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Original article

COMPOUND HETEROZYGOZITY ASSOCIATED PATHOGENESIS IN HYPERTROPHIC CARDIOMYOPATHY

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ABSTRACT

ABSTRACT-The article deals with the molecular and insilico analysis of compound heterozygosity to understand the complex interactions and effects of the genetic compounds in HCM.

Introduction

Hypertrophic Cardiomyopathy (HCM) was first described in detail in 1958 [1]. HCM remains the most common cardiovascular genetic disorder, with an incidence of 1/500 in the general population [2]. In the absence of secondary causative factors, like hypertension, left ventricular hypertrophy of the myocardium is primarily due to genetic factors. Patients exhibit marked diversity in their morphological features and clinical manifestations, ranging from no symptoms to heart failure and sudden death [3]. Clinical heterogeneity is manifested as diastolic dysfunction, arrhythmogenic substrates leading to ventricular arrhythmias, small-vessel disease leading to subendocardial ischemia, and left ventricular outflow tract obstruction which underlines the complex pathophysiology of HCM [4, 5]. Hence, HCM is a disease with a multitude of potential cardiac pathologies, resulting in a diverse range of clinical outcomes.

HCM was the first cardiovascular disorder identified with a positive familial history. A positive family history in presence of specific "malignant" gene mutations along with symptoms like history of resuscitated cardiac arrest, a left ventricular wall thickness greater than 30 mm, syncope, and nonsustained ventricular tachycardia on 24-hour ambulatory ECG monitoring are all considered important risk factors for sudden death in HCM [6, 7].

About 13 genes have been primarily implicated in the disease manifestation and progression, viz, in decreasing order of disease occurrence, β –MHC (Myosin Heavy Chain), MyBPC3 (Myosin binding Protein C), cTnT (Cardiac Troponin T), TM (Tropomyosin), cTnI (Cardiac Troponin I), CSRP3 (Cardiac Muscle LIM protein), TCAP (Telethonin), MYL2 (Regulatory light chain), MYL3 (Essential Light chain), ACTC (Actin), TTN (Titin), α –MHC (Myosin heavy chain) and cTnC (Cardiac Troponin C). Specific genes and specific mutations dictate the severity and pattern of hypertrophy, age of disease onset and progression to heart failure. For example studies have shown that MYBPC3 gene mutations predispose the individual to HCM earlier than individuals carrying MYH7 gene mutations [8]. Troponin T (TNNT2) mutations, though associated with mild left ventricular hypertrophy, are reported to cause sudden deaths [9]. This apart, recent studies report significant proportion of patients harboring multiple mutations [10-12].

Considering an example of insertion/deletion polymorphism of the angiotensin-converting enzyme gene being implicated in a number of cardiovascular diseases, including cardiac hypertrophy, progression to heart failure, and sudden death [13]; it can be stated that there are several polymorphisms which play a secondary genetic role like regulatory factors such as gene promoter regions altering gene expression or key enzymes important in normal cardiovascular biology [14]. Most recently, there is an emerging recognition that a proportion of patients carry 2 (multiple) independent disease-causing gene mutations (ie, not polymorphisms), leading to more severe clinical outcome. These mutations can occur in the same gene (compound mutation) or in 2 different genes (double mutation).

Since, HCM is an autosomal dominantly inherited disease, the concept of polygenic mutations challenges the well-accepted paradigm of a single mutation in a single gene being the direct cause of an autosomal dominant disease [8].

Atleast 5% of families carry 2 distinct disease causing mutations especially in sarcomeric genes [10-12]. Although there is a clear association of multiple mutations with a more severe clinical phenotype in HCM, familial LQTS, and FH, further genotype-based prospective studies are required to fully evaluate the use of multiple mutation information for risk stratification of patient and families.

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While much of the research work published focuses on families/patients with compound heterozygous mutations, there is very little importance given to the role of non-synonymous/synonymous SNPs in the disease etiology. Specially, synonymous SNPs are mostly ignored due to their negligible impact on disease progression. However, a HCM patient may develop the disease due to a conglomeration of sSNPs that they may carry in the absence of any mutations in any of the disease causative genes. Each SNP may affect the final protein product in one of the 4 molecular steps which lead to protein synthesis, namely, micro RNAs, m RNA secondary structure and translational efficiency, alternative splicing and codon bias.

Certain synonymous sites are also strongly conserved during evolution which further supports the fact that synonymous SNPs are not to be ignored in disease pathology.

The present study tries to emphasise on the importance of synonymous SNPs as possible causatives in the disease pathogenesis especially when present as compound heterozygotes/genetic compounds.

METHODS

A total of 100 HCM cases confirmed based on medical history, physical examination; ECG and Echo were included in this study against 100 controls who have no family history/history of cardiovascular diseases/symptoms. These cases were referred to the cardiology units of CARE Hospitals, Hyderabad and controls were collected from a local PHC, Hyderabad. Informed consent was obtained from all participating individuals along with Institutional ethics committee clearance and ethical clearance from CARE Hospitals, Hyderabad.

Total genomic DNA was isolated from the whole blood of patients and controls [15] followed by PCR based SSCP analysis. Polymerase chain reaction (PCR) was carried out in 0.2ml tubes with a final volume of 25ul. Each tube contained 100ng of genomic DNA, 50pmoles each of forward and reverse primer, 0.5-1 unit of Taq DNA polymerase enzyme, 200uM of dNTP, 1X PCR buffer (Tris-HCl 10mM (pH 9.0), 50mM of KCl, 1.5mM of MgCl2) and water to make up the final volume. Amplification was carried out in an Eppendorf – Mastergradient thermocycler. Initial denaturation was carried out at 950 C for 5min, followed by denaturation step at 950 C for 30 sec. Annealing temperature (53-59 0C) for 30 sec and extension was carried out at 720 C for 1min. A final extension step of 720 C was for 5 min at the end of the reaction [16].

