A cardiac myosin binding protein C mutation in the Maine Coon cat with familial hypertrophic cardiomyopathy

Kathryn M. Meurs[1,*], Ximena Sanchez[2], Ryan M. David[2], Neil E. Bowles[2], Jeffrey A. Towbin[2], Peter J. Reiser[3], Judith A. Kittleson[4], Marcia J. Munro[4], Keith Dryburgh[1], Kristin A. MacDonald[4] and Mark D. Kittleson[4]

[1]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, 601 Vernon Tharp Street, Columbus, OH 43210, USA, [2]Department of Pediatric Cardiology, 6621 Fannin, Baylor College of Medicine, Houston, TX 77030, USA, [3]Department of Oral Biology, College of Dentistry, The Ohio State University, 4195 12th Street, Columbus, OH 43210, USA and [4]Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

Hypertrophic cardiomyopathy (HCM) is one of the most common causes of sudden cardiac death in young adults and is a familial disease in at least 60% of cases. Causative mutations have been identified in several sarcomeric genes, including the myosin binding protein C (*MYBPC3*) gene. Although numerous causative mutations have been identified, the pathogenetic process is still poorly understood. A large animal model of familial HCM in the cat has been identified and may be used for additional study. As the first spontaneous large animal model of this familial disease, feline familial HCM provides a valuable model for investigators to evaluate pathophysiologic processes and therapeutic (pharmacologic or genetic) manipulations. The *MYBPC3* gene was chosen as a candidate gene in this model after identifying a reduction in the protein in myocardium from affected cats in comparison to control cats (*P* < 0.001). DNA sequencing was performed and sequence alterations were evaluated for evidence that they changed the amino acid produced, that the amino acid was conserved and that the protein structure was altered. We identified a single base pair change (G to C) in the feline *MYBPC3* gene in affected cats that computationally alters the protein conformation of this gene and results in sarcomeric disorganization. We have identified a causative mutation in the feline *MYBPC3* gene that results in the development of familial HCM. This is the first report of a spontaneous mutation causing HCM in a non-human species. It should provide a valuable model for evaluating pathophysiologic processes and therapeutic manipulations.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a clinically heterogeneous myocardial disease characterized by increased left ventricular (LV) mass due to an increase in wall thickness in the absence of apparent pressure overload or metabolic stimuli and histologically by myofibrillar and myocyte disarray (1,2). It has an estimated prevalence of one in 500 humans and is one of the most common causes of sudden cardiac death in young adults (1). In at least 60% of cases,

it is a familial disease. Spontaneous causative mutations have been identified in several genes that encode sarcomeric proteins including the alpha and beta myosin heavy chains, cardiac myosin binding protein C (*MYBPC3*), cardiac troponins T, I and C, alpha tropomyosin, the essential and regulatory light chains, actin and, most recently, titin (1–9). Mutations within the genes that encode for the sarcomeric proteins may lead to the development of the HCM phenotype by affecting either protein function or protein structure or both (1,9–12).

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*To whom correspondence should be addressed at: Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA 99164-6610, USA. Tel: +1 5093350738; Fax: +1 5093350880; Email: meurs@vetmed.wsu.edu

HCM is the most common cardiac disease identified in domestic cats (13). A colony of a feline model of familial HCM has been produced in the Maine Coon cat (13). Because of the identification of over 240 mutations in genes that encode for sarcomeric proteins in humans, we hypothesized that a mutation in one of these genes would be responsible for familial HCM in this animal model (1). After identifying a reduction in the cMyBP-C protein in affected cats, we identified a mutation in the feline gene that is predicted to alter the protein conformation of this gene and results in sarcomeric disruption. This is the first report of a sarcomeric gene mutation in a species other than human being. As the first spontaneous large animal model of this familial disease, feline familial HCM provides an extremely valuable model for investigators to evaluate pathophysiologic processes and therapeutic (pharmacologic or genetic) manipulations.

