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CONTRIBUTION OF SARCOMERIC GENE VARIANTS TO THE PREDICTION OF SUDDEN CARDIAC DEATH RISK IN FAMILIAL HYPERTROPHIC CARDIOMYOPATHY

AİLESEL HİPERTROFİK KARDİYOMİYOPATİDE SARKOMERİK GEN VARYANTLARININ ANİ KARDİYAK ÖLÜM RİSKİNİN ÖNGÖRÜLMESİNE KATKISI

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ABSTRACT

Objective: Hypertrophic cardiomyopathy (HCM) is one of sudden cardiac death (SCD) causes. This study aimed to identify high-risk pathogenic variants for SCD in the three sarcomeric genes with the most frequent mutations in HCM.

Material and Method: The study included 12 adult HCM index cases with a family history of SCD and/or HCM, and 31 of their family members. All the participants were evaluated with detailed cardiac examinations. The exonic regions of the *MYH7*, *MYBPC3* and *TNNT2* genes were analysed using CorTAG HCM1 resequencing arrays.

Results: Six pathogenic variants causing amino acid substitutions were found in 8 of the index cases with HCM. Five of them were identified as previously defined missense variants of Val698Ala, Arg719Trp, Met822Leu and Arg663Cys (in three cases) in the *MYH7* gene, and Arg102Trp in the *TNNT2* gene. For the first time in an HCM family with a history of late-onset SCD, Tyr525Asn and c.*27-21G> A variants in the *MYBPC3* gene were identified as compound heterozygous. These variants were not present in control subjects (n=777) from the Turkish population.

Conclusion: In this study, novel variants in the *MYBPC3* gene were identified in an HCM family with SCD history. However, there was no clear association between pathogenic variants and the risk of SCD.

Keywords: Sarcomeric gene variant, familial hypertrophic cardiomyopathy, sudden cardiac death

ÖZET

Amaç: Hipertrofik kardiyomiyopati (HKM), ani kardiyak ölümün (AKÖ) nedenlerinden biridir. Bu çalışmada, HKM'de en sık mutasyon bulunan üç sarkomerik gende, AKÖ için yüksek riskli patojenik varyantların belirlenmesi amaçlandı.

Gereç ve Yöntem: Çalışmaya, AKÖ ve/veya HKM için aile öyküsü olan 12 yetişkin HKM'li indeks vaka ve 31 aile üyesi dahil edildi. Tüm katılımcılar, kardiyolojik olarak değerlendirildi. *MYH7*, *MYBPC3* ve *TNNT2* genlerinin ekzonik bölgeleri, CorTAG HCM1 dizileme sistemi kullanılarak analiz edildi.

Bulgular: HKM'li indeks vakaların 8'inde, amino asit değişimine neden olan 6 farklı patojenik varyant bulundu. Bunlardan beşinin, *MYH7* genindeki Val698Ala, Arg719Trp, Met822Leu ve Arg663Cys (üç vakada) ve *TNNT2* genindeki Arg102Trp değişimlerinin daha önce tanımlanmış yanlış anlamlı patojenik varyantlar olduğu belirlendi. İleri yaşta AKÖ öyküsü olan bir HKM ailesinde, *MYBPC3* geninde Tyr525Asn ve c.*27-21G>A varyantlar bileşik heterozigot olarak ilk defa tanımlandı. Bu varyantlar, Türk popülasyonu kontrol örneklerinde (n=777) saptanmadı.

Sonuç: Bu çalışmada, AKÖ öyküsü olan bir HKM ailesinde yeni varyantlar tanımlandı. Ancak, HKM ailelerinde saptanan patojenik varyantlar ile AKÖ riski arasında net bir ilişki bulunamadı.

Anahtar Kelimeler: Sarkomerik gen varyantı, ailesel hipertrofik kardiyomiyopati, ani kardiyak ölüm

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INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a cardiac disease characterized by left ventricular hypertrophy, causing sudden cardiac death (SCD) or progressive heart failure (1-3). HCM exhibits familial aggregation usually with an autosomal dominant inheritance, occurring in about 1 in 500 individuals (1). The first gene localization of familial HCM was mapped to the locus of ß-MyHC on chromosome 14q12 in a large French-Canadian family in 1989 (4). From this date, more than 1400 variants in the sarcomeric and non-sarcomeric genes in HCM have been identified using various techniques (5-9).

