Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

Sarcomeric gene mutations in phenotypic positive hypertrophic cardiomyopathic patients in Indian population

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ARTICLE INFO

Original paper

Article history:
Received: August 17, 2021
Accepted: December 08, 2021
Published: December 30, 2021

Keywords:
autosomal dominant; sudden cardiac death; cardiac hypertrophy; cardio-pathophysiology; SSCP

ABSTRACT

HCM is a monogenic cardiac disorder with a high risk of sudden cardiac death, heterogeneous phenotypic expression and genetic profile. HCM is expressed as autosomal dominant in fashion with the prevalence of 1:500 in the general population. The main objective of the current study was to unravel the mutation status in sarcomeric genes in urbanizing Pune population. HCM patients were recruited from Bharti hospital and Poona hospital and research centre, Pune after being screened by 2-D echocardiography. DNA was extracted from whole blood samples and PCR amplification was performed for selected exons from pre-selected genes, amplimers of >300 b.p were restriction digested and the SSCP technique was optimized for maximum result output. HCM patients shows the maximum prevalence of mitral regurgitation (23.3%) while the minimum prevalence was left auricular diameter (10%). Maximum variation spectrum was present in MYBPC3 genes as most of them were "benign" type as per Polyphen-2 tool status. Mutations in the MYH7 gene produce a prominent impact on splicing by the creation of a new SRP40 binding site (Exon Splicing Enhancer) as predicted by Human Splicing Finder 3.1. I736T mutation in the MYH7 gene results in replacement of β-strand by α-helix upstream from mutation site which may have a profound impact on protein tertiary structure as predicted by Polyphen-2 tool (probably damaging-1.00). Also, two 'novel' mutations and one 'novel' variation were reported in the present study. Thus, the MYBPC3 gene shows maximum mutation load among other sarcomeric genes. Double gene mutations do not represent much severe pathophysiology as compared to single gene mutated and genotypic negative HCM patients.

DOI: http://dx.doi.org/10.14715/cmb/2021.67.6.1

Introduction

Hypertrophic cardiomyopathy (HCM) is a type of cardiac disorder mainly characterized by concentric or symmetric septal hypertrophy with the predominant interventricular septum (1). HCM shows a prevalence of 1:500 in the general population and is inherited in an autosomal dominant fashion. Individuals positive with HCM have a 50% probability of inheriting mutations to offspring. De novo mutations may result in sporadic cases in probands with genotype-negative parents (2). Most of the mutations (90%) present in the MYBPC3 gene affect the physical and functional properties of the concerned proteins (2). These mutations are mainly missense or frameshift mutations affecting several amino acids which results in different products (2). The presence of peak left ventricular obstructive gradient >30 mmHg in HCM patients has a prognostic significance in predicting the risk of sudden cardiac death (SCD) and heart failure (3). HCM probands that are genotypically positive have about 70% of mutations in MYH7 and MYBPC3 genes, while other genes tropomyosin, α-actin, troponin I and troponin T genes account for only 1-5% (3). Genotype penetration and hypertrophic severity are dependent on the type of mutation and gene location e.g. mutations in the MYH7 gene are associated with moderate-severe hypertrophy of left ventricle, onset at a younger age. Mutations in the MYBPC3 gene are related to favorable disease course, less left ventricular hypertrophy, slower development which in turn result in a good prognosis (4). TNNT2 gene mutations are associated with minimal left ventricular hypertrophy (5). Thus this

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study unravels the mutation spectrum in phenotypically positive HCM patients and the impact of these mutations on the pathophysiology of cardiac functioning.

Materials and methods

Patient identification and ethics permission

For the current study, the approval from the Institutional Human Ethics Committee of Savitribai Phule Pune University and Bharati Vidyapeeth deemed university Medical college (Ref: BVDV/MC/44) was granted. 30 HCM patients were recruited from Bharati Hospital and Poona hospital and research centre, Pune. All the patients were screened by 2-D echocardiography and patients showing interventricular septum >13mm were taken as phenotypically positive for the current study (Table 1). Patients showing aortic stenosis and hypertension were excluded from the current study. Control samples (n=30) of the same ethnic background and without a family history of cardiac disorders were recruited for the current study.

