Short communication

Variable cardiac myosin binding protein-C expression in the myofilaments due to MYBPC3 mutations in hypertrophic cardiomyopathy☆

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ARTICLE INFO

ABSTRACT

Background: Mutations in *MYBPC3* are the most common cause of hypertrophic cardiomyopathy (HCM). These mutations produce dysfunctional protein that is quickly degraded and not incorporated in the myofilaments. Most patients are heterozygous and allelic expression differs between cells. We hypothesized that this would lead to cell-to-cell variation in cardiac myosin binding protein-C (cMyBP-C, encoded by *MYBPC3* gene) protein levels.
Methods: Twelve HCM patients were included (six had no sarcomere mutations (HCM$_{smn}$) and served as the control group and six harbored mutations in the *MYBPC3* gene (MYBPC3$_{mut}$). Western blot and RNA sequencing analysis of cardiac tissue lysates were performed to detect overall cMyBP-C protein and mRNA levels. Cellular expression of cMyBP-C and α-actin was obtained by immunofluorescence staining. Quantification of cell-to-cell variation of cMyBP-C expression between cardiomyocytes was measured by determining the ratio of cMyBP-C:α-actin stained area of each cell.
Results: Protein and mRNA analysis revealed significantly reduced cMyBP-C levels in MYBPC3$_{mut}$ patients compared with HCM$_{smn}$ patients (0.73 ± 0.09 vs. 1.0 ± 0.15, $p < .05$; 162.3 ± 16.4 vs. 326.2 ± 41.9 RPKM, $p = .002$), without any sign of truncated proteins. Immunofluorescence staining of individual cardiomyocytes in HCM$_{smn}$ patients demonstrated homogenous and equal cMyBP-C:α-actin staining ratio. In contrast, MYBPC3$_{mut}$ patients demonstrated inhomogeneous staining patterns with a large intercellular variability per patient. Coefficient of variance for cMyBP-C/α-actin staining for each patient showed a significant difference between both groups (17.30 ± 4.08 vs. 5.18 ± 0.65% in MYBPC3$_{mut}$ vs. HCM$_{smn}$, $p = .02$).
Conclusion: This is the first study to demonstrate intercellular variation of myofilament cMyBP-C protein expression within the myocardium from HCM patients with heterozygous *MYBPC3* mutations.