To the amplified products,equal amount of formamide loading dye was added and denatured at 95oC for 10 min and immediately cooled (snap cooling) on ice to prevent renaturation of the single strands. The samples were then loaded on 11% Polyacrylamide gel (PAGE) and electrophoresis carried out at 160V [17]. The gel was silver stained for band pattern observation. SSCP for band variations were repeated and further confirmed by commercial sequencing (373 DNA Analyser, Macrogen, Korea).

Hardy-Weinberg equilibrium was established by adopting the X2 test (SNPstats software), for all polymorphisms. Odds ratios, with 95% confidence intervals were calculated to compare allele and genotype frequencies. The extent of linkage disequilibrium (LD) was expressed in terms of the maximum likelihood estimate of disequilibrium, D9. Statistical analysis was carried out using SNPstats software. For all tests, significance level was set as

Free softwares available online were used to construct m-RNA secondary structures (Brodskii et al, 1995); to analyse splice site variations [18, 19]; estimate codon-usage bias; protein secondary and tertiary structure predictions [20].

RESULTS

Of all the 100 patients studied along with available family members, 22 cases carried atleast one mutation in one of the sarcomeric genes apart from several other polymorphisms in these genes (Table.1).In the present study, the maximum number of cases discussed are compound heterozygous variations in MYH7 and MYBPC3 (Hotspot) genes, as reported earlier. The familial cases exhibited genetic anticipation, a phenomenon where a genetically inherited disease manifests itself at an early age in each generation, similar to nucleotide repeat syndromes.

The 22 familial cases are separately discussed w.r.t their compound heterozygousity with its impact on their molecular changes (as predicted insilico) which may consequently effect disease progression.

* - Refer to table.1 and Fig.1 for respective illustrations.

#- Refer to only table.1 for the respective illustration.

*P47:

Such high penetrance and early age of onset in the family can be attributed to 3 reasons:

1. Compound heterozygosity of 2 mutations in Exon 34 of MYH7. Though one of them is synonymous yet they affect splicing by converting a silencer binding site to a cryptic enhancer binding site for 2 proteins SC35 and SRp40.

2. The same SRp 40 is again affecting disease since a cryptic binding site is created for SRp 40 and binding site is lost for SRp 55.Also m- RNA secondary structure predictions reveal a slightly less stable structure for mutant compared to wildtype.

3. Several cryptic binding sites are created at positions 114,115,116 and 118 for different splicing protins like SF2/ASF and hnRNP A1 motif.However, there is no change in m-RNA secondary structure.

Conclusion:

Creation/loss of splicing sites/cryptic splice sites for the specific spliceosome proteins seems to be the main reason for the early age of onset and high penetrance in the proband. However it is not sure as to whether all these compound mutations seen in this proband are inherited. But, it could be predicted that SRp40 being a major splicing protein should be playing a major role in the progression and severity which ultimately leads to death of the affected individuals in the family.

*P64:

The notable point in this case is that for both unrelated synonymous mutations in 2 different genes,ie, MYBPC3 and MYH7, the binding sites for SF2/ASF spliceosome is disrupted. There is a creation of new enhancer sites and disruption of silencer sites for splicing. Also, codon usage bias is very distinctly observable in both cases where the wildtype codon usage frequency (CAG-0.73) is much higher than the variant codon usage frequency (CAA-0.27). Codon usage bias estimation helps to predict the reason behind the disease for a silent mutation.

Table.1: Epidemiological and clinical variables in HCM cases exhibiting compound heterozygosity

			3-E 26			ygote	(0.24)	(0.18)					
105 B1	HCM	55/ M	MYBPC 3 E-24	Non synonymous mutation	**D770N**		NA	NA	2.2	1.6	Nil	Nil	Mother, 2 male siblings and son are affected
			MYBPC 3 E-31	Synonymous SNP	**1096 A/G**		GAG (0.58)	GAA (0.42)					
			MYBPC 3-E 14	Intronic variation	**IVS14-25C>T**	Hetero zygote	NA	NA					
			MYH7 E-20	Intronic variation	**IVS19-56 A/G**		NA	NA					
			MYH7 E-3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
FHC 122	HOCM	23/F	MYH7-E 4	Silent mutation	**6350C>T (Y6350Y)**	Hetero zygote	TAC (0.57)	TAT (0.43)	1.1	2.1	82	Nil	NA
			MYH7-E 3	Synonymous SNP and intronic variation	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
					IVS2-25 G/T	Hetero zygote	NA	NA					
			MYH7-E 19	Intronic variation	**IVS 19+92 A/G**		NA	NA					
			MYH7-E 20	Intronic variation	**IVS 19-56 A/G**		NA	NA					
FHC 128	HOCM	36/ M	MYH7-E 22	Missense mutation	**R870H**	Hetero zygote	NA	NA	2.1	3.2	Nil	40	NA
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			TNNI3-E 3	Intronic SNP	**IVS2-8T/A**	Homo zygote	NA	NA					
FHC-139	HOCM	37/ M	MYBPC 3-E 34	Intronic deletion mutation	**25 bp deletion in I-33**		NA	NA	1.3	2.0	Nil	Nil	Mother sudden death(SCD?)
			MYBPC 3- E 24	Intronic variation	**IVS24'+18 C/G and +35 G/T**		NA	NA					
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			TNNI3-E 5	Silent mutation	**2560 G>T (R68R)**	Hetero zygote	CGG (0.20)	CGT (0.08)					
			MYH7-E 19	Intronic variation	**IVS 19+92 A/A**	Homo zygote	NA	NA					
FHC 136	HCM	49/ M	MYH7-E 4	Silent mutation	**6350C>T (Y6350Y)**	Hetero zygote	TAC (0.56)	TAT (0.44)	1.3	2.0	Nil	Nil	NA

