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SHORT REPORT

Genetic and phenotypic characterization of mutations in myosin-binding protein C (MYBPC3) in 81 families with familial hypertrophic cardiomyopathy: total or partial haploinsufficiency

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Mutations in the *MYBPC3* gene, encoding the sarcomere protein myosin-binding protein C, are among the most frequent causes of autosomal dominant familial hypertrophic cardiomyopathy (FHC). We studied the frequency, type, and pathogenetic mechanism of *MYBPC3* mutations in an unselected cohort of 81 FHC families, consecutively enrolled at a tertiary referral center. Nine mutations, six of which were novel, were found in 10 (12.3%) of the families using single-strand conformation polymorphism and DNA sequencing. A frameshift mutation in exon 2 clearly suggests that haploinsufficiency is a pathogenetic mechanism in FHC. In addition, splice site mutations in exon 6 and intron 31, a deletion in exon 13, and a nonsense mutation in exon 25, all lead to premature termination codons, most likely causing loss of function and haploinsufficiency. Furthermore, there were two missense mutations (D228N and A833 T) and one in-frame deletion (ΔLys813). A considerable intrafamilial variation in phenotypic expression of *MYBPC3*-based FHC was noted, and we suggest that mutations influencing stability of mRNA could play a role in the variable penetrance and expressivity of the disease, perhaps via partial haploinsuffciency.
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Introduction

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant cardiac disorder characterized by unexplained myocardial hypertrophy, predominantly of the interventricular septum.[1,2] The clinical phenotype varies from asymptomatic over limited symptoms to severe heart failure or the occurrence of serious arrhythmias or sudden cardiac death.[3]

FHC has been associated with more than 200 mutations in 10 known genes encoding cardiac sarcomere proteins.[4] Furthermore, mutations in other genes (eg mitochondrial DNA and *PRKAG2*) have been associated with a cardiac phenotype resembling hypertrophic cardiomyopathy.[5,6] Mutations in *MYH7*, encoding β-myosin heavy chain, and in *MYBPC3*, encoding myosin binding protein C, are the most prevalent and probably account for 20–40% of FHC cases.[4,7]

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We studied the occurrence of *MYBPC3* mutations in a cohort of 81 consecutive patients in a nationwide Danish FHC study. We found nine mutations, six of which were novel, in 10 families. In three families, mutations in both *MYBPC3* and *MYH7* were found. Several of the mutations undoubtedly result in total or partial haploinsufficiency.

Materials and methods

Patients

In all, 81 unrelated FHC families were remitted consecutively from The National University Hospital, Rigshospitalet, Copenhagen, a tertiary referral center for FHC. DNA samples from 100 blood donor samples were used as controls.

Clinical evaluation

Clinical examinations were performed as previously described.[8] All probands fulfilled conventional diagnostic criteria for hypertrophic cardiomyopathy.[9]

Mutation analyses

Mutation analyses were performed by single-strand conformation polymorphism/heteroduplex analysis (SSCP/HA)[10] or capillary array single-strand conformation polymorphism (CAE-SSCP)[11,12] of amplicons covering all 34 coding exons of *MYBPC3* using intronic primers from[13] or defined from the gene sequence (GenBank Accession No. U91629). The sequence of primers and PCR conditions are available upon request. Genetic variants with frequencies above 1% in controls were considered polymorphisms. Seven other FHC-associated genes (*ACTC, MYH7, MYL2, MYL3, TNNI3, TNNT2*, and *TPM1*)[11,14,15] were screened for mutations in the genotyped probands.

Messenger RNA transcript analysis

Ectopic mRNA expression of MYBPC3 in peripheral blood lymphocytes was studied.[16–18] RNA was extracted from fresh blood using an RNA purification kit (Qiagen, Germany). RT-PCR was performed using MMLV Reverse Transcriptase (Stratagene, LA Jolla, CA, USA) and exon specific primers. In case of amplification with the 5′-proximal primer set, MYBPC1EF (5′-CGACCAGGGATCTTACGCATG-3′) and MYBPC12ER (5′-GGGTGCCTGCCGTAGGATCTC-3′) a second, nested PCR amplification was applied on the primary amplicon using primers MYBPC294F (5′-CGACCAGGGATCTTACGCAGT-3′) and MYBPC897R (5′-CCTCA TGGCTATCACTGATCCG-3′). For amplification of the 3′-proximal region, primers MYBPC29EF (5′-CGCTCGCC GCGTGCATTCAG-3′) and MYBPC33ER (5′-CCGTCAAAG GGGCAGGGCTTTC-3′) were applied. RT-PCR products were cloned into pCR-Script (SK) (Stratagene) and sequenced.

