Expression Patterns of Cardiac Myofilament Proteins – Genomic and Protein Analysis of Surgical Myectomy Tissue from Patients with Obstructive Hypertrophic Cardiomyopathy

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ABSTRACT

Background: Mutations in myofilament proteins, most commonly MYBPC3-encoded myosin binding protein C and MYH7-encoded β-myosin heavy chain, can cause hypertrophic cardiomyopathy (HCM). Despite significant advances in structure-function relationships pertaining to the cardiac sarcomere, there is limited knowledge of how a mutation leads to clinical HCM. We therefore set out to study expression and localization of myofilament proteins in left ventricular tissue of patients with HCM.

Methods and Results: Frozen surgical myectomy specimens from 47 patients with HCM were examined and genotyped for mutations involving 8 myofilament-encoding genes. Myofilament protein levels were quantified by western blot with localization graded from immunohistochemical staining of tissue sections. Overall, 25/47 (53%) patients had myofilament-HCM including 12 with MYBPC3-HCM and 9 with MYH7-HCM. Compared to healthy heart tissue, levels of myofilament proteins were increased in patients manifesting a mutation in either gene. Patients with a frameshift mutation predicted to truncate MYBPC3 exhibited marked disturbances in protein localization as compared to missense mutations in either MYBPC3 or MYH7.

Conclusions: In this first expression study in human HCM tissue, increased myofilament protein levels in patients with either MYBPC3 or MYH7-mediated HCM suggest a poison peptide mechanism. Specifically, the mechanism of dysfunction may vary according to the genetic subgroup suggested by a distinctly abnormal distribution of myofilament proteins in patients manifesting a truncation mutation in MYBPC3.

Word Count: 6103

Key Words: Cardiomyopathy, Hypertrophy, Genetics, Protein
BACKGROUND

Hypertrophic cardiomyopathy (HCM) is the most common heritable cardiovascular disease affecting 1 in 500 individuals. Clinically, the disease is characterized by unexplained thickening of the myocardial wall in the absence of any known cause of hypertrophy (i.e. hypertension) and is the most common cause of sudden cardiac arrest (SCA) in young athletes.

Over the past two decades it has become apparent that like the phenotype, the genetic basis of this disease is diverse. With the identification of over 20 HCM-associated genes, our understanding of the basis for the disease has significantly advanced. In addition to mutations within the myofilament, mutations have also been discovered in glycogen storage disease associated genes, genes encoding proteins localized to the Z-disc and, most recently, the involvement of proteins involved in calcium handling. Although the genetic spectrum of HCM has expanded considerably, mutations in MYBPC3-encoded myosin binding protein C and MYH7-encoded β-myosin heavy chain continue to comprise the two largest genetic subtypes accounting for eighty percent of genetically explained HCM.

In part due to the high prevalence of mutations in HCM, both MYBPC3 and MYH7 have been studied to elucidate their roles in the structure and function of the sarcomere. Notably, their precise interaction is necessary for the stability of the sarcomere as well as for adrenergic modulation of contraction. While the N-terminus of MYBPC3 is understood to act as a restraint on MYH7, the C-terminal portion of the protein is important in stabilizing the thick filament and localizing MYBPC3 to the A band. The importance of the C-terminus of MYBPC3 is interesting in light of the observation that the majority of HCM-associated MYBPC3 mutations involve frameshifts which are predicted to result in a truncated protein product.
The intimate association of MYBPC3 with MYH7 and the recognition that their corresponding genes are the two most common genotypes in HCM prompts the question as to how a mutation would affect levels and sub-cellular localization of these proteins in HCM patients. We hypothesize that a mutation in *MYBPC3* or *MYH7* causes the mutant protein to perturb the wild type protein resulting in disrupted localization and impairment of their roles in mediating or regulating contraction in myocardium.

**METHODS**

*Study population.* Between July 1998 and May 2002, 209 patients underwent surgical septal myectomy to relieve symptoms of obstructive HCM refractory to pharmacotherapy. Of these, 47 unrelated patients consented to participate in this study. Following receipt of written consent for this Mayo Foundation Institutional Review Board-approved protocol, a portion of myectomy tissue was divided and either flash frozen in liquid nitrogen or fixed in formalin for paraffin embedding.