Blood collection and DNA extraction

Written informed consent was taken from all the participants. 10ml blood samples were received from HCM patients as well as from control individuals by a trained phlebotomist and samples were immediately processed for DNA extraction. DNA extraction was done by the method of phenol: chloroform: isoamyl alcohol. Briefly, 300µl of blood was used for DNA extraction and mixed with 800µl of 1X SSC (saline-sodium citrate) buffer at room temperature. Samples were centrifuged at 10,000 rpm for 2 minutes at room temperature. 20µl of 10% SDS and 10ul proteinase K was also added to the sample and pipetting was done back forth to mix the constituents. After incubation at 55 °C for 1 hour, phenol-chloroform isoamyl alcohol was added to the solution and vortexed for 30 sec. 1ml of chilled ethanol was added to the solution and constituents were mixed thoroughly until DNA is visible. DNA quantification was done by Nanodrop (Bio spectrophotometer Basic, Eppendorf, Germany).

Primer designing and PCR amplification

Primers were obtained from http://genepath.med.harvard.edu/~depalma/outdated-mutdb/index.html. All the primers amplified the intron-exon boundary. The reaction contains 2X Master Mix (10µl), dNTPs (200µM), $MgCl_2$ (1.5mM), Taq polymerase 5U/µl (1U), PCR Buffer (10X), Forward primer 10pmol/µl, Reverse primer 10pmol/µl, DNA template 100-200ng/µl, Molecular grade H_2O (10µl). Amplified products were run on 2% agarose gel and were visualized under gel doc system (Alpha Imager HP, Cell BioSciences).

Restriction digestion of amplified PCR product

PCR products larger than 300 b.p were digested by diluting (three times that of sample volume) with molecular grade water, buffer 2 µl, and 1µl restriction enzyme was added. Digested products were visualized on 2% agarose gels and documented.

Single-Strand Conformational Polymorphism (SSCP) and PAGE

20µl of Phenol: chloroform: isoamyl alcohol was added to the PCR mixture, vortexed for 30 sec. and centrifuged at 10,000 rpm for 2 min at room temperature. The upper watery layer was aspirated and transferred in a fresh tube. 10µl of formamide was added and pipetted back and forth until all constituents are mixed. Samples were denatured at 95^0C for 10min. and snap-chilled on ice to prevent renaturation of amplified product. 20µl of the sample was loaded in each well on 8-10% polyacrylamide gels. Each exon was optimized on different gel concentrations to obtain maximum results (Figure 1) (Table 2).

Silver staining of polyacrylamide gels

Silver staining was done as per the method of (6). Photography was done by digital camera for future records.

PCR purification and Sanger sequencing

Digested PCR products showing aberrant bands concerning control samples were processed for PCR purification by Sure Clean Plus purification kit (Bioline, India). Samples were visualized on 2% agarose gels and processed for bi-directional Sanger sequencing (Macrogen, South Korea).

In silico Analysis

Chromatograms were visualized on Finch TV 1.4.0 and bases showing quality value >20 were processed further. To check whether the particular variation is

"novel" in status, dbSNP, 1000 genomes browser and ExAC browser beta data were cross-verified. Secondary protein structure prediction was done by the PSIPRED tool. To validate the impact of missense mutations on protein functionality, the PolyPhen-2 tool was used. To find the impact of variations on splicing, Human Splicing Finder (HSF-3.1) was used.

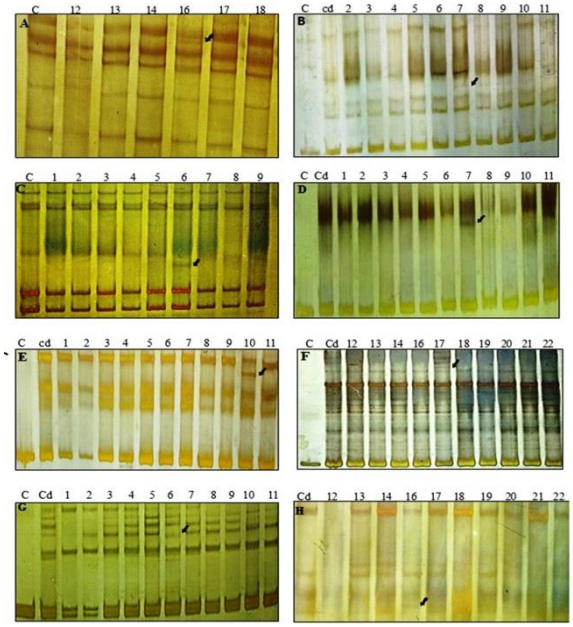

Figure 1. SSCP analysis of HCM patients showing aberrant band shift with respect to control samples.
C= control undenatured; Cd= control denatured; A= MYBPC3 gene (exon 07); B= MYH7 gene (exon 20); C= MYH7 gene (exon 25); D= MYBPC3 gene (exon 21); E= MYBPC3 gene (exon 33); F= TPM1 (exon 07); G= MYL3 gene (exon 03); H= MYL2 gene (exon 07)

