MYOSIN BINDING PROTEIN C GENE *(MYBPC3)* MUTATIONS IN HYPERTROPHIC CARDIOMYOPATHY

PhD Thesis

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LIST OF PUBLICATIONS

Publications directly related to the thesis:

I. **Tóth T,** Sepp R, Orosz A, Nagy V, Pálinkás A, Hőgye M, Csanády M, Forster T. A myozinkötő C-fehérje gén *(MYBPC3)* mutációszűrése magyar hypertrophiás cardiomyopathiás betegekben. *Cardiologia Hungarica* 2009; 39:318-24.

II. **Tóth T,** Sepp R, Orosz A, Nagy V, Pálinkás A, Hőgye M, Csanády M, Forster T. Myozinkötő C fehérje *(MYBPC3)* génmutációt hordozó hypertrophiás cardiomyopathiás családok klinikai és genetikai analízise. *Magy Belorv Arch* 2010; 63: 35-40.

III. Csanády M, Sepp R, **Tóth T,** Orosz A, Nagy V, Hőgye M, Forster T. A myozin kötő C fehérje gén *(MYBPC3)* mutációjának azonosítása veleszületett süketnémasággal társult hypertrophiás cardiomyopathiában. *Orvostudományi Értesítő* 2008; 81:23-25.

IV. **Tóth T,** Nagy V, Faludi R, Csanády M, Nemes A, Simor T, Forster T, Sepp R. The Gln1233ter mutation of the myosin binding protein C gene: causative mutation or innocent polymorphism in patients with hypertrophic cardiomyopathy? *Int J Cardiol* 2011; 153(2):216-9. IF: **7.078**

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II. **Tóth T,** Orosz A, Nagy V, Hőgye M, Csanády M, Forster T, Sepp R. A myozin kötő C-fehérje gén *(MYBPC3)* mutációanalízise hypertrophiás cardiomyopathiában. *Cardiologia Hungarica* 2008; 38: B48.

III. **Tóth T,** Orosz A, Nagy V, Hőgye M, Forster T, Csanády M, Sepp R: Mutation analysis of the myosin binding protein C gene *(MYBPC3)* in hypertrophic cardiomyopathy

Slovenska Kardiologija 2008; 5: 34 (FC7-10). Abstracts of 16th Annual Meeting of the Alpe Adria Association of Cardiology.

IV. **Tóth T,** Orosz A, Csanády M, Hőgye M, Forster T, Sepp R: Klinikai és genetikai szűrés veleszületett süketnémasággal társult hypertrophiás cardiomyopathiában. *Orvostudományi Értesítő* 2009; 1:59.

V. **Tóth T,** Orosz A, Nagy V, Hőgye M, Forster T, Csanády M, Sepp R. Myozin kötő C fehérje *(MYBPC3)* génmutációt hordozó családok klinikai és genetikai analízise. *Cardiologia Hungarica* 2009; 39: A54.

VI. Sepp R, Losonczi L, **Tóth T,** Nagy V, Orosz A, Kádár K, Hőgye M, Fekete Gy, Csanády M, Forster T. Kóroki géneloszlás magyar hypertrophiás cardiomyopathiás betegekben. (Disease gene distribution in Hungarian patients with hypertrophic cardiomyopathy). *Cardiologia Hungarica* 2010; 40: G101.

VII. **Tóth T,** Nagy V, Faludi R, Hőgye M, Csanády M, Simor T, Forster T, Sepp R. A myozinkötő C fehérje gén *(MYBPC3)* Gln1233ter eltérése hypertrophiás cardiomyopathiában: kóroki mutáció vagy ártatlan polymorfizmus? (The Gln1233ter alteration of the myosin binding protein C gene *(MYBPC3)* in hypertrophic cardiomyopathy: causative mutation or innocent polymorphism?). *Cardiologia Hungarica* 2010; 40: G102.

VIII. Sepp R, Losonczi L, **Tóth T,** Nagy V, Orosz A, Kádár K, Hogye M, Fekete Gy, Csanády M, Forster T. Prevalence of sarcomeric gene mutations in Hungarian patients with hypertrophic cardiomyopathy. *J Kardiol* 2010; 17 (Suppl A): 15.

IX. **Tóth T,** Nagy V, Faludi R, Hogye M, Csanády M, Simor T, Forster T, Sepp R. The Gln1233ter alteration of the myosin binding protein C gene *(MYBPC3)* in hypertrophic cardiomyopathy: causative mutation or innocent polymorphism? *J Kardiol* 2010; 17 (Suppl A): 15.

LIST OF ABBREVIATIONS

ACTC1: alpha-cardiac actin gene

ACTN2: alfa actinin-2 gene

ASH: asymmetric hypertrophy

AVR: aortic valve replacement

CSRP3: muscle LIM protein gene

dbSNP: Single Nuleotide Polymorphism Database

DDD: dual-chamber pacemaker

Del: deletion

DHPLC: denaturing high performance liquid chromatography

ECG: electrocardiogram

fs: frame-shift

HCM: hypertrophic cardiomyopathy

HGMD: Human Gene Mutation Database

HTX: heart transplantation

ICD: implantable cardioverter defibrillator

JPH2: junctophyllin-2 gene

LV: left ventricle, left ventricular

LVEDD: left ventricular end-diastolic diameter

LVEF: left ventricular ejection fraction

LVESD: left ventricular end-systolic diameter

LVmax: maximum left ventricular wall thickness

LVOT: left ventricular outflow tract

MRI: magnetic resonance imaging

MYBPC3: myosin binding protein C gene

MYH7: beta myosin heavy chain gene

MYL2: regulatory myosin light chain gene

MYL3: essential myosin light chain gene

MYOZ2: myozenin 2 gene

MVR: mitral valve replacement

PCI: percutaneous coronary intervention

PCR: polymerase chain reaction

PLN: phospholamban gene

PM: pace-maker

PTSMA: percutaneous transluminal septal myocardial ablation

SAM: systolic anterior motion of the anterior leaflet of the mitral valve

SCD: sudden cardiac death

SSCP: single strand conformation polymorphism

TCAP: telethonin gene

TNNI3: troponin I gene

TNNT2: troponin T gene

TPM1: alpha tropomyosin gene

TTN: titin gene

VCL: vinculin/metavinculin gene

ZASP/LBD3: cypher-LIM binding domain 3 gene

1. INTRODUCTION

1.1. Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a primary disease of the myocardium characterised primarily by left ventricular myocardial hypertrophy, predominantly involving the interventricular septum [reviewed in detail in (1-4)]. The cavity of the left ventricle is typically narrow. According to current literature data the disease is more frequent, then it was previously thought as its prevalence was shown to be 1/500-1000 (5). Clinically the patients may be asymptomatic, but the development of symptoms in form of dyspnoea, chest pain, palpitation or syncope is more typical. Arrhythmias are frequent and the risk of sudden cardiac death is also increased.

1.2. Molecular genetics of hypertrophic cardiomyopathy

Genetic studies have shown that in most cases HCM is a hereditary disease with a typical autosomal dominant inheritance, variable penetrance and expression. Using molecular genetic methods specific alterations in genes encoding for mainly sarcomere proteins were found to cause the disease [reviewed in detail in (6,7)]. The most important affected genes implicated in the disease include the beta myosin heavy chain- (*MYH7*) (8), the alpha tropomyosin- (*TPM1*) (9), the troponin T- (*TNNT2*) (9), the myosin binding protein C- (*MYBPC3*) (10,11), the troponin I- (*TNNI3*) (12), the essential- (*MYL3*) and the regulatory myosin light chain- (*MYL2*) (13), the alpha-cardiac actin- (*ACTC1*) (14) and the titin (*TTN*) genes (15) (*Table 1*). Based on the above, HCM can be considered as the disease of the sarcomere (9). Other genes, affecting genes encoding for the sarcomere Z-disk [muscle LIM protein- (*CSRP3*) (16); telethonin- (*TCAP*) (17); myozenin 2- (*MYOZ2*) (18); vinculin/metavinculin- (*VCL*) (19); alfa actinin-2- (*ACTN2*) (20); cypher-LIM binding domain 3 (*ZASP/LBD3*) genes (21)], and encoding for Ca^{++}-handling proteins [junctophyllin-2- (*JPH2*) (22,23); phospholamban (*PLN*) genes (24)] has been also implicated causing HCM in selected cases (*Table 2*). Mutations affecting sarcomere genes are present in 40-60% of HCM patients. In 5-10% of the cases mutations affect genes which may lead to HCM phenocopies (i.e. Fabry disease, Danon disease, transthyretin amyloidozis, etc.) (25). In the remaining 20-25% of the cases the cause of HCM is still unknown (2).

Although HCM is a genetically heterogeneous disease, the most commonly affected sarcomere gene that can cause HCM is the myosin binding protein C gene (*MYBPC3*). According to literature data on screening different patient populations with HCM the prevalence of mutations affecting the *MYBPC3* gene is 15-25% (26-41). Since the identification of the first *MYBPC3* gene mutation nearly 600 other mutations have been identified in the gene. Our team published the first *MYBPC3* gene mutation in a Hungarian patient in 2001 (42).

Gene	**Symbol**	**Locus**	**Prevalence (%)**
beta myosin heavy chain	*MYH7*	14q12	15-25
myosin binding protein C	*MYBPC3*	11p11.2	15-25
troponin T	*TNNT2*	1q32	<5
troponin I	*TNNI3*	19q13.4	<5
troponin C	*TNNC1*	3p21-p14	<1
alpha-tropomyosin	*TPM1*	15q22	<5
alpha cardiac actin	*ACTC1*	15q14	<1
essential myosin light chain	*MYL3*	3p21.31	<1
regulatory myosin light chain	*MYL2*	12q24.21	<2
titin	*TTN*	2q31	<1
AMP activated protein kinase, γ2 regulatory subunit	*PRKAG2*	7q34-q36	<1

Table 1. Affected sarcomere genes and their prevalence in hypertrophic cardiomyopathy.