*Mutational Analysis.* DNA was extracted from the myectomy tissue using the Purgene DNA extraction kit (Gentra, Inc, Minneapolis, MN). Polymerase chain reaction (PCR) was performed on the 34 translated exons of *MYBPC3* and the 38 translated exons of *MYH7* including all flanking splice junctions. In order to exclude patients manifesting compound heterozygosity, mutational analysis was performed on 6 additional myofilament genes which, together with *MYBPC3* and *MYH7*, comprise commercially available HCM genetic tests. Each individual exon was evaluated for sequence variation using denaturing high performance liquid chromatography (DHPLC, Transgenomic, Omaha, Nebraska). All samples with an abnormal elution profile were directly sequenced (ABI Prism 377; Applied Biosystem, Foster City, California) to characterize the precise genetic variant. Furthermore, a panel of 400 reference alleles from 200 Coriell...
Repository DNA samples were examined to exclude variants as common polymorphisms. Primer sequences, PCR, and DHPLC conditions are available upon request.

**Protein Extraction from Myocardial Myectomy Tissues.** Whole protein lysate was extracted from 20 mg of myocardial tissue for each patient. Tissue was manually homogenized in ice-cold RIPA buffer containing 50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS. Immediately prior to lysis, a protease inhibitor cocktail was added consisting of 1 mM PMSF, 5 μg/ml Aprotinin, 5 μg/ml Leupeptin, and 1% Na deoxycholate. Samples were centrifuged at 1000XG for 15 minutes at 4°C and supernatant was collected for further analysis.

Healthy flash frozen heart tissue was obtained from the interventricular septum of a disease-free, accidental death victim (Biochain, Hayward, CA). As with the HCM myectomy tissue, healthy tissue was also flash frozen in liquid nitrogen. Due to the post-mortem ascertainment of this tissue, we assessed tissue quality by examining overall protein profiles with coomassie stain.

**Western Blot Analysis.** Protein concentrations were determined using the BCA™ Protein Assay Kit (ThermoScientific, Rockford, IL). For each sample, 10 μg of denatured and reduced protein lysate was resolved by 4-15% SDS-polyacrylamide gel electrophoresis using a Mini-PROTEAN electrophoresis system (Bio-Rad, Hercules, CA). A minimum of three wells were reserved to run the lysate derived from the normal cardiac tissue specimen. Proteins were transferred to a nitrocellulose membrane and detected with the WesternLightning™ Chemiluminescence reagent (Perkin Elmer, Boston, MA) which, in conjunction with an HRP-labeled antibody, produces luminescence. X-ray film captured and imaged the labeled protein.
which was then quantified utilizing Labworks™ Image Analysis Software and normalized to GAPDH.

**Antibodies.** Multiple antibodies were used for western blotting. Polyclonal myosin binding protein C (1:10,000) was previously developed in Dr. Moss’ laboratory. Monoclonal antibodies were purchased that recognize myosin heavy chain (ab15)(1:15,000)(Abcam, Cambridge, MA) and GAPDH (MAB374)(1:10,000)(Millipore, Billerica, MA). In addition, a monoclonal antibody recognizing the N-terminus of MYBPC3 was made by immunizing rabbits against a stretch of amino acids within human MYBPC3 (LLKRDSTPRDSKLEA; amino acids 298-315) (available upon request). Horse-radish peroxidase-conjugated rabbit anti-mouse (ab6728)(1:5000) and goat anti-rabbit (ab6721)(1:5000) antibodies were purchased from Abcam.

**Immunohistochemistry.** Formalin fixed, paraffin embedded myectomy tissue blocks were sectioned at 5 μm for immunohistochemical staining. Deparaffinization with xylene and subsequent rehydration with graded ethanol preceded heat induced epitope retrieval with EDTA buffer (pH 8) in a Lab Vision PT Module (Fremont, CA). The staining procedure was carried out by an automated immunohistochemistry-staining machine (DAKO Techmate 500, DAKO, Denmark) using the Envision program. The antibodies against myosin binding protein C and myosin heavy chain were diluted 1:1000 and scored for two parameters which assessed overall homogeneity of protein expression utilizing a whole slide imaging platform. Evaluation of intercellular distribution was observed at a lower magnification (20X) followed by assessment of the intracellular distribution at a higher magnification (400X). Interpretation of the final scores was done blinded to the genotype and all results were confirmed by a cardiac pathologist (DVM). The initial score was based on the average percentage of sarcoplasm showing disruption of protein expression: 0-Normal (0% Loss); 1-<25% Loss; 2-25 to 50% Loss; 3- 50-75% Loss; 4-75-100% Loss. The second score utilized the same scoring system, but now analyzed the overall percentage of
the cell population showing disruption of staining. These two scores were multiplied and converted to a percentage ultimately representing the total disruption in protein localization with 0 representing normal distribution and a score of 16 indicating 75-100% disruption of the protein.