Results

Mutation screening

Nine mutations were detected in 10 families with one mutation present in two families (Table 1). Three of the mutations (g5256G>A (Glu258Lys; Figure 1), g16153G>A (Ala833Thr), and g15919insG) have previously been characterized.[13,19] The six novel mutations consisted of a missense mutation (g5166G>A (Asp228Asn)) and a nonsense mutation (g16182C>G; Tyr842Ter), three exonic deletions (g2432delT, g10080-96delCTGACCGTGGAACTGG, and g16086delAAG (DelLys813) and one intronic deletion (g20931-37delCCTGTCA(−8-(−)14)).

The small size of most of the genotyped families makes it difficult to establish a causal relationship between the

Table 1 Mutations in *MYBPC3* and their tentative pathogenetic mechanism

Family	*Mutation*	*Intron/exon*	*Region*	*Type*	*Tentative result*	*Experimental*	*Tentative functional consequence*
ZV	g2431ΔT[a]	Exon 2	N-terminus	Deletion	Frameshift PTC (next codon)	2 mRNA species wt and mutant	PTC and HI
ZJ	g5166G>A[a]	Exon 6	C1-Ig motif	Missense	D228N	ND	Altered function/HI
ZI	g5256G>A[b]	Exon 6	C1-Ig motif	Missense	Exon skipping PTC/E258K	2 mRNA species wt, and exon 6 skipped	PTC and HI
ZL	g10080-96ΔCT..GG[a]	Exon 13	C3-Ig motif	Deletion	Frameshift + PTC	ND	PTC and HI
ZF and ZP	g15919insG[b]	Exon 24	C6-Fn-III motif	Insertion	Frameshift or exon skipping/PTC−	ND	PTC and HI
ZX	G16086ΔAAG[a]	Exon 25	C6-Fn-III motif	Deletion	DelK	ND	Altered function/HI
E	g16153G>A[a]	Exon 25	C6-Fn-III motif	Missense	A833T	ND	Altered function/HI
C	g16182C>G[a]	Exon 25	C6-Fn-III motif	Nonsense	PTC/Y842Ter	ND	PTC and HI
ZH	g20931-37[a] ΔlCCTGTCA (−8(−)14)	Intron 31	C9-Fn-III motif	Deletion	Affects splicing	2 mRNA species: wt and exon 32 skipped	PTC and HI

References: [a]Present study; [b]Niimura *et al*.[13] PTC, premature termination codon; FS, Frameshift; WT, normal allele; NMD, Nonsense-mediated mRNA decay; HI, total or partial haploinsuffiency; ND, not determined.