Although, in recent times, more than 40 genes have been identified in HCM patients by new genetic sequencing (NGS) (8, 10), HCM is mostly due to pathogenic single nucleotide variants (SNVs) (53-85%) in the *MYH7* gene encoding cardiac ß-myosin heavy chain (ß-MyHC), the *MYBPC3* gene encoding cardiac myosin binding protein-C (cMyBP-C) and the *TNNT2* gene encoding cardiac troponin T (cTnT) (2, 3, 11).

In this study, our aim was to identify novel high-risk pathogenic variants in the *MYH7, MYBPC3* and *TNNT2* genes in Turkish HCM families with SCD history, and also to perform retrospective clinical evaluations of variant carriers.

MATERIAL AND METHOD

The participants of study

The study included 12 index HCM cases (8 male/4 female; age range at diagnosis 16–67 years) with a family history of HCM and/or SCD, and their 31 family members. Study subjects were evaluated with a clinical history, physical examination, electrocardiography and echocardiography. This study was conducted in accordance with the ethics standards of the Ethics Committee of the University of Istanbul, Istanbul Faculty of Medicine and with the Helsinki Declaration (1964), and informed consent was obtained from the study subjects.

Variant detection

The genomic DNA samples were extracted from peripheral leukocytes. The coding regions and flanking intronic sequences of the *MYH7, MYBPC3* and *TNNT2* genes were screened in 12 index cases using array-based resequencing (CorTAG HCM1 Mutation Detection Assay based on Affymetrix CustomSeq Resequencing Arrays). This assay comprised primer sets for the long-range amplification of all coding regions and intron flanks. Pools of fragmented PCR-products were then hybridized and analysed with high-density oligonucleotide probe arrays. All novel variants and previously reported pathogenic variants were confirmed with Sanger sequencing after touchdown polymerase chain reaction (PCR) and control of the amplicons by 2% agarose gel electrophoresis. Primer sequences are shown in Table 1. The individual and possible pathogenic variants were researched in index cases and family members using the RFLP-PCR method or direct Sanger sequencing. The usage restriction enzymes in the RFLP-PCR method for genotyping of variants were listed in Table 2. The selection of the variant specific restriction enzyme and the lengths of the fragments were determined using the software RestrictionMapper (http://www.restrictionmapper.org.)

Database analysis

dbSNP, ClinVar, VarSome, American College of Medical Genetics and Genomics-ACMG Standards (12) for interpretation classification of variants, and HGMD Professional (Human Genome Mutation Database; BIOBASE) were used to describe the variants in the sarcomeric genes. The variants detected in our HCM patients were also screened in 777 distinct individuals from the in-house exome database of the Advanced Genomics and Bioinformatics Research Center (IGBAM) as a control group.

In silico analysis

The pathogenicity and conservation scores of variants were checked in VarSome using *in silico* tools such as MutationTaster, DANN, SIFT, PROVEAN and GERP. Moreover, Alamut (Interactive Biosoftware, trial version 2.6, Rouen, France) was used to predict whether the novel intronic variants change the characteristics of the splice signals and exonic splicing enhancers (ESE) binding sites on genes. ESEs predictive tools were used to identify the ESEs site for serine/arginine proteins such as SF2/ASF, SC35, SRp40, and SRp55. And also, in this software, the splice site variants were analysed using five prediction tools (SpliceSiteFinder-like, NNSPLICE, GeneSplicer, Human Splicing Finder and MaxEntScan). Default thresholds were used for all the analyses.