Table 2. Optimized conditions of exons on different gel concentration

% gel conc.	SSCP Conditions	Gene type and Exon no.	Band shift
8	95 °C-10m →snapchill →20µl sample →4°C→80 V →12hrs.	MYBPC3 (21,33,), MYH7 (20,25), MYL3 (03)	YES
10	----------	MYBPC3 (07), MYL2 (07), TPM1 (07)	YES

95 °C- Denaturing condition, 4 °C- sample run temperature, 12hrs- sample run duration

Results and discussion

In the present study, of all comorbidities in HCM patients maximum percentage of mitral regurgitation (23.3%), tricuspid valve regurgitation (20%), grade I diastolic dysfunction (13.3%) and left auricle dilated (10%) was observed. On sequencing all exons of MYBPC3, MYH7, TPM1, MYL2 and MYL3 genes, maximum variations were found in the MYBPC3 gene, a mutation in exon 07 causing T>C transition at g.9213 which results in S236G (rs3729989), C>G transversion in exon 21 of intronic variant at g.11789 (rs10769254), G>A transition in exon 33 of intronic variant at g.20167 (rs373904644). MYH7 gene shows two missense mutations one reported in exon 20 located at g.9945 (rs727503261) results in T>C transition (I736T) and a novel mutation in exon 25 results in A>T transversion at g.18433 causes I1066F mutation. TPM1 gene shows one novel mutation (C>G transversion) in exon 07 at c.699 resulting in K233N mutation. MYL2 and MYL3 genes show one intronic variant in exon 07 (C>T transition) at g.12383 (rs2233260) and in exon 03 (G>C transversion) at g.262364 (novel) respectively (Figure 2) (Table 3).

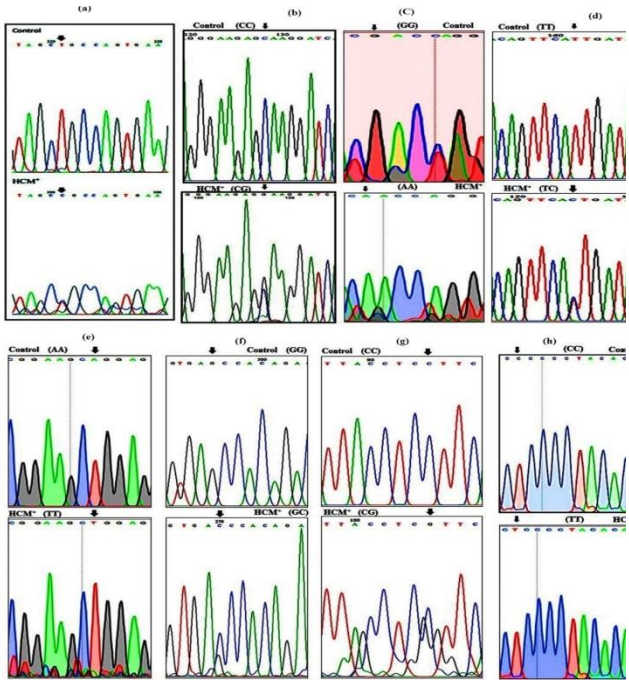

Figure 2. Electropherograms of HCM phenotypic and genotypic positive patients. **(a)** Missense heterozygous mutation at g.9213 (T>C) results in serine236glycine in exon 07 of the MYBPC3 gene. **(b)** A heterozygous intronic variant at g.11789 (C>G) in exon 21 of the MYBPC3 gene results in splice site changes. **(c)** A homozygous intronic variant at g.20167 (G>A) in exon 33 of the MYBPC3 gene may have an impact on splice site changes. **(d)** Missense heterozygous mutation at g.9945 (T>C) in exon 20 of MYH7 gene causes Isoleucine736 Threonine **(e)** A novel missense mutation (A>T) at g.18433 in exon 25 of MYH7 gene results in Isoleucine1066Phenylalanine. **(f)** A novel heterozygous

intronic variant at g.262324 (G>C) in exon 03 of the MYL3 gene may have an impact on splice site changes. **(g)** A heterozygous missense mutation at c.699 (C>G) in exon 07 of the TPM1 gene results in Lysine233Asparagine. **(h)** A homozygous Intronic variant (C>T) in exon 07 in the MYL2 gene at g.12383 is reported (rs2233360) in status.