**Statistical Analysis** Student t test and Fisher exact tests were used to compare clinical characteristics among the genotypes utilizing the JMP statistical software (JMP 6.0, 2005; SAS Institute Inc, Cary, NC). Student t test was also utilized to compare localization patterns in different genotype groups. Generalized estimating equations, using a gamma distribution and a log link, were used to determine variation in levels of myosin binding protein C and myosin heavy chain protein. This type of analysis was used because the distribution of protein levels was highly skewed and could not be transformed to the normal distribution. The gamma distribution provided a much better fit than any other distribution available. Levels observed in healthy heart tissue were compared to patients having a missense mutation in either protein and to those having a truncation mutation in MYBPC3.

**RESULTS**

**Clinical and Genetic Analysis of Cohort.** The demographics of this cohort (n = 47, 25 male) are summarized in Table 1. The mean age at diagnosis was 33.8 ± 19.9 years with a mean left ventricular wall thickness (LVWT) of 24.2 ± 7.7 mm. Mutational analysis of 8 HCM-associated myofilament genes revealed that 25/47 (53%) were mutation positive including 12 with MYBPC3-HCM and 9 with MYH7-HCM. For the purpose of the study, we focused on these 21 patients manifesting a single mutation in either MYBPC3 or MYH7. Due to small quantity and low quality of tissue, 5 patients were excluded from further experimental analysis; the excluded patients are annotated in Table 2.

Consistent with prior investigations, the 22 patients who were sarcomere genotype negative were significantly older and exhibited less hypertrophy than those with myofilament-
HCM (Table 1). Similar to our previous findings, septal morphological subtype strongly predicted the presence of a myofilament mutation.

*Spectrum and Prevalence of MYBPC3- and MYH7-HCM.* Table 2 summarizes the clinical profiles of the 21 patients having either MYBPC3-HCM or MYH7-HCM. While all mutations in MYH7 were missense mutations, 7 of the 12 (58%) mutations accounting for MYBPC3-HCM were insertion/deletions or splice site mutations predicted to result in an in-frame deletion (case 6) or C-terminal truncation (cases 7-12).

Examination of the clinical phenotypes of these patients failed to reveal significant genotype-phenotype differences between MYBPC3-HCM and MYH7-HCM, however those with a mutation in MYBPC tended to be older than those with a mutation in MYH7 (Table 1). In comparison to the myofilament-negative group, MYH7-HCM patients were significantly younger at diagnosis (24.3 ± 24.7 vs. 41.1 ± 19.1 years; p = 0.03) and had greater hypertrophy as evidenced by LVWT (24.3 ± 7.1 vs. 21.8 ± 5.2 mm; p = 0.05).

*Spectrum of MYBPC3 insertion/deletions and splice site mutations.* Table 3 presents detailed protein characteristics for those individuals with an insertion/deletion mutation or mutation at the canonical splice site. Investigation of the cardiac transcriptome permitted elucidation of the precise slicing defect: three transcripts in case 7 and two transcripts in case 8 (data not shown). Case 6 had a splice defect which yielded an in-frame deletion of 89 amino acids resulting in a protein product lacking the CO domain. A summary of proteins that would result including the domains present in the truncated proteins are indicated (Table 3).

*Levels of Protein Expression*

Analysis of whole protein lysate from patients with either MYBPC3- or MYH7-mediated HCM revealed that a mutation results in increased protein levels as compared to healthy tissue.
Loading for individual samples was controlled by running a replicate gel for coomassie and quantitative normalization was performed utilizing GAPDH (Figure 1). In each subgroup, the levels of myosin binding protein C and myosin heavy chain were significantly increased when compared to that observed in healthy heart tissue (Figure 2). Although the antibody against myosin heavy chain recognizes the beta and alpha isoforms, it is still plausible that this increase may be secondary to activation of the fetal gene program.