RESULTS

In this study, disease-causing variants of the *MYH7, MYBPC3* and *TNNT2* genes in 12 HCM families were searched, and pathogenic variants were identified in eight of them (Figure 1). Twelve index cases had a left ventricular maximal wall thickness >15 mm, and had a family history of SCD and/or HCM. In the genetic analysis of three sarcomeric genes of the index cases, a total of fifteen single nucleotide variations (SNV) were detected. Only six (five already defined and one novel) of these SNVs were pathogenic variants causing amino acid substitutions. Two novel uncertain significance intronic variants as well as seven non-pathogenic variants were also detected (Table 1). Minimal allele frequencies, gene localizations, primer sequences, restriction enzyme type for RFLP-PCR analysis of these variants are demonstrated in Table 1. The minimum allele frequencies of all SNVs in the Turkish population were checked in 777 controls of the in-house exome data-

Table 1: The general information of the identified SNVs in index cases with HCM

The variant position on reference transcript	SNV-ID	MAF	Amino acid substitution	Clinical significance (HGMD)	ACMG Classification	Localization	FP-5'>3'	RP-5'>3'	RE (Amplicon length)	Index cases
NM_000257.3 (*MYH7*)										
c.639+32G>T	novel intronic	0		vus	PM2, BP4	7. intron	cagttgtggattttgggtgt	ggaagagagtggtgatgagt	Bse MII (514 bp)	TK1
c.1062C>T	rs735712	51/1000	p.Gly354Gly	benign	PM1, BA1, BP6, BP7	12. exon	ggtatgggagtctcagaacc	agaagagagatgactgctga	Hae II (337 bp)	T1, A1, N1
c.1755C>A	rs201860580	1/1000	p.Ile585Ile	VUS, benign	PM1, BP6, BP7	16. exon	agcctactaccttaacaccc	aacctgacattgaagtggtg	SfaN I (526 bp)	TK1
c.1579-53C>T	rs45448096	1/1000		likely benign	BS1, BP4	16. intron	agcctactaccttaacaccc	aacctgacattgaagtggtg	ApoI (526 bp)	A1
c.1987C>T	rs397516127	0.0000165/2#	**p.Arg663Cys**	pathogenic (CM973126)	PM1, PM2, PM5, PP2, PP3	18. exon	tcatctctgtgacttctcga	atgtccatcagagtgcctta	Non* (424 bp)	K2, M3, N1
c.2093T>C	rs397516130	0	**p.Val698Ala**	likely pathogenic (CM054010)	PM1, PM2, PP2, PP3, PP5	19. exon	tcagaacccagaacttcagt	aggaaaagcatcagaggagt	Alf I (518 bp)	H1
c.2155C>T	rs121913637	0	**p.Arg719Trp**	pathogenic (CM941086)	PM1, PM2, PM5, PP2, PP3, PP5	19. exon	tcagaacccagaacttcagt	aggaaaagcatcagaggagt	Non* (518 bp)	D4
c.2464A>C	rs730880742	0	**p.Met822Leu**	pathogenic (CM138708)	PS1, PM1, PM2, PM5, PP2, PP5	22. exon	tatgaacacactgcagtcac	tcctctgactgaaggaacaa	EcoR II (536 bp)	KY1
NM_000256.3 (*MYBPC3*)										
c.472G>A	rs3729986	33/1000	p.Val158Met	benign	PM1, PP2, BA1, BP4, BP6	4. exon	tgggtgacagagcaagac	ctctccatgtcccctctct	BspH I (442 bp)	AO1
c.706A>G	rs3729989	0.100/12114#	p.Ser236Gly	benign (CM043536)	PM1, PP2, BA1, BP4, BP6	6. exon	ggggattacaggcctgag	ctaccctgcaagccttgg	Bbv I (406 bp)	M3, Y1**, N1
c.786C>T	rs11570058	61/1000	p.Thr262Thr	benign	BA1, BP6, BP7	7. exon	gtcatgaatgggcaagtctg	cagtgctgggatttggag	Gsu I (651 bp)	M3, Y1**, N1
c.1573T>A	novel exonic	0	**p.Tyr525Asn**	likely pathogenic	PM1, PM2, PP2, PP3	17. exon	tccctctctctctccttt	caagccctaaagcctcatg	Non* (803 bp)	T1
c.*27-21G>A	novel intronic	0		vus	PM2, BP4	34. intron	gttccatgtttgtttccagc	agacattgtttcttgaggcc	Non* (852 bp)	T1
NM_000364.3 (*TNNT2*)										
c.304C>T	rs397516456	0.00000824/1#	**p.Arg102Trp**	pathogenic (CM971501)	PM1, PM2, PM5, PP2, PP3, PP5	9. exon	gtagagcataggtgggatgg	gagacctcacacctagctg	Bsr I (458 bp)	Y1
c.601-296C>T	rs374006913	2/1000		benign	BS1, BS2, BP4	12. intron	catgtctctccttgcctttg	ggaaaatatgtgaggcagtcc	Hae II (699 bp)	AO1