RESULTS

Clinical description

Twenty-three (16 affected, seven unaffected) Maine Coon cats from a colony with familial HCM, as previously described, and 100 unaffected control cats were evaluated. The pedigree of the cats in the colony studied has been published previously (13). Disease status of adult cats was identified by repeated echocardiographic examinations and the median of LV wall thickness and interventricular wall thickness was 7 mm (range: 6–9 mm; normal = 3–5 mm) in affected cats. Most affected cats also had systolic anterior motion of the mitral valve and left atrial enlargement. Papillary muscle hypertrophy was frequently noted.

Sarcomeric protein concentrations are altered

Myocardial samples were obtained from the LV free wall of eight affected cats at the time of death due to euthanasia for refractory heart failure, or as soon after death as possible from cats that died suddenly, as well as from three apparently healthy unrelated cats. Myocardial proteins were evaluated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Two proteins were noted to be absent or greatly reduced, and one protein was noted to be increased in the affected cats in comparison to the normal cats. The identification of the proteins that were reduced or increased in the affected cats was tentatively determined by analysis with MALDI mass spectrometry and by entering the peptide mass information into the NCBI database. The proteins reduced were identified as cMyBP-C and myomesin, an M-band protein. The protein that was increased in the affected cats was identified as anomalously migrating beta myosin (Fig. 1). Western blot analysis confirmed the identification of the reduced proteins (Fig. 2). When the proteins from the SDS–PAGE were evaluated quantitatively by densitometry, both the cMyBP-C and myomesin proteins were significantly reduced in the affected cats in comparison to the control cats (cMyBP-C, $P < 0.001$ and myomesin, $P = 0.011$) (Fig. 3). A weak inverse correlation ($r = -0.341$) was observed between the amount of anomalously migrating myosin and the cMyBP-C for affected cats.
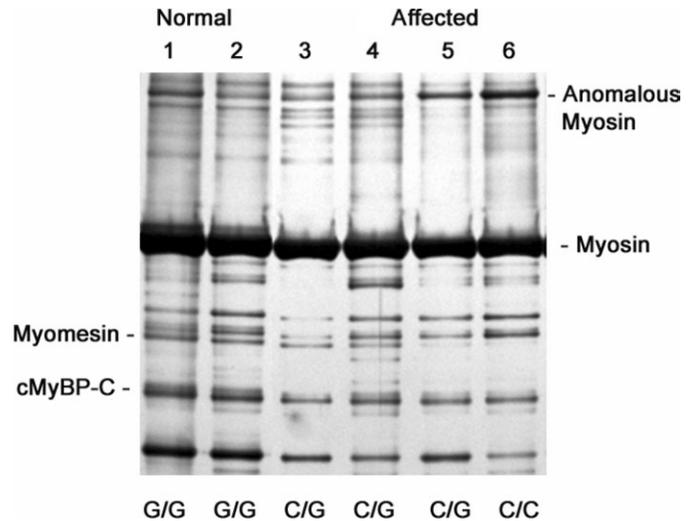

Figure 1. SDS–PAGE analysis of LV (free wall) myocardial samples from normal (lanes 1 and 2) and affected (lanes 3–6) cats. The cMyBP-C and myomesin proteins are reduced and the anomalously migrating beta myosin appears to be increased in comparison to the normal cats. The genotypes of the cats are shown below the lanes as G/G (normal cat), C/G (affected heterozygote) and C/C (affected homozygote).
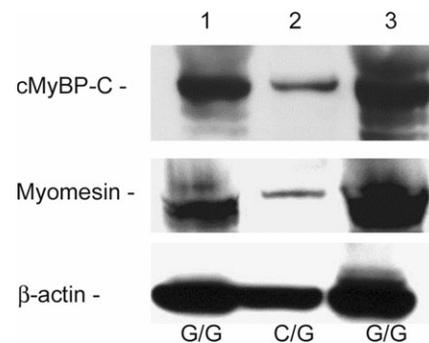

Figure 2. Immunoblot performed to confirm the identification of the abnormal proteins. Lanes 1 and 3 contain myocardial samples from normal cats, lane 2 contains a myocardial sample from an affected (heterozygote) cat. The genotypes of the cats are shown below the lanes as G/G (normal cat) and C/G (affected heterozygote).