**Patterns of Protein Localization**

In order to determine how a mutation might affect the localization of these proteins, we performed immunohistochemistry on paraffin embedded tissue sections. Within healthy heart tissue, we observed that staining with either protein had an even, homogenous pattern throughout the entire tissue section (Figure 3a). Patients with a missense mutation in either protein had a small degree of disruption in the even distribution (Figure 3b and 3c), but were not significantly different from normal. Interestingly, analysis of these proteins in patients with a mutation resulting in the truncation of MYBPC3 showed a marked disturbance in protein localization. In these patients, both proteins demonstrated a heterogeneous pattern suggesting regional variations in expression or incorporation of protein (Figure 3d and 3e). This irregular staining pattern in patients with a truncation mutation was confirmed by staining with a monoclonal antibody that specifically recognizes the intact N-terminus of MYBPC3. (Figure 3d and 3e). Interestingly, the heterogeneity in the staining did not correlate with the degree of disarray and was observed throughout the tissue section. Furthermore, the presence of striations in areas of light staining confirms that these are cardiomyocytes and excludes the presence of fibrosis as an explanation for decreased staining (Figure 4a-c). Figure 5 summarizes the expression score data from the three groups of interest based upon the percent disruption in either myosin binding protein C
or myosin heavy chain (Figure 5b). Greater disruption of both proteins was observed in patients having a MYBPC3 mutation resulting in premature truncation MYBPC3.

DISCUSSION

Despite the fact that over 20 genes have been implicated in the pathogenesis of HCM, the majority of genetically established HCM is explained by mutations within MYBPC3 or MYH7. In vitro work and animal models have guided our understanding of how a mutation in either protein may lead to disease yet little is known about the effects of these mutations in the human heart. As a tertiary referral center for the surgical treatment of HCM, our unique ability to collect and flash freeze human cardiac tissue from myectomies was essential for our characterization of the expression and localization patterns of myosin heavy chain and myosin binding protein C in myocardium from patients with HCM.

MYBPC3 and MYH7 are the principal constituents of the thick filament and are essential for proper function of the cardiac sarcomere. In fact, the activity of MYH7 is regulated/modulated by MYBPC3 which has dual roles in stabilizing the sarcomere and regulating contraction. In mice lacking myosin binding protein C, septal hypertrophy and impaired contractile function were observed whereas in the human, the vital necessity for myosin binding protein C is evidenced by neonatal lethality when both alleles encode for a premature truncation.

Our studies of protein levels in human HCM illustrate that both proteins are present at normal to increased levels when compared to healthy tissue. This data suggests that the disease is not mediated by a decrease in total protein in these patients and mirrors what has been observed previously, both in animal models as well as in human myectomy tissue. Though we were unable to identify the truncated MYBPC3 product in patients manifesting an
insertion/deletion or splice site variation, this protein product may be present at levels that are too low to be detected by Western blot. It is still plausible that minute quantities may lead to disease as evidenced in a homozygous knock-in mouse expressing truncated C-protein.\textsuperscript{30}

One potential avenue by which a mutation in a protein could poison the cell is through mis-localization caused by the inability to properly interact with partner proteins. Our immunohistochemistry study revealed that the effect of a missense mutation on the localization of either protein was modest as shown by an even distribution of protein throughout the cardiomyocyte and tissue. In contrast, in myocardium from patients predicted to have a truncation in MYBPC3, a heterogeneous expression pattern was observed. Even within an individual cardiomyocyte, the protein appears to be localized to discrete zones in patterns that affect myocardial contraction. This mis-localization of the protein in human tissue may be secondary to the absence of a complete C-terminus which is necessary for proper incorporation of the protein into the sarcomere.\textsuperscript{15,37}

We surmise that this disrupted localization of MYBPC3 in patients with a truncation mutation may be due to disruption of the cooperative assembly of proteins known to exist within the sarcomere.\textsuperscript{38} While degradation of truncated MYBPC3 through the ubiquitin proteasome system has been observed,\textsuperscript{39} it is plausible that as the proteasome ages, truncated MYBPC3 could integrate into the sarcomere. Previous work by others has demonstrated that truncation of the C-terminus can result in mis-incorporation of the protein and a diffuse pattern throughout the sarcomere.\textsuperscript{15} This mis-incorporation of mutant protein may consequently act as a nidus for aggregation of wild type protein at these locations.