			3-E 26			ygote	(0.24)	(0.18)					
105 B1	HCM	55/ M	MYBPC 3 E-24	Non synonymous mutation	**D770N**		NA	NA	2.2	1.6	Nil	Nil	Mother, 2 male siblings and son are affected
			MYBPC 3 E-31	Synonymous SNP	**1096 A/G**		GAG (0.58)	GAA (0.42)					
			MYBPC 3-E 14	Intronic variation	**IVS14-25C>T**	Hetero zygote	NA	NA					
			MYH7 E-20	Intronic variation	**IVS19-56 A/G**		NA	NA					
			MYH7 E-3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
FHC 122	HOCM	23/F	MYH7-E 4	Silent mutation	**6350C>T (Y6350Y)**	Hetero zygote	TAC (0.57)	TAT (0.43)	1.1	2.1	82	Nil	NA
			MYH7-E 3	Synonymous SNP and intronic variation	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
					IVS2-25 G/T	Hetero zygote	NA	NA					
			MYH7-E 19	Intronic variation	**IVS 19+92 A/G**		NA	NA					
			MYH7-E 20	Intronic variation	**IVS 19-56 A/G**		NA	NA					
FHC 128	HOCM	36/ M	MYH7-E 22	Missense mutation	**R870H**	Hetero zygote	NA	NA	2.1	3.2	Nil	40	NA
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			TNNI3-E 3	Intronic SNP	**IVS2-8T/A**	Homo zygote	NA	NA					
FHC-139	HOCM	37/ M	MYBPC 3-E 34	Intronic deletion mutation	**25 bp deletion in I-33**		NA	NA	1.3	2.0	Nil	Nil	Mother sudden death(SCD?)
			MYBPC 3- E 24	Intronic variation	**IVS24'+18 C/G and +35 G/T**		NA	NA					
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			TNNI3-E 5	Silent mutation	**2560 G>T (R68R)**	Hetero zygote	CGG (0.20)	CGT (0.08)					
			MYH7-E 19	Intronic variation	**IVS 19+92 A/A**	Homo zygote	NA	NA					
FHC 136	HCM	49/ M	MYH7-E 4	Silent mutation	**6350C>T (Y6350Y)**	Hetero zygote	TAC (0.56)	TAT (0.44)	1.3	2.0	Nil	Nil	NA

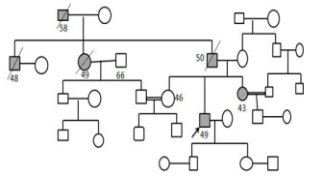
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			MYBPC 3- E 22	Intronic variation	**IVS 22+56 C/T**		NA	NA					
			MYBPC 3-E 24	Intronic variation	**IVS23-5C/T**		NA	NA					
FHC 110	HCM	50/ M	MYH7-E 21	Silent mutation	**T786T**	Hetero zygote	ACG (0.12)	ACT (0.24)	1.0	2.1	Nil	Nil	NA
			MYBPC 3-E 31	Silent SNP	**1096 A/G**		GAG (0.58)	GAA (0.42)					
			MYBPC 3- E 22	Intronic variation	**IVS22+118 G>A**		NA	NA					
			TNNI3-E 3	Intronic SNP	**IVS2-8T/A**	Homo zygote	NA	NA					
FHC 143	HOCM	65/ M	MYH7-E 21	Silent mutation	**T786T**	Hetero zygote	ACG (0.12)	ACT (0.24)	1.0	1.8	NA	30	NA
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			MYBPC 3-E 5	Non synonymous mutation	**V158M**	Hetero zygote	NA	NA					
			MYBPC 3-E 14	Intronic variation	**IVS14-25C/T**	Hetero zygote	NA	NA					
			TNNI3-E 3	Genetic compounds of intronic variations	**IVS2-8T/A &IVS3-21G/A**		NA	NA					
FHC 88	HCM	39/ M	MYH7-E 23	Silent mutation	**N923N**		AAC (0.54)	AAT (0.46)	2.0	1.2	NA	NA	NA
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			MYH7-E 12	Synonymous SNP	**9636 G/A**		AAG (0.13)	AAA (0.07)					
			MYH7-E 19	Intronic variation	**IVS 19+92 A/A**		NA	NA					
FHC 140	HCM	52/ M	MYH7-E 7	Synonymous SNP	**7647 A/G (A199A)**		GCA (0.23)	GCG (0.11)	1.1	1.7	NA	NA	father had HTN and died suddenly. Mother also has HTN and brother died of unknown cause at the age of 1yr.
			MYH7-E 3	Synonymous SNP and intronic variation	**5909T/C and IVS2-25 G/T**	Hetero zygote	ACC (0.36)	ACT (0.24)					
						Hetero zygote	NA	NA					

			MYH7-E 19	Intronic variation	**IVS19+92 A/G**		NA	NA					
			MYBPC3-E 31	Synonymous SNP	**1096 A/G**		GAG (0.58)	GAA (0.42)					
			TNNT2-E 14	Synonymous SNP	**A12750G (E246E)**		GAA (0.42)	GAG (0.58)					
FHC 137	HOCM	36/M	MYBPC3-E 15	Intronic variation	**IVS15-13 G/A**		NA	NA	1.2	2.1	53	Nil	None affected
			MYH7-E 3	Synonymous SNP	**5909T/C and IVS2-25 G/T**	Hetero zygote	ACC (0.36)	ACT (0.24)					
						Hetero zygote	NA	NA					
			MYH7-E 19	Intronic variation	**IVS19+92 A/G**		NA	NA					
			TNNI3-E 3	Intronic SNP	**IVS2-8T/A**		NA	NA					
FHC 102	HCM	33/F	MYBPC3-E 19	Intronic variation	**IVS19+92 A/G**		NA	NA	1.2	1.7	Nil	Nil	1 affected member and 1 sudden death.
			MYBPC3-E 24	Intronic variation	**IVS24+18 C/G;35G/T**		NA	NA					
			MYBPC3-E 14	Intronic variation	**IVS14-25C>T**	Hetero zygote	NA	NA					
			MYBPC3-E 5	Missense mutation	**V158M**	Hetero zygote	NA	NA					
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
FHC 144	HCM	55/F	MYBPC3-E 24	Intronic variation	**IVS24+18 C/G;35G/T**		NA	NA	1.3	2.0	Nil	Nil	1 affected daughter
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
			TNNT2-E 14	Synonymous SNP	**A12750G (E246E)**		GAA (0.42)	GAG (0.58)					
FHC 54	Familial ASH	13/M	TNNI3-E 3	Truncating mutation	**Lys 36 stop codon**		NA	NA	1.1	1.8	92	Nil	2 affected family members
			MYBPC3-E 22	Intronic variation	**IVS22+56 C/T**		NA	NA					
FHC 145	HCM	64/F	TNNI3-E 5	Synonymous SNP	**G2560T (R68R)**		CGG (0.20)	CGT (0.08)	1.0	1.4	Nil	Nil	NA
			MYH7-E 3	Synonymous SNP	**5909T/C**	Hetero zygote	ACC (0.36)	ACT (0.24)					
FHC	HCM	43/	MYH7-	Silent and	**Q1704Q**		CAG	CAA	1.3	1.5	Nil	Nil	NA