Identification of a *MYBPC3* mutation

Because of the marked and consistent reduction in myocardial *MYBPC3* concentration in affected cats, the *MYBPC3* gene was targeted for analysis. DNA sequencing revealed a single base pair change (G to C) in codon 31 (exon 3) in affected cats (Fig. 4). This changed a conserved amino acid from alanine (A) to proline (P) (A31P) in each of the Maine Coon cats with HCM, but none of the unaffected Maine Coon or control cats. Affected cats were either heterozygous ($n = 10$) or homozygous ($n = 6$) for the mutation based on direct DNA sequence analysis. Computer protein structure analysis predicted a reduction in the alpha helix and an increase in random coils in this region of the molecule in the affected cats (Fig. 5).
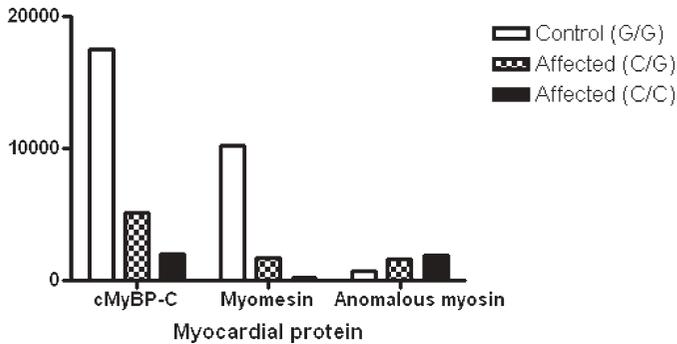
Figure 3. Quantitative (arbitrary densitometry units) SDS–PAGE analysis of myocardial proteins in normal and affected cats. Both the cMyBP-C and myomesin proteins were significantly reduced in the affected cats in comparison to the control cats (cMyBP-C, $P < 0.001$ and myomesin, $P = 0.011$). A weak inverse correlation ($r = -0.341$) was observed between the amount of anomalously migrating myosin and the cMyBP-C for affected cats. The genotypes of the cats are shown as G/G (three normal cats), C/G (seven affected heterozygotes) and C/C (one affected homozygote).
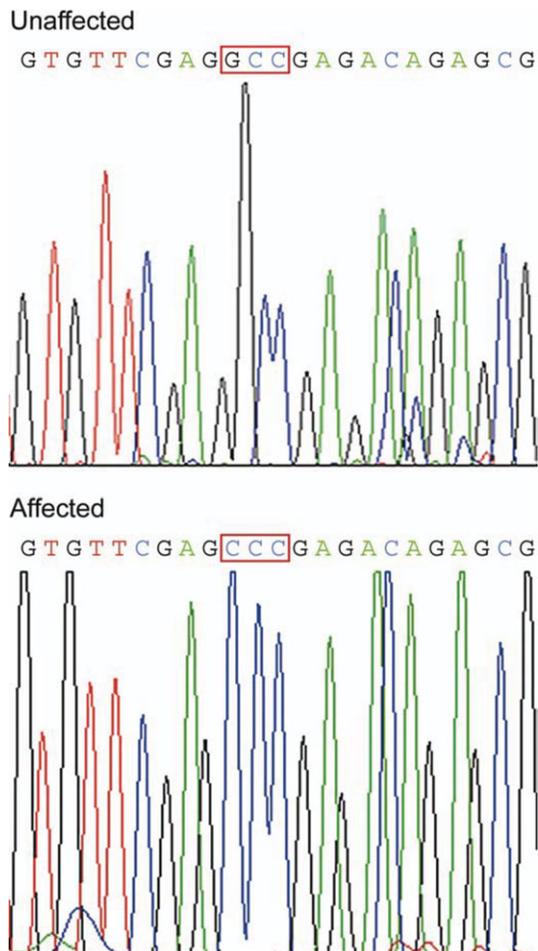

Figure 5. Computer protein structure analysis predicted a reduction in the alpha helix (blue) and an increase in random coils (purple) in the amino acid region from 10 to 20 (altered amino acid is 16) of the molecule in affected cats compared to normal cats.

