Evidence From Human Myectomy Samples That MYBPC3 Mutations Cause Hypertrophic Cardiomyopathy Through Haploinsufficiency*

Steven Marston, O’Neal Copeland, Adam Jacques, Karen Livesey, Victor Tsang, William J. McKenna, Shapour Jalilzadeh, Sebastian Carballo, Charles Redwood, Hugh Watkins

Rationale: Most sarcomere gene mutations that cause hypertrophic cardiomyopathy are missense alleles that encode dominant negative proteins. The potential exceptions are mutations in the MYBPC3 gene (encoding cardiac myosin-binding protein-C [MyBP-C]), which frequently encode truncated proteins.

Objective: We sought to determine whether there was evidence of haploinsufficiency in hypertrophic cardiomyopathy caused by MYBPC3 mutations by comparing left ventricular muscle from patients undergoing surgical myectomy with samples from donor hearts.

Methods and Results: MyBP-C protein and mRNA levels were quantitated using immunoblotting and RT-PCR. Nine of 37 myectomy samples had mutations in MYBPC3: 2 missense alleles (Glu258Lys, Arg502Trp) and 7 premature terminations. No specific truncated MyBP-C peptides were detected in whole muscle homogenates of hypertrophic cardiomyopathy tissue. However, the overall level of MyBP-C in myofibrils was significantly reduced (P<0.0005) in tissue containing either a truncation or missense MYBPC3 mutation: 0.76±0.03 compared with 1.0±0.05 in donor and 1.01±0.06 in non-MYBPC3 mutant myectomies.

Conclusions: The absence of any detectable truncated MyBP-C argues against its incorporation in the myofiber and any dominant negative effect. In contrast, the lowered relative level of full length protein in both truncation and missense MYBPC3 mutations argues strongly that haploinsufficiency is sufficient to cause the disease.

Myosin binding protein-C (MyBP-C) is a component of the thick filaments of striated muscles. The human cardiac muscle isoform, encoded by MYBPC3, is composed of 11 globular domains, 8 with homology to IgI and 3 with fibronectin III (Figure 1). MyBP-C is likely to have both structural and regulatory roles within the sarcomere, and recent data have suggested that MyBP-C has a role in relaxation and stretch activation. The physiological importance of MyBP-C has been further highlighted with the discovery of mutations in MYBPC3 as the most commonly identified cause of hypertrophic cardiomyopathy (HCM), typically being found in ~20% to 25% of patients screened; more than 150 different mutations have been reported. In striking contrast to all other HCM disease genes, approximately two-thirds of MYBPC3 mutations are predicted to generate a truncated protein product. At present, it is not known whether the autosomal dominant nature of the MYBPC3 mutations results from haploinsufficiency (indicating that functional loss of one copy of the gene cannot be compensated) or a poison peptide effect (by which the mutant proteins interfere with normal sarcomere function). Functional studies on HCM mutants of other proteins have given clear evidence of a poison peptide effect. Published studies on the heart muscle of individual patients with different MYBPC3 truncation mutations did not find truncated protein, but one study suggested reduced MyBP-C content. Data from transgenic mouse models that overexpress truncated cMyBP-C have been conflicting, with support for both mutant protein incorporation and haploinsufficiency. Mice with both alleles of MyBP-C knocked out are viable, in one model, heterozygous null mice show a slight decrease in MyBP-C expression and a late-onset hypertrophy phenotype, consistent with a haploinsufficiency mechanism. In this report, we have searched for truncated peptides and reduced MyBP-C quantity in myofibrils from control and affected human heart tissue and find a consistently lower MyBP-C expression in the patients with either truncation or missense MYBPC3 mutations.

Methods

We obtained human heart muscle from donor hearts and interventricular septum from HCM patients at surgical myectomy. Genotyping and mRNA analysis was by standard methods. MyBP-C protein was detected in muscle homogenates and myofibrillar fractions using an antibody specific to the N-terminal region of MyBP-C, and the MyBP-C content was quantified relative to the actin content using an anti-actin antibody.

An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

We screened for MYBPC3 mutations in a series of left ventricular septum samples from HCM patients undergoing septal myectomy to relieve left ventricular outflow tract obstruction. In 9 of the 39 patients, mutations in MYBPC3, with convincing evidence that they were responsible for HCM, were identified (Figure 1). Two carried previously described missense alleles Glu258Lys (sample code M10) and Arg502Trp (MA); 7 had premature terminations, truncating in domains C3 (same mutation present in M8, MI, MT;
predicted molecular mass, 52 kDa), C5 (M9, 90 kDa), C7 (M15, 97 kDa; M25, 114 kDa), and C10 (M6, 140 kDa).