The bolds are missense pathogenic variants, MAF: Minor allele frequency of in-house data (or public data, #ExAC), HGMD: Human Genome Mutation Database, RE: the restriction enzyme used in RFLP-PCR method, Non*; confirmed by PCR and Sanger sequencing, FP: forward primer, RP: reverse primer, ** homozygote for related variant, the abbreviations is according to ACMG guideline (Richards *et al.* 2015), PM1; location in a mutational hot spot and/or critical functional domain, PM2; absent from control databanks, PM5; novel missense variant, BA1; allele frequency is greater than 5%, PP2; the relation with mechanism of disease for missense variant, PP3; multiple lines of computational evidence support a deleterious effect, PP5; reputable source recently reports variant as pathogenic, PS1; same amino acid change as a previously established pathogenic variant regardless of nucleotide change, BP4; impact on gene in computational analysis, BS1; allele frequency is greater than expected for disorder, BP6; in recently reports variant as benign, BP7; a silent variant, BS2; the observation in a healthy adult individual for a disorder with full penetrance expected at an early age.

Table 2: The clinical characteristics of index cases and variant carriers in HCM families.

IDs of cases	Current age or age of death, years	Age of diagnosis, years	Gender	Diagnosis	Syncope/ presyncope	Shortness of breath (NYHA)	Left atrium size (cm)	LV end-diastolic diameter (cm)	LV end-systolic diameter (cm)	LV maximal wall thickness (cm)	LV ejection fraction (%)
Y2	46	35	M	1	0	I	3.74	3.90	2.29	2.7	73
Y1	43	32	M	1	0	I	3.21	3.65	2.05	2.20	76
T1	79	67	F	1	0	II	5.05	4.18	2.41	2.86	74
T4	47	47	F	0	0	I	3.10	4.60	2.80	1.00	69
T5	43	43	M	1	0	I	3.58	4.78	2.70	1.60	75
KY1	45	31	F	2	0	I	5.9	4.94	2.70	2.18	78
KY2	24	24	M	0	0	I	2.80	4.20	2.50	1.00	65
KY4	56	42	M	2	0	I	4.40	3.60	1.40	2.32	92
KY8	26	26	M	2	0	I	4.20	3.60	1.50	2.26	88
KY9	20	20	F	2	0	I	4.30	4.40	2.40	2.24	67
KY10	18	18	M	2	0	I	4.20	4.60	2.80	2.40	69
KY12	40	40	M	0	0	I	3.50	4.90	3.20	1.10	65
KY13	20	20	M	0	0	I	3.10	4.80	3.00	1.10	66
KY15	15	15	M	0	0	I	2.80	3.70	2.30	1.10	70
KY16	41	41	M	1	0	I	4.30	3.70	2.00	1.80	80
D4	32	22	M	1	0	I	4.10	5.04	3.43	2.81	68
D1	67	57	M	1	0	I	4.30	5.00	3.30	2.30	65
H1	59	39	F	1	0	II	4.80	4.70	2.60	2.39	75
H3	32	20	F	1	0	I	2.77	4.25	2.14	2.34	87
N1	55	45	M	1	1	II	4.60	4.70	3.40	2.50	55
M3	27	19	M	1	0	I	4.14	4.90	3.27	1.73	62
M1	44	39	F	1	0	I	4.35	4.47	2.41	2.37	77
K2	17	16	M	2	0	I	3.61	3.10	1.07	2.10	93
K1	42	42	M	1	0	I	3.70	4.05	2.45	2.40	71
HT1	54	54	M	3	1	I	4.60	4.14	1.70	2.20	89
A1	28	28	M	1	1	II	4.88	4.49	2.78	3.30	69
TK1	54	54	M	1	0	I	4.31	3.90	2.55	1.90	66
AO1	66	57	F	2	0	III	5.66	3.06	2.29	2.13	52

Diagnosis groups; 1, nonobstructive asymmetric septal hypertrophy; 2, obstructive asymmetric septal hypertrophy; 3, nonobstructive concentric hypertrophy, the bolds are index cases.