Figure 4. DNA sequencing identified a single base pair change (G to C) in codon 31 of the *MYBPC3* gene in the affected cats ($n = 16$) but not in any of the unaffected family members ($n = 7$) or control cats ($n = 100$). A homozygous affected cat is displayed.
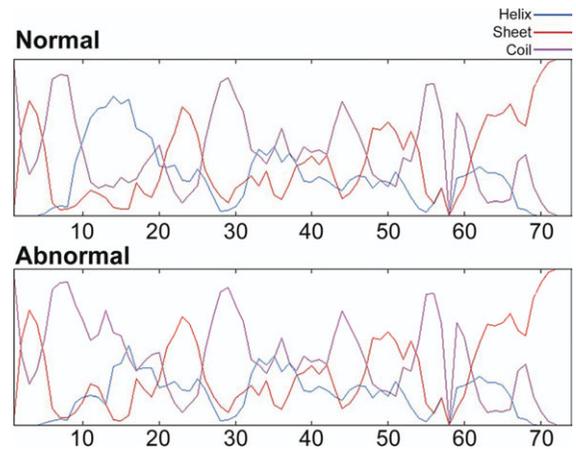
Sarcomeric protein organization is altered

Immunofluorescence analysis of sarcomeric proteins in LV sections from affected and unaffected cats revealed significant disruption in several sarcomeric proteins in affected cats, with reductions in staining intensity of cMyBP-C, myomesin, titin and cardiac actin (Fig. 6A–D). Staining for myosin heavy chain (Fig. 6E) and connexin 43 (data not shown) were normal.

MYBPC3 mRNA is increased

Although the cMyBP-C protein was noted to be reduced by SDS–PAGE, western blot analysis and immunofluorescence, quantification of *MYBPC3* mRNA by reverse transcription, real-time PCR in three affected (two heterozygous and one homozygous) cats and three unaffected cats determined that the amount of mRNA in affected cats was increased 1.25–3-fold (HPRT and actin as housekeeping genes) (Fig. 7).