Regardless the mechanistic basis for the maldistribution of MYBPC3, the juxtaposition of sarcomeres with and without MYBPC3 would be expected to profoundly affect myocardial
function during a twitch. Previously, myocardium lacking MYBPC3, was shown to have an accelerated stretch activation response, suggesting that MYBPC3 normally constrains cross-bridges and thereby reduces both the probability of binding to actin and the rate of force development. Ablation of MYBPC3 would relieve this constraint and accelerate the rate of force development. In myocardium in which the incorporation of MYBPC3 is not uniform, as in the patients with a truncation yielding MYBPC3 allele, sarcomeres without MYBPC3 would develop force more rapidly than sarcomeres with normal or greater amounts of MYBPC3. This mismatch in contraction kinetics would set up an oscillating system of reciprocal stretch activation during a twitch: Zones without MYBPC3 would quickly develop more force than zones with MYBPC3; because of the force differential, the former would stretch the latter and thereby stretch-activate the zone with MYBPC3. Since stretch activation occurs with a time delay, the net effect would be to delay the time to full relaxation of the twitch. Moreover, because of its faster kinetics, the zone without MYBPC3 will begin to relax before the zone with MYBPC3. This again sets up a force differential between the zones, so that the zone without MYBPC3 is now stretch activated by the zone with MYBPC3. This asynchronous reciprocal stretch activation is similar to what is observed in the antagonist asynchronous flight muscles of insects. In the present case the oscillation would rapidly decay due to the intracellular sequestration of Ca2+, but the net effect is a slowing of relaxation, which notably, is a distinct functional feature in mice lacking MYBPC3.

Accordingly, a more severe clinical phenotype might be expected among patients with a premature truncation in MYBPC3 compared to those with MYBPC3 missense mutations. However, in this study, the phenotypes of these two subsets were similar between the 7 patients
with truncations and the 5 patients with missense mutations in MYBPC3. A much larger sample size with longitudinal follow-up will be necessary to further explore this possibility.

CONCLUSION

This study is the first characterization of the localization and expression patterns of myofilament proteins in the largest known cohort of patients from whom flash frozen myectomy tissue has been collected. The levels of myosin binding protein C and myosin heavy chain in patients with either MYBPC3 or MYH7-mediated HCM support the notion that that these mutations lead to dysfunction and disease through a poison peptide mechanism. More specifically, the mechanism may vary according to the genetic subgroup, a conclusion that is suggested by the distinctly abnormal distribution of MYBPC3 in patients manifesting a truncation mutation in this gene.

ACKNOWLEDGEMENTS

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DISCLOSURES

Dr. Ackerman is a consultant for PGxHealth.
REFERENCES


FIGURE LEGENDS

Figure 1. SDS polyacrylamide gels and ECL blots of myofilament proteins from a healthy individual or patients manifesting a mutation. Each lane is labeled either as normal or with the corresponding case (See Table 1) in which a mutation was identified in (A) MYBPC3 or (B) MYH7. Coomassie staining of the gel illustrated overall protein profiles and GAPDH served as a quantitative loading control to which other proteins were normalized. Myosin binding protein C and myosin heavy chain blots are shown for each individual.

Figure 2. Protein levels in cardiac tissue of patients with MYBPC3-HCM or MYH7-HCM. Levels of myosin heavy chain (A) and myosin binding protein C (B) are shown. Samples were run in triplicate with 3 to 4 normal samples run on each gel for comparison to diseased tissue. Statistically significant differences from normal were determined by generalized estimating equations, using a gamma distribution and a log link (*, p<0.001). Values are represented as adjusted least square means ± S.E., after adjusting for the sample and gel effects.