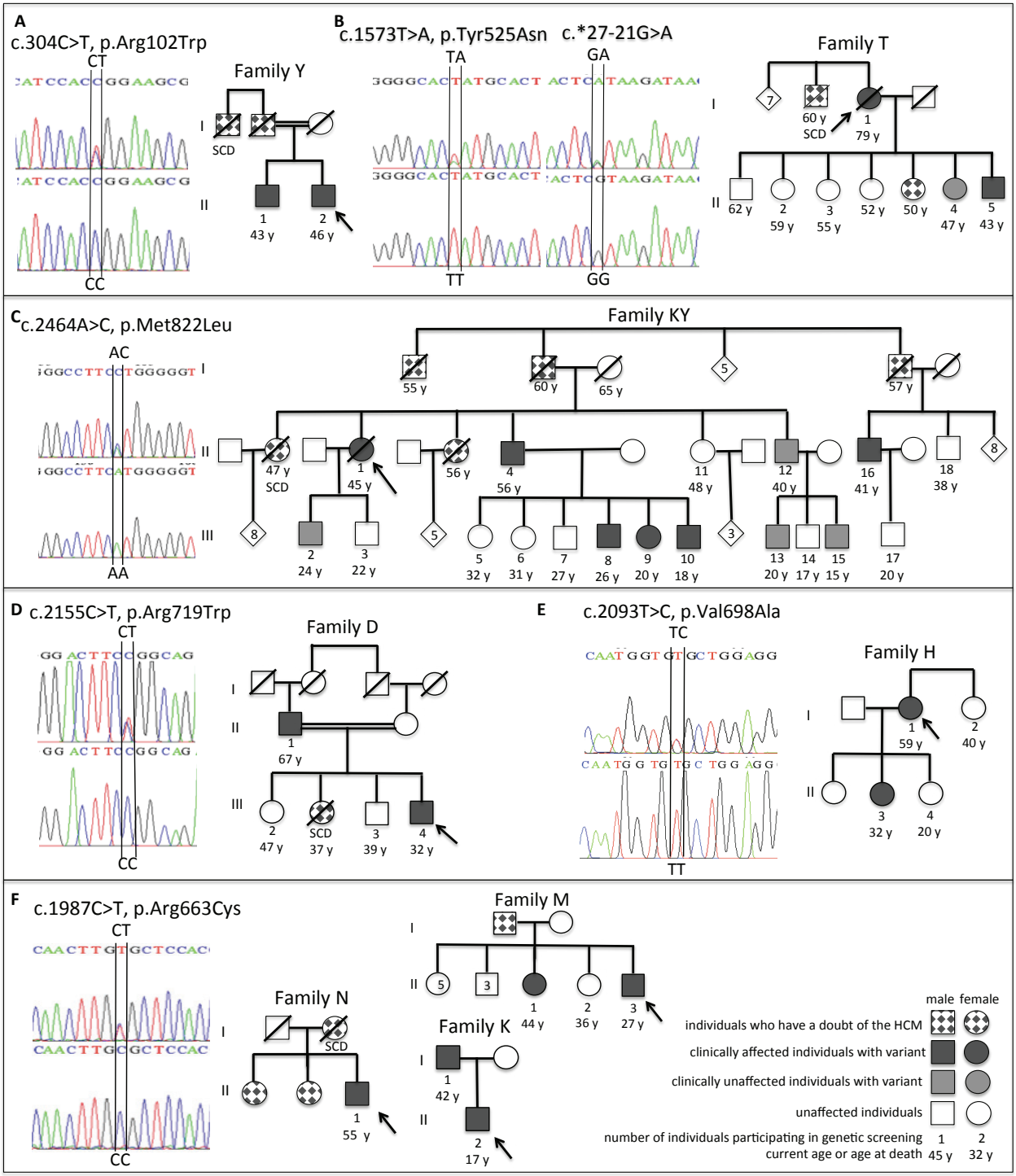

Figure 1: The family screening of the identified clinical significance variants in the *TNNT2* (A), *MYBPC3* (B) and *MYH7* (C-F) genes. Index cases are indicated with arrows.