Immunoblots were carried out on whole tissue homogenates from the myectomy samples using an antibody specific to the N terminus (C0-C2) of MyBP-C (see Online Figure I). At moderate loading (2 μg of tissue), MyBP-C was detected as a single band (Figure 1B). We did not observe any bands corresponding to the expected truncated protein in M6, M8, M9, M15, M25, MI, or MT at moderate (Figure 1B) or high (Online Figure II) loading. Loading tests indicated that the antibody could detect a concentration of <3% of the main bands.

The quantity of MyBP-C in myofibrils was determined in myectomy samples and compared with nonfailing donor heart muscle (Figure 2). The quantity of MyBP-C relative to actin was consistent between donor heart samples and the mean ratio was used to normalize all of the data. An MyBP-C/actin content significantly lower than donor was found in every myectomy sample containing a MYBPC3 mutation, including the 2 missense mutations (Figure 2B and Online Figure III and Online Table II). The mean MyBP-C/actin ratio in myofibrils of all samples with MYBPC3 mutations was 24% lower than donor tissue, but the ratio was unaltered in myectomy samples that did not have a MYBPC3 mutation (myectomy/donor 1.01 ± 0.06).

To examine whether reduced amounts of mutant message contributed to the lower total MyBP-C protein content, wild-type/mutant MYBPC3 mRNA ratios were measured in 4 of the samples using a real-time PCR assay with allele-specific probes (Figure 2C). A moderate decrease in the relative abundance of the mutant transcript in comparison with the wild type was found in 3 samples, including the missense mutation sample M10.

### Abbreviations and Acronyms

<table>
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<th>Description</th>
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<tr>
<td>HCM</td>
<td>hypertrophic cardiomyopathy</td>
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<tr>
<td>MyBP-C</td>
<td>myosin binding protein-C</td>
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</table>

### Discussion

We have been able to systematically assess the effect of both truncation and missense HCM-causing MYBPC3 mutations in human heart muscle by studying a series of 9 samples obtained from patients undergoing surgical myectomy in comparison with donor heart and myectomy samples without a MYBPC3 mutation. In samples with MYBPC3 truncation mutations, we show that no truncated MyBP-C proteins are detectable, either incorporated (as determined by analysis of myofibril fractions) or indeed unincorporated into the sarcomere (from analysis of homogenates). In the analysis of all heart samples of patients bearing MYBPC3 mutations, we find a 24% lower MyBP-C content, thus arguing strongly for haploinsufficiency as the disease mechanism for both truncation and missense mutations. This agrees with, and extends, certain earlier observations.7–9 For the truncation mutants, the measured modest reductions in mRNA (Figure 2C), possibly caused by nonsense-mediated mRNA decay, cannot account for the undetectable levels of mutant MyBP-C protein, and thus degradation of the truncated protein is likely, possibly via the ubiquitin–proteasome system as earlier proposed.14

The presence of normal MyBP-C mRNA from the remaining wild-type allele is apparently not sufficient to yield a full complement of MyBP-C protein; this is in contrast to some other contractile proteins, for example, a heterozygous α-tropomyosin knockout mouse has the normal level of protein in the heart.15 Our surprising finding that missense mutations can cause MyBP-C haploinsufficiency may also be explained by proteolysis of the mutant protein (as reported for

![Figure 1. Location of MyBP-C mutations and detection of MyBP-C protein in muscle homogenates. A, Domain structure of MyBP-C showing the location of the MyBP-C mutations detected in the myectomy samples. B, Whole homogenates of heart muscle probed with anti MyBP-C. A total of 2 μg of whole muscle homogenate was separated by SDS-PAGE, and MyBP-C was detected using a rabbit polyclonal antibody. Arrows indicate the predicted position of truncated peptides for M25 (114 kDa), M15 (97 kDa), M9 (90 kDa), and M8 (52 kDa). MA and M10 are missense mutations; M5 has no MyBP-C mutation. A single ~140-kDa band was observed in all myectomy samples, including those with predicted MyBP-C truncations.](http://circres.ahajournals.org/doi/fig/10.1161/CIRCRESAHA.108.193544)
1 heterologously expressed MYBPC3 missense mutant\(^6\), although a modest reduction in mutant mRNA (as suggested by the M10 data in Figure 2C) could also account for the reduced full-length protein; the mechanism by which a reduced mRNA level of a missense allele is achieved is unclear. It remains possible that the missense mutant protein does incorporate and has an additional deleterious effect.