Disease outcome relates to genotype

The phenotype of the affected cats evaluated in this study varied from moderate to severe HCM in cats whether they had one or two affected alleles. Most of the cats had echocardiographic evidence of HCM by 2–3 years of age, but one female (heterozygote) did not have echocardiographically identifiable disease until 7 years of age. The clinical outcome of the disease did vary with the genotype with a larger number of cats with a homozygous mutation developing moderate to severe disease and dying of their disease at 4 years of age or less, four of them suddenly (Table 1). One of these cats appeared echocardiographically normal but died unexpectedly under anesthesia at 4 years of age. Of the 10 cats with a heterozygous mutation, three are still alive at 8–12 years of age with moderate disease and only one died suddenly, a larger number of these cats developed severe HCM and died of heart failure. One died of an unrelated cause.
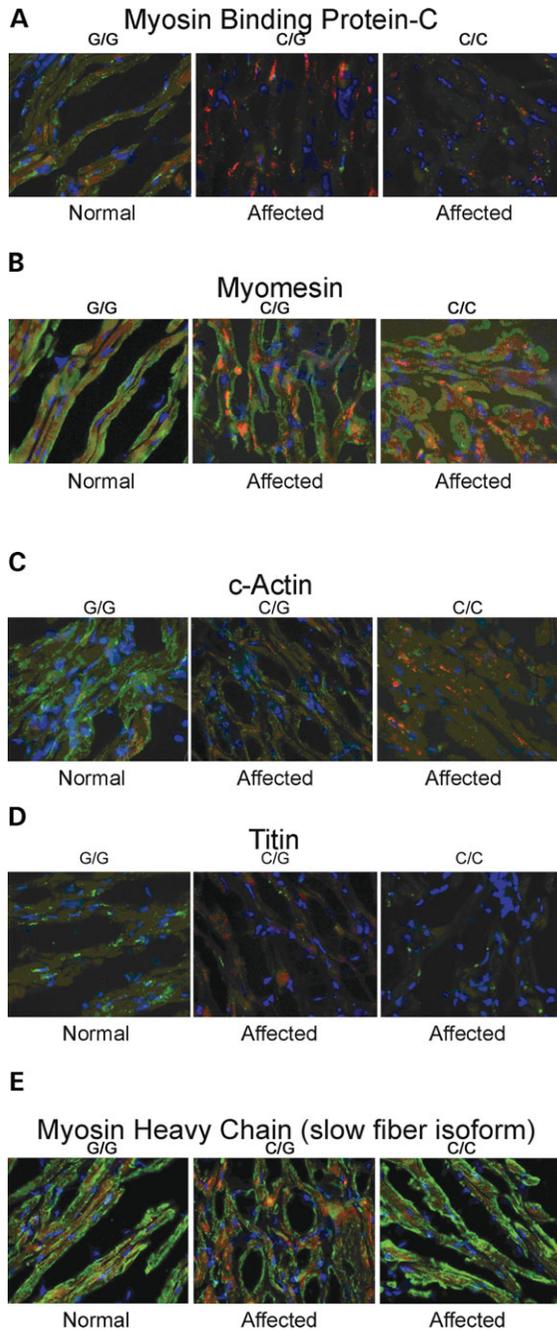
Figure 6 (A–E). Immunofluorescent staining of left ventricular free wall sections from affected and unaffected cats. Analysis of sarcomeric proteins in left ventricular sections from affected and unaffected cats revealed significant disruption to several sarcomeric proteins, with reductions in staining intensity of cMyBP-C (A), myomesin (B), cardiac actin (C) and titin (D) in affected cats. However, staining for myosin heavy chain (E) and connexin 43 (data not shown) were normal. Proteins of interest are stained green, while phalloidin and DAPI staining are red and blue, respectively.

Figure 7. Histogram of the ratio of *MYPBPC3* message RNA for three affected cats (two heterozygous and one homozygous) to three unaffected cats demonstrating that the amount of message RNA was increased 1.25–3-fold in affected cats. The HPRT and feline actin genes were used as housekeeping genes.

Table 1. The clinical outcome of the disease appeared to vary with the genotype although the number of cats in each group was small and should be cautiously interpreted

Phenotype	Sudden death	Congestive heart failure	Died of non-cardiac disease
G/C	1/10	5/10	1/10
C/C	4/6	0/6	1/6

DISCUSSION

In this study, we have identified a previously unreported *MYBPC3* mutation that changes a conserved amino acid in a purebred domestic cat model of familial HCM. To our knowledge, this is the first k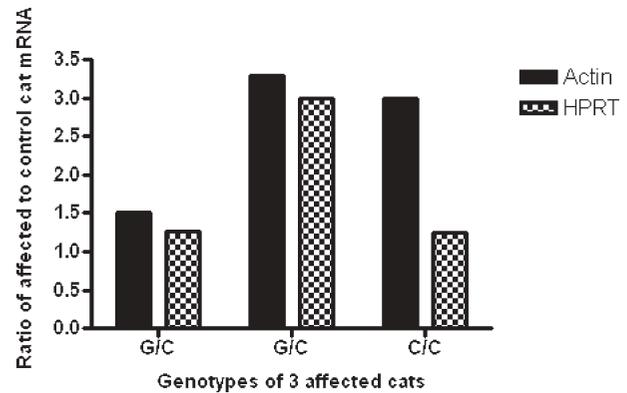nown spontaneous cause of familial HCM identified in a species other than *Homo sapiens*. Although mutations in exon 3 of the *MYBPC3* have been reported as causative for familial HCM previously in human beings, this particular mutation has never been reported (14). The amino acid affected is located in the linker region between domains C0 and C1 of the protein. The functional aspects of this area are not well understood, however, there is evidence that domain C0 and the C0–C1 linker region may bind to myosin and/or actin (15–18). The observations that the mutation identified in this model changes the computed structure of this protein in this region, and was associated with disruption of several sarcomeric proteins may suggest a change in the interaction of the abnormal cMyBP-C protein with corresponding cardiac proteins.