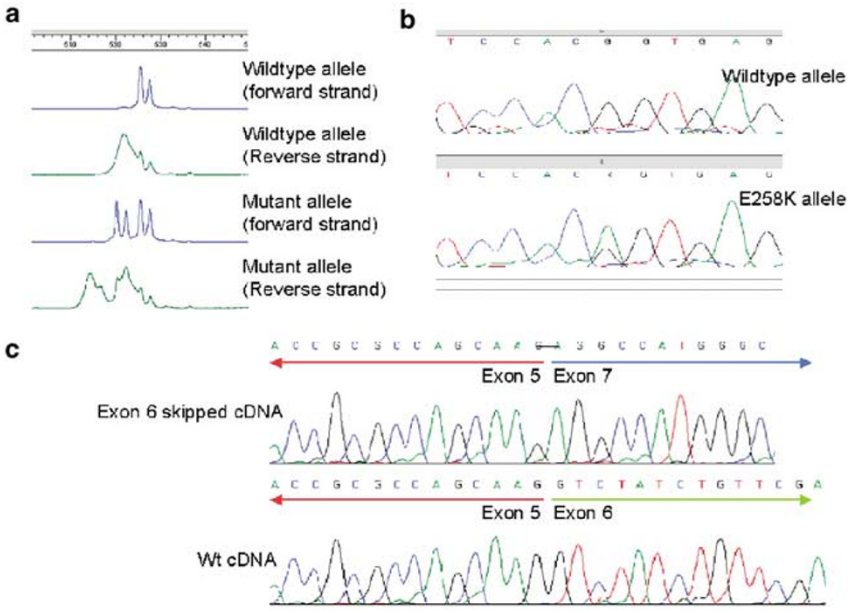

Figure 1 Detection of g5256G>A (Glu258Lys) mutation and consequent exon skipping. (**a**) Capillary array SSCP, where the upper two panels represent the wild-type chromatograms (forward (upper) and reverse (lower), respectively); the two lower panels represent the mutant chromatograms (forward (upper) and reverse (lower), respectively); (**b**) DNA sequence of the wild-type control (upper panel) and the heterozygote (G>A substitution) (lower panel); (**c**) DNA sequence of RT-PCR clone containing the splice site mutation; upper panel illustrates sequence where exon 6 has been skipped and lower panel the normal cDNA sequence.

occurrence of a specific mutation and FHC. *MYBPC3* and *MYH7* mutations were found in the families E, G and XI, where the latter two carried very rare variants in *MYBPC3* of unknown significance besides disease-associated mutations in *MYH7*.

Messenger RNA studies

In order to determine the consequences of mutations, we performed analysis of ectopically expressed *MYBPC3* mRNA in the absence of cardiac tissue. Nested RT-PCR performed on DNA from a g2432ΔT mutation carrier using exon-specific primers MYBPC1EF/MYBPC12ER followed by nested PCR with primers MYBPC294F/MYBPC897R) resulted in one band on a gel, migrating as an approximately 500 bp DNA fragment. Cloning and sequencing revealed that both alleles were present.

Nested RT-PCR analysis in a g5256 mutation carrier, from family ZI, using the primer pair MYBP294F and MYBPC897R for reamplification, yielded two dominant PCR products corresponding to the wild-type allele and an allele with exon 6 skipping (Figure 1c). Exon skipping resulted in a frameshift and a premature termination codon, and, most likely as a consequence in haploinsufficiency.

The deletion in intron 31 (g20931-37delCCTGTCA (−8(−)14)) was shown by RT-PCR using MYBPC29F and MYBPC33R primers to result in both a normally spliced transcript and a transcript with exon 32 skipped.

The remaining splice-site mutations with a possible splice variation, IVS8-20C>A and IVS16-6G>A, and the insertion/deletion mutations could not be analyzed as fresh blood or cardiac tissue from the mutation carriers could not be obtained.

Clinical evaluation of patients with *MYBPC3* mutations

Among the 44 carriers with mutations solely in *MYBPC3*, there were 25 carriers (57%) that had a maximum LVD⩾13 mm and 14 carriers (32%) with HCM symptoms or prior myectomy. In three families, ZF, ZH, and ZP, no major cardiac events had occurred, and in the other families, one to two members had experienced a cardiac event. Three of the probands with a *MYBPC3* mutation (C, ZL, and ZV) had a debut age under 18 years with severe hypertrophy. A detailed clinical description of patients with digenic inheritance is shown in Table 2. No specific phenotype could be attributed to these patients.

Discussion

The prevalence of *MYBPC3* mutations in Denmark, 10/81 (12.3%) is similar to the frequency of 13.6% (15/110) recently reported in a study of Caucasian patients[20] and

Table 2 Clinical/characteristics in families with mutations in both *MYBPC3* and *MYH7*