Although haploinsufficiency has not been observed with mutations in other HCM genes, we propose that in the case of MYBPC3 mutations, it is sufficient to cause the disease. Functional studies, in which MyBP-C has been partially extracted from fiber preparations to a similar extent to the reduction observed in myectomy tissue, suggest that the measured reduction in protein content is sufficient to have a significant effect on contractility.\(^{17,18}\) The depletion of MyBP-C protein in different samples is not equivalent, and we suggest this may contribute to the observed spectrum of disease severity.

Acknowledgments
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Disclosures
None.

References


Key Words: cardiomyopathy mutation myocardial contractility
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SUPPLEMENT MATERIAL

Evidence from human myectomy samples that MYBPC3 mutations cause hypertrophic cardiomyopathy through haploinsufficiency.

METHODS

Collection and Storage of Human Myocardium

Hypertrophic Cardiomyopathy Human myocardial samples were obtained from patients with hypertrophic cardiomyopathy undergoing surgical septal myectomy for relief of left ventricular outflow tract obstruction. The samples were frozen in liquid nitrogen and stored for later use. Local ethical approval was obtained from University College London Hospitals and Royal Brompton and Harefield ethics committees for collection of tissue samples. The HCM patients clinical phenotypes were characterised by obtaining detailed clinical histories and examinations. All patients had cardiac investigations including, 12-lead ECG, chest X-ray, holter monitor, cardiopulmonary exercise test, two-dimensional transthoracic echocardiography, transoesophageal echocardiography, cardiac catheterisation and coronary angiography (Online Table I) 1,2.

Non-Failing donor heart muscle. Tissue samples were supplied by Prof. C Dos Remedios, University of Sydney, Australia. Ethical approval was obtained from The Brompton, Harefield & NHLI, London and St Vincent’s Hospital, Sydney. The investigation conformed with the principles outlined in the Declaration of Helsinki. Non-failing heart tissue (donor) was obtained from donor hearts where no suitable transplant recipient was found. These were obtained from patients with no history of cardiac disease, a normal cardiac examination, normal ECG and normal ventricular function on echocardiography within 24 hours of heart explantation. Myocardium was immediately frozen in liquid nitrogen and stored for later analysis. Clinical and functional characteristics of troponin from these samples has been previously reported 3.
### Online Table I: Clinical details, myectomy patients in this study

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Clinical Data from patients who underwent a surgical myectomy SCD=sudden cardiac death, AS=alcohol septal ablation, PPM=permanent pacemaker, ICD=implantable cardioverter defibrillator, VSD=ventricular septal defect, LVWT=left ventricular wall thickness, ST-septal thickness, LVESD=left ventricular end diastolic dimension, LVEDD=left ventricular end systolic dimension, FS=fractional shortening, SAM=systolic anterior motion of the mitral valve, MR=mitral regurgitation, LVOT=left ventricular outflow tract, VT=ventricular tachycardia, VE=ventricular ectopics, ETT=exercise testing, SR=sinus rhythm, LVH=left ventricular hypertrophy, RAD=right axis deviation, LAD=left axis deviation, NYHA=New York Heart Association.
Genotyping of HCM Patients

Blood samples were collected and genetic analysis performed on the genomic DNA extracted. Local ethical approval was obtained for collection of blood samples; mutation screening of coding regions and splice sites of the MYBPC3 gene (exon 1-34) was undertaken using ‘Hi-res Melting’ analysis (LightScanner) and bi-directional fluorescent sequencing using big dye terminators and ABI3730.

Preparation of whole muscle homogenates and myofibrillar fraction from human heart muscle

Human heart samples (50mg) were removed from liquid nitrogen and immediately pulverised in a LN$_2$ cooled percussion mortar. Pulverised human heart muscle was manually homogenised in 1.5ml of a wash buffer containing 5mM NaH$_2$PO$_4$, 5mM Na$_2$HPO$_4$ pH 7.0, 0.1M NaCl, 5mM MgCl$_2$, 0.5mM EGTA, 0.1% Triton X-100 and 5mM DTT with 2µg/ml each of the protease inhibitors E64, chymostatin, leupeptin and pepstatin A. This whole homogenate was used to assay for truncated peptides. For myofibrils the homogenate was then centrifuged at 16,500xg for 5 minutes and the supernatant discarded. The wash-homogenisation-sedimentation step was repeated three more times until the pellet was pale yellow. The myofibrillar pellet was then dissolved in SDS-gel solution for analysis by SDS-PAGE.