1. Introduction

Hypertrophic cardiomyopathy (HCM), the most common genetic cardiomyopathy with an estimated prevalence of 1:200 [1], is characterized by isolated, asymmetric left ventricular hypertrophy (LVH), diastolic dysfunction and an increased risk on arrhythmias. Mutations in the genes encoding the sarcomeric thick filament components cardiac myosin binding protein-C (cMyBP-C) and β-myosin
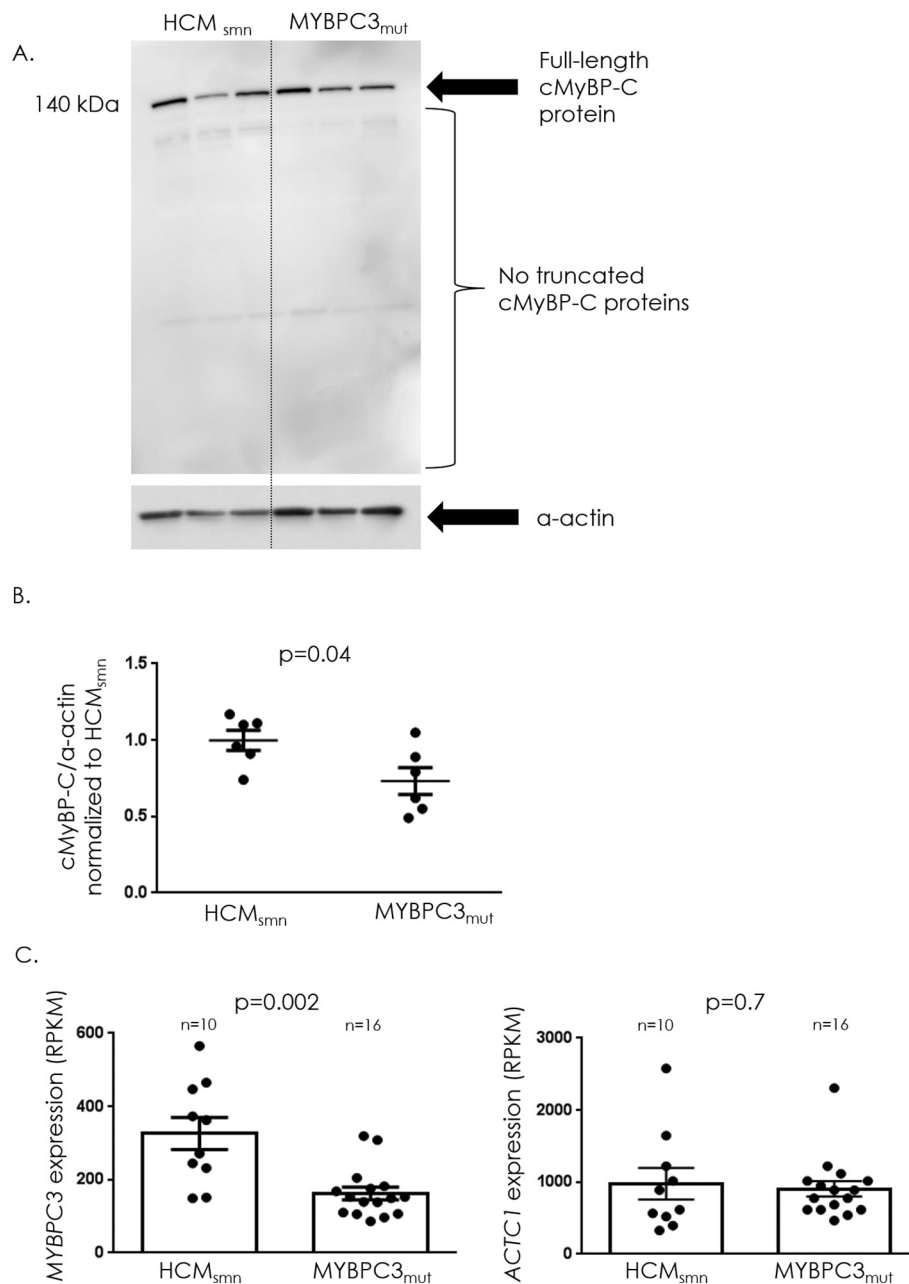
Fig. 1. Protein and mRNA analysis. A. Western immunoblot of MYBPC3$_{mut}$ ($n = 6$) and HCM$_{smn}$ ($n = 6$) patients showing only expression of the full-length cMyBP-C protein with no signs of truncated proteins formed in MYBPC3$_{mut}$ patients. B. Significantly lower expression of cardiac myosin binding protein-C normalized to α-actin in six MYBPC3$_{mut}$ and HCM$_{smn}$ patients. cMyBP-C/α-actin in HCM is set to 1. C. Reduced mRNA expression level of *MYBPC3* in MYBPC3$_{mut}$ patients ($n = 16$) compared with HCM$_{smn}$ ($n = 10$). *ACTC1* mRNA levels were not changed.

heavy chain (*MYBPC3* and *MYH7*, respectively) account for ~80% of mutations [2].

Two disease mechanisms are described in HCM: 1) poison polypeptides, in which point mutations directly result in changes in protein function, and 2) haploinsufficiency, where the mutated allele produces truncated proteins and the other allele cannot fully compensate. Most HCM-associated *MYBPC3* mutations (MYBPC3$_{mut}$) are predicted to lead to truncated proteins [3]. These truncated proteins are suspected to be degraded rapidly since they could not be detected in myectomy samples from HCM patients [4]. This means that all protein which is expressed originates from the healthy allele. The loss of full-length cMyBP-C proteins leads to a reduction of cMyBP-C expression of 30% (i.e. haploinsufficiency) [4].

It has recently been shown that when expressing a gene, cells transcribe both alleles independently. Allelic transcription occurs in a stochastic manner, where one cell might favor one allele while the next cell favors the other allele and this preference can change over time [5]. This burst-like, stochastic on/off-switching of allele transcription should average out over time, but in case of heterozygosity with protein product of different stability, it can lead to variable expression of healthy and mutant proteins. This was recently shown by Kraft and colleagues [6,7]. Patients with heterozygous HCM-associated *MYH7* gene mutation demonstrated a variable healthy:mutant ratio of mRNA expression in cardiomyocytes from the same heart [6]. This was postulated to result in a variable expression of *MYH7* mutant protein. In case of *MYBPC3* truncating mutations where the mutant allele does not produce functional protein [4,8], averaging out over time does not occur and cell-to-cell differences in cMyBP-C protein expression are

expected. The objective of this study was to investigate whether the most common cause of HCM, heterozygous *MYBPC3* mutations give rise to a variable myofilament cMyBP-C expression pattern.