Figure 3. Immunohistochemistry of myosin heavy chain and myosin binding protein C in patients with myofilament HCM. (A) Homogeneous pattern of expression myosin heavy chain and myosin binding protein C in normal heart tissue. (B) Even distribution noted in Case 20 having a missense mutation in MYH7 and (C) Case 4 having a missense mutation in MYBPC3. Splotchy pattern of distribution in patients with a truncation mutation in MYBPC3 is demonstrated in (D) case 9 and (E) case 7 with the polyclonal antibody (middle panel) and the new monoclonal antibody (right panel). Score is represented in upper right hand corner for staining with myosin heavy chain and the polyclonal MYBPC3 antibody. Scores ranged from 0
(100% homogeneity of protein throughout the tissue) to 16 (complete disruption of protein staining). All images shown are at 20X magnification and scale bars indicate a 50 μM distance.

**Figure 4: High magnification illustrating intracellular disruption of protein expression.**

(A) 200X magnification confirms homogeneous expression of myosin binding protein C in normal heart tissue. (B) 200X magnification illustrates heterogeneous expression of MYBPC3 in Case 7 manifesting a splice mutation. (C) 400X magnification of Case 7 showing striations are still present with lighter staining (asterisk) and distinct from fibrosis (arrow).

**Figure 5. Quantification of Localization Patterns.** Scores were averaged for both the distribution of myosin binding protein C (A) and myosin heavy chain (B) and converted to a percentage representing the total disruption of the protein in the tissue section. This takes into account both intracellular as well as intercellular distribution pattern of the protein. Statistically significant differences were determined by unpaired Student’s t-test and represented as the mean ± S.D.
Table 1. Clinical Characteristics of HCM Myectomy Cohort

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<th>MYBPC3 Mutation</th>
<th>MYH7 Mutation</th>
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<td>32.5 ± 13.9</td>
<td>24.3 ± 24.7 *</td>
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<td>≤ 30 yrs, n (%)</td>
<td>20 (43%)</td>
<td>13 (52%)</td>
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<td>Age at Myectomy, yrs</td>
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<td>HCM, n (%)</td>
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<td>LVWT, mm</td>
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Values are mean ± SD or n (%). LVWT – left ventricular wall thickness; NS – not statistically significant; A–in a first-degree relative; B–LVWT ≥ 25 mm; LVOT–left ventricular outflow tract. Statistically significant differences to genotype negative group were confirmed by unpaired Student’s t-test (*, p<0.05).
Table 2. Clinical Profiles of HCM Patients with Mutations in MYBPC3 or MYH7

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LVWT – left ventricular wall thickness; A- in a first degree relative; † - indicates patient samples not used in expression studies
Table 3. Mutations and predicted protein of patients having a genetic variation resulting in a truncated protein.

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<thead>
<tr>
<th>Genetic Variation</th>
<th>Affected Exon</th>
<th>Predicted Protein Size</th>
<th>Intact Domains</th>
<th>Consequence of Mutation</th>
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<td>140 kD</td>
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Figure 1

A

B

Coomassie

GAPDH

Myosin binding protein C

Myosin Heavy Chain

Normal 6 7 8 9 11 12 2 4

Normal 13 14 15 17 18 19 20 21
Figure 2

A

- Y-axis: Myosin Heavy Chain
- Legend: MYBPC3 Truncation Mutation (n=6), Missense Mutation (n=10), Normal Cardiac Tissue

B

- Y-axis: Myosin Binding Protein C
- Legend: MYBPC3 Truncation Mutation (n=6), Missense Mutation (n=10), Normal Cardiac Tissue
Figure 3

A

Myosin Heavy Chain

NORMAL

MYBPC3 Polyclonal

MYBPC3 Monoclonal

B

M922K-MYH7

1

1

C

W792R-MYBPC3

1

2
Figure 4

A  NORMAL

B  g>a int+1 Exon 7  x200

C  g>a int+1 Exon 7  x400
Figure 5

A  

Percent Disruption

p < 0.05

Myosin Binding Protein C

- MYBPC3 Truncation Mutation (n=6)
- MYBPC3 Missense Mutation (n=2)
- MYH7 Missense Mutation (n=8)

B  

Percent Disruption

p = 0.07

Myosin Heavy Chain

- MYBPC3 Truncation Mutation (n=6)
- MYBPC3 Missense Mutation (n=2)
- MYH7 Missense Mutation (n=8)
Expression Patterns of Cardiac Myofilament Proteins - Genomic and Protein Analysis of Surgical Myectomy Tissue from Patients with Obstructive Hypertrophic Cardiomyopathy


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