141		M	E 34	Missense genetic compound mutations			(0.73)	(0.27)					
					Q1683D		NA	NA					
			MYH7-E 19	Intronic variation	**IVS19+92 A/G**		NA	NA					
			TNNT2-E 16	Intronic variation	**IVS15-89C/T**		NA	NA					
			MYBPC3-E 5	Missense mutation	**V158M**	Heterozygote	NA	NA					
			MYBPC3-E 26	Silent mutation	**V849V**	Homozygote	GTC (0.24)	GTT (0.18)					
FHC 108 B1	HCM	28/M	MYBPC3-E 19	Frameshift mutation	**D570fs**		NA	NA	1.4	2.0	Nil	Nil	NA
			MYBPC3-E 5	Missense mutation	**V158M**	Heterozygote	NA	NA					
			MYBPC3-E 14	Intronic variation	**IVS14-25C>T**	Heterozygote	NA	NA					
			MYBPC3-E 26	Silent mutation	**V849V**	Homozygote	GTC (0.24)	GTT (0.18)					
			MYBPC3-E 24	Intronic variation	**IVS24+18 C/G;35G/T**		NA	NA					
			MYH7-E 3	Synonymous SNP	**5909C/T**	Heterozygote	ACC (0.36)	ACT (0.24)					
			MYH7-E 19	Intronic variation	**IVS19+92 A/G**		NA	NA					
			MYH7-E 20	Intronic variation	**IVS19-56 A/G**		NA	NA					
			TNNI3-E 3	Genetic compounds of intronic variations	**IVS2-8T/A &IVS3-21G/A**		NA	NA					
FHC 26	HOCM	26/F	MYBPC3 E-34	Intronic Deletion mutation	**25 bp deletion in I-33**		NA	NA	1.2	2.8	NA	13	NA
			MYBPC3 E-22	Intronic variation	**IVS 22+56C/T**		NA	NA					
			MYH7 E-3	Synonymous SNP	**5909T/C**	Heterozygote	ACC (0.36)	ACT (0.24)					
			MYH7 E-19	Intronic variation	**IVS19+92 A/A**		NA	NA					
P64	HOCM&	9/F	MYH7-	Synonymous	**Gln1704Gln(G>A)**		CAG	CAA	1.5	0.9	92	60	Cousin (SCD);
	Noonan syndrome		E 34				(0.73)	(0.27)		6			Consanguineous
			MYBPC3-E 26	Synonymous	**V849V**	Homozygote	GTC (0.24)	GTT (0.18)					

Fig.1 Pedigrees of familial cases

P47



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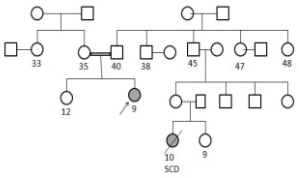

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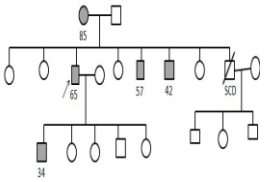

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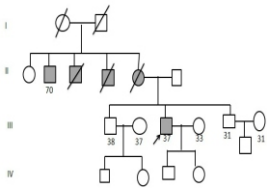

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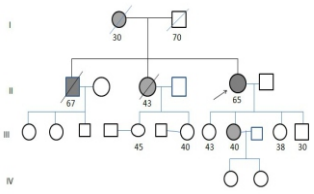

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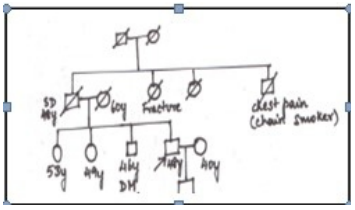

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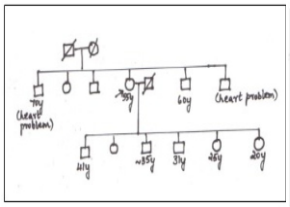

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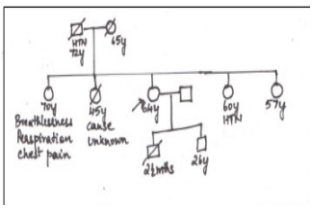

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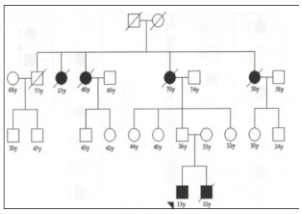
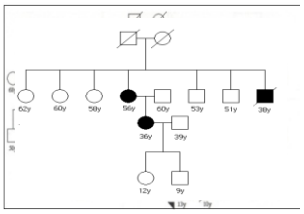