Gene	**Symbol**	**Locus**	**Prevalence (%)**
muscle LIM protein	*CSRP3*	11p15	<1
telethonin	*TCAP*	17q12	<1
myozenin 2	*MYOZ2*	4q26	<1
vinculin/metavinculin	*VCL*	10q22.1	<1
alfa actinin-2	*ACTN2*	1q42	<1
cypher-LIM binding domain 3	*ZASP/LBD3*	10q22.3	1-5
junctophyllin-2	*JPH2*	20q12	<1
phospholamban	*PLN*	6q22.1	<1

Table 2. Affected Z-disk and Ca^{++}-handling genes and their prevalence in hypertrophic cardiomyopathy.

1.3. Structure and function of the myosin binding protein C (cMyBP-C)

The structure and sequence of the human *MYBPC3* gene have been determined in 1997 (43). It encompasses more than 21 kbp and is composed of 34 coding exons. The gene encodes cardiac myosin binding protein C (cMyBP-C) which is a multi-modular structural protein component of the sarcomere [reviewed in detail in (44-47)]. The cMyBP-C is a polypeptide, which belongs to the intracellular immunoglobulin superfamily. The protein consists multiple domains, and can be found in the myofibrillum of every striated muscle tissue of the body, binding to the thick filament of the sarcomere, except the Purkinje cells of the heart. The protein itself is located in the 'C' („crossbridge") zone of the 'A' band of the sarcomere, 43 nm from each other, as a form of 7-9 stripes (*Figure 1*). The cMyBP-C protein is made up of eight immunoglobulin I (IgI) and three fibronectin III (FnIII) domains (C0-C10) (*Figure 2*).

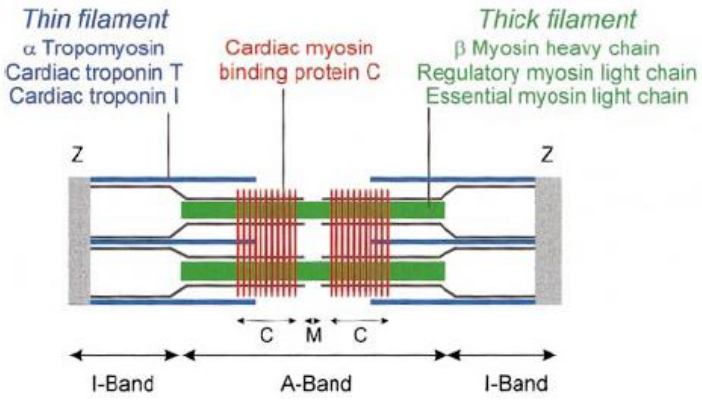

Figure 1. The localization of cardiac myosin protein binding C in the sarcomere (Source: Bonne G et al. Circ Res 1998; 83: 580-93.)

There are three known isoforms of the MyBP-C protein in the human body: the fast skeletal muscle, the slow skeletal muscle (originally that was mentioned as MyBP-C-X) and the cardiac isoform. The different isoforms of the protein are encoded by separate genes; the fast skeletal type is encoded by *MYBPC2* on chromosome 19, and the slow skeletal type is encoded by *MYBPC1* on chromosome 12. The cardiac type is encoded by the *MYBPC3,* located on chromosome 11. All isoforms are fibre type specific, the cardiac type can be found only in the heart. The cardiac isoform differs in 3 basic aspects from the skeletal muscle types. First, the cardiac type has an extra immunoglobulin I motif on the N-terminal (C0), the function of which is still unclear. Second, there is a proline-rich

region in the middle of the C5 IgI domain in the cardiac type. Third, there are two more phosphorylation sites in the linker region that consist of nearly 100 amino acids and links to each other the C1 and the C2 domains (*Figure 2*). Although the precise role of the cMyBP-C protein in the sarcomere structure and in the process of contraction-relaxation is still unclear, it appears that it has both structural and regulatory function.

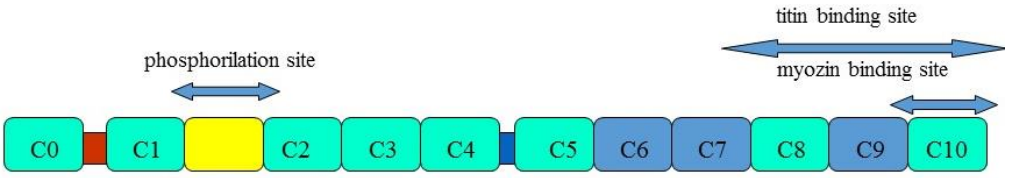

Figure 2. Schematic representation of the structure of the cardiac myosin protein binding C. The eleven domains of the cMyBP-C protein (C0-C10) including the eight immunoglobulin I (IgI) domains (green) and the three fibronectin III (FnIII) domains (blue) are shown. The phosphorylation, myosin- and titin binding sites are indicated.

1.4. Mutations affecting the *MYBPC3* gene in hypertrophic cardiomyopathy

Using linage analysis, the presence of an HCM locus was mapped in 1993 to chromosome 11 (11p11.2) (48). Further research identified the *MYBPC3* gene as the causative gene of the locus in 1995 when mutations were found in different families with HCM in the *MYBPC3* gene (10,11). In one of the families a partial duplication of the gene was identified that contained 18 base pairs, which extended the mutant protein by 6 amino acids. In three other families a "splice site" mutation was described which caused an early termination of the protein and resulted in a truncated protein.

Ever since numerous mutations have been identified that can affect the gene: the professional version of the Human Gene Mutation Database (HGMD, www.hgmd.cf.ac.uk) lists 614 *MYBPC3* gene mutations. Early studies screening for mutations in the *MYBPC3* gene found a 13-26% prevalence of *MYBPC3* gene mutations in patients with HCM. Erdmann et al. who investigated 110 patients found a 13.6% prevalence in a German HCM cohort (49), while Van Driest et al., screening 389 patients, and Richard et al., who analysed 197 patients, reported an occurrence of 18% (37) and 26%, respectively (35).

Approximately two-thirds of the *MYBPC3* mutations result in a truncated protein. This phenomenon can be attributed to either so-called „splice-site" mutations, or nucleotide insertions or deletions, which all can cause a shift in the reading frame. This will lead to

incorporation of nonsense sequences which will usually end by an early stop codon activation. Besides, several missense mutations were identified which result in the change of only one amino acid (44,47,50).

1.5. The p.Gln1233Ter mutation of the *MYBPC3* gene

Some of the *MYBPC3* mutations have a debated consequence. Such a mutation is the p.Gln1233Ter *MYBPC3* mutation of the gene which is caused by a C-T transition in exon 33 of the *MYBPC3* gene (c.3697C>T). The mutation leads to the initiation of a premature stop codon instead of glutamine at codon 1233 (p.Gln1233Ter). This presumably causes the loss of the terminal 48 amino acids of the myosin binding protein C which contains binding sites for myosin and titin.

The p.Gln1233Ter mutation of *MYBPC3* has been reported by three groups in the literature. In a report, originating from Germany, the mutation has been described in two families with Turkish origin, in which three mutation carriers were identified (49). Haplotype analysis suggested a founder effect in the apparently unrelated families. However, affected-to-affected transmission of the mutation was not possible to prove owing to the small family size and availability of samples. Another report came from Australia, where the p.Gln1233Ter mutation was identified in one patient in a “compound” form in association with another *MYBPC3* mutation (p.Gln1233Ter/p.Arg326Gln) (51). Again, transmission of the mutation from affected-to-affected was not demonstrated. In a third report, originating from the USA, the p.Gln1233Ter mutation has been also identified in a HCM proband, but also in normal controls with an incidence of 2%, and, therefore, it was regarded more a polymorphism (37). Therefore, the importance of the mutation has been questioned based on two arguments; 1) no affected-to-affected transmission has ever been demonstrated; and 2) presence in apparently normal controls in some populations. Of note, with regard to the latter observation, the mutation was neither detected in 50 normal controls in the German group (49), nor in 300 negative controls in the Australian group (51).

2. AIMS

Previous to our work, no information were available with regard to the occurrence, prevalence, or distribution of *MYBPC3* gene mutations in Hungarian patients with hypertrophic cardiomyopathy. Similarly, no data existed on possible specific genotype-phenotype correlations, penetrance rates or specific expression of the disease in a Hungarian HCM patient cohort.

Therefore, in my PhD work I aimed to:

1. Identify mutations affecting the myosin binding protein C gene *(MYBPC3)* in Hungarian patients with hypertrophic cardiomyopathy;

2. Establish the prevalence rate of *MYBPC3* mutations in Hungarian HCM patient population;

3. Conduct clinical and genetic screening of family members of patients with *MYBPC3* gene mutations;

4. Analyze genotype-phenotype correlations of *MYBPC3* mutations identified in Hungarian HCM patients;

5. Characterize specific *MYBPC3* mutations identified in the Hungarian HCM cohort.

3. PATIENTS AND METHODS

3.1. Patients

3.1.1. Hungarian patients with hypertrophic cardiomyopathy screened for mutations in the *MYBPC3* gene

We analyzed forty-five unrelated patients with hypertrophic cardiomyopathy. In all cases collection of case history data, physical examination, overview of available clinical documentation, 12-lead ECG and transthoracic echocardiography were carried out. In selected cases patients were hospitalized for detailed in-hospital cardiology assessment (24-hour Holter monitoring, stress test, semi-supine bicycle stress echocardiography, cardiac MRI, coronarography, haemodynamic study). In all cases the diagnosis of HCM was based on internationally accepted diagnostic criteria (2-4).

The demographic characteristics, the main data on the course of the disease, as well as on the main echocardiographic characteristics of the patients are shown in *Table 3*. The average maximum left ventricular wall thickness were 22±6 mm, in 40 cases typical asymmetric septal hypertrophy, in 3 cases apical HCM were identified. In 15 cases significant resting left ventricular outflow tract obstruction (greater than 30 mmHg) were present. In the 45 analyzed probands during the average 8±7 years (median: 6 years) follow-up period 9 deaths occurred, 8 of them were sudden cardiac death. In 6 patients ICD implantation, in 2 cases DDD pacemaker implantation, in 3 cases percutaneous transluminal septal myocardial ablation (PTSMA), in 2 cases surgical myomectomy were carried out. In one case HCM transformed into dilated phase, and the patient underwent heart transplantation.