S236G mutation is shown by a proband of 51 years of age with systole/diastole-120/80mmHg, IVS-24mm, LVEF-60%, LA diameter-30mm, LVPWD-17mm, LVIDs-30mm, LVIDd-51mm. Proband shows slit-like left ventricular cavity, LV cavity obliteration during systole, LV diastolic dysfunction, sclerotic aortic valve, trivial aortic regurgitation, Minimal and tricuspid mitral regurgitation, mild pulmonary hypertension, allergic bronchial asthma, LAD-type III, normal slow flow, RCA-dominant normal, PLV-ostial plaque and asymmetrical septal hypertrophy without a family history of sudden cardiac death. This mutation results in the breakage of the binding site for SF2/ASF (IgM-BRCA1) and the creation of a new site for 9G8 protein as both are exons splicing enhancer proteins which may affect the splicing process as per Human splicing finder 3.1. This mutation was found as benign type as per Polyphen-2 (0.0) and SIFT scores (1.00) (Figure 3). I736T mutation is present in the proband of 67 years of age showing IVS-14mm, LVEF-60%, LA diameter-22mm, aorta-28mm, LVPWD-14mm, LVIDd-34mm, moderate concentric LVH, Grade I LV diastolic dysfunction, No RWMA, and asymmetrical septal hypertrophy without a family history of sudden cardiac death. A new binding site for SRp40 protein (exon splicing enhancer) upstream from the variation site is created which may alter splicing as per Mutation Taster software. With respect to control, mutated sample show loss of β-strand and replacement by α-helix upstream from mutation site which may have a profound impact on protein tertiary structure (Figure 4) as predicted by Polyphen-2 tool score (probably damaging-1.00). This mutation is highly conserved across different vertebrate groups as the mutation is present in the conserved region thus, having more impact on protein stability as compared to the non-conserved region (Figure 5).

Proband of 19 years of age with I1066F mutation shows systolic/diatolic-90/60mmHg, IVS-26mm, LVEF-60%, LA diameter-42mm, LVPWD-12mm, LVIDs-21mm, LVIDd-42mm and hypertrophic obstructive cardiomyopathy with a family history of HCM as his father shows asymmetrical septal hypertrophy. This variation results in breakage of exon splicing enhancer site of 9G8 protein in mutant motif as compared to reference motif and may have a potential alteration of splicing as also predicted by mutation taster software. Polyphen-2 predicts a 'benign' type of mutation (score-0.250) as this mutation is present in a less conserved region as per multiple alignments of amino acids (Figure 6).

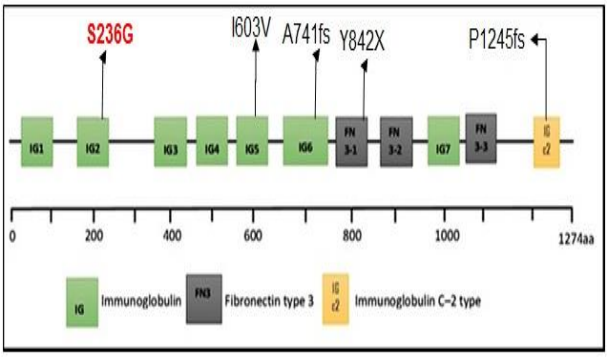

Figure 3. Localization of a known mutation (S236G) in this study and mutations reported in MYBPC3 protein domains.

Mutation in TPM1 gene (K233N) is present in the proband of 42 years of age (Figure 7) with systolic/diastolic- 110/70 mmHg, IVS-13mm, LVEF-60%, LA diameter- 42mm, LVPWD-11mm, LVIDs-32mm, LVIDd-45mm. Proband shows dilated LA, LV, global LV hypokinesia, moderate LV systolic dysfunction, mild mitral regurgitation, mild tricuspid regurgitation, mild pulmonary hypertension, tobacco chewer, focal area of gliosis in the left occipital region. Mural thickening of proximal intracranial part of the left ventricular artery causing significant narrowing. The patient has extreme tachycardia, deep S waves in lateral leads, infero-anterior ST changes. PR-199, QRS-88, QT-360, QTC-377, P axis-40 with asymmetrical septal hypertrophy and family history of sudden cardiac death. Mutation results in breakage of the binding site at variation site in SRp40 protein which may alter splicing. K233N mutation is present at the periphery of the coiled-coil region of TPM1 protein thus plays an important role in interaction with actin molecules and protein stability (Figure 8). This mutation is probably damaging (score-0.995) as per Polyphen-2 tool score and SIFT online tool prediction (score 0.000). This mutation site is highly conserved in vertebrates as well as in invertebrates (round worms).

