Disrupted Sarcomere Stoichiometry and Heterogeneous Expression of Mutant Sarcomere Proteins in Human Hypertrophic Cardiomyopathy

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Background:

Hypertrophic cardiomyopathy is the most common heritable cardiovascular disease, affecting 1 in 500 individuals. HCM is characterized by cardiac hypertrophy and a broad clinical spectrum that includes heart failure, arrhythmias, and sudden cardiac death. Heterozygous mutations in sarcomere genes account for the majority of cases of HCM. Despite the identification of >1,000 mutations in these genes, and a growing body of knowledge on the molecular mechanisms of disease, fundamental questions about the expression levels of these mutant proteins and the functional relationship between co-expressed mutant and wild-type proteins in HCM human hearts remain unanswered. Previous studies of in vitro and animal model systems, along with limited data in human HCM hearts, have led to the widely accepted hypothesis that sarcomere gene mutations that result in premature protein truncation (>50% of cMyBPC mutations) cause haploinsufficiency, while missense mutations in full-length proteins (majority of non-cMyBPC mutations) exert their effects through a dominant-negative mechanism, maintaining sarcomere protein stoichiometry. In the latter case, the conventional belief is that the ratio of mutant to wild-type proteins is ~50:50 but this remains largely unsupported by experimental data in human HCM. Here we study sarcomere gene and protein expression in septal myectomy specimens from HCM patients with known genotype established by clinical genetic testing.

Table 1. Demographics and clinical data for HCM patients undergoing myectomy

	Age (y)	% male	Maximum wall thickness (mm)	Ejection fraction (%)	LVOT gradient (mmHg)
Control	53 +/- 4	0.50	11.8 +/- 0.8	57 +/- 2	-
HCM					
No sarcomere mutations	50 +/- 5	0.60	20.6 +/- 1.3	71 +/- 2	80 +/- 17
Non-MyBPC mutations	41 +/- 6	0.50	19.2 +/- 0.7	71 +/- 3	75 +/- 19
MyBPC mutations	38 +/- 3	0.53	26.0 +/- 2.4	71 +/- 4	79 +/- 13

Age and gender are comparable among all groups. Wall thickness, ejection fraction and left ventricular outflow tract gradients are comparable among all genotyped subgroups of HCM patients.

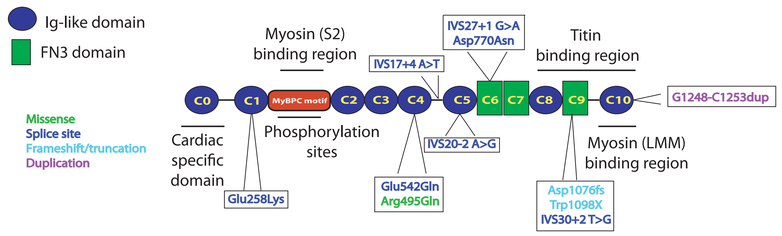

Figure 1. Schematic representationa of cMyBPC and localization of human mutations. cMyBPC consists of 8 immunoglobulin (Ig)-like domains and 3 fibronectin (FN3) domains, with binding sites for myosin and titin. HCM-associated cMyBPC mutations in this study are distributed throughout the protein. Table 2 indicates mutations in other sarcomere proteins and their domain localization.

Table 2. Other sarcomere gene mutations

Gene	Mutation	Protein domain
MyH7	Ala797Thr	Between head and tail
MyH7	Asp1096Tyr	Tail
MyH7	Arg1606His	Tail
Tpm1	Ile284Val	Terminal codon
Tnnt2	Asp86Ala	Tropomyosin (T1) binding
MyL2	His161Arg	C-terminal EF hand

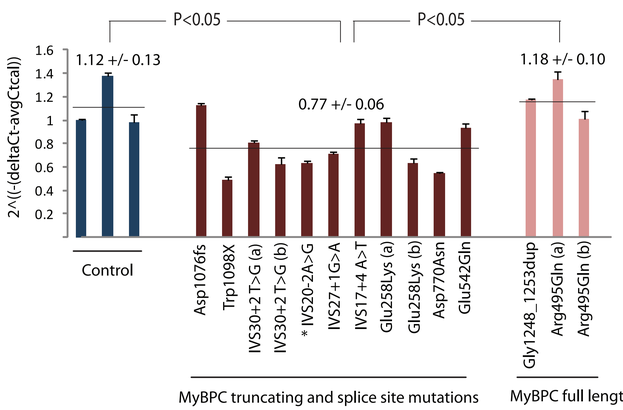

Figure 2. Total cMyBPC mRNA expression in human HCM myectomy specimens from patients with cMyBPC %mutations. Quantitative rtPCR was performed using Taqman hydrolysis assays. A Taqman hydrolysis probe recognizing the exon 2-3 junction within human cMyBPC was used to quantify total mRNA expression. In all experiments, β-actin was used as an endogenous referent gene. Experiments were performed on the 7000 Sequence Detection System (Applied Biosystems). The relative mRNA amount was estimated according to the comparative Ct method with the 2-ΔΔCt formula in comparison to non-failing control samples. Mutations identified in more than one patient and are designated (a) and (b). cMyBPC transcript abundance was significantly reduced in patient samples with truncating or splice site mutations compared to controls or cMyBPC full length mutations (P<0.05). * indicates apical sample obtained at time of left ventricular assist device for end-stage heart failure.