base and also in public databases (ExAC) (Table 1). The pathogenic variants causing amino acid substitutions and two novel intronic variants (c.639+32G>T in *MYH7* and c.*27-21G>A in *MYBPC3*) had not been previously identified in this Turkish in-house database. The main clinical data of the 12 index cases and other clinically diagnosed family members with HCM is summarised in Table 2.

The pathogenic variants of *TNNT2* and *MYBPC3* genes

The previously described Arg102Trp missense variant (also called Arg92Trp) in the *TNNT2* gene was found in two brothers with HCM and a positive family history for SCD in Family Y with consanguineous marriage (Figure 1A). The uncle of the index case had SCD at a young age without clinical diagnosis.

Two novel p.Tyr525Asn and c.*27-21G>A compound heterozygous variants in the *MYBPC3* gene were identified in another Turkish family with HCM and a positive family history for SCD (Figure 1B). The c.*27-21G>A variant in *MYBPC3* was segregated with a novel Tyr525Asn missense variant in Family T. A genetic analysis could not be performed in a member of Family T with SCD at 60 years old. Index case (T1), with compound heterozygous variants, had mild clinical HCM and possible late onset hypertrophy. Cases T4 and T5 had these compound variants but only T5 had HCM, and T4 with a normal echocardiography (Table 2). Cases T2 and T3 had no variant with normal echocardiographic findings (Figure 1B).

The pathogenic variants of *MYH7* gene

The other four variants causing amino acid substitutions in the *MYH7* gene were previously reported missense pathogenic variants in patients with HCM according to variant databases. The Met822Leu variant in exon 22 of the *MYH7* gene was screened in 18 members of Family KY with a positive family history for SCD (Figure 1C). Ten family members were found to have this variant, out of which 6 had severe cardiac hypertrophy. Other 4 asymptomatic pathogenic variant carriers (called as KY2, KY12, KY13 and KY15) had normal echocardiographic findings. The unaffected family members without this variant had no cardiac disease. However, asymptomatic case KY12 and his two sons (called KY13 and KY15) had this variant. The Met822Leu variant site was conserved among the genomes of 35 mammals (GERP score; 4.6), and was determined as disease causing *in silico* tools (MutationTaster, FATHMM and SIFT).

The Arg719Trp variant in the *MYH7* gene was determined in an index case with cardiac defibrillator (implanted for documented ventricular tachycardia) in Family D (Figure 1D). Although, this pathogenic variant seems to be associated with a positive family history for SCD (sister with SCD at age 37 without documentation of HCM), the father (HCM documented) of the index case had a good prognosis without cardiac events.

The Val698Ala pathogenic variant was found to be related with severe cardiac hypertrophy in Family H (Figure 1E). However, there was no SCD history in this family.

The Arg663Cys pathogenic variant was identified in index cases of three unrelated families (Figure 1F). These three index cases (N1, K2 and M3) also have additional individual variants (Table 1). The HCM diagnosis age of this variant carriers (n=4) was less than 42 years old in Family K and Family M. This variant was also found in the index case (N1) of Family N (mother with SCD without HCM diagnosis). The cases of two other families (Family M and Family K) without a history of SCD had mild clinical forms of HCM. The clinical characteristics of the cases diagnosed HCM that were called N1, M1, M3, K1 and K2 in these families are shown in Table 2.