Missense mutations have more pathophysiological impact in HCM patients as compared to intronic variants in this study. Missense mutation patients show lower LVEF (56.25±7.5%) as compared to intronic variant patients (60%). Left auricular diameter was increased (34±9.7 mm) in missense mutated probands as compared to intronic variant probands (32±8.3 mm). Missense variants show higher diastolic dysfunction in terms of higher 'A' value m/sec than intronic variant patients (Table 4).

Table 4. Impact of gene variations on heart pathophysiology

	Type of variation	
	Missense type	Intronic type
IVS (mm)	19.25±6.7	20.75±5.3
LVEF (%)	56.25±7.5	60
LA (mm)	34±9.7	32±8.3
LVPWD (mm)	13.5±2.64	15.5±3.1
E value m/sec	0.62±0.2	0.68±0.2

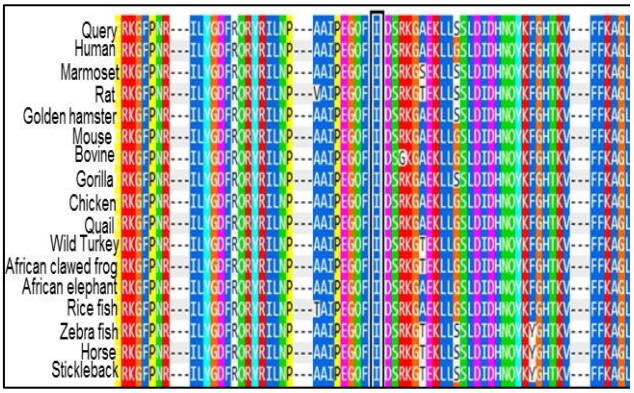

Figure 5. Multiple alignments of amino acids for I736T mutation. Box shows variation site.

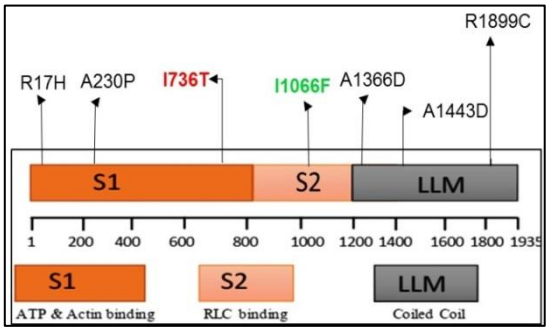

Figure 6. Localization of one novel (I1066F) and reported mutation (I736T) in MYH7 protein domains.

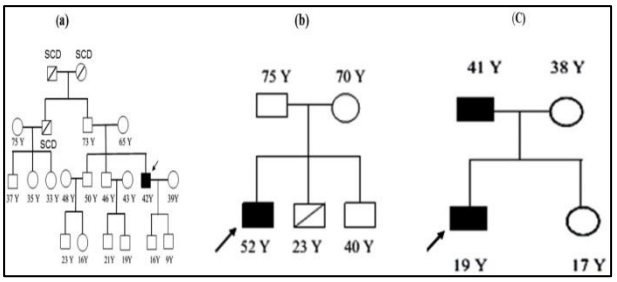

Figure 7. Pedigree analysis of HCM patients showing cases of sudden cardiac deaths **a.** proband shows K233N mutation with a family history of sudden cardiac deaths **b.** proband shows rs373904644 variation **C.** proband shows double mutation with a family history of HCM as a father shows asymmetrical septal hypertrophy.

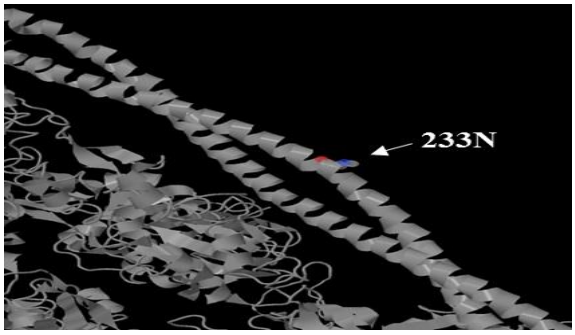

Figure 8. 3-D structure of tropomyosin showing localization of mutation in the periphery of α-helix by JSmol tool.