Both the cMyBP-C and myomesin proteins were decreased in the myocardium of affected cats in this study. In previous studies that evaluated *MYBPC3* mutations and familial HCM, the *MYBPC3* mutations frequently resulted in a frameshift that was predicted to produce a truncated protein, however, measurable quantities of the truncated protein were not detectable (9,12). A recent study demonstrated that truncated cMyBP-C proteins appear to be rapidly degraded by the ubiquitin–proteasome system as opposed to incorporation into the sarcomere of the abnormal protein (19). In the study presented here, we hypothesize that changes in the protein structure of cMyBP-C may alter the ability of the protein to be properly integrated into the sarcomere and that a similar degradation system may be involved in the reduction of the abnormal protein. This is supported by the finding that the affected

cats actually had a 1.25–3 increase in *MYBPC3* message produced in conjunction with the observed decrease in protein. Myomesin, a smaller (185 kDa) anchoring protein in the M-band that interacts with both titin and myosin in the assembly and stabilization of myofibrils, was also found to be reduced in the affected cats. Additionally, a proportion of cardiac myosin migrated anomalously. The abnormal behavior of the myomesin and the myosin is likely due to the significant interactions observed between these proteins and the cMyBP-C protein (20). Both myomesin and the cMyBP-C are built into the cytoskeletal lattice with titin before myosin, even though the sarcomeric myosin heavy chain is one of the first myofibrillar proteins expressed (21). It could be hypothesized that myomesin was partially degraded in these cats due to failure to be properly incorporated into the sarcomeric complex. The correct assembly of this cytoskeletal scaffold appears to be an important prerequisite for correct thick filament assembly and the integration of the contractile apparatus into the myofibril (21). The immunohistochemical analyses suggest that this mutation leads to disruption of the scaffold, as indicated by the aberrant staining of myomesin and titin in addition to cMyBP-C. However, the immunohistochemical analysis of myosin did not demonstrate significant disruption of this protein. Although cMyBP-C protein is not needed for formation of myosin filaments, it has been previously suggested that it is probably needed for them to form normally, as without the normal content of cMyBP-C protein, synthetic myosin filaments were observed to be thicker and longer and to have a more heterogeneous thickness (22). This could be an explanation for the anomalous migration of the myosin detected by SDS–PAGE. Such aberrant electrophoretic mobility of a protein on a SDS–PAGE has been observed for proteins that undergo post-translational modifications (23–25). The mechanism for the anomalous migration of myosin in this study is unclear, but it might be speculated that the reduced or abnormal cMyBP-C protein prevented normal formation and integration of a proportion of the myosin into the thick filaments and that myosin that is not integrated normally may migrate anomalously (26). However, sufficient unaffected myosin remained to be detected by immunohistochemistry.

Affected cats in this study had some variability of phenotype from mildly affected to severe hypertrophy. Some cats developed congestive heart failure and some died suddenly. Although it is tempting to suggest that these variations may be based on gene dose, the number of affected cats in this study is too small to suggest that disease outcome is related to the homozygosity or heterozygosity of the mutation.