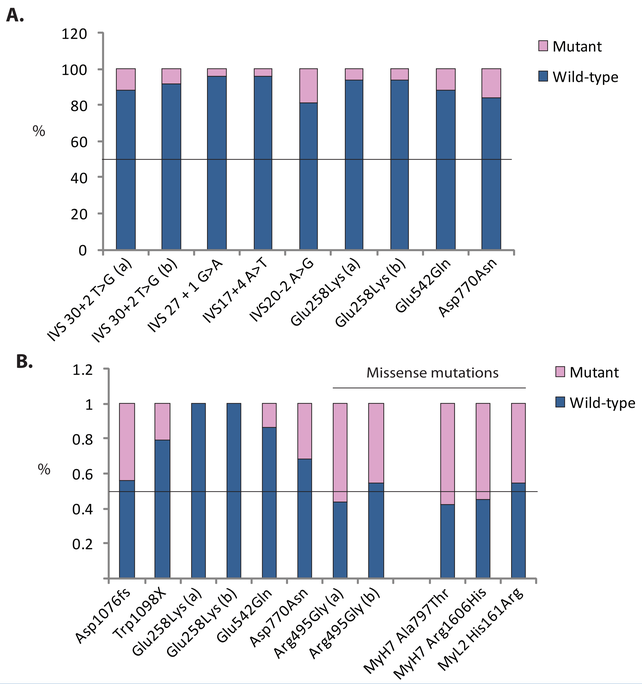

Figure 3. Relative expression of wild-type and mutant mRNAs. A. Quantitative rtPCR was performed using allele-specific Taqman hydrolysis probes to determine the proportions of wild-type and mutant cMyBPC transcripts in samples with splice site mutations. The mutant fraction constituted <20% of total mRNA expression in all samples, suggesting degradation by nonsense-mediated RNA decay. **B.** The proportions of wild-type and mutant transcripts in samples with insertions, truncations, and missense (or exon splice site) mutations were analyzed on the Sequenom MassARRAY® platform. PCR amplification followed by a single-base extension reaction was performed using a probe oligo adjacent to the mutation site. Extension products were purified and analyzed by mass spectrometry. The area under each peak on the resulting spectrogram is proportional to the quantity of allele-specific product . Missense mutations all showed ~50:50 ratio of wild-type:mutant. Ratios were more highly variable in other samples.

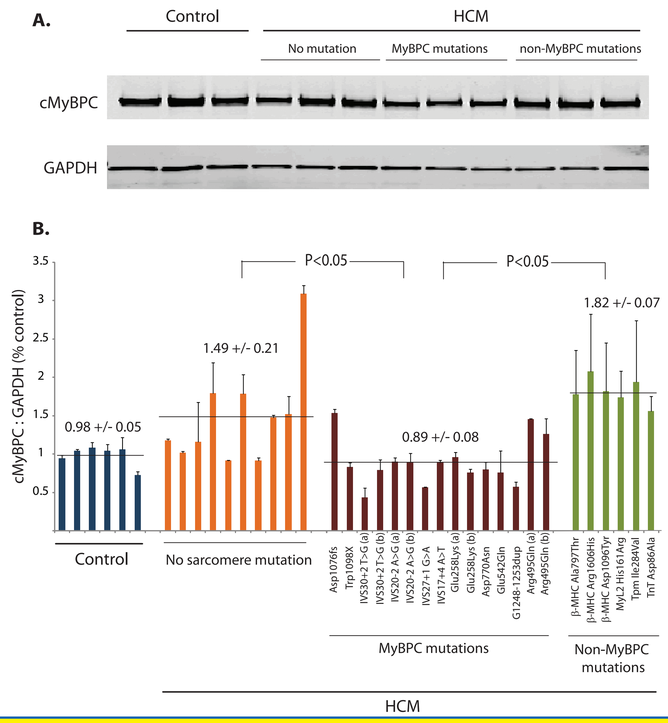

Figure 4. Protein quantification of total cMyBPC. A. Representative immunoblot comparing expression of total cMyBPC in heart tissue from HCM patients without sarcomere mutations, patients with non-cMyBPC sarcomere mutations, patients with cMyBPC mutations, and non-failing donors as controls, using an N-terminal custom cMyBPC antibody. No truncated mutant cMyBPC mutant proteins were detected. **B.** Densitometric analysis of cMyBPC expression relative to GAPDH, and expressed as a percentage of the mean of control samples. Total MyBPC expression was significantly decreased in HCM hearts with cMyBPC mutations compared to those without sarcomere mutations (P<0.05) and those with non-MyBPC sarcomere mutations (P<0.05). Despite a reduction in total protein in cMyBPC mutation samples, the remaining cMyBPC localized appropriately to the A-band by immunofluorescent imaging and was present in the myofilament cellular fraction (data not shown). There were no significant differences in expression of other sarcomere proteins among groups (data not shown).