Western blotting.

The MyBP-C content in whole muscle homogenates and in the myofibrillar fraction was measured in western blots of SDS-PAGE. Gel-electrophoresed proteins were transferred to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, RPN 303D) with a Hoeffer semi-dry electroblotter (Semi-phor TE70) at 6mA and 200 mV for 2 hours at 4°C. Membranes were blocked for 1 hour at room temperature in blocking buffer [1% dried milk powder, 0.1% Tween 20 and 1 x phosphate buffered saline (137 mM NaCl, 27 mM KCl, 4.3 mM Na$_2$HPO$_4$·7H$_2$O, 1.4 mM KH$_2$PO$_4$, pH 7.3)]. Blots were incubated overnight in rabbit polyclonal antibody to MyBP-C at 1/20,000 (gift from Samantha Harris) normalised to tissue quantity by including a rabbit polyclonal anti-actin antibody (Sigma A2066, 1/2000). MyBP-C and actin were detected using anti-rabbit HRP and ECL and visualised with a cooled CCD camera. 12-bit TIFF images were analysed with GeneQuant software (Syngene). Tests with recombinant MyBP-C fragments showed that the antibody cross-reacted specifically with the N-terminal region (C0-C2, see Online Figure I).
mRNA quantitation

RNA was extracted from 30 mg of frozen myectomy tissue using the RNeasy Fibrous Tissue kit (Qiagen) according to manufacturers’ instructions. Reverse transcription was carried out using oligo dT primers. The following Taqman probes specific for the wild type (FAM labelled) and mutant (YAK labelled) allele for samples M6, M9, M10 and M15 were synthesised:

M6:  5’-CGCCTGGAGGTGCAGTGCC (wild type)  
5’-CGCCTGGAGGTGTGAGTGCC (mutant)
M9:  5’-CCTGCAGTACAGTTGGGAGCCGC (wild type)  
5’-CTGCACAGTACAGGTTGGGAGCCGC (mutant)
M10:  5’-CTCACTGTCCACGAGGCCTGGGCA (wild type)  
5’-CTCACTGTCCACAAGGCCTGGGCA (mutant)
M15  5’-TCGCTGGGGGGACCGATAGGC (wild type)  
5’-GGTTCGCTGGGGGTCCGATAGGC (mutant)

qPCR carried out using a Roche 480 Lightcycler. The difference in the number of cycles between wild type and mutant to reach threshold (ΔCt method) was used to calculate the ratio of wild type to mutant mRNA with the assumption that the efficiencies of the wild and mutant reactions were identical.
RESULTS

Online Figure I
Demonstration of specificity of the MyBP-C antibody for the N-terminal domains C0-C2 upstream of all predicted chain terminations.
Online Figure II

Western blot of 15µg of whole muscle homogenate separated by SDS-PAGE and probed with antibody to MyBP-C. Bands at 95, 60 and 45kDa represent non-specific labelling of myofibrillar proteins or breakdown of MyBP-C and were the same in all samples. Arrows indicate the predicted position of truncated peptides in M25, M15, M9 and M8.
Online Figure III

Complete dataset of MyBP-C content determinations in myectomy samples.

Means and standard errors of these data are plotted in Figure 2C. Red asterisk indicates missense mutations.
Online Table II

Statistical analysis of data.

MyBP-C content is significantly less than the donor control (=1, t-test) and in most cases significantly greater than 0.5.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quantity relative to donor ± sem</th>
<th>n</th>
<th>P single group v.s. 0.5</th>
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Determination of the transcripts produced by the Glu258Lys mutation

An earlier report found that the Glu258Lys mutation has an effect on splicing in that two transcripts were shown to be generated by this mutation in peripheral blood lymphocyte cDNA, one full length bearing the expected missense mutation and the other in which exon 6 is skipped. In order to test whether the Glu258Lys mutation affects splicing in the heart, cDNA was amplified by PCR using the MYBPC3 primers 5’-ACTGCAGAACATATGATTGGCCTCTCTTGATGCGG and 5’-ATGGCTATCCTACTGATCCGGACCACCTCCAGC; this was predicted to give a 417bp product for the normally spliced transcript and a 249bp product if exon 6 were skipped. Our data show no evidence of the 249bp product suggesting that this mutation generates only a full length transcript including the Glu258 mutation in the myocardium.
REFERENCES