In silico analysis of novel intronic variants

Two novel clinically uncertain significance variants in the intron 7 of *MYH7* (c.639+32G>T) and in the intron 34 of *MYBPC3* (c.*27-21G>A) were identified in two HCM patients with a family history for SCD (TK1 and T1 called index cases, respectively). The conservation score among the genomes of 35 mammals were calculated as 3.51 for c.639+32G>T and 4.75 for c.*27-21G>A in the GERP *in silico* tool. It was observed that these variants were preserved evolutionarily. The splicing effect of these variants was investigated with *in silico* analysis using the Alamut software, which integrates several prediction tools (SpliceSiteFinder-like, NNSPLICE, GeneSplicer, Human Splicing Finder, MaxEntScan) and also SR proteins binding site prediction tools (ESEfinder and RESQUE-ESE). In exogenous splice enhancers (ESEs) site tools, these intronic variants have been found to alter motif scores for binding sites of SR proteins (Figure 2A and 2B). The splice site tools of Alamut predicted that the donor splice site scores of c.*27-21G>A variant had disappeared (Figure 2C). However, there was no difference in the splice site scores in the c.639+32G>T variant (data not shown). The association of the c.639+32G>T variant with HCM could not be determined precisely in TK1 because other family members were not available.

The polymorphic variants of *TNNT2, MYBPC3* and *MYH7* genes

The clinically benign variants with the exception of pathogenic exonic and two novel intronic variants were detected in index cases. The information of the gene localization, the effects on codon and allele frequencies of these SNVs are given in Table 1.

This polymorphic variants in index cases were defined according to ACMG (14) (Table 1).

The Ser236Gly missense variant in the *MYBPC3* gene, previously associated with HCM and also available in the Human Genome Mutation Database (HGMD) was identified in three index cases called M3, Y1 and N1.

DISCUSSION

In this study, disease-causing variants in three HCM-associated sarcomeric genes (*MYH7, MYBPC3* and *TNNT2*) were identified in 67% of the Turkish families with HCM

Figure 2: *MYH7* c.639+32G>T and *MYBPC3* c.*27-21G>A variants with potential effect on the exonic splicing enhancers (ESE) binding site (A and B) and the splicing site (C) as predicted using the Alamut software. The exonic sequences are shown as blue boxes. The splicing site scores from each mutation prediction tool are displayed in blue vertical bars for the 5' (donor) sites, and as green vertical bars for the 3' (acceptor) sites.

using sequence-based re-sequencing. The association between these pathogenic variants and SCD risk in familial HCM was also investigated with a retrospective clinical analysis. To date, there is no population-based study to demonstrate the prevalence of hypertrophic cardiomyopathy (HCM) and no large-scale mutation screening

studies for HCM in Turkey. In previous studies, reported mutations of the *MYH7* gene have been demonstrated in Turkish HCM patients (13, 14). In the study performed by Kucukates et al., the Arg403Gln missense mutation in *MYH7* was observed in 8 of 32 cases in 3 families (13). In another study, previously reported four mutations in *MYH7* were not found in 18 HCM patients (14). A novel insertion mutation in *MYBPC3* was detected in a Turkish family with HCM in the mutation screening of the *MYBPC3* and *MYH7* genes using PCR and Sanger sequencing (15). In addition to pathogenic variants, the identification of functional variants may be important for the modifier or founder effect (16) of variants in the disease phenotype. Therefore, in this study, all individual variants of the index cases were presented, but the population-specific effects of these variants in larger Turkish case-control studies could not be investigated.

The autosomal dominant inherited HCM is a complex disease that is characterized by an heterogenous clinical and genetic expression. It is caused primarily by missense mutations, although causative nonsense, frame-shift, and in-frame insertion/deletion mutations have also been observed, particularly in sarcomeric genes (3, 17). As a matter of fact, we have identified missense pathogenic variants in this study. According to the HGMD public database and Pubmed screening with a specific keyword, the Met822Leu missense variant in the *MYH7* gene has not been reported previously. However, we found one publication (18) that identified this pathogenic variant with the Alamut software and VarSome, and also another publication (19) that claims to identify the same variant for the first time in the HGMD Professional database. Since the clinical details of variant carriers were not mentioned in these studies, we could not compare them with the findings of our patients. Therefore, further experimental studies should be conducted to demonstrate the clinical significance and the association of this variant with SCD.