3.1.2. Family members of patients with hypertrophic cardiomyopathy carrying *MYBPC3* gene mutations for clinical and genetic investigations

Family members of 5 index patients were investigated in whom 5 different, specific *MYBPC3* gene mutations were identified by our previous investigations [c.3697C>T (p.Gln1233Ter); c.821+1G>A; c.2864_2865delCT (p.Pro955ArgfsTer95); c.1776_1777delGT (p.Ser593ProfsTer11); c.3407_3409delACT (p.Tyr1136del)]. Family members of the index patient carrying the c.431_432delGT (p.Gly144AlafsTer8) mutation were not available for family screening.

total number of patients, n (%)	45 (100%)
male, n (%)	27 (60%)
mean age (at diagnosis, year)	38±15
familiarity proven, n (%)	18 (40%)
death, n (%)	9 (20%)
cause of the death, n (%)	
sudden cardiac death	8 (89%)
other (suicide)	1 (11%)
atrial fibrillation, n (%)	11 (24%)
intervention	
ICD implantation, n (%)	6 (13%)
Pacemaker implantation, n (%)	2 (4%)
PTSMA, n (%)	3 (7%)
PCI, n (%)	2 (4%)
Myomectomy, n (%)	2 (4%)
HTX, n (%)	1 (2%)
Echocardiography data	
Maximal left ventricular wall thickness (mm)	22±6
hypertrophy localization, n	ASH: 40, apical: 3, other: 2
left atrium diameter, mm	45±11
LVEDD, mm	45±11
LVESD, mm	28±13
EF, %	61±12
LVOT gradient at rest, mmHg	26±29
LVOT gradient at rest >30 mmHg, n (%)	15 (30%)

Table 3. The main demographic, clinical and echocardiographic parameters of the patient population with HCM screened for MYBPC3 gene mutations. ICD: implantable cardioverter defibrillator, PTSMA: percutaneous transluminal septal myocardial ablation, PCI: percutaneous coronary intervention, HTX: heart transplantation, LV: left ventricle, ASH: asymmetric hypertrophy, LVEDD: left ventricular end-diastolic diameter, LVESD: left ventricular end-systolic diameter, EF: ejection fraction, LVOT: left ventricular outflow tract.

Altogether, 62 family members (30 male, 32 female, age: 40±18 years) were investigated in the 5 families. In all cases collection of case history data, physical examination, overview of available clinical documentation, 12-lead ECG and transthoracic echocardiography were carried out. In selected cases patients were hospitalized for detailed in-hospital cardiology assessment (24-hour Holter monitoring, stress test, semi-supine bicycle stress echocardiography, cardiac MRI, coronarography, haemodynamic study). In all cases diagnosis of HCM were based on internationally accepted diagnostic criteria (2-4), in case of the family members the McKenna criteria were considered (52).

3.2 Methods

3.2.1. Genetic analysis

In accordance with legal regulations all investigated patients and family members gave written informed consent to molecular genetic analyses. DNA isolation was carried out using standard methodology from peripheral blood samples. The complete coding sequence and exon-intron junctions of the *MYBPC3* gene (1-35 exons) was amplified by polymerase chain reaction (PCR) from the sample DNAs (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) with specific primer pairs described in the literature (10). In general, the samples were amplified in 25 µl PCR reaction using 100 ng DNA template with optimized PCR protocols. The PCR products were analysed by 'single strand conformation polymorphism' (SSCP) or 'denaturing high performance liquid chromatography" (DHPLC) chromatography mutation analysis methods (*Figure 3*), which were based on the different temperature-dependent separation of the DNA sample carrying a different nucleotide (variant). The DHPLC analysis was carried out on a Helix (Varian Inc, Palo Alto, USA) DHPLC equipment. The optimum melting temperature of all specific PCR fragments were defined by using the DHPLC Melt Programme (http://insertion.stanford.edu/melt.html). After PCR the denatured samples were renatured by slow cooling (1°C/min). The elution curves of the samples were analysed by the programme of the equipment (Star Reviewer, version 2.0). Every abnormal chromatogram was sequenced using the ABI PRISM 310 automated sequencer.

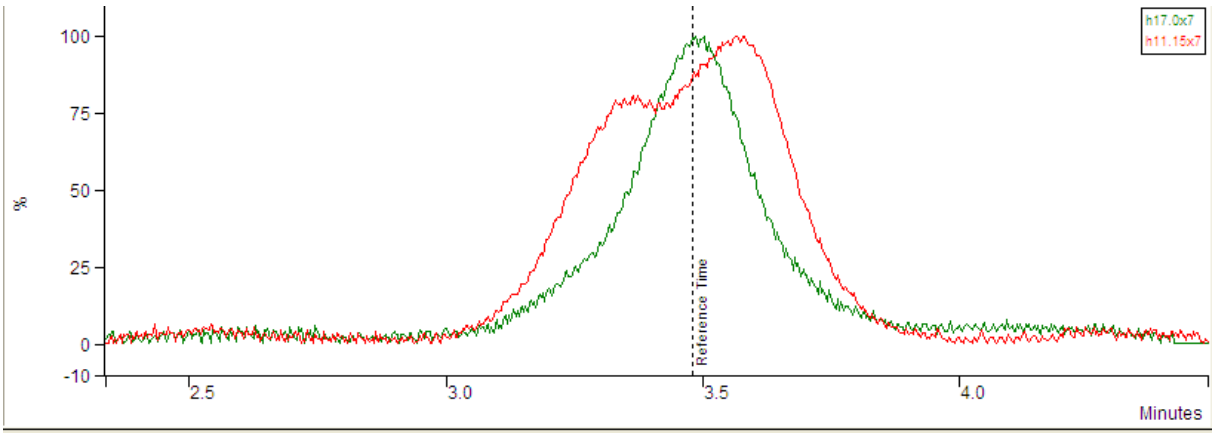

Figure 3. Denaturing high performance liquid chromatography (DHPLC) analysis of a normal (green) and mutant (red) PCR fragment. The method is based on the different temperature-dependent separation of the DNA sample carrying a different nucleotide (variant). After PCR the denatured samples are renatured by slow cooling than eluted from a DNA-binding column at an optimum melting temperature of the specific PCR fragments. If a nucleotide change (i.e. variant) is present in the sample, the elution curves are different.

3.2.2. Bioinformatics

Assessing the nucleotide variants and determining the mutations the Ensemble database of the European Molecular Biology Laboratory- European Bioinformatics Institute was used (www.ensemble.org). For reporting variants, LRG_386 was used as a stable genomic reference framework for describing sequence variants for this gene (transcript: LRG_386t1, protein: LRG_386p1). The dbSNP database (Single Nuleotide Polymorphism Database) of the National Centre for Biotechnology Information was used as a variation reference (http://www.ncbi.nlm.nih.gov/SNP/), while the HGMD database (Human Gene Mutation Database) of the at the Institute of Medical Genetics in Cardiff was used as a mutation reference.

4. RESULTS

4.1. Identification of *MYBPC3* mutations in Hungarian patients with hypertrophic cardiomyopathy

4.1.1. Mutation data

In the 45 analyzed patients with HCM we identified 6 (13%) different causative *MYBPC3* mutations (*Table 4 and Figures 4-5*). One of the mutations was a nonsense (stop codon) mutation in exon 33 of the gene (c.3697C>T, p.Gln1233Ter, *Figure 4, Panel A and B*). Another mutation was a splice site mutation at the junction of exon and intron 7 (c.821+1G>A, *Figure 4, Panel C and D*). In other three cases three 2 base-pair microdeletions, affecting exons 27, 18 and 4 were found, which resulted in 'frame-shift' [exon 27: c.2864_2865delCT (p.Pro955ArgfsTer95, *Figure 4, Panel E and F*); exon 18: c.1776_1777delGT (p.Ser593ProfsTer11, *Figure 5, Panel A and B*); exon 4: c.431_432delGT, (p.Gly144AlafsTer8, *Figure 5, Panel E and F*)]. The sixth identified mutation was a 3 base-pair microdeletion affecting exon 31, which did not cause 'frame shift' but led to the deletion of a single amino acid (c.3407_3409delACT, p.Tyr1136del, *Figure 5, Panel C and D*). Each mutation was present in heterozygous form. Three of the mutations were previously reported in the literature (p.Gln1233ter, c.821+1G>A, c.2864_2865delCT) while the other three were novel mutations. Besides the causative mutations we identified several intronic polymorphisms, that do not affect the coding sequence of the gene, furthermore, several 'silent' mutations that do not change the sequence of the amino acids (intron 2: g.1505G>C; intron 21: g.13170A>G; intron 21: g.13090A>T; intron 33: g.20756C>T).

patient	affected exon	affected codon	nucleotide change	amino acid change	reported	HGMD No.	dbSNP No.	reference
H 16.0	33	1233	c.3697C>T	p.Gln1233ter	yes	CM014069	rs397516037	37, 49, 51
H 11.0	exon/ intron7	-	c.821+1G>A	-	yes	CS982276	rs397516073	37, 49, 58
H 65.0	27	955	c.2864_2865delCT	p.Pro955ArgfsTer95	yes	CD982813	rs397515990	49, 58
H 92.0	18	592-593	c.1776_1777delGT	p.Ser593ProfsTer11	no	-	rs730880713	
H 76.0	31	1136	c.3407_3409delACT	p.Tyr1136del	no	-	rs730880674	
H 55.0	4	144	c.431_432delGT	p.Gly144AlafsTer8	no	-	rs397516047	

Table 4. Identified MYBPC3 mutations in the Hungarian HCM patient cohort. Del: deletion, fs: frame-shift, HGMD: Human Gene Mutation Database, dbSNP: Single Nucleotide Polymorphism Database.