The identification of the first sarcomeric gene mutation in a non-human species is highly significant and completes the development of this animal model of familial HCM. Our findings should increase the ability of investigators to use this model to address some of the remaining questions regarding HCM, such as the mechanism by which this specific mutation leads to the development of hypertrophy, the effect of modifiers on clinical phenotype and prognosis and the optimal effects of therapy on these variables. Additionally, evaluation of this model with a unique mutation within the domain 0–1 linker may aid in providing information about the structure and function of this domain.

MATERIALS AND METHODS

This study was conducted in accordance with the 'Position of the American Heart Association on Research and Animal Use' and under the guidelines of the Animal Care and Use Committee of the University of California at Davis.

Animal procurement and determination of phenotypic expression

Feline echocardiographic studies were performed using an Acuson 128XP/10 ultrasound machine (Siemens, Malvern, PA, USA) and a 7 MHz transducer using standard views (27). The cats were unsedated and restrained in right and left lateral recumbency on a Plexiglas table. Standard right parasternal long-axis and short-axis views plus left apical and left cranial views were examined (27). Measurements of diastolicLV wall thickness were made from the two-dimensional image.Cats were definitively diagnosed with HCM when severe papillary muscle hypertrophy was present and/or a region of the LV wall or the entire wall ofthe LV was ≥6 mm thick (27).

SDS–PAGE electrophoresis analysis and immunoblotting

The preparation of protein samples and methods for preparing gels and the running conditions were as described previously by Reiser and Kline (28) and Blough *et al.* (29). Samples were weighed and homogenized in sample buffer, consisting of 8 M urea, 2 M thiourea, 0.05 M Trizma base, 0.075 M dithiothreitol, 3% (w/v) SDS, pH 6.8 and 0.004% (w/v) Bromophenol blue. Homogenization of the samples in this buffer, with the high concentrations of urea and thiourea, coupled with homogenization, virtually ensured complete extraction of protein from the samples. Stacking gels consisted of 4% total acrylamide (acrylamide:bis = 50:1) and 5% (v/v) glycerol (pH 6.8). Separating gels consisted of 7% total acrylamide (acrylamide:bis = 50:1) and 5% (v/v) glycerol (pH 8.8). Protein loads were ~12 μg per gel lane. The gels were run in a Hoefer SE600 unit at 250 constant volts for 15 h at 8°C. A set of molecular weight standards was loaded in one lane to verify the identification of the bands. After electrophoresis, the gels were silver-stained and evaluated by densitometry for quantitation of the specific proteins (28). Protein bands of interest were evaluated by loading gels with ~20× more total protein, staining the gel with Coomassie blue and analyzing the excised bands by MALDI mass spectrometry (Ohio State Mass Spectrometry and Proteomics, Columbus, OH, USA).

A preliminary gel (12% acrylamide) was run, stained and scanned to test uniformity of protein loads. The actin band of this gel was scanned and quantitated. The coefficient of variation of the actin band was 12.1%, indicating that protein loads were reasonably uniform.

Immunoblotting was performed to confirm the identity of the proteins (cMyBP-C, myomesin, myosin heavy chain) of interest in the stained gels. Proteins were separated by SDS–PAGE (as described earlier) and transferred to nitrocellulose. Blots were incubated with an anti-myosin heavy chain

antibody (MF 20, Developmental Studied Hybridoma Bank, University of Iowa, Iowa City, IA, USA) diluted at 1:50, a rabbit polyclonal anti-rat myosin binding protein C antibody (gift from Dr Samantha Harris, University of Wisconsin, Madison, WI, USA) at 1:500 dilution or a mouse monoclonal anti-chicken myomesin antibody B4 (1:500 dilution) (gift from Dr H.M. Eppenberger, Institut für Zellbiologie, ETH-Zürich, Switzerland). The blots were washed with TBST three times, incubated with an anti-mouse alkaline phosphatase-conjugated secondary antibody (1:6667 dilution, Promega, Madison, WI, USA) and washed again three times with TBST. Color development was performed with NBT and BCIP (Promega) as substrates.

