene (exon 9; *p.E111**). The most frequently detected alterations were heterozygous missense single nucleotide changes. Only three sequence variants in exon 12 of *NEBL* gene and

one in exon 9 *TNNT2* gene were detected in homozygosity. From the total number of detected sequence variants, 58.14% were attributed as a novel based on their absence in databases. Sanger sequencing approach identified 14 unique alterations, confirmed only in one patient.

In fact, 11 sequence variants (three known with rs identification and eight novel) were evaluated as potential pathogenic, based on results of all used variant effect predictor software (Mutation Taster, PhD-SNP, Polyphen2, PredictSNP, SIFT, SNAP2). The predicted deleterious sequence variants and their molecular genetic characteristics are summarized in Tables 3 and 4. Clinical characteristics of patients (mean age of 55.7±18.42 years) with detected known potentially pathogenic sequence variants are in Table 5. The most frequent alteration rs121913629 with pathogenic allele and detected deleterious potential (detected only in patients with hypertrophic cardiomyopathy) was confirmed in exon 23 of MYH7 gene.

Finally, by comparing our data with Ensembl database (www.ensembl.org) we identified two variants, from the total number of 43 variants, which were previously associated with cardiomyopathy phenotype (rs730880758; rs121913629).

4 | DISCUSSION

Hypertrophic cardiomyopathy is clinically and genetically heterogeneous disease. Although many genes and mutations are known or suspected to cause hypertrophic cardiomyopathy, the candidate

TABLE 3 Molecular genetic characteristics of potentially pathogenic sequence variants detected in our study and noted in databases

Gene	rs ID	Pos.	Seq.	AA	Anc/Mut	no P/C	Phen	MAF	Clin Sig
MYH7	rs730880758	chr14:23424095	c.2734A>C	p.Lys912Gln	G/T	1/0	CMP	0.0006	LPA
MYH7	rs121913629	chr14:23423984	c.2845G>A	p.Glu949Lys	C/T	9/0	FHCM	0.0006	PA
SCN5A	rs137854616	chr3:38566465	c.3978C>G	p.Gly1262Ser	G/A	1/0	Bs	0.0032	PA

AA, amino acid change; Anc/Mut, ancestral/mutated allele; LPA, sequence variant with likely pathogenic allele; MAF, minor allele frequency (according to <http://db.systemsbio.org/kaviar/cgi-pub/Kaviar.pl>); NA, not analyzed; no. P/C, number of patients/controls; Ns, nonspecific phenotype; PA, sequence variant with pathogenic allele; Phen, phenotype; Pos., position Hg38/1.

Gene	Seq	AA	Alter.	no P/C
MYH7	c.2736G>T	p.Lys912Asn	chr14:23893302C>A	1/0
MYH7	c.2870T>G	p.Leu957Arg	chr14:23893168A>C	13/8
MYH7	c.2876T>G	p.Leu959Arg	chr14:23893162A>C	1/0
MYH7	c.2885C>A	p.Ala962Asp	chr14:23893153G>T	3/0
MYBPC3	c.1037G>C	p.Arg346Pro	chr11:47367811C>G	1/0
TNNI3	c.473T>G	p.Leu158Arg	chr19:55665474A>C	1/0
TNNT2	c.342G>T	p.Lys114Asn	chr1:201334358C>A	18/6
TNNT2	c.346G>A	p. Glu116Lys	chr1:201334354C>T	4/3

AA, amino acid change; Alter., alteration on chromosomal level; Anc/Mut, ancestral/mutated allele; no. P/C, number of patients/controls.

TABLE 4 Molecular genetic characteristics of novel potentially pathogenic sequence variants detected in our study

TABLE 5 Clinical characteristics of patients with detected known potentially pathogenic sequence variants

Specimen	Gender	Age	LVOTO	LVEF	NYHA	Family history	ICD	SV
KMP 1	F	39	NO	60%	I.	NO	NO	rs121913629
KMP 4	F	69	NO	60%	II.-III.	NO	NO	rs121913629
KMP 8	F	60	YES	55%-60%	—	NO	NO	rs121913629 rs137854616
KMP7	M	36	YES	60%	—	NO	NO	rs121913629
KMP 8	M	62	YES	60%	—	NO	NO	rs121913629
KMP 11	F	57	NO	60%	I.	YES	NO	rs121913629
KMP 15	F	82	YES	60%	III.	YES	YES	rs730880758
KMP 16	F	60	YES	39%-40%	I.	NO	YES	rs121913629
KMP 18	F	71	NO	60%	I.-II.	NO	NO	rs121913629
KMP 22	M	21	NO	60%	—	NO	YES	rs121913629

LVOTO, left ventricular outflow tract obstruction; LVEF, left ventricular ejection fraction; ICD, implantable cardioverter defibrillator; SV, known potentially pathogenic sequence variants.

genetic alterations for faster diagnosis of hypertrophic cardiomyopathy are not defined. A large number of mutations were detected in genes, mostly encoding sarcomeric proteins.^{5,6} Pathogenic variants in these genes were identified in multiple studies, which provided definitive evidence of disease association for these genes. However, non-sarcomeric genes can be equally responsible for the number of hypertrophic cardiomyopathy cases.¹² There is a tendency of reevaluation of the role of non-sarcomeric genes in hypertrophic cardiomyopathy etiopathogenesis.¹³ In this study, we analyzed selected exons of sarcomeric and non-sarcomeric genes with the purpose to identify genetic variants with pathogenic potential in hypertrophic cardiomyopathy patients from Eastern Slovakia. The sequence variants identified in this study may be further investigated in order to elucidate molecular genetic mechanisms of hypertrophic cardiomyopathy and improve the management, diagnosis, and treatment of Slovak patients and high-risk relatives with hypertrophic cardiomyopathy.