Id	*Age(years)*	*Sex*	*Symptoms/interventions*	*Max LVD (mm)*	*Location*	*LVOT (mmHg)*	*ECG*	*MYBPC3*	*MYH7*
XI:III-1	61	M	NYHA 2	18	Septal	32	RE-sc:5	g6181C>A+V896M	D778E
XI:III-3	35	M	SCD	NA	NA	NA	NA	g6181C>A+V896M	D778E
XI:IV-1	40	M		17	Ant wall	8		g6181C>A+V896M	D778E
XI:IV-2	39	M	Syncope, NSVT/ICD	18	Mid-sept	60	RE-sc:2		D778E
XI:IV-3	36	F	NYHA 2	14	Septal	41	RE-sc:5, Sokolow	g6181C>A+V896M	D778E
XI:IV-4	38	M	NYHA 2	15	Septal	9	Sokolow		D778E
G:II-1	65	M	Myectomy	15	Septal	1	RE-sc:3	g10899G>A	R694C
G:III-1	41	M		12		6		g10899G>A	
G:III-3	36	M		16	Septal	9	RE-sc:3, Sokolow		R694C
G:III-5	41	M		16	Septal	10		g10899G>A	R694C
G:IV-2	11	M		8		5	Sokolow		R694C
G:IV-3	11	F		8		6	RE-sc:3, Sokolow	g10899G>A	R694C
E:II-1	65	F	Myectomy	23	Septal	91	LBBB	A833T	L390V
E:II-8	78	F		23	Septal	10	Q-waves	A833T	L390V
E:II-10	70	F	NYHA 2	21	Septal	90	RE-sc:1		L390V
E:III-1	43	M		11		4		A833T	
E:III-4	40	F		7		6		A833T	
E:III-7	53	M		17	Septal	4		A833T	
E:III-12	52	F		10		6			L390V
E:III-14	46	M		14	Septal	7			L390V
E:IV-1	10	M		7		4	Sokolow	A833T	

NYHA: New York Heart Association heart failure classification. SCD: Sudden cardiac death. NSVT: Non-sustained ventricular tachycardia. ICD: Intracardiac defibrillator. RE-sc.: Romhilt-Estes score. Sokolow: Sokolow criteria for left ventricular hypertrophy fulfilled. LBBB: Left bundle branch block. NA, data not available.

10.8% (14/130) in a Japanese study[13] but much lower than recent reports where 24% of Finnish HCM cases carried *MYBPC3* mutations[21] and in a French study with a prevalence of 42% *MYBPC3* mutations among 124 genotyped patients.[4]

In agreement with other studies, we find that late onset is characteristic, but in some families, children may be affected.[13,20] The relatively low proportion of mutations leading to single amino-acid substitutions is similar to the findings in the Caucasian study,[20] contrary to the findings in a Japanese and a recent French study.[4,13] The g15919insG mutation, previously described by Niimura *et al*,[13] was found in two families (ZF and ZP) and could be a founder mutation, analogous to the Turkish, Finnish and South African founder mutations.[20,22] Haplotype analysis using STRs in the flanking regions as well as SNP analysis in the *MYBPC3* shows that the two probands are not closely related.[23]

The finding of three families with mutations in both *MYBPC3* and *MYH7* is surprising, but the phenomenon of digenic inheritance in these genes has previously been described in recent French and European studies.[4,24] These findings should lead to a re-evaluation of all patients diagnosed with HCM based on *MYBPC3* mutations, since they may carry the true disease-causing mutations on other genes.

Haploinsufficiency

Six out of the 10 mutations found in this study resulted in generation of a premature termination codon (Table 1). For at least three of these (g2432ΔT, g5256G>A, and g10080-96delCT…GG), and possibly also the remaining three, the translation product will be so short as to prevent effectively its incorporation in the sarcomere; thus in these three cases, there will be a quantitative rather than a qualitative deficiency of the sarcomere – haploinsufficiency – as mRNAs containing premature termination codons are often rapidly degraded through the so-called nonsense mediated mRNA decay.[25] However, the splice variants that were observed may be artifacts due to ectopic expression in leukocytes, and final proof of aberrant splicing for mRNA or protein studies may only be documented in cardiac tissue, which has not been available in the present cases.

It is interesting that the most seriously affected families, ZV, ZI, and ZL are the ones where haploinsufficiency as a pathogenic mechanism is mostly likely. However, only large studies comparing clinical phenotypes with mutations and polymorphisms, in large families, and corresponding studies of transcription and translation, can provide the certain knowledge we need to have in order to use the genetic workup clinically. Presently, as our study documents, mutations in *MYBPC3* should be studied carefully before being assigned as disease causing.

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