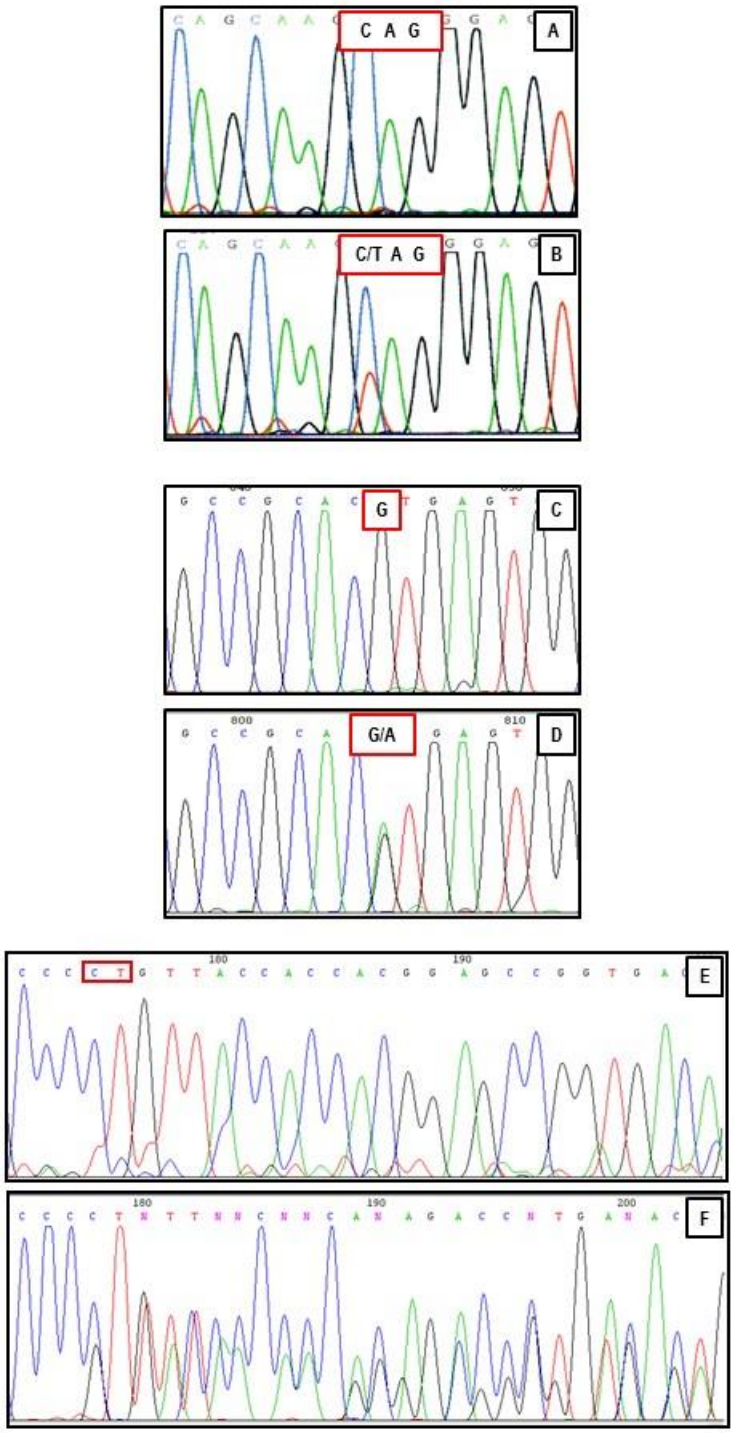

Figure 4. Electropherograms of MYBPC3 gene mutations identified in Hungarian HCM patients. Panel A: a C to T transition in exon 33 in patient H 16.0 (c.3697C>T) resulting in nonsense (stop codon) mutation p.Gln1233Ter. Panel C: a G to A transition at the junction of exon and intron 7 in patient H 11.0 (c.821+1G>A) resulting in a splice site mutation. Panel E: a CT 2-bp deletion in exon 27 in patient H65.0 (c.2864_2865delCT) resulting in mutation p.Pro955ArgfsTer95. The mutation sites are highlighted in red brackets. Panel B, D and F illustrate the corresponding normal sequences.

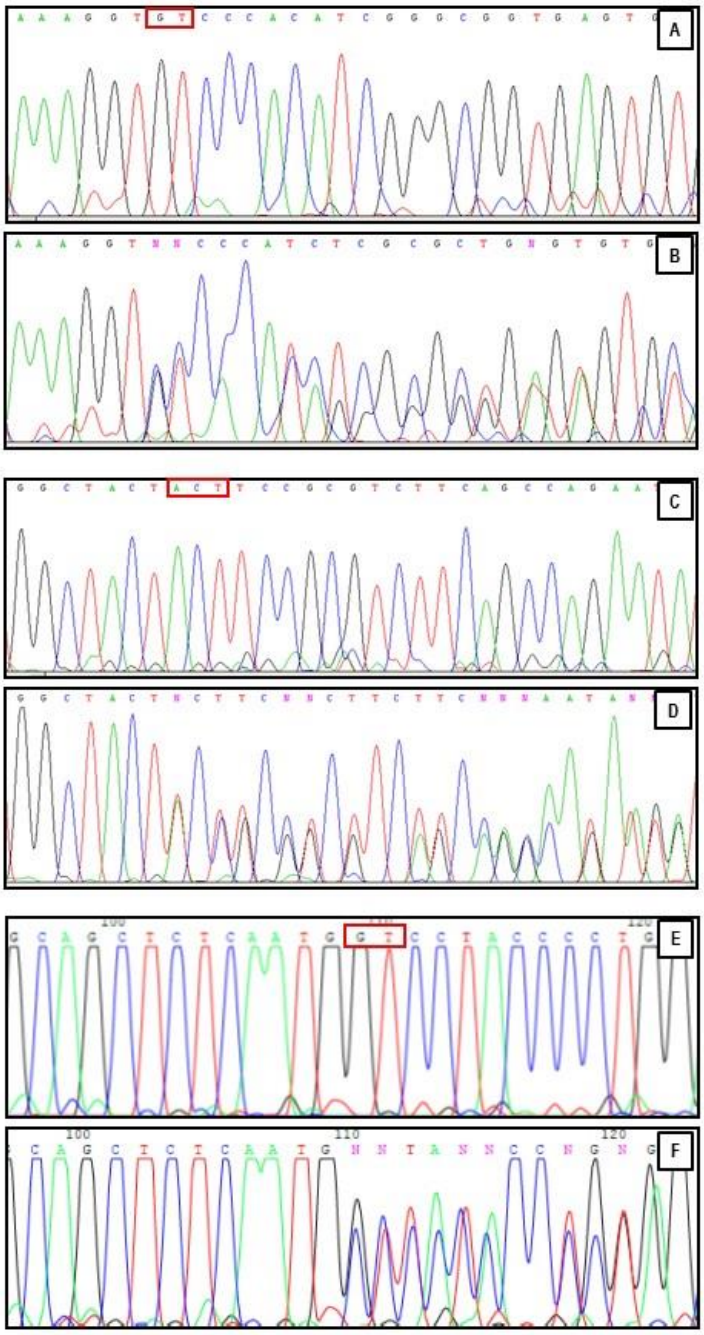

Figure 5. Electropherograms of MYBPC3 gene mutations identified in Hungarian HCM patients. Panel A: a GT 2-bp microdeletion in exon 18 in patient H 92.0 (c.1776_1777delGT), leading to mutation p.Ser593ProfsTer11; Panel C: an ACT 3-bp microdeletion in exon 31 in patient H 76.0 (c.3407_3409delACT), leading to the deletion of a single amino acid, p.Tyr1136del; Panel E: a GT 2-bp microdeletion in exon 4 in patient H 55.0 (c.431_432delGT), leading to mutation p.Gly144AlafsTer8. The mutation sites are highlighted in red brackets. Panel B, D and F illustrate the corresponding normal sequences.

4.1.2. Clinical data

The main clinical parameters of the patients with *MYBPC3* mutations are shown in *Table 5*.

patient ID	mutation	gender	age (at diagnosis, year)	LVmax (mm)	hypertrophy localisation	follow up
H 16.0	p.Gln1233Ter	female	43	32	ASH	death due to stroke at the age of 49 years
H 11.0	c.821+1G>A	male	21	26	ASH	transformation into dilated phase, AF
H 65.0	p.Pro955ArgfsTer95	female	54	25	ASH	alive, no significant progression
H 92.0	p.Ser593ProfsTer11	male	43	16	ASH	SCD at the age of 59 years
H 76.0	p.Tyr1136del	male	54	26	ASH	SCD at the age of 56 years
H 55.0	p.Gly144AlafsTer8	female	40	24	ASH	alive, no significant progression

Table 5. The main clinical parameters of the index patients carrying MYBPC3 gene mutations. ASH: asymmetric septal hypertrophy; SCD: sudden cardiac death, LVmax: maximum left ventricular wall thickness.

4.1.2.1. c.3697C>T, p.Gln1233Ter mutation

We identified the p.Gln1233Ter mutation of the *MYBPC3* gene (*Figure 4, Panel A and B*) in a female proband (H 16.0) who was diagnosed with HCM at age 43 after presenting with signs of diastolic heart failure. Echocardiography revealed marked septum hypertrophy (maximum left ventricular wall thickness: 32 mm) without left ventricular outflow tract obstruction. During follow up thinning of the septum with the decrease of the left ventricular ejection fraction and increase of mitral regurgitation was noted. She died suddenly at age 49, due to cerebral embolism from a left ventricular thrombus, according to autopsy data. The case history of the patient - who was the first patient in Hungary with a *MYBPC3* mutation identified - has already been described in detail in a previous publication (42).

4.1.2.2. c.821+1G>A mutation

The second causative *MYBPC3* mutation was identified in a 54-year-old male patient (H 11.0), who was first hospitalized due to chest pain at the age of 21. HCM was diagnosed with moderate septal hypertrophy (maximal left ventricle wall thickness: 26 mm) and with the phenomenon of ’systolic anterior motion’ (SAM) on echocardiography. Past medical history of the patient revealed that that he was also deaf-mute (19). During last follow-up, at the age of 53, septal thickness of 22 mm, enlarged left atrium (61 mm), enlarged left

ventricular dimensions (LV end-diastolic diameter: 59 mm, LV end-systolic diameter: 36 mm), reduced left ventricular ejection fraction (48%) were measured with the disappearance of SAM phenomenon and the development of atrial fibrillation. The clinical characteristics of the case was published previously as Csanady's cardiomyopathy (53) and was included into the London Dysmorphology Database.

Genetic analysis revealed a G-A transition at the border of exon 7 and intron 7 of the *MYBPC3* gene that affects the splice donor site of exon 7 (c.821+1G>A, *Figure 4, Panel C and D*). The mutation also affects the recognition site of restriction endonuclease *Eco72I*, so the mutation was verified by endonuclease digestion (*Figure 6*).