FHC-102



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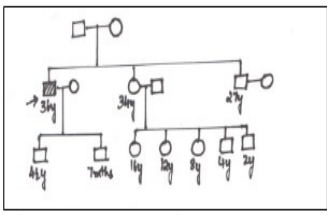

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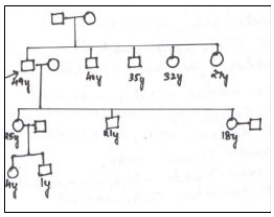

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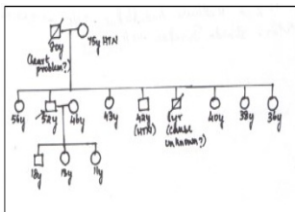
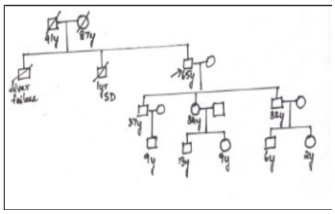


FHC-143



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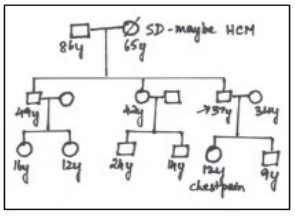

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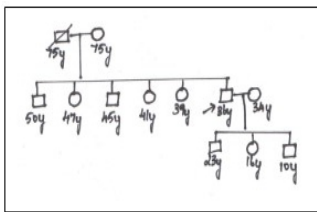
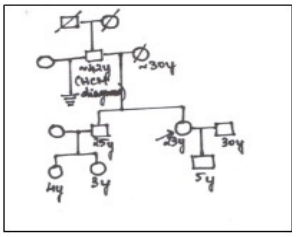


FHC-122



*P2:

This case revealed a compound heterozygous mutation in MYBPC3 along with 2 SNPs,one intronic and other exonic.The mutations and exonic variants being synonymous in nature,seem to mostly affect the spliceosome complexes,especially, SF2/ASF protein and SRp 55 in all 3 variations with slight variations in m-RNA secondary structure and its stability. Codon usage bias estimation proved to be an added contributor to the disease in this case. Similar to the case of P64, the proband also died early at the age of 17 and his brother died at age 10 of SCD.

*P38:

The compound heterozygous condition in MYH7 results in a decrease of alpha helices in protein structure which could have drastic effects on its interaction with other sarcomeric proteins. Also, both mutations put together affect major spliceosome complexes like SRp40 and SC35.These could be the major reasons for dominant negative effect of the variations in the proband whose family again shows male predominance as in the case of P47. This apart the protein structure of MYBPC3 in this domain is affected by an increase in beta sheets. The splicing enhancer and silencer binding sites have switched places between themselves at positions 63 and 66.

#P60:

In this case,both itronic variations in MYBPC3 occur as genetic compounds, which affects binding sites for SRp40 and SRp55.This same effect is also created due to the C>T SNP in exon 26 of MYBPC3.The secondary structure of m-RNA due to these 2 intronic SNPs are the same with slight instability of mutant (-51.1 Kcal/mol) compared to wildtype(-55Kcal/mol).In the exon 5 variation of V158M, the protein structure of MYBPC3 in this domain is affected by an increase in beta sheets. Though family history for this person was unavailable at the time of sampling, it would be difficult to rule out the possibility of familial inheritance considering the genetic compounds for intronic and exonic mutations in this proband.

*FHC-141:

A 48 year old male case of non-obstructive HCM with father having died suddenly at the age of 60 while exercising. His 46yr old brother has HTN/DM, DM being inherited from the mother. The genetic compound mutations in MYH7 results in drastic changes in spliceosome complexes, viz, SF2/ASF; SC35/SRp40; hnRNP1.Codon usage bias created by CAG/CAA(0.73/0.27) transition may be causal to increase the penetrance and early age at onset and severity of HCM. The other SNPs observed in MYH7 and TNNT2 may not have any significant effects. But, mutations in MYBPC3 in this case show significant results,viz, codon usage bias, protein folding changes in respective domains due to variations with disruptions in spliceosome complex, namely, SF2/ASF and hnRNP1 which are also affected in MYH7 mutations in this case. Thus, the compound heterozygous mutations in MYH7 and MYBPC3 must be acting synergistically to predispose this proband to HCM.

*FHC-144:

This case is a 50yr old female who has 2 elder brothers (>60yrs) with heart problems but no other familial history of CVDs. Except a single mutation in TNNT2 all other 3 variations are SNPs, all of them exhibiting significant variations in energy levels and was observed only in the exon 14 of TNNT2 mutation. Both the exonic variations in MYH7 and TNNT2 revealed a codon usage bias tilted towards the wildtype codon .Similar results were also observed wrt t-RNA usage frequency for the respective codons. Hence, this is a mild case of hypertrophy which is also supported by clinical features, genetic screening and absence of familial history.

*FHC-102:

A 33 yr old female presented with HCM. Her mother is affected and her maternal uncle died of sudden death. She has the same molecular picture as FHC-144, but also carries additionally, 2 variations, one an intronic mutation and other an exonic missense mutation. The intronic variant creates some disturbances to the spliceosomes:SRp40 and SRp55, apart from destabilizing the m-RNA secondary structure slightly. The exonic mutation results in an increase in the length of the β-sheets which might in turn have an effect on the protein folding. Also, the splicing analysis revealed that the splicing enhancer and silencer sites have switched places and hence might not have a very significant effect on the translation of protein. This case being familial, the disease could be attributed to a large extent on the 2 additional variants the proband carries apart from those which have been analysed in the case of FHC-144.

*FHC-140:

This is a 52yr old male patient whose father is suspected to have died of SCD. He is a carrier of 2 mutations and 3 polymorphisms. The variations in exons 19 and 31 of MYH7 and MYBPC3 genes respectively, have absolutely no effect as per insilico predictions. Both the synonymous mutations surprisingly result in loss of splice sites few bp downstream to the variation, though present in different genes (MYH7 and TNNT2). Codon usage bias and t-RNA usage frequencies are higher for the wildtype codon in all the variations. The disease progression in this patient could then be attributed to the combined effects of all these polymorphisms.