MYBPC3, MYH7 and other sarcomeric gene groups (OSG) including TPM1, MYL2 and MYL3 mutated probands show the same left ventricular ejection fraction values (60%). OSG affected probands have higher interventricular septum diameter (21±4.04mm) as compared to MYH7 mutated probands (20±6mm) and MYBPC3 affected patients (19±2.8mm). Left auricular diameter was higher in OSG affected patients (38±6.9mm) as compared to MYH7 mutated patients (32±14.4mm) and MYBPC3 gene affected patients (28.6±6.1mm). MYBPC3 mutated probands show higher levels of left ventricular posterior wall diameter (16.6±2.5mm) with respect to MYH7 affected (13±1.4mm) and OSG mutated probands (13.3±3.2mm). OSG mutated probands show higher levels of left ventricular internal dimensional diasystole (46±4.5mm,) with respect to MYBPC3 affected (40±9.5mm) and MYH7 gene mutated probands (38±5.6mm). Double mutated probands show a similar pattern of the interventricular septum (21.3±6.4) as that of genotypic negative probands (21.3±7.8), while the single gene mutated probands shows lower values

(16±4.2). Left ventricular posterior wall diameter was higher in single gene mutated probands (15±5.6) as compared to double gene mutated (14.3±2.5) and genotypic negative probands (13.3±3.9). Genotypic negative probands show lower ejection fraction % (58.7±6.9) as compared to double and single gene mutated probands (60%). Thus, Double gene mutations in the current study do not show worse pathophysiology in HCM patients may be due to the presence of mutations in the less impactful site in sarcomeric proteins (Figure 9).

MYBPC3 shows the maximum amount of variations in this study as compared to other sarcomeric gene variations. I736T and K233N mutations were more deleterious thus may have a profound impact on protein structure and stability.

MYBPC3 gene mutations are frequently present in most HCM cases representing 30-40% of all HCM mutations (7). Patients associated with MYBPC3 mutations have decreased penetrance, better prognosis, later disease onset, lifelong expectancy. These mutations mostly affect myosin and titin binding sites, whereas missense mutations preserve these binding sites (8). Most of the MYHC mutations are located in the S1 region in HCM probands and their families. ATP-binding pockets mutations of MYHC protein (Thr124Leu, Phe244Leu) may either alter the water structure or binding of phosphate groups in the active site which in turn decreases the catalytic activity of the S1 fragment (9). Arg403Gln mutation at the actin-myosin interface may impact actin and myosin binding by the closure of the connection between actin and ATP binding sites. Mutations on two reactive thiols (Phe513Cys, Gly584Arg) results in conformational changes during sarcomere contraction or alter the conformation in this region. S2 region mutation Leu908Val may lead to defective force transmission from myosin heads to thick filaments (9). Tropomyosin contains α-helical molecules in a thin filament groove. Asp175Asn and Glu180Gly mutations changes surface charge in TPM1 that has been involved in Ca^{++} sensitive TnT binding. Asp175Asn mutated skinned fibers show higher Ca^{++} sensitivity as compared to controls (ΔpCa_{50}=0.09) but no significant change in cooperativity, maximum force and maximum shortening velocity (10). Thus α-Tm mutations increase force generations at submaximal Ca^{2+} concentrations in HCM patients (11). Mutation dosage in the current study does not show much impact on pathophysiology in clinical output, the presence of two mutations as compared to single mutated HCM probands does not significantly change the clinical output or HCM pathogenesis (12). (13) found that 2.5 fold higher incidence of sudden cardiac death with positive family history, severe cardiac hypertrophy and higher LV wall thickness in patients with multiple mutations. Different studies showed that compound heterozygous, double or homozygous mutations have a higher sudden cardiac death rate, more severe left ventricular hypertrophy and episodes of cardiac arrest in family members (14-16).

Conclusions

Mitral regurgitation was the most prevalent type of comorbidity in HCM patients in the current study. I736T and K33N mutations were more deleterious in terms of cardio-pathophysiology as compared to other benign mutation types. Other sarcomeric groups including TPM1, MYL2 and MYL3 genes were having the worst cardiac outcome as compared to MYH7 and MYBPC3 group and needs to unravel further. Also, double mutated probands do not show severe complications in terms of cardio-physiology as compared to single mutated and genotypic negative patients in this study.

Acknowledgements

Authors thank DST PURSE, DRDP and CAS for partial financial support. SAA is also thankful to UPE-II (CSSH) and SPPU for providing fellowship.

Interest conflict

None.