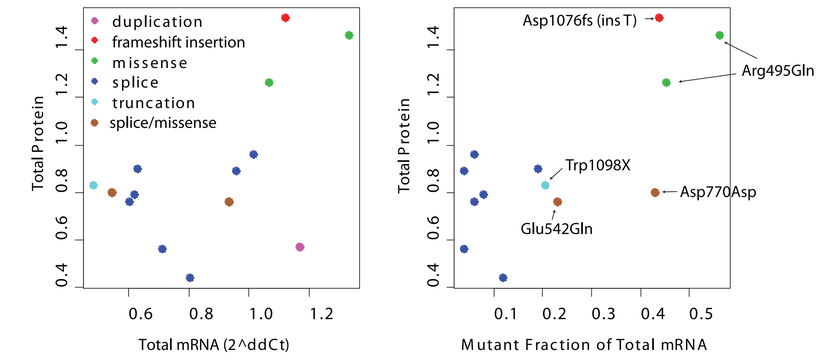

Figure 5. A linear regression model of cMyBPC protein expression as a function of the total cMyBPC mRNA and the fraction of that RNA comprised of the mutant cMyBPC copy. The model indicated a high degree of overall statistical significance (p = 0.002). The model gave an R^2 value of 0.65, indicating that 65% of the variation in cMyBPC protein content in samples with cMyBPC mutations could be explained by these two factors.

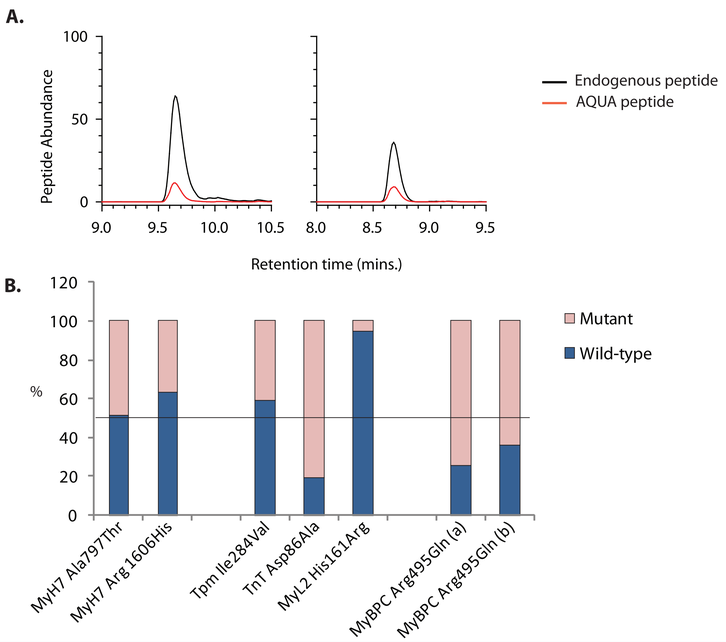

Figure 6. Absolute quantification of abundance (AQUA) methodology used to compute the stoichiometric ratios of missense sarcomere mutant proteins relative to wild-type proteins. Wild-type and mutant AQUA peptides were synthesized using heavy Lys or Arg isotopes. Endogenous cMyBPC, MyH7, cTnT, Tpm, and cTnT proteins from human HCM hearts were gel extracted and digested with trypsin or Lys-C. Peptide mixtures were spiked with a known concentration of the corresponding AQUA peptides and analyzed in duplicate by LC/SRM. **A**. Representative chromatogram showing peak areas for endogenous peptides and AQUA peptides. **B**. Molar ratios of endogenous mutant : wild-type peptides are highly heterogenous, with the proportion of mutant peptide ranging from 6-80%. Truncated cMyBPC peptides were not detectable by mass spectroscropy. Missense peptides Glu258Lys and Asp770Asn, and duplication mutant Gly1248_1253dup were also not detecable. Mutant Glu542Gln was detectable and is in the process of quantification by AQUA.

Findings:

1. Total cMyBPC mRNA expression is significantly reduced in human hearts harboring cMyBPC truncation and splice site mutations. This finding correlated with a reduced proportion of nonsense mutant transcripts (<20% of total), implicating nonsense-mediated RNA decay of mutant transcripts.

2. In contrast, mRNA expression of missense or duplication mutations in cMyBPC was not reduced and the proportion of mutant transcripts was ~50% of the total for cMyBPC and other missense sarcomere mutants.

3. cMyBPC protein was modestly, but significantly, reduced in hearts with cMyBPC mutations compared to other HCM hearts, but not to controls. 65% of the variation in cMyBPC protein content could be explained by total mRNA and the fraction of that mRNA comprised of mutant transcript.

4. Molar ratios of missense mutant to wild-type proteins were highly variable, likely reflecting allele-specific differences in inherent protein stability or sarcomere incorporation efficiency.

Implications:

Sarcomere gene and protein expression patterns are highly heterogeneous in human HCM hearts, and implicate mutation-specific mechanisms of disease.