*FHC-122:

A 23yr old female patient diagnosed with HOCM harbored 3 intronic SNPs and a novel synonymous mutation (6350 C>T) in MYH7 gene. Except for codon bias observed for all 4 variants and a significant difference in wildtype and mutant m-RNA structures in one intronic SNP, no other pathogenic effects were found insilico. She having inherited the variant from her father who is also a confirmed case of HCM, we predict the role of some other modifier genes/the combined effects of the codon usage bias/ genetic anticipation which might influence the disease phenotype.

#FHC-108B1:

This particular case of non-obstructive HCM, is of the utmost interest considering the fact that the index case has 9 genetic compounds-1frameshift, 1 missense, 1 synonymous mutation and 7 other polymorphisms in MYH7,MYBPC3 and TNNI3 genes. The frameshift mutation produces a 570 aa long truncated MyBPC3 protein – lacking the titin & myosin binding sites. Family history of SCD with mother being affected. The missense mutation V158M revealed an increase in the length of the β-sheets, which might in turn have an effect on the protein folding. This was further confirmed by tertiary structure prediction by Pymol visualiser. The variation also resulted in the shift of the enhancer motif from position 63 to 66, and the silencer motif from position 66 to 63. The intronic variation IVS14-25 C/T results in a new binding site

for SRp 40 and loss of binding site for SRp50 spliceosomes, which being very imortant regulators of pre-m-RNA splicing will have a concordant effect on translation. No differences by insilico predictions were observed for SNPs in MYH7 and TNNI3 (Table.1). The E24 mutation in MYBPC3 resulted in significant differences in energy and ensemble diversity observed for m-RNA sec structures--262 and -256 Kcal/mol respectively for wildtype and mutant forms. Diversity is 257 and 203 resp. However there is no diff in alternative splice site prediction due to the mutation. With respect to the MYH7 mutation in E3, (5909C/T homozygote), no significant energy differences were spotted in m-RNA sec structures, but conformational changes in structures were observed. Though, statistically, this SNP is not fond to be significantly associated, codon bias could possibly be a compounding factor with respect to other SNPs/ mutations carried by this patient.

*FHC-105:

A case of HCM with familial history of ASH without obstruction; male predominance in the family, the proband has a major mutation D770N in the MYBPC3 gene, though not inherited, resulting in loss of splice donor site which could lead to a loss of myosin and titin binding sites since the protein would be truncated. None of the other family members carried this mutation suggesting that this patient may be a compound heterozygote for other causative mutations carried in the family. For the MYBPC3 gene mutation in E31, no significant association was reported, which is supported by no splicing differences and insignificant differences in m-RNA structure stability except for codon usage bias. Same is the case with the intronic SNP in E20 of MYH7 gene which has no significant association with the disease. However, m-RNA sec structure variation without sig energy differences along with codon usage bias could be a contributing factor for the role of E3 SNP in MYH7 (5909 C/T heterozygote) which is statistically significant.

FHC-26:

This is a case of HOCM who has a 25 bp deletion in Intron 33 of MYBPC3 apart from 2 other intronic variations and compound heterozygous variations of exon and intron in Exon 3 of MYH7. The 25 bp deletion resulted in splicing deficiency due to loss of splicing branch point. Both proband and son had HOCM (deletion present) and another son(deletion absent hence mild) had SH without obstruction all other siblings experienced neonatal deaths and available family members didn't carry this mutation proving that this mutation only adds to the phenotype. This genetic variant is novel and reported only in south India and seems to be segregating with the disease. The Exon 19 intronic variation in MYH7 was not found to be statistically significant, the insilico results also supporting the same. Compound exonic and intron variations in Exon 3 of MYH7 were statistically proven to be associated significantly with HCM which is also proven by the differences in wildtype and mutant m-RNA secondary structure and codon usage for the mutated codons. Being heterozygous, it could be having a dominant negative effect also on the disease progression. This clearly reveals why other affected family members had mild hypertrophy since these SNPs may be pathogenic but do not influence severity.

*FHC-139:

A male HCM patient whose mother died of sudden cardiac arrest and had no affected family members could have primarily developed the disease because of the I33 novel deletion in MYBPC3, since the other mutations, mostly intronic seem to have no particular effect on the m-RNA splicing/secondary structure, that is also supported statistically. As in the previous cases described above, the exon and intronic variations in E3 of MYH7 produces only conformational changes in m-RNA sec structure and also codon usage bias. As with the E24 intronic mutation in MYBPC3, no splicing pattern differences were observed but significant conformational and energy differences were observed.

*FHC-128:

This is a family with male HOCM case harboring a novel heterozygous mutation R870H, in the rod region of the myosin heavy chain protein. This patient had worked in the army for 10 yrs and hence might have developed such severe hypertrophy as a result of severe physical stress, which may rule out any case of inheritance given that familial information is unavailable. Heterozygote being more severely hypertrophied might be due to different pathways involved in hypertrophic heart failure and also dominant negative effect.

* FHC-136:

A male HCM case with no family history of cardiac disorders was found to be a genetic compound for 2 silent mutations in MYH7 gene and 2 intronic variations in MYBPC3 gene. Only the intronic variant IVS23-5C/T in MYBPC3 showed to be a splicing enhancer and also significantly associated with HCM while the other 2 MYH7 SNPs were affecting only codon usage and m-RNA secondary conformations.