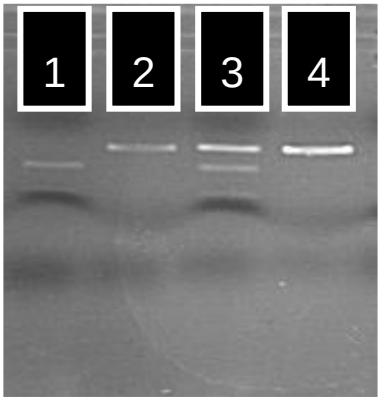

Figure 6. Eco72I restriction fragment analysis of the c.821+1G>A mutation. Due to the mutation, a restriction site is lost for the restriction enzyme Eco72I, and after digestion additional, undigested fragments are seen in the mutant samples. Lane 1: wild type, Eco72I digested; lane 2: wild type, non-digested; lane 3: mutant, Eco72I digested; lane 4: mutant, non-digested.

4.1.2.3. c.2864_2865delCT, p.Pro955ArgfsTer95 mutation

The third *MYBPC3* gene mutation was identified in a 68-year-old female patient (H 65.0) who was first hospitalized at the age of 54 due to chest pain. On echocardiography, non-obstructive HCM was diagnosed that caused moderate left ventricle hypertrophy (maximum LV wall thickness: 25 mm). Risk stratification indicated low risk for sudden cardiac death. Using combined beta-blocker and calcium channel blocker therapy the patient is in a stable clinical status, no progression was seen at the last follow up. In her family one of her brothers also suffers from HCM.

In exon 25 of the *MYBPC3* gene a 2 base-pair CT microdeletion was identified by molecular genetic testing (c.2864_2865delCT, p.Pro955ArgfsTer95, *Figure 4, Panel E and*

F). The mutation is a frame-shift mutation that causes a p.Pro955Arg amino acid change after the normal alanine amino acid in position 954, which is followed by the incorporation of 95 aberrant amino acids before the translation stops at this point due to a hidden stop codon (*Figure 7*).

4.1.2.4. c.1776_1777delGT, p.Ser593ProfsTer11 mutation

The fourth *MYBCP3* gene mutation was identified in a 43-year-old male patient (H 92.0) whose disease manifested with symptoms of chest pain occurring at rest, dyspnea and pre-syncope. On echocardiography mild septal hypertrophy (maximal left ventricular wall thickness: 16 mm) and a non-significant left ventricular outflow tract gradient was detected. Over the years his disease progressed into dilated phase with increasing left ventricular parameters and decreasing left ventricular ejection fraction. At his last follow-up echocardiography parameters showed septal thickness of 15 mm, left atrial diameter of 55 mm, left ventricular end-systolic diameter of 44 mm, left ventricular ejection fraction of 31%. The development of atrial fibrillation was also noted. His death occurred at the age of 59 suddenly.

Molecular genetic analysis detected a 2 base-pair (GT) microdeletion in exon 18 of the *MYBPC3* gene (c.1776_1777delGT, *Figure 5, Panel A and B*). The mutation leads to a frame shift that causes a p.Ser593Pro amino acid change after the normal lysine amino acid in position 592 followed by incorporation of 11 aberrant amino acids before the translation stops due to a hidden TGA stop codon (p.Ser593ProfsTer11, *Figure 7*).

4.1.2.5. c.3407_3409delACT, p.Tyr1136del mutation

The p.Tyr1136del mutation of the *MYBPC3* gene was detected in a male patient (H 76.0) who was diagnosed with HCM at age of 54 years after an accidental discovery of a heart murmur. Echocardiography showed significant asymmetric septal hypertrophy (maximal left ventricular wall thickness: 26 mm) with a non-significant LV outflow tract gradient (18 mmHg). Risk stratification indicated a low risk profile, however, during his follow-up loss of consciousness occurred 2 times under exercise. His death occurred at the age of 56 suddenly. Mutation analysis indicated a 3 base-pair (ACT) microdeletion in exon 31 of the *MYBPC3* gene (c.3407_3409delACT, *Figure 5, Panel C and D*). Interestingly, the mutation leads to an in-frame deletion of only one amino acid, tyrosine, in position of 1136 (p.Tyr1136del, *Figure 7*).

4.1.2.6. c.431_432delGT, p.Gly144AlafsTer8 mutation

The p.Gly144AlafsTer8 mutation of the *MYBPC3* gene was detected in a female patient (H 55.0) with HCM, who was diagnosed with HCM at age of 40 years, when she was hospitalized because of chest pain and dyspnea. Echocardiography revealed a maximum left ventricular wall thickness of 24 mm with the appearance of non-obstructive HCM. During her follow up left ventricular outflow tract obstruction (43 mmHg) and subsequent enlargement of the left atrium was detected (53 mm).

Her genetic analysis confirmed a GT microdeletion (c.431_432delGT, *Figure 5, Panel E and F*) in the exon 4 of the *MYBPC3* gene. The mutation leads to a p.Gly144Ala amino acid change after the normal asparagine amino acid in position 143 followed by incorporation of 8 aberrant amino acids before the translation stops due to a hidden stop codon (p.Gly144AlafsTer8, *Figure 7*).

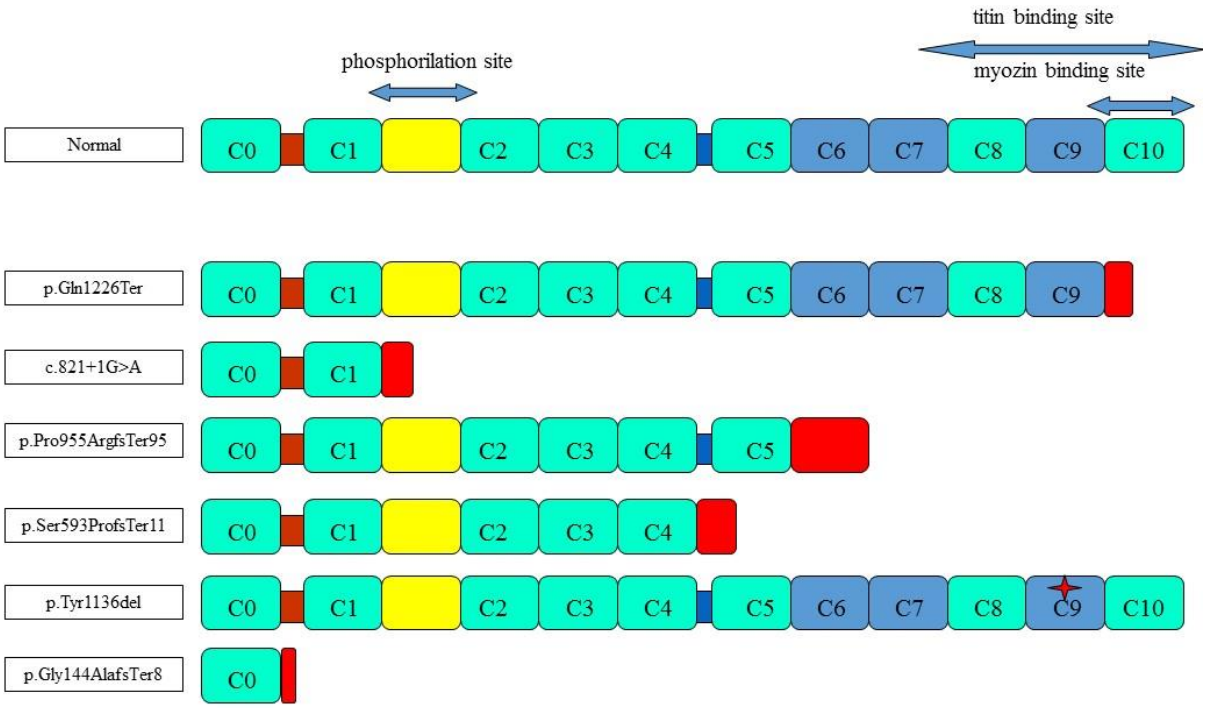

Figure 7. Presumed consequences of the MYBPC3 gene mutations identified in Hungarian patients with HCM. The eleven domains of the cMyBP-C protein (C0-C10), including the eight immunoglobulin I (IgI) domains (green) and three fibronectin III (FnIII) domains (blue) are shown. The phosphorylation, myosin- and titin binding sites are indicated. The presumed truncated parts of the mutant protein are shown in red.

4.2. Clinical and genetic investigations of families of patients with hypertrophic cardiomyopathy carrying *MYBPC3* gene mutations

4.2.1. Mutation data

Mutation carrier status was verified in 30 of the 62 (48%) investigated family members (p.Gln1233Ter: 3/7, c.821+1G>A: 7/20, p.Pro955ArgfsTer95: 15/30, p.Ser593ProfsTer11: 2/2, p.Tyr1136del: 3/3; *Table 6*). The mutation occurred in heterozygous form in every family member.

family	mutation	screened family member (n)	mutation carrier (n)	clinically affected (n)
H 16	p.Gln1233Ter	7	3	1
H 11	c.821+1G>A	20	7	5
H 65	p.Pro955ArgfsTer95	30	15	2
H 92	p.Ser593ProfsTer11	2	2	1
H 76	p.Tyr1136del	3	3	1
All		62	30	10

Table 6. Main data of the genetic and clinical screening in the MYBPC3 gene mutation carrier families.

4.2.2. Clinical data

Clinical diagnosis of HCM could be verified in 10 of the 30 (33%) mutation carrier family members, including the index patients. The main clinical parameters of the mutation carrier index patients and the affected family members are included in *Table 7*. HCM was verified in 5 of the 7 mutation carrier family members in family H 11, and in 2 of the 15 mutation carrier family members in family H 65. In the remaining three families (H 16, H 76, H 92) there was no clinically manifest family member with HCM, except for the index cases.

In family H 65, the proband's brother (H 65.3) was verified with HCM whose disease was diagnosed at age of 76. His disease was characterised by asymmetric septal hypertrophy, the maximum left ventricle wall thickness was 17 mm, without outflow tract gradient. During risk stratification low risk was verified. He deceased at the age of 83 which was unrelated to HCM (gastric cancer). Family details regarding family H 11 is described under 4.3.

In clinically affected mutation carriers the diagnosis of HCM was established at the age of 27 the earliest, and at the age of 76 the latest. Nine patients were diagnosed with HCM

above the age of 40, and 6 patients above the age of 50 years. In case of the clinically affected mutation carriers the average age at the diagnosis was significantly higher than in case of the clinically not affected family members (51±13 vs. 38±17 years, p=0.028).