2. Methods

2.1. Study population

Studies were performed in septal myectomy samples from six patients with frameshift and missense mutations in *MYBPC3* (MYBPC3$_{mut}$) and six sarcomere mutation negative, phenotype positive patients (HCM$_{smn}$) (see Fig. 2 for genetic information). All patients were symptomatic with an increased resting left ventricular outflow tract gradient despite optimal medical treatment and were indicated to undergo septal myectomy. All samples were immediately frozen and stored in liquid nitrogen. The study protocol was approved by the local ethics committees, and written informed consent was obtained.

For expanded methods see the Online Supplemental Material.

3. Results

3.1. Study population

The study population consisted of twelve HCM patients (six MYBPC3$_{mut}$; six HCM$_{smn}$) who underwent a surgical septal myectomy procedure. MYBPC3$_{mut}$ were significantly younger and were more often men compared to HCM$_{smn}$ patients (29 ± 7 vs. 61 ± 7 years, $p < .001$ and 6 males vs. 4 males, $p = .02$, respectively). Although all patients underwent a septal myectomy procedure to relief left ventricular outflow tract obstruction, MYBPC3$_{mut}$ subjects demonstrated a significantly higher septal wall thickness compared to HCM$_{smn}$ (28 ± 8 vs. 18 ± 3 mm, $p = .02$), see Table S1 for additional clinical details.

3.2. Reduced cMyBP-C protein and mRNA level in cardiac tissue with MYBPC3 mutation

MYBPC3$_{mut}$ patients were heterozygous for frameshift ($n = 5$) and missense ($n = 1$) mutations. As shown before [4], no truncated protein products were detected (Fig. 1A). Haploinsufficiency was confirmed since total cMyBP-C protein level was significantly lower in MYBPC3$_{mut}$ compared with HCM$_{smn}$ patients (0.73 ± 0.09 vs. 1.0 ± 0.15, $p = .04$) (Fig. 1B). MYBPC3$_{mut}$ patients revealed a significant lower mRNA level of *MYBPC3* gene expression compared with HCM$_{smn}$ ($p = .002$ for *MYBPC3*) while *ACTC1* expression was unchanged ($p = .7$, Fig. 1C).

3.3. Immunofluorescence staining of cardiomyocytes

As the truncated protein products of the nonsense *MYBPC3* mutations were not detected, any cMyBP-C protein expression present in MYBPC3$_{mut}$ cardiomyocytes has to originate from the healthy allele. Therefore the protein expression ratio of healthy:mutant allele was quantified by measuring the amount of cMyBP-C. Fig. 2 depicts a representative cardiomyocyte of a HCM$_{smn}$ (panel A) and MYBPC3$_{mut}$ (panel B) patient. The cardiomyocyte in Fig. 2A demonstrates a homogenous cMyBP-C (white) and α-actin (green) staining, while MYBPC3$_{mut}$ staining was heterogeneous (Fig. 2B). Cell width in both patient groups was similar and excluded the possibility that the absence of cMyBP-C staining could be caused by insufficient antibody penetration (see Fig. S4). More rod shaped cells were seen in MYBPC3$_{mut}$ compared to HCM$_{smn}$ (Fig. S5). Fig. 6 of the Supplemental Material shows additional images of cardiomyocytes of cMyBP-C staining in HCM$_{smn}$ and MYBPC3$_{mut}$ patients.

3.4. Cell-to-cell variability of cardiac myosin binding protein-C protein levels in MYBPC3$_{mut}$

To quantify the variability of cMyBP-C protein level between cardiomyocytes from the same patient, cMyBP-C:α-actin staining area ratio was measured in 15 to 27 cardiomyocytes per patient, as depicted in Fig. 2C and D. HCM$_{smn}$ patients demonstrate homogenous and equal ratio of cMyBP-C:α-actin ratio of stained area in all cardiomyocytes (Fig. 2C). In contrast, MYBPC3$_{mut}$ patients displayed a large intercellular variability of cMyBP-C staining relative to α-actin (Fig. 2D). The variation of the cMyBP-C staining pattern demonstrated a large difference in staining pattern between MYBPC3$_{mut}$ compared with HCM$_{smn}$ cells (Fig. S7, panels A, B and C), and cMyBP-C free patches varied in length (Fig. S7, panel D). Distribution of cMyBP-C/α-actin stained ratio showed a normal distribution (Fig. S8). The variation in ratio of cMyBP-C/α-actin stained area per cell per patient was quantified by determining the coefficient of variance of cMyBP-C/α-actin ratio for each patient. This demonstrated a significant difference in variation of protein expression between the cardiomyocytes in both patient groups (5.18 ± 0.65 vs. $17.30 \pm 4.08\%$, $p = .02$) (Fig. 2E).