FHC-110:

He is a 50 yr old HCM case with no family history of cardiac diseases. The MYBPC3 gene variations and TNNI3 variant along with the MYH7 E19 intronic variant have no significant effects on any molecular process both insilico and statistically. The E21 novel silent mutation results in a higher t-RNA frequency and correspondingly higher codon frequency of the mutant codon which might correlate to increased translational efficiency than the normal. However, the E3 mutation in MYH7 leads to a conformational change in m-RNA secondary structure without much change in its dynamics. This increase in translational efficiency with codon bias along with different conformation of m-RNA could lead to a conclusion that the evolutionary selection of an RNA-structure destabilizing allele would be compensating for a destabilizing amino acid substitution within a mutant protein structure (gkr 165 pdf)

*FHC-143:

A 65 yr old HOCM male whose family history is unavailable. Being a carrier for several mutations in the 3 primary genes (MYBPC3, MYH7 and TNNI3) responsible for HCM, he has developed it late probably because its not inherited. The compound intronic variations in TNNI3 are found to have no effect on RNA secondary structures, computationally. Similar to the case of FHC 108B1, the MYBPC3 E14 and E5 SNPs (IVS14-25 C/T and V158M) show similar effects, when determined insilico. Also, as discussed above, in case of FHC-110, the MYH7 E21 novel synonymous mutation leads to greater translational efficiency and may contribute to pathogenicity correlating to severity.

FHC-88:

This particular HCM male case is similar to FHC-122 with MYH7 compound mutations alone, 3 being synonymous SNPs and one being intron variant. The intronic SNP of MYH7 E19, has no significant effect both statistically and computationally. Except for the 5909T/C, where RNA secondary structure is seen without energy differences, the other synonymous SNPs seem to be effecting the disease through codon usage bias.

*FHC-137:

HOCM male case with no family history of cardiac events was molecularly assessed and found to harbour 4 intronic SNPs in 3 primary genes of HCM and one silent mutation. The E15 intronic variant IVS15-13 G/A, was observed only in this patient. Though it is close to splice acceptor site, there was no variation in splice score. Hence,unlikely to be the sole cause of HOCM. The E3 compound of intronic and exonic SNPs were found to be statistically and computationally associated significantly in the diseased phenotype through codon bias usage and structural changes in m-RNA. The other 2 intronic variants as shown in Table.1, have no significant effects on HCM.

*FHC-145:

A female HCM case who has few family members with cardiovascular disease related symptoms but no disease as such (Fig.1). This case is a genetic compound for 2 silent mutations in TNNI3 and MYH7. But, both variations resulted in insignificant differences in secondary structure of m-RNA and seem to affect only codon usage bias. Also, the Troponin C binding domain is predicted to be affected since this mutation lies in that region of Troponin I. All these factors together with some familial influence is predicted as the reason for HCM with respect to this case.

*FHC-54:

A 13 yr old boy affected by familial ASH and with a history of SCDs, presents a genetic compound of a truncated mutation which leads to a premature stop codon at the 40th amino acid. He is also a carrier for an intronic mutation, IVS22+56 C/T, in exon 22 of MYBPC3. The truncated mutation leads to helices and strands instead of coils in the 40 amino acid long mutant protein, as per secondary protein structure prediction, insilico (Psipred software). 3D model prediction of Troponin I wildtype and mutant reveals that the mutant protein is composed of only strands which makes a protein highly unstable and hence immediately initiates proteosome and ubiquitin mediated degradation, leading to a loss of expression of the gene and a null protein. The intronic mutation has no effect on splicing but leads to a small difference in m-RNA secondary structure with minor changes in structure which may only have minor effect on the disease compared to the effect which a truncated mutant protein will have on the disease.

From all the above data, the following epidemiological variables were found to be in common:

· The most affected age group- 25-55 yrs. Males are most commonly affected as supported by the pedigrees also where most affected family members are also males. This observation has already been reported previously. This study only reiterates that fact.

· Genes commonly involved- Hotspot genes MYBPC3 and MYH7; TNNI3.

· Types of HCM found to be most common- HCM and HOCM.

· Most frequently mutated exons-

MYBPC3: Exons 5, 14, 22, 24, 26

MYH7: Exons 3, 19, 34. Exon 3 seems to be very highly mutable since it is mutated in every other case

TNNI3: Exon 3

· Effect of family history- 17 cases out of 22 (77%) had familial history of HCM

The missense mutations in the MYBPC3 protein identified in these 22 cases lies in domain 1 (V158M mutation in Exon 5), domain 4 (D570fs mutation in exon 19) and domain 5 (D770N mutation in exon 24). The synonymous nucleotide changes which result in silent amino acid replacements are found to be in domains 2 and 6 of the protein.

Though the D770N mutation lies in the motif 5 which has a cardiac specific region, it doesn't lie in the 28 amino acid conserved region. Hence, it may not have a drastic effect on the intra protein folding which is mediated by the domains 3-7.The missense mutations S1685N, R870H, Q1683D in MYH7 mutations are present in coiled coil region of myosin heavy chain. COIL prediction software analysis revealed that the mutations have no effect on the coils.

Also, none of the mutations in Troponin I found in these cases have any missense changes.

DISCUSSION

Compound heterozygosity is a condition of having two heterogeneous recessive alleles at 2 different locations within the same gene or in different genes that can cause a genetic disease. As compound heterozygotes, the cases are likely to have less penetrance and are less deleterious than a classic homozygous case. As a result, compound heterozygotes , often manifest the disease with less severe symptoms. However, environmental factors may also determine the clinical outcome and clinical variations associated with a disease.

HCM patients with homozygous and double or compound heterozygous mutations typically present with more severe left ventricular hypertrophy and a higher incidence of sudden cardiac death events among family members, compared to individuals with single-gene mutations. Consequently, cases with multiple mutations are also significantly younger at diagnosis and more commonly present with childhood-onset hypertrophy. This is because the presence of 2 mutations has an additive effect in terms of molecular pathogenesis. This supports the notion of a mutation "dosage effect," where a larger amount of defective protein overruns normal sarcomere function and results in a more severe clinical course [8]. In HCM, multiple mutations mostly involve the β - MHC and MyBPC3 genes [10-12]. Mutations in the MyBPC3 gene mainly lead to truncated proteins which gives rise to a relatively mild phenotype. Many a time, childhood HCM occurs in association with another underlying condition, although 2 HCM related neonatal deaths were previously reported due to compound homozygous truncating mutations, also pure HCM in neonates is rare [21].