In our study, two novel SNVs in the intron 7 of *MYH7* (c.639+32G>T) and in the intron 34 of *MYBPC3* (c.*27-21G>A) were determined. These variants were analysed to determine possible-binding sites of ESEs for splice-site recognition and for serine/arginine proteins (20) using the Alamut software. *In silico* analysis predicted that c.*27-21G>A variant has a possible impact on mRNA splicing but no effect for the c.639+32G>T variant. These results indicate that *in vitro* studies are required to study the possible impacts of intronic variants on mRNA splicing and expression. However, in this study, the possible effects of these novel defined variants could not be supported by *in vitro* functional studies.

Furthermore, we identified one novel missense variant, in exon 17 of the *MYBPC3* gene (Tyr525Asn). So far, the different mutations related with HCM were reported in the same codon as the Tyr525His (21), the Tyr525Ser (22) and the Tyr525Term (23). The cases with a mild HCM form carrying both Tyr525Asn and c.*27-21G>A variants as compound heterozygote had late-observed SCD in Family T. This intronic variant in the 3'-UTR of the *MYBPC3* transcript suggests that it may affect the mutant allele expression.

In a previous study, similar to our results with family D, it was determined that the *MYH7* Arg719Trp mutation in four families increased the risk of death associated with HCM and caused the malignant phenotype (24). The Val698Ala mutation was firstly identified in Australian proband with hypertrophic cardiomyopathy but there is no clinical information for comparison to our patients (25). The Arg663Cys mutation was described as a "hot spot", and identified nine of the 58 HCM patients (26). This present finding was similar to our results. Indeed, in this study, we found this pathogenic variant in three of the 12 index patients who were not related to each other. In addition, we determined that these three cases had other individual variants besides the main pathogenic variant, and the risk of SCD and cardiac symptoms seems to be different among these three families. These additional polymorphic individual variants might be causing different functional effects on cardiomyocytes with a pathogenic sarcomeric protein.

In our study, the pathogenic variants were not detected in 4 of 12 patients with familial HCM, possibly because disease-causing variants can be found in other HCM-associated genes. In NGS studies applied in recent years, it has been determined that pathogenic variants in both the exonic and intronic regions of more than 40 candidate genes were found to cause the HCM phenotype (3, 8-10). The use of next generation sequencing for the diagnosis of multigenic diseases such as HCM appears to be advantageous to identify all variants. However, as the number of sequenced genes increases, variant analysis becomes difficult, so firstly, candidate genes with a high mutation detection rate can be searched. It is known that 53-85% of HCM is caused by pathogenic variants in the *MYH7, MYBPC3* and *TNNT2* genes (2, 3, 11). The molecular diagnosis rate of this study was 66%, as in the expected range. As supported by this study's results, a positive family history affects the genetic diagnosis rate of HCM.

In recent studies, as in this study, it has not been confirmed that there is a clear prognostic association between the pathogenic variants and the risk of SCD in HCM (3, 17). Prospective clinical follow-up of these patients with variants will clarify the prognostic importance of these findings. Future advances in genotype-based risk stratification are expected to shed light on the management of these patients, particularly in terms of sudden cardiac death (3).

Genetic testing in HCM patients is mainly used for the identification of family members at risk of the disease and for genetic counselling (27). Although the mutation-screening strategy in families with HCM is cost-effective (28), the issue still needs to be further evaluated for reproduction, competitive sports and professional career counselling. As a result, despite the fact that most HCM patients have mild clinical symptoms, some patients may suffer from SCD and end-stage heart failure. Therefore, it is important to define the individual variants for a strong genotype-phenotype correlation of HCM observed incomplete penetrance and variable expressivity. On the other hand, experimental studies using approaches such as gene-editing and allele-specific RNA silencing on HCM treatment in recent years are promising (29, 30).

In conclusion, novel variants related with familial HCM and SCD history were identified but the association of these pathogenic variants and SCD was not clear. Variants rather helped other family members to get a clinical diagnosis. Determination of all individual variants in HCM-related genes may be important to demonstrate the inter variant effects on the clinical and structural differences of HCM families with the same pathogenic variant.

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