Author Contribution

SAA done all the experimental work and also contributed to manuscript writing. CC recruits and screened HCM patients and finalized the manuscript. RB helps in clinical data interpretation and finalizing the draft. VW designed the work and also contributed to finalizing the draft.

Abbreviations

SCD (sudden cardiac death), HCM (Hypertrophic cardiomyopathy), SSCP (single-strand conformational

polymorphism), LVPWD (left ventricular posterior wall diameter)

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Table 1. 2-D echocardiography of phenotypic and genotypic HCM positive patients

Proband	Age (yrs.)	Sex	Syst/Dias	IVS (mm)	EF (%)	LA (mm)	LVPWd (mm)	LVIDs (mm)	LVIDd (mm)	E (m/sec)	A (m/sec)	Mitral valve	Aortic valve	Pulm. valve	Type
P16	51	M	120/80	24	60	30	17	30	51	0.45	0.8	normal	normal	normal	ASH
P6	19	M	90/60	26	60	42	12	21	42	---	---	---	----	---	HOCM
P7	67	F	---	14	60	22	14	---	34	0.8	1.1	normal	normal	normal	ASH
P10	52	M	130/70	19	60	34	19	----	35	0.8	0.5	normal	normal	normal	HNOCM
P17	42	M	110/70	13	45	42	11	32	45	----	----	normal	normal	normal	ASH

ASH= Asymmetrical septal hypertrophy; HOCM= Hypertrophic obstructive cardiomyopathy; HNOCM= Hypertrophic non-obstructive cardiomyopathy

Table 3. Prediction of the functionality of gene variations in HCM positive patients.

Proband	Gene	Exon	variation	Position	Type	Aminoacid	Status	Mutation Taster	Predict SNP	SIFT	PolyPhen 2
P16	MYBPC3	07	T>C transition	g.9213	Missense	S236G	rs3729989	Splice site change	Neutral (-1.0000)	Tolerated (1.00)	Benign (0.0)
P6	MYH7	25	A>T transversion	g.18433	Missense	I1066F	**Novel**	Splice site change	Neutral (-1.0000)	Demaging (0.001)	Benign (0.25)
P7	MYH7	20	T>C transition	g.9945	Missense	I736T	rs727503261	Amino acid change	Demaging (1.0000)	Demaging (0.01)	Probably demaging (1.00)
P10	MYBPC3	33	G>A transition	g. 20167	Intron variant	---	rs373904644	Splice site change	Neutral (-1.0000)	-----	------
P17	TPM1	07	C>G transversion	c.699	Missense	K233N	**Novel**	-----	----	Demaging (0.00)	Probably demaging (0.995)
P16	MYL2	07	C>T transitions	g.12383	Intron variant	----	rs2233260	----	----	-----	----
P6	MYL3	03	G>C transversion	g.262324	Intron variant	-----	**Novel**	Splice site change	-----	-----	----
P7	MYBPC3	21	C>G transversion	g.11789	Intron variant	----	rs10769254	Splice site change	----	----	-----