Mutations in the β -MHC and MyBP-C genes have also been reported to occur together with mutations in the cTnT, cardiac troponin I (cTnI), and cardiac actin (ACTC) genes. Double mutations of β -MHC with ACTC, and MyBP-C with cTnI are reported to cause severe childhood-onset hypertrophy, when each mutation in the single heterozygous state causes only mild HCM

heterozygous patients display more clinically severe disease compared to single heterozygous patients. This suggests that, for individuals with a positive family history and an early age of onset of the disease, the search for additional mutations should not stop with the identification of an initial putative HCM-causing defect [12]. Identification of 2 disease-causing mutations in a subgroup of patients will help to plan out prevention strategies and more aggressive treatment or initiation of therapies such as β -blockers or insertion of an implantable cardioverter-defibrillator [8].

Generally, loss-of-function variants lead to reduced or nil protein output due to non-sense mediated decay (NMD) if the termination codon is introduced at least 50–55 nucleotides upstream of the 3′ end of the penultimate exon [23], but there are also instances of NMD escape. Such variants do not result in a complete loss-of function and instead lead to mRNAs that escape NMD and are translated in heterozygous individuals, resulting in a dominant-negative effect [24, 25].

The primary focus of this study was to understand the effects of compound heterozygous mutations/SNPs on splicing and spliceosomes; m-RNA secondary structure conformations and dynamics; protein secondary and 3-D structures through computational predictions using online bioinformatic tools to relate them to the aspects of disease progression like age of onset, severity, effects of familial predisposition, etc.

60% of mutations that cause disease do so by disrupting splicing ; are located in noncoding regions, such as those affecting 5′ and 3′ ss, branch sites or polyadenylation signals, exonic as well as intronic splicing enhancers and silencers [26-28]. These are mostly cis-acting mutations which affects splicing thru spliceosomes and more than 50% of such mutations disrupt at least one of the target motifs for the SR proteins (SF2/ASF, SRp40, SRp55 and SC35) found in ESEs [29]. The rest can be explained on the basis of secondary structure rearrangements. Likewise, secondary structure explains why mutations that change splicing motifs sometimes show no splicing effect. These polymorphisms may lead to a disruption of the splicing code, thereby causing the splicing apparatus to utilize cryptic ss nearby or to skip one or more exons [30]. The observation that splicing affects phenotype and inturn the severity of HCM shows its prominence as a contributor to phenotypic variability and disease susceptibility[31-33].

In our study, all the SNPs/mutations involved in splicing, were found to affect SR proteins and the ASF/SF2 family of spliceosomes.

ASF/SF2 is an essential sequence specific splicing factor involved in pre-mRNA splicing [34-36], with a critical function in heart development [37], embryogenesis, tissue formation, cell motility, and cell viability in general [38, 39], and post-splicing activities, such as mRNA nuclear export and translation [40].

SR proteins are a highly conserved family of essential splicing factors like SC35, SRp40, SRp55, ASF/SF2 which promote initial steps in spliceosome binding to pre-m-RNA. This family was found to play a major role in TNNT2 gene exon 5 skipping consequently leading to the creation of an enhancer within the CDS [41].

Two main mechanisms are thought to be important factors in determining the efficiency of mRNA translation: (i) the ease of unwinding the mRNA structure at the 5′ end and (ii) codon usage. Use of low-efficiency codons near the initiation site aids the efficiency of translation which prevents ribosomal traffic jams near the initiation site.

Unusual codon usage is a side effect of selection for reduced mRNA structure [42. Codon usage bias is more likely to occur at sites which encode conserved amino acids [43, 44]. The rate of selection of aminoacyl-tRNA by different codons is- more preferred codons select their aminoacyl-tRNAs more quickly than more rarely used codons [45]. Surprisingly, though most genes are under selection to increase the use of preferred codons, some are found to be under selection in the opposite direction, reason for which is not determined [46, 47]. Though unlikely to explain the entire phenomenon, the use of unpreferred codons may possibly be selected for in cases where there is a need to stall translation. For example, inclusion of rare codons in intra domain regions would be advantageous, because this may allow for slowdown in translation, which will allow better domain folding and prevent detrimental effects.

This may be exemplified in our study considering the case FHC-110. The structure of mRNA molecules is determined by their sequence, and in order to maintain their structure some properties of their sequence may be under selection. Selection would also act on regulatory elements, such as transcription factor binding sites, RNA localization elements, translation initiation sites, and splicing signals, may be contained within coding sequences [48]. The 5′-end structure in allelic-dependent manner has been reported to control the in vitro translation rate [49]. The region in the vicinity of the start codon may potentially play a more significant role when coming to translational efficiency [50, 51].

This study has helped to give a whole new dimension to the way SNPs/mutations have been identified and analysed with respect to cardiomyopathy in the Indian context. It helps us understand how important synonymous SNPs or mutations are and how protein folding and other molecular processes will drive a pathological change in the cardiac sarcomere. The study also reinstates that the main genes involved in cardiomyopathy are MYBPC3, MYH7 and TNNI3, which have been reported to carry several hotspot mutations/SNPs.

CONCLUSION

We present the molecular and insilico analysis of compound heterozygosity in 22 cases, most of them being familial cases. This study broadens the spectrum of understanding the complex interactions and effects of the genetic compounds in an individual HCM case, with genetic anticipation, codon usage bias, penetrance, splicing and m-RNA conformational changes playing major roles in molecular pathogenesis of cardiomyopathy. Hence, comprehensive genetic testing might provide important insights into risk stratification and potentially indicate the need for differential surveillance strategies based on genotype and compound heterozygosity.

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