The Sanger sequencing approach applied in our study identified 43 sequence variants in selected exons of six analyzed cardiomyopathy genes, namely, *MYBPC3* (exons 12, 24, 25), *MYH7* (exon 23), *NEBL* (exon 12), *SCN5A* (exons 12, 20, 21), *TNNI3* (exon 7), and *TNNT2* (exon 9) genes. Heterozygous missense single nucleotide changes were the most common types of detected sequence variants. Our findings are in consistency with the results of other authors.^{6,9,14} Among 43 identified sequence variants, four variants were identified in homozygosity (three sequence variants in exon 12 of *NEBL* gene and one in exon 9 of *TNNT2* gene). All these variants were predicted to be benign by *in silico* prediction tools and further testing of individuals who are homozygous is recommended. In fact, only ~9% of detected variants were synonymous single nucleotide changes that might act as phenotype modifiers.

From the total number of detected sequence variants, we identified several novel non-synonymous variants. Both novel and known variants were predicted by *in silico* prediction tools. The harmful effect was confirmed in 11 variants. From the 25 novel sequence variants detected in our cohort of patients, 8 were predicted to be damaging. The functional effect of novel, potentially pathogenic sequence variants, needs to be further investigated. These sequence variants might be potential disease mutations or modifiers, but we cannot exclude their interactions and a potential effect of benign variants to hypertrophic cardiomyopathy phenotype.

The aim of our investigation was to detect known genetic alterations in positive association with hypertrophic cardiomyopathy. Two detected deleterious variants, *rs730880758* and *rs121913629*, were associated with cardiomyopathy phenotype according to Ensembl database (www.ensembl.org) and one known variant detected in *SCN5A* gene was associated with Brugada syndrome in previous studies.¹⁵ These variants were detected only in 10 patients with a mean age of 55.7±18.42 years and not in a group of controls. The youngest patient was only 21 years old. The majority of detected known pathogenic sequence variants were related to female gender. It is claimed that patients with a confirmed mutation in sarcomeric genes are predominantly female.¹⁶ Only in one

patient were detected two known potentially pathogenic sequence variants, *rs121913629* and *rs137854616*, respectively. All patients were symptomatic and left ventricular outflow tract obstruction was confirmed in half of them. Positive family history was reported in two patients with known deleterious sequence variants and we recommend whole-genome sequencing analysis of patients with positive family history and genetic testing of first-degree family members.

In our study, we identified 14 unique sequence variants. On the other hand, the most frequent variant confirmed only in patients with hypertrophic cardiomyopathy was *rs121913629* which leads to the exchange of glutamic acid to lysine at position 949 in hotspot region of *MYH7* gene (exon 23). This sequence variant was firstly detected by Watkins et al.¹⁷ in one family with familial hypertrophic cardiomyopathy. Sequence variant was associated with the late onset of disease and severe left ventricular hypertrophy. These cardiomyopathy hallmarks have also been confirmed in our mutation carriers. Several studies have shown an association of sequence variant *rs121913629* with the different phenotypes and their heterogeneity.¹⁷⁻²⁰

A large portion of patients harbors multiple sequence variants. It is assumed that the presence of multiple mutations might be a marker of disease severity.²¹ The most frequently detected combination of sequence variants was *p.Glu949Lys* and *p.Leu957Arg* in exon 23 of *MYH7* gene, respectively. The sequence variants detected in almost all patients (22 of 23 patients) were found in *TNNT2* gene. The majority of identified sequence variants were confirmed in exon 23 of *MYH7* gene. Our data are consistent with other investigations and confirm the predominant role of *MYH7* gene in the etiopathogenesis of hypertrophic cardiomyopathy.^{22,23}

In conclusion, the findings of our study indicate that the Sanger sequencing method is a useful approach for the identification of potentially pathogenic sequence variants associated with hypertrophic cardiomyopathy, and our results may be beneficial in the risk evaluation of individuals with disease diagnosis or with possible susceptibility to develop hypertrophic cardiomyopathy.

5 | CONCLUSION

The aim of the study was to analyze selected exons of sarcomeric and non-sarcomeric genes with the purpose to identify potentially pathogenic genetic variants in the patients with hypertrophic cardiomyopathy from Eastern Slovakia. In our study 43 sequence variants, due known and novel were detected. From the total number of variants identified in each analyzed exon, 11 were predicted to be potentially pathogenic, but we did not observe any strong mutation hotspot. The results of this study emphasize the importance of the whole-genome sequencing approaches in hypertrophic cardiomyopathy etiology investigations. The sequence variants identified in this study might be further investigated in order to improve the management, diagnosis, and treatment of Slovak patients with hypertrophic cardiomyopathy.

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ETHICAL APPROVAL

All procedures performed in this study were in accordance with ethical standards of the research committee of University of Presov and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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