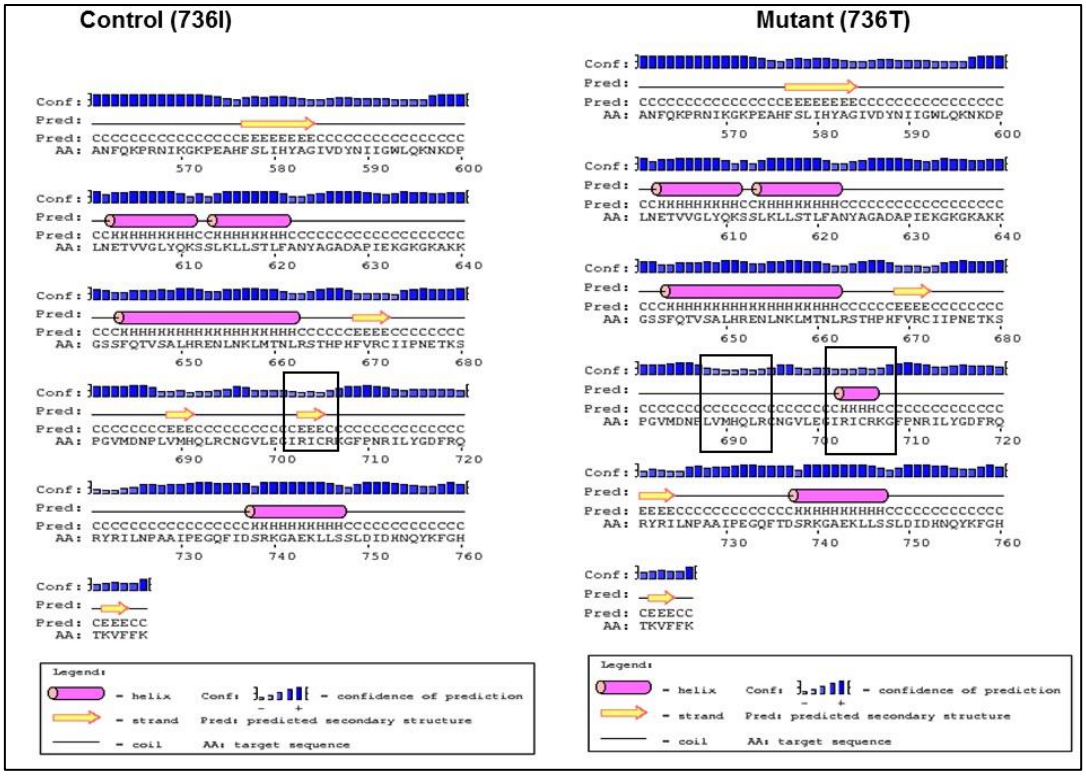

Figure 4. Secondary protein structure prediction shows replacement of β-strand by α-helix in I736T mutation by PSIPRED tool.

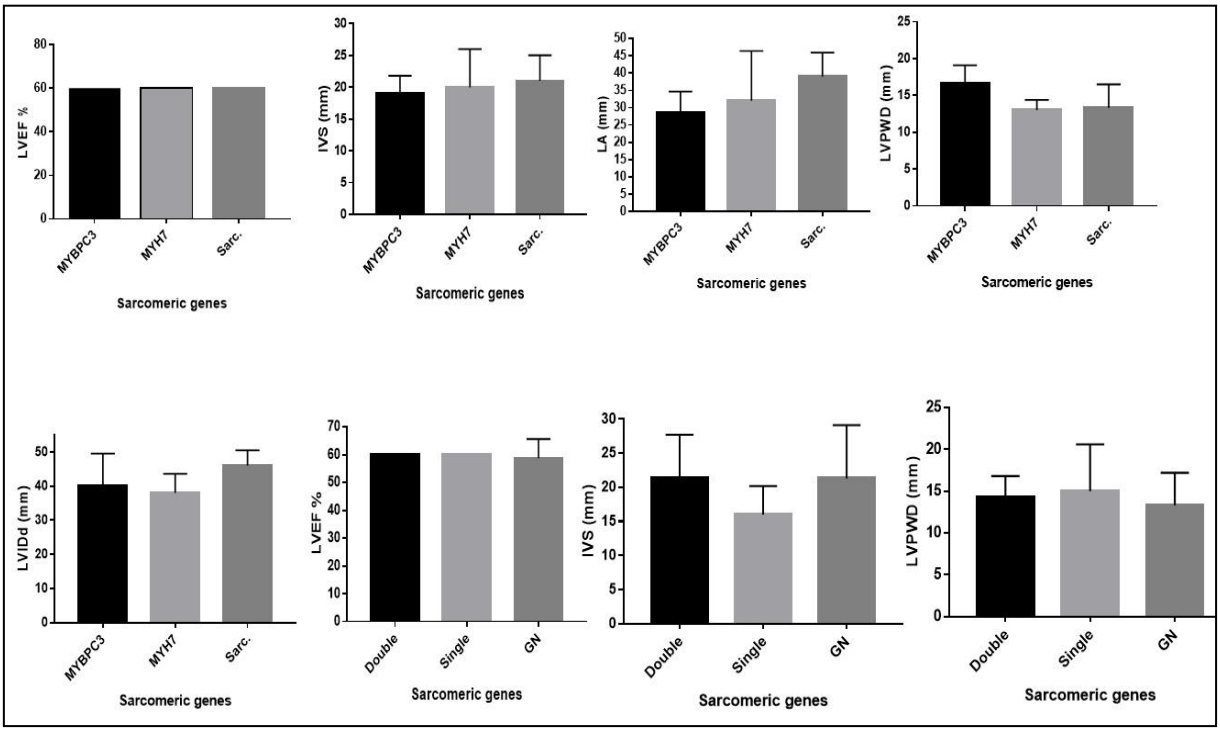

Figure 9. Impact of gene mutations on pathophysiology of cardiac output in HCM patients**.** GN= Genotypic negative; Sarc. (OSG) = TPM1, MYL2, MYL3 genes.