4. Discussion

This is the first study to demonstrate intercellular variation of cMyBP-C myofilament protein expression due to mutations in *MYBPC3* within the myocardium from HCM patients.

The concept underlying variable expression is that a cell predominantly transcribes one allele of a gene, which is "chosen" at random, which was demonstrated by single cell sequencing [9]. In healthy subjects it does not matter from which allele a cell transcribes its protein as this process occurs stochastically. In case of a mutant allele and a wild-type allele (such as in heterozygous HCM patients), this will lead to a certain number of cardiomyocytes with a relatively high level of mutant protein and other cardiomyocytes with very little mutant protein. This was recently shown to occur at the mRNA level in the *MYH7 R723G* mutation [6]. As *MYH7* mutations are point mutations, this variable expression pattern was not validated on protein levels yet. Western blot analysis in this study confirmed previous findings that the truncated protein products of the mutated allele are degraded in the cardiac tissue [10] (Fig. 1A).

The amount of healthy cMyBP-C in a MYBPC3$_{mut}$ cardiomyocyte is the result of the amount of transcription from the healthy allele, the translation efficiency and the protein turnover rate. Helms and colleagues demonstrated a paradoxical upregulation of mRNA in samples with truncating mutations, which was assumed to be a compensatory mechanisms as an attempt to maintain full-length cMyBP-C [11]. However, we observed a decreased expression of cMyBP-C at mRNA and protein level in MYBPC3$_{mut}$ confirming previous observations [4,8]. In accordance, transduction of full-length and truncating mutant *MYBPC3* in engineered heart tissue, to mimic a heterozygous mutation, showed cMyBP-C haploinsufficiency [12]. It has recently been shown by Lewis and colleagues that for maintenance of the sarcomere, a highly defined spatial control of protein translation and degradation is present [13]. They showed that at individual sarcomeres throughout the cell, protein translation and degradation machinery was present. In MYBPC3$_{mut}$ we know that transcription and therefore translation of *MYBPC3* is impaired, but also that there are changes in protein degradation, and therefore turnover [14]. Changes in microtubule architecture are known to occur in HCM [15], which are important for delivery of the translation and degradation machinery throughout the cell [13]. The loss of spatial control of protein translation and degradation in conjunction with the lower levels of transcription likely all contribute to the diverse cMyBP-C staining patterns observed in the cardiomyocytes from MYBPC3$_{mut}$. In HCM$_{smn}$ homogeneous expression of cMyBP-C with low levels of cell-to-cell variation were seen, indicating that the observed effects are specific for *MYBPC3* mutations. These