During the 10±8 years (median: 8 years) follow-up period 6 patients died out of the 10 clinically affected mutation carriers (60%) at the average age of 58±10 years, 9±4 years after

patient ID	mutation	gender	age (at diagnosis, year)	LVmax (mm)	hypertrophy localisation	follow up
H 16.0	p.Gln1233Ter	female	43	32	ASH	death due to stroke at the age of 50
H 11.0	c.821+1G>A	male	21	26	ASH	transformation into dilated phase, AF
H 11.1	c.821+1G>A	male	63	26	ASH	SCD at the age of 72
H 11.15	c.821+1G>A	male	42	26	ASH	SCD at the age of 51
H 11.22	c.821+1G>A	male	52	13	ASH	alive, no significant progression
H 11.3	c.821+1G>A	male	59	15	ASH	AF, endocarditis, AVR, MVR
H 65.0	p.Pro955ArgfsTer95	female	54	25	ASH	alive, no significant progression
H 65.3	p.Pro955ArgfsTer95	male	76	17	ASH	death at the age of 83, unrelated to HCM
H 92.0	p.Ser593ProfsTer11	male	43	16	ASH	SCD at the age of 59
H 76.0	p.Tyr1136del	male	54	26	ASH	SCD at the age of 56

Table 7. The main clinical parameters of the clinically manifest probands and family members with HCM who carries the MYBPC3 gene mutation. ASH: asymmetric septal hypertrophy; SCD: sudden cardiac death, LVmax: maximum left ventricular wall thickness, AVR: aortic valve replacement; MVR: mitral valve replacement.

the diagnosis. In 4 cases the cause of the death was sudden cardiac death, in one case it was stroke and in the last case the death was unrelated to HCM (gastric cancer). In case of the 20 clinically unaffected mutation carriers no death occurred.

4.3. Observation of extreme phenotypic variability in a HCM family carrying the *MYBPC3* c.821+1G>A gene mutation

In family H 11 five patients were diagnosed with manifest HCM (*Figure 8*). The index patient's case history was already detailed under 4.1.2.2. The index patient's father (H 11.1) was diagnosed with the disease at the age of 63 (interventricular septal thickness: 26

mm), and died suddenly at the age of 72. In case of the younger brother of the index patient (H 11.15) hypertrophic cardiomyopathy has been developed over the time, in the form of typical ASH. A manifest cochlear hearing loss was also associated, diagnosed at the age of 48 (the cochlear loss of hearing was unequivocal left sided, on the right it was moderate). During cardiology follow-up, the echocardiography parameters or symptomatic status of the patient showed no progression, nevertheless, at the age of 51 he died suddenly while sleeping. In case of the older brother of the index patient (H 11.22) we also observed the development of

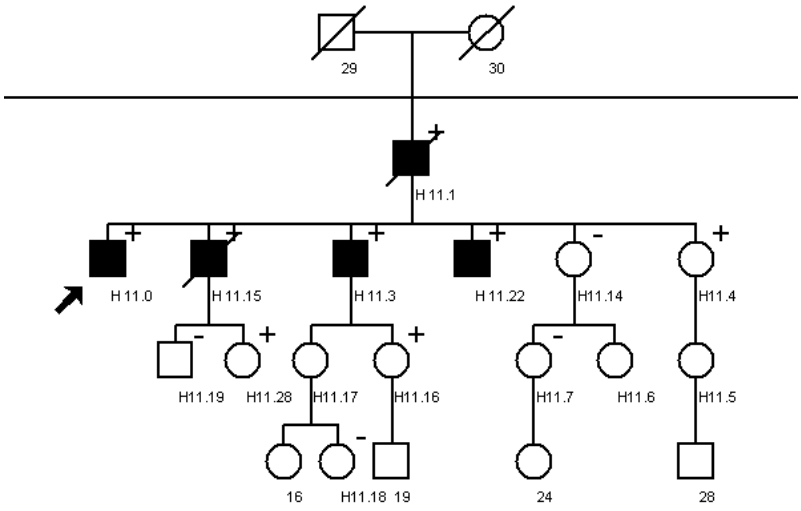

Figure 8. Part of pedigree of the MYBPC3 c.821+1G>A mutation carrier HCM family. Squares and circles denote males or females, respectively; filled symbols represent clinically affected family members. Arrow points to the index patient. Deceased individuals are slashed. Mutation carriers are labelled with a plus (+) sign, non-carriers with a minus (–) sign.

hypertrophic cardiomyopathy in the form of asymmetric septal hypertrophy. Beside the current medical therapy the cardiac status of the patient is stable. The younger brother of the index patient (H 11.3) was also proved to have manifest hypertrophic cardiomyopathy. In his case the typical non-obstructive asymmetric septal hypertrophy was diagnosed at the age of 59 in a cardiology screening. One year later he was hospitalised several times due to cardiac decompensation and paroxysmal atrial fibrillation. Shortly thereafter bacterial endocarditis developed which necessitated aortic and mitral valves replacement. Besides family members manifesting HCM we observed a symptom-free mutation carriers (H 11.4, H 11.28, H 11.16), who are completely free of signs or symptoms of the disease, and has a normal ECG and echocardiography parameters. Thus, we observed an extremely variable

phenotype with regard to the *MYBPC3* c.821+1G>A gene mutation, including symptom-free mutation carrier, sudden cardiac death, progression into dilated phase and complications of infective endocarditis.

4.4. Analysis of the p.Gln1233Ter mutation of the *MYBPC3* gene in three carrier families

Further studies identified the *MYBPC3* p.Gln1233Ter mutation in three, apparently not-related index patients, including patient H 16.0, whose case history was given in 4.1.2.1. In the second, male index patient (H 214.0, see family tree, *Figure 9 and Table 8*) the diagnosis of HCM was done at age 33, based on cardiology assessment initiated because of frequent occurrence of syncope. Echocardiography showed moderate septum hypertrophy (21 mm), with systolic anterior movement of the anterior mitral valve leaflet and a left ventricular peak outflow tract gradient of 55 mmHg which rose to 88 mmHg on the Valsalva manoeuvre. Invasive measurement also verified significant resting (45-58 mmHg) and provocable (158-165 mmHg) obstruction. Based on his clinical condition and findings myectomy and later PM implantation was performed. In the third, female index patient (H 109.0, see family tree, *Figure 9 and Table 8*) HCM was diagnosed at age 62, based on echocardiography which was initiated because heart enlargement was detected on a routine chest X-ray film in the otherwise symptom-free patient. Echocardiography revealed moderate left ventricular hypertrophy, most pronounced at the interventricular septum (23 mm), and with left ventricular outflow tract peak resting gradient of 35 mmHg.

patient	sex	clinical status	age (at diagnosis or last FU, yrs)	LVmax (mm)	follow up
H 16.0	F	affected	43	32	died of stroke at age 49
H 16.1	F	not-affected	34	10	alive, without major symptoms
H 16.3	M	not affected	18	8	alive, without major symptoms
H 214.0	M	affected	33	21	myectomy, PM implantation
H 214.1	F	affected	14	33	alive, without major symptoms
H 214.2	M	not affected	37	12	alive, without major symptoms
H 109.0	F	affected	62	23	alive, without major symptoms
H 109.2	F	affected	38	18	alive, without major symptoms

Table 8. Major clinical parameters of the MYBPC3 p.Gln1233Ter mutation carrier family members. FU: follow up, LVmax: maximal left ventricular thickness.

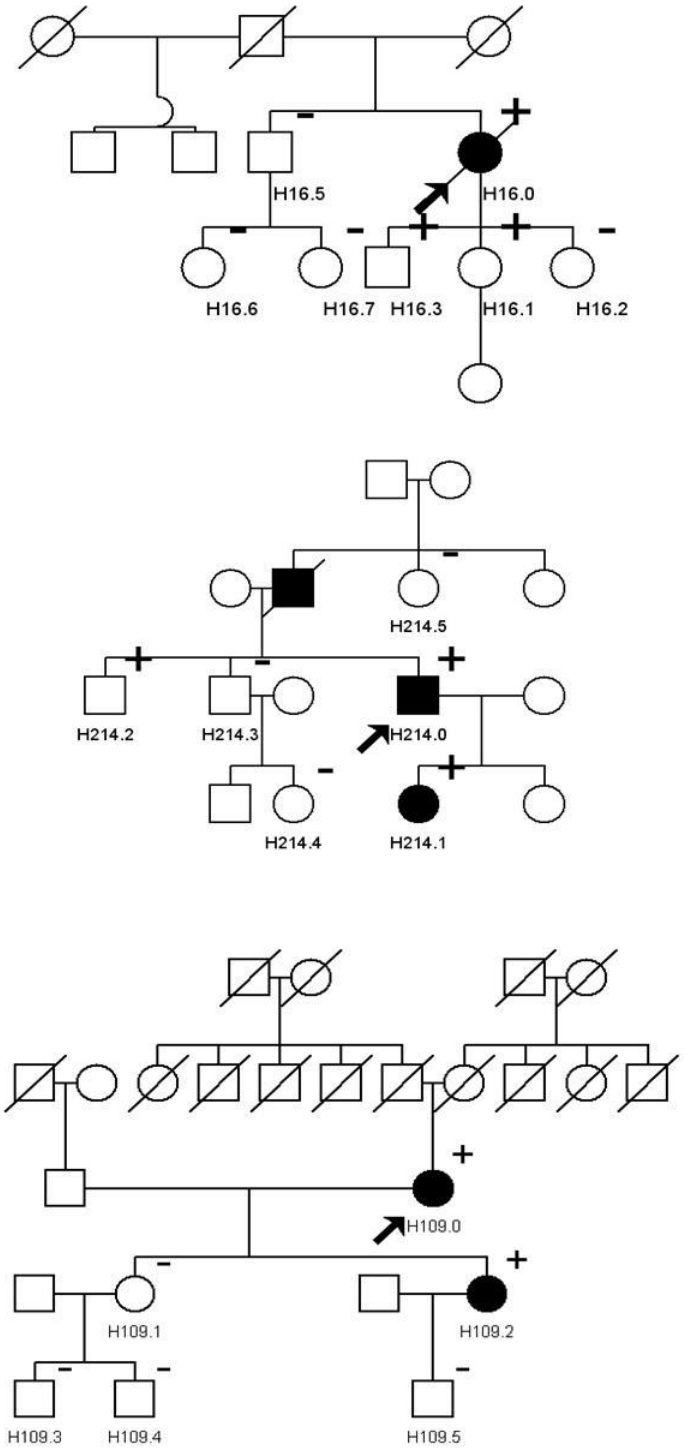

Figure 9. Pedigrees of the MYBPC3 p.Gln1233Ter mutation carrier HCM families. Squares and circles denote males or females, respectively; filled symbols represent clinically affected family members. Arrow points to the index patients. Deceased individuals are slashed. Mutation carriers are labelled with a plus (+) sign, non-carriers with a minus (–) sign.