Mutation analysis

DNA was extracted from peripheral lymphocytes from all cats as previously described (30). Oligonucleotides were designed for amplification of the 38 exons of the feline *MYBPC3* gene, using known human sequences (GenBank accession no. U91629) and Primer3 software (31). Annealing temperatures were optimized for each exon and individual exons were amplified at $95°C$ (5 min) followed by 40 cycles of $94°C$ (20 s), optimized annealing temperature (20 s) and $74°C$ (39 s). Amplified samples were sequenced using an ABI377 (Applied Biosystems, Foster City, CA, USA) sequencer and compared for base pair changes. Sequences were analyzed for species conservation with mouse, cow, dog and human being by comparison to their respective GenBank accession nos (NM008653, XM583653, XM540744, U91629).

Structural analysis

Protein structure predictions were performed using the GOR4 (PBIL, France) and the Protein Structure Analysis software programs (BMERC, Boston, MA, USA).

Immunohistochemistry

Frozen myocardial sections (7 μm) were cut from the left ventricle. Unfixed sections were stained using cMyBP-C (gift from Dr Samantha Harris, University of Wisconsin, Madison, WI, USA), myomesin (gift from Dr H.M. Eppenberger, Institut für Zellbiologie, ETH-Zürich, Switzerland), myosin (clone NOQ7.5.4D, Sigma M8421), connexin-43 (clone CXN-6, Sigma C8093), actin (clone AC-40, Sigma A4700) and titin (clone T11, Sigma T9030) antibodies. Each primary antibody was diluted 1:500 in PBS, pH 7.2 containing 5% BSA and then added to the sections. The sections were incubated for 1 h at room temperature. The slides were washed for 10 min three times in $1×$ PBS pH 7.2 at room temperature. The sections were then incubated with secondary antibody (Alexa-488-anti-mouse conjugated secondary antibody (Invitrogen, Carlsbad, CA) diluted 1:1000 in PBS pH 7.2 containing 5% BSA for 1 h at room temperature. The slides were washed three times in $0.1×$ PBS pH 7.2 and mounted with Cytoseal 280 mounting medium (Stephens Scientific, Riverdale, NJ, USA) prior to observation.

Real-time PCR

Messenger RNA was purified and quantitated from LV myocardial samples of three affected (one homozygous and two heterozygous) and three unaffected cats with a Quickprep Micro mRNA purification kit (Amersham Bioscience, Piscataway, NJ, USA).

Single-step reverse transcription, real-time PCR was performed on purified mRNA. Probes were designed to be complementary to a segment located in exon 22 of *MYBPC3* (F-AACCTCCCAAGATCCACCTGG, R-CTGCGTGATAGCCTTCTGCC) and two housekeeping genes, the feline actin gene (GenBank accession no. AB005557) and a HPRT gene (GenBank accession nos L77488, L77489) as previously described (32). In brief, a mixture of all reagents required for RT–PCR was prepared to include: 12.5 μl SYBR green reaction buffer (Qiagen, Valencia, CA), 10 μl RNase-free water, 0.65 μl 20 μM forward primer, 0.65 μl 20 μM reverse primer, 2.0 μl purified mRNA ($≤250$ ng/reaction) and 0.25 μl reverse transcriptase. Samples were run in triplicate on a Stratagene Mx3000P (Stratagene, La Jolla, CA, USA) in 96-well MicroAmp optical plates (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed at $50°C$ for 30 min, followed by inactivation of the reverse transcriptase at $95°C$ for 15 min, and 40 cycles of $94°C$ (15 s), $57°C$ (30 s), $72°C$ (30 s). Relative quantities were calculated using the Stratagene instrument software.

Statistical analysis

The Student's *t*-test was used to evaluate differences in protein quantity between affected and unaffected cats. A Pearson correlation was used to determine a correlation between quantity of cMyBP-C and anomalously migrating myosin. Significance was defined as an alpha of <0.05.

Conflict of Interest statement. The authors have no conflict of interest to disclose.

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