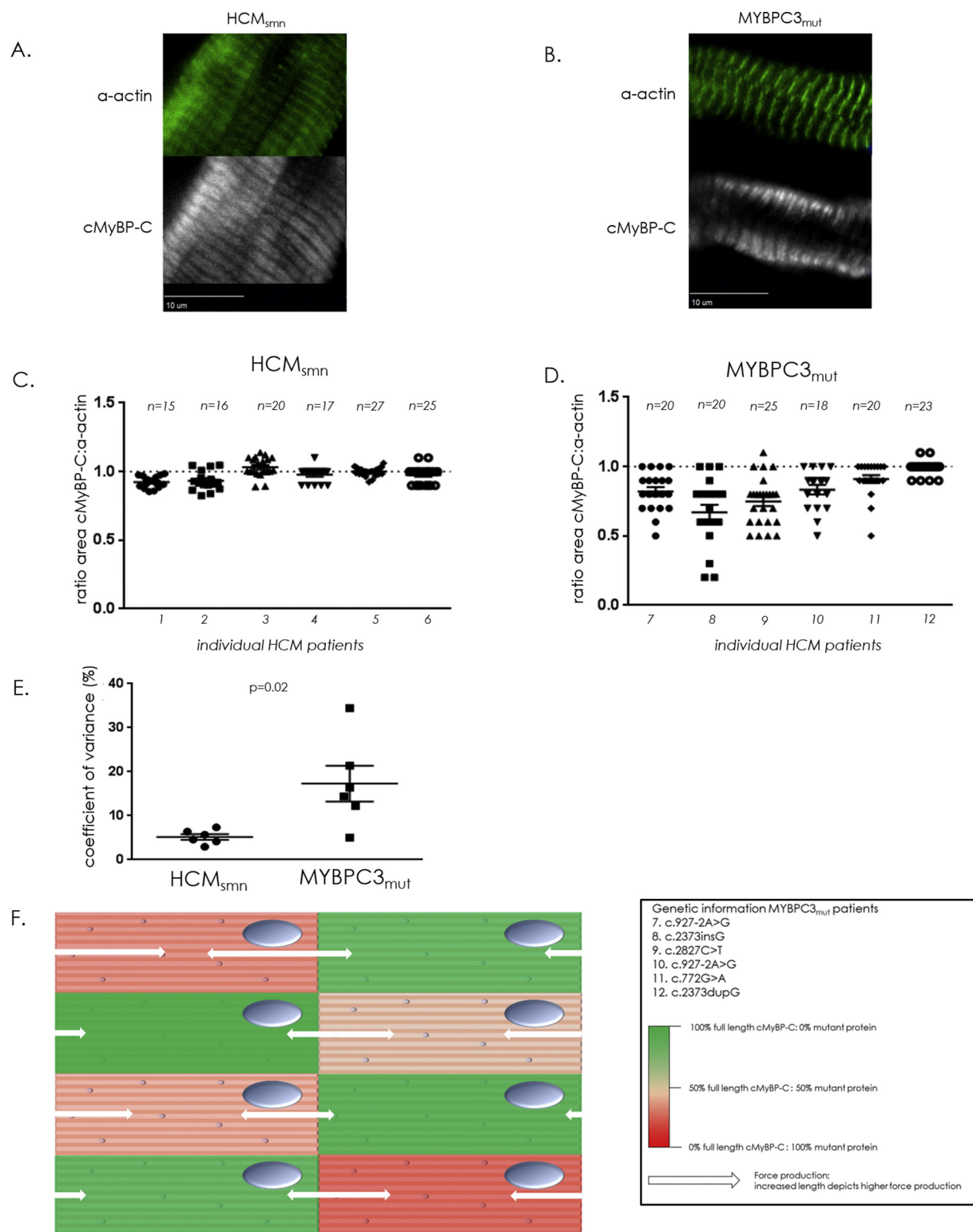
Fig. 2. Cell-to-cell variability. Typical examples of cardiomyocytes with double-staining of cardiac myosin binding protein-C and α-actin in a HCM$_{smn}$ (panel A) and MYBPC3$_{mut}$ (panel B) patient. Heterogeneous expression of cardiac myosin binding protein-C in MYBPC3$_{mut}$, compared to HCM$_{smn}$ patients in which staining of cardiac myosin binding protein-C is homogenously distributed. C. Heterogeneity of the cMyBP-C:α-actin ratio in HCM$_{smn}$ and (D) MYBPC3$_{mut}$ patients. E. Heterogeneity of the cMyBP-C:α-actin staining area per patient demonstrated a significant variability in MYBPC3$_{mut}$ ($p = .02$). F. The hypothesized consequences of cell-to-cell variation of cMyBP-C protein expression. Different contractile properties between each cardiomyocyte may lead to inhomogeneous contraction and relaxation and myofibrillar disarray.

observations are in line with staining patterns of cMyBP-C protein throughout cardiomyocyte from HCM patients with *MYBPC3* mutations. Cells with *MYBPC3* mutation demonstrated greater disruption in staining of thick filament proteins compared to *MYH7* mutations [16]. The experiments performed in this study utilized immunofluorescence analysis on mechanically isolated single cells. With the current analysis we were not able to quantify haploinsufficiency in individual cardiomyocytes, as we did in cardiac tissue (Fig. 1). The functional consequences of this variable expression of cMyBP-C remain to be determined. Loss of cMyBP-C in mouse studies have shown that severe cardiac dysfunction occurs [17]. It may be speculated that intercellular variation of cMyBP-C protein levels lead to inhomogeneous contraction and relaxation and may be involved in the formation of myofibrillar disarray (Fig. 2F). As aging reduces the quality of protein degradation pathways and increases the degree of allelic imbalance in cardiomyocytes [18], an age-dependent progression of cellular protein variability may contribute to HCM development.

5. Conclusion

This is the first study which demonstrates the intercellular variation of cMyBP-C myofilament protein levels in cardiomyocytes of MYBPC3$_{mut}$ patients.

Disclosures

None to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2018.08.023.

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