Clinical and genetic family screening of the three families indicated 8 mutation carriers (including the index patients) among the 19 family members screened in the three families (*Figure 9* and *Table 8*). Of the 8 mutation carriers the clinical diagnosis of HCM was established in 5 family members (*Table 8*). Importantly, affected-to-affected transmission of the mutation was seen in two of the three families. Haplotype analysis did not prove a founder effect. The mutation was not identified in 149 apparently normal healthy controls (neither in 218 patients with dilated cardiomyopathy, nor in 97 other patients with hypertrophic cardiomyopathy; 928 chromosomes in total).

5. DISCUSSION

5.1. Identification of *MYBPC3* mutations in Hungarian patients with hypertrophic cardiomyopathy

According to literature data, the most frequently affected gene causing HCM is the *MYBPC3* gene. Initial studies reported a prevalence rate of 13-26% for the occurrence of *MYBPC3* gene mutations in HCM patient cohorts. Erdmann et al. reported a 13.6% prevalence (28) in the 110 patients she investigated, while Van Driest et al. found in 18% (37) of 389 patients, and Richard et al. found in 26% (35) of the 197 patients they assessed. Later studies on disease gene distribution in HCM confirmed a 10-35% occurrence rate of *MYBPC3* gene mutations in HCM (*Table 9*). The most recent figure for *MYBPC3* gene involvement is coming from studies which used next generation sequencing technology for genotyping. Lopés et al. studied 223 unrelated patients with HCM, in whom they analyzed coding, intronic and regulatory regions of 41 cardiovascular genes, by means of massive parallel resequencing. In the cohort they identified 46 *MYBPC3* gene variants, which gives a prevalence rate of 21% (54).

Cohort	Patients, No.	Screened genes, No.	Sarcomeric gene mutation (%)	*MYBPC3* gene mutation (%)	Sequencing methodology
Richard et al. (35)	197	9	63	26.4	SSCP+DS
Morner et al. (33)	46	8	28	20	SSCP /DHPLC
Erdmann et al. (28)	108	6	33.3	18.5	SSCP+DS or RFLP
Van Driest et al. (37-39)	389	8	37.7	18.2	DHPLC+DS
Olivotto et al. (34)	203	8	62	34	DHPLC+DS+RFLP
Garcia-Castro et al. (29)	120	5	26.7	16	DS
Andersen et al. (26)	90	11	36	10	SSCP+DS
Laredo et al. (31)+ Rodriguez-Garcia et al. (36)	130	2	23	15	SSCP+DS+RFLP
Millat et al. (32)	192	4	48	25	DHPLC+DS
Waldmuller et al. (40)	236	2	41	24	DS
Brito et al. (27)	77	5	53	35	DS
Gruner et al. (30)	471	8	35	14.9	DS
Zou et al. (41)	200	8	51	18	DS

Table 9. Studies on investigating gene mutations frequencies in different HCM cohorts. DHPLC, denaturing high performance liquid chromatography DS, direct sequencing; RFLP, restriction fragment length polymorphism; SSCP, single strand conformation polymorphism.

In the 45 analyzed Hungarian patients with HCM we identified 6 different causative *MYBPC3* mutations establishing a 13% prevalence rate of the *MYBPC3* gene mutations in the Hungarian HCM patient population. This occurrence rate is very well in agreement with literature data. Considering our previous data, where we found that the mutation rate of the beta myosin heavy chain gene (*MYH7*) in the Hungarian patient population was 5% (55), and in 100 Hungarian patients with HCM we found no troponin I (*TNNI3*) (56) or troponin T (*TNNT2*) mutations (57), the *MYBPC3* gene seems to be the most commonly affected gene in the Hungarian patient population with HCM.

Two of the mutations identified by us were point mutations (p.Gln1233Ter, c.821+1G>A), the others were 2-3 base pairs microdeletions (c.2864_2865delCT, c.1776_1777delGT, c.431_432delGT, c.3407_3409delACT). Three of the microdeletions produced "frame-shift" mutations that resulted in the incorporation of aberrant amino acids and then the activation of a stop codon, leading to truncated proteins at the C terminal end.

The c.821+1G>A point mutation that affects the junction site of exon/intron 7 is a classical splice-site mutation which also results in the activation of a hidden stop codon and the formation of a truncated protein, similarly to the p.Gln1233Ter point mutation which is a stop codon mutation. Due to the stop codon activation the translation of the protein stops, and exons located distally from the stop codon will be not transcribed and the C terminal part of the protein will be truncated. Except the p.Tyr1136del mutation all the mutations' probable effect is the deletion of the distal part of the protein, which part is responsible for the myosin and titin binding function of the protein. Direct evidence for the above could have been provided from the analysis of mRNA extracted from the heart muscle, but as no native myocardium was available it was not possible to directly prove it.

The fourth microdeletion is an "in-frame" deletion which causes the deletion of only one amino acid (p.Tyr1136del). At positions 1135-1136 of the cMyBP-C protein, which is part of the C9 motif, two Tyr locates. Both are evolutionally conserved amino acids that highlights a functionally important role [the Tyr1135 is identical in the human, in the mouse, in the chicken and in the fugu (Japanese pufferfish, *Takifugu rubripesis*); the Tyr1136 is identical in the human and in the mouse, in the chicken Phe, in the fugu Val can be found in identical position].

Three of the mutations we identified have already been published in the literature (p.Gln1233Ter, c.821+1G>A, p.Pro955ArgfsTer95). The p.Gln1233Ter mutation had been

already reported by three research teams. Erdmann et al. detected the mutation in two families with Turkish origin, where three mutation carrier were identified (49). The disease of the mutation carriers started between at the age of 25-41, their maximum left ventricular wall thickness were 17-20 mm. In four additional family members who were clinically relevant but genetically not tested, sudden cardiac death occurred. By the haplotype analysis of the mutant allele it was possible to detect that the apparently unrelated families inherited a common "founder" haplotype (so-called "founder" mutation). Van Driest et al. detected the p.Gln1233Ter mutation in the patient population of the Mayo Clinic, interestingly, they detected the variant in 2% of the 400 control reference alleles and on this basis they regarded the variant as a rare polymorphism (37). According to the 2005 publication of Ingles et al. the p.Gln1233Ter/Arg326Gln "compound" mutation of the *MYBPC3* gene was detected in only one member of a family, in whom the maximum left ventricular wall thickness was 28 mm (51). The c.821+1G>A mutation was also reported by three research group (discussed under 5.3). The p.Pro955ArgfsTer95 mutation is also known in the literature. It was first mentioned in the article of Niimura et al. published in 1998 (58), where it was proved in a four-generation family. Eighteen mutation carriers were described, from whom 8 family members were clinically affected. In that family 3 deaths occurred due to HCM. In the paper of the Mayo Clinic this mutation was also described (37). The three other mutations out of the six mutations we detected previously were not published (p.Ser593ProfsTer11, p.Gly144AlafsTer8, p.Tyr1136del). Although the p.Ser593ProfsTer11 mutation was not previously published, there is a report in the literature on the p.Val592fs mutation which is almost identical with the one we identified. The latter was described in a couple of Japanese families and was proven that it is a "founder" mutation (59). Interestingly, 23% of the mutation carrier 30 manifest patients with HCM transformed into end-stage dilated form. In case of our p.Ser593ProfsTer11 mutation carrier patient we also observed the progression into dilated phase, the development of atrial fibrillation and sudden cardiac death.

5.2. Clinical and genetic investigations of families of patients with hypertrophic cardiomyopathy carrying *MYBPC3* gene mutations

Analysing families of index patients with *MYBPC3* gene mutations we found that 30 of the 62 (48%) screened family members were mutation carriers, which ratio is in agreement

with autosomal dominant nature of inheritance. Among the 30 mutation carrier patients, we observed the development of HCM in 10 patients, including the index patients (33%).

With regard to the clinical presentation of the *MYBPC3* gene mutations earlier publications reported about non-significant left ventricular hypertrophy, mild symptoms and good prognosis. Charron et al. in their 1998 publication described the investigation of 76 patients with *MYBPC3* gene mutation, whom they found that the prognosis of patients with *MYBPC3* gene mutations was significantly better (no death under the age of 40), the symptoms of the disease occurred significantly later and the thickness of left ventricle was significantly less compared to patients with HCM carrying beta myosin gene mutation (60). Investigating 48 patients carrying the *MYBPC3* gene mutation Richard et al. perceived 55% benign, 35% intermediate and 10% malignant appearance forms in terms of cardiac complications including HCM related death due to sudden cardiac death, heart failure or stroke, heart transplantation and resuscitated cardiac death (35). Subsequent publications have found no difference between patients carrying *MYBPC3* mutations and patients carrying other HCM mutations. Erdmann et al. investigating 29 patients carrying *MYBPC3* mutations and 11 patients carrying beta myosin heavy chain mutations found no significant difference between the appearance of the disease and its main clinical parameters (extent of left ventricular hypertrophy, myectomy, necessity of septal ablation) (49). Investigating 63 patients carrying the *MYBPC3* mutations and 61 patients carrying beta myosin heavy or light chain mutations Van Driest et al. similarly found no substantive difference with regard to the clinical appearance (age at time of diagnosis, extent of left ventricle hypertrophy, necessity of myectomy or ICD implantation) (37).

In a recent meta-analysis, looking at genotype-phenotype correlations, Lopés et al. pooled patients from eighteen publications (corresponding to a total of 2459 patients). They reported that the presence of any sarcomere gene mutation was associated with a significantly younger age at presentation (38.4 vs. 46.0 years), a family history of HCM (50.6% vs. 23.1%), family history of SCD (27.0% vs. 14.9%). and greater maximal LV wall thickness (21.0 vs. 19.3 mm). There were no differences when the two most frequently affected genes, *MYBPC3* and *MYH7*, were compared (61).

In our patient population we observed a malignant appearance in the 10 clinically affected patients carrying *MYBPC3* mutations, 6 of them deceased at average age of 58 years. Five of the 6 above-mentioned deaths were probably HCM-related, including 4 cases of sudden cardiac death, and one case of stroke. In two additional patients, serious, HCM-related

progressive disease were observed with a transition into dilated phase and development of endocarditis. The 20 clinically not manifest family members carrying the mutation showed no signs or symptoms of the disease, seemed to be asymptomatic and no death occurred. The latter observation also suggest that not the mutation carrier status itself, but the clinical manifestation and characteristics of HCM determine prognosis.

Interestingly, in patients carrying *MYBPC3* gene mutation, HCM was possible to be diagnosed above 50 years which means prolonged clinical manifestation or long asymptomatic stage. That delayed clinical manifestation of the *MYBPC3* gene mutations is well documented in the literature. Niimura et al. investigating 212 mutation carrier members of 16 families found that it was possible to verify HCM in 58% of mutation carriers under the age of 50, and the penetrance of the mutation also did not reach 100% above the age of 60 (58). It is in sharp contrast with the HCM caused by mutations of the beta myosin heavy chain gene or the troponin T gene, in which the disease was almost fully penetrant by the age of 30.

In our patient population we also found 5 mutation carrier family members above the age of 50, and 2 family members above the age of 60, in whom HCM had not been developed yet. The oldest age when HCM was diagnosed was 76 years. The clinical relevance of these observations is that in young adults it cannot be stated that a family member did not inherit the disease, as in *MYBPC3* gene mutation carriers the disease manifests subsequently, maybe at an older age. In case of the above family members cardiology screening is recommended every 3-5 years or in case of complaints.

5.3. Observation of extreme phenotypic variability in a HCM family carrying the *MYBPC3* c.821+1G>A gene mutation

The *MYBPC3* c.821+1G>A mutation was first published by Niimura et al. (due to the different exon numbering in the form of Int8DSG+1A) in a two-generation family where 9 mutation carriers were observed and 5 of them were clinically affected (58). There was no death in the family related to HCM. In the families analysed by Erdmann et al. that mutation was also described in two unrelated German families where 5 mutation carriers were found (49). The disease occurred at the age of 24-59, the left ventricular wall thickness was 19-24 mm, and there was 1 myomectomy, 1 ICD implantation and 2 septal alcohol ablations among the family members. By the analysis of the patients' mRNA 2

aberrant transcriptions were detected in one case with the loss of the exon 7 and in the other case the exon 7/8, furthermore, with the activation of a hidden stop codon in the exon 9. About the two apparently unrelated German families a common ‘founder’ haplotype was identified. That mutation was also noticed in the report from the Mayo Clinic (37).

In the large Hungarian HCM family we investigated with the *MYBPC3* c.821+1G>A gene mutation we observed an extremely variable phenotype, including symptom-free mutation carriers, sudden cardiac death, progression into dilated phase and complications of infective endocarditis.

Early investigations looking at phenotype–genotype correlations in HCM observed a high degree of phenotypic variability which appeared to be partly explained by genetic heterogeneity (i.e. involvement of different disease genes). Some genes, or some particular mutations, were reported to be associated with a high risk for sudden cardiac death (e.g. *TNNT2* mutations or *MYH7* p.Arg403Gln mutation), modest LV hypertrophy (*TNNT2* mutations), or delayed cardiac expression (*MYBPC3* mutations) (58,60,62-64). Subsequent studies, based primarily on individual patients rather than families, have reported exceptions and more heterogeneous cardiac expression within a given gene or mutation. With currently available methods for phenotyping, no clear and consistent correlations are detected for most of mutations. Our ability to draw more accurate genotype–phenotype correlations over the spectrum of disease will improve in the future as more robust and quantitative methods to assess phenotype are identified, and as we are able to follow larger genotyped cohorts over time. At a broader level, patients with complex genotype and multiple simultaneous mutations may have more severe or early disease expression, related to a gene dosage effect. This was clearly described in small families (51,65-67) and recently suggested in a larger study. Finally, some studies have observed that patients with a pathogenic sarcomere mutation had an increased risk of cardiovascular events, especially heart failure, compared with patients with negative genetic testing and no sarcomere mutation (34,68,69).

5.4. Analysis of the *MYBPC3* p.Gln1233Ter mutation in three carrier families

The *MYBPC3* p.Gln1233Ter mutation had a debated consequence in the literature. The importance of the mutation has been questioned based on the arguments that no affected-to-affected transmission has ever been demonstrated and the mutation was found to be

present in apparently normal controls in some populations. In our study we were able to detect the p.Gln1233Ter mutation in three HCM families, and affected-to-affected transmission of the mutation was demonstrated in two of the three families. We also proved that the mutation was absent in a large cohort of control samples (928 chromosomes in total). Based on the above findings we suggested that the p.Gln1233Ter mutation of the myosin binding protein C gene is a true causative mutation for hypertrophic cardiomyopathy, because affected-to-affected transmission of the mutation has been shown for the first time in two families, and it was not found in a large number of control samples. In addition, the characteristics of the mutation, being a stop codon mutation, also suggested a causative role. The former identification of the mutation in some control population in a previous report may have indicated more the presence of silent mutation carriers without signs and symptoms of the disease. The latter was also demonstrated in our families, as three of the 8 mutation carriers did not manifest the disease by the age of 18–37 years.

Causation of a given genetic variant is impossible to prove with 100% certainty in most ‘real life’ clinical cases. However, the American College of Medical Genetics and Genomics and the Association for Molecular Pathology have recently developed a guideline to give recommendations on this issue (70). This report recommends the use of specific standard terminology—“pathogenic,” “likely pathogenic,” “uncertain significance,” “likely benign,” and “benign”—to describe variants identified in genes that cause Mendelian disorders. Moreover, this recommendation describes a process for classifying variants into these five categories based on criteria using typical types of variant evidence (e.g. population data, computational data, functional data, and segregation data). On the basis of the above guideline, there are multiple lines of evidence to characterize the identified p.Gln1233Ter mutation we report as ‘pathogenic’. The variant possess the following characteristics: 1. Null (nonsense) variant (PVS1: very strong evidence of pathogenicity); 2. absent from controls (PM2: moderately strong evidence of pathogenicity); 3. protein length changes as a result of in-frame deletions/insertions in a no-repeat region or stop-loss variants (PM3: moderately strong evidence of pathogenicity); 4. cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease (PP1: supporting evidence of pathogenicity); 5. patient’s phenotype or family history is highly specific for a disease with a single genetic aetiology (PP4: supporting evidence of pathogenicity).

The *MYBPC3* p.Gln1233Ter variant is currently listed with a comment of "with pathogenic allele" in the dbSNP database, and with comment of 'pathogenic' in the NCBI ClinVar database. According to the ExAC (ExAc Aggregated Populations) database, which provides data on the genotype of 60,706 unrelated individuals sequenced as part of various disease-specific and population genetic studies, the minor allele frequency of the p.Gln1233Ter variant is 0.000008%, pointing out that this variant is almost non-existent in the general population, which also strengthen the malignant nature of this variant.

6. SUMMARY AND CONCLUSIONS

1. We identified known and novel *MYBPC3* mutations in Hungarian patients with hypertrophic cardiomyopathy.

Six causative myosin binding protein C gene mutations were identified in 45 Hungarian patients with HCM. Similarly to the existing literature knowledge, most of the mutations are predicted to cause shorter-than-normal, truncated proteins. The clinical appearance of the mutations was heterogeneous with high mortality rate in the index patients.

2. We observed a prevalence of 13% of *MYBPC3* gene mutations in the Hungarian HCM patient cohort, which makes the gene the most frequently affected gene in the Hungarian HCM population.

The identification of 6 different causative *MYBPC3* mutations out of 45 Hungarian patients with HCM establish a 13% prevalence rate of the *MYBPC3* gene mutations in the Hungarian HCM patient population. Taking our previous data on disease gene distribution in Hungarian HCM population into consideration, the *MYBPC3* gene seems to be the most commonly affected gene in the Hungarian patient population with HCM.

3. We observed that HCM caused by *MYBPC3* mutations may manifest at older ages in adulthood and its appearance is not typical at young age. Once the disease develops its clinical course is not benign but has a high mortality rate and may carry and increased risk for sudden cardiac death and progression into dilated phase.

According to our data hypertrophic cardiomyopathy caused by the myosin binding protein C manifests especially at older ages, and its appearance is not typical at younger ages. Many patients were diagnosed with HCM above the age of 40, or even above the age of 50 years. The course of HCM caused by *MYBPC3* gene mutations which has already been manifested can be overtly malignant leading to sudden cardiac death or progression into the dilated phase. The latter observation also suggest that not the mutation carrier status itself, but the clinical manifestation and characteristics of HCM determine prognosis.

4. An extremely variable phenotype of the *MYBPC3* c.821+1G>A mutation was observed.

In the large Hungarian HCM family we investigated with the *MYBPC3* c.821+1G>A gene mutation we observed an extremely variable phenotype, including symptom-free mutation

carriers, sudden cardiac death, progression into dilated phase and complications of infective endocarditis.

5. Observing that the *MYBPC3* p.Gln1233Ter mutation was transmitted from affected-to-affected in two carrier families and that it was not present in a large number of controls we provided evidence that the variant is a causative mutation for HCM.

In our work we were able to detect the *MYBPC3* p.Gln1233Ter mutation in three carrier families, and affected-to-affected transmission of the mutation was demonstrated in two of the three families. We also proved that the mutation was absent in a large cohort of control samples (928 chromosomes in total). Based on the above findings we suggested that the p.Gln1233Ter mutation of the myosin binding protein C gene is a true causative mutation for hypertrophic cardiomyopathy. This observation is still holding true in the light of recent genetic data coming from large population studies.

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9. APPENDIX