I, Animesh Tandon, hereby submit this original work as part of the requirements for the degree of Master of Science in Clinical and Translational Research.

It is entitled:
Dystrophin genotype-cardiac phenotype correlations in Duchenne and Becker muscular dystrophy using cardiac magnetic resonance imaging

Student’s name: Animesh Tandon

This work and its defense approved by:

Committee chair: Erin Nicole Haynes, Ph.D.

Committee member: Zhiqian Gao, Ph.D., MSPH

Committee member: Michael Taylor, M.D., Ph.D.
Dystrophin genotype-cardiac phenotype correlations in Duchenne and Becker muscular dystrophy
using cardiac magnetic resonance imaging

A thesis submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

Master of Science
in Clinical & Translational Research

In the Department of Environmental Health
Division of Epidemiology & Biostatistics
of the College of Medicine
April, 2014

by

Animesh Tandon

Doctor of Medicine, University of Michigan Medical School, May 2008
Bachelor of Arts, Washington University in St. Louis, May 2003

Committee Chair: Erin Haynes, DrPH
Abstract

Background: Duchenne and Becker muscular dystrophy (DBMD) are caused by mutations in dystrophin. Cardiac manifestations vary broadly across the population, making prognosis difficult. Current dystrophin genotype-cardiac phenotype correlations are limited. For skeletal muscle, the reading-frame rule suggests that in-frame mutations tend to yield milder phenotypes.

Methods: A translational model was applied to patient-specific deletion, indel, and nonsense mutations to predict exons and protein domains present within the truncated dystrophin protein. Patients were dichotomized into predicted present and predicted absent groups for exons and protein domains of interest. Development of myocardial fibrosis (represented by late gadolinium enhancement (LGE)) and depressed left ventricular ejection fraction (LVEF) on CMR was compared.

Findings: Patients (total n=274) with predicted present cysteine-rich domains (CRD) (n=34), C-terminal domains (CTD) (n=30), and both actin-binding domain 1 and cysteine-rich domain (ABD1+CRD) (n=21) had a decreased risk of LGE and trended toward greater freedom from LGE. Patients with in-frame mutations exactly overlapped with those with CTD predicted present. Patients with predicted present C-terminal domain/in-frame mutations and N-terminal actin-binding+cysteine-rich domains trended toward decreased risk of and greater freedom from depressed LVEF.

Interpretation: Genotypes previously implicated in altering DBMD cardiac phenotype were not significantly related to LGE and depressed LVEF. Patients with predicted present cysteine-rich domain, C-terminal domain/in-frame mutations, and N-terminal actin-binding+cysteine-rich domains trended toward greater freedom from LGE and depressed LVEF, suggesting that the DBMD reading-frame rule may be applicable to the cardiac phenotype. Genotype-phenotype correlations may help predict the cardiac phenotype for DBMD patients and guide future therapies.
Acknowledgements

I would like to thank my wife Maria Tandon, my mother Neeru, father Rakesh, and sisters Maneera and Sanjana for their continual support for my continual schooling.

I would like to thank Dr. Michael Taylor for his mentorship and support.

I would like to thank Drs. Lynn Jefferies and Kan Hor for their support of my research and this project.

I would like to thank Dr. Jeanne James, Dr. Jeffrey Towbin, and the Heart Institute at Cincinnati Children’s Hospital Medical Center for their support for my pursuing this master’s degree.

I would like to thank Dr. Brenda Wong, Zhiqian Gao, the CCHMC Division of Neurology, and the Heart Institute Research Core for their support of this project.

I would like to thank Drs. David Kwiatkowski, Benjamin Landis, and Ryan Moore for their support.
Table of Contents
Abstract ......................................................................................................................................................... ii
Acknowledgements ...................................................................................................................................... iv
Abbreviations .............................................................................................................................................. vii
Introduction ................................................................................................................................................... 1
Methods ........................................................................................................................................................ 3
  Study population ....................................................................................................................................... 3
  Genotype characterization ........................................................................................................................ 3
  Cardiac phenotype .................................................................................................................................... 4
  Ventricular function imaging .................................................................................................................... 5
  Late gadolinium enhancement imaging .................................................................................................... 5
  Data analysis ............................................................................................................................................. 5
Results ........................................................................................................................................................... 6
  Study cohort .............................................................................................................................................. 6
  Dystrophin genotype-cardiac phenotype correlations ............................................................................... 6
Discussion ..................................................................................................................................................... 7
  Dystrophin genotype-cardiac phenotype correlations ............................................................................... 7
Limitations .................................................................................................................................................... 9
Conclusions ................................................................................................................................................... 9
References ................................................................................................................................................... 10
Table ........................................................................................................................................................... 13
  Table 1. Relationship of cardiac phenotype markers to dystrophin genotypes...................................... 13
Figures ........................................................................................................................................................ 15

Figure 1. Graphical representation of predicted present base pairs. ....................................................... 15

Figure 2A. Freedom from late gadolinium enhancement (LGE); cysteine-rich domain. ...................... 16

Figure 2B. Freedom from late gadolinium enhancement (LGE); C-terminal domain. ....................... 17

Figure 2C. Freedom from late gadolinium enhancement (LGE); N-terminal actin-binding domain and cysteine-rich domain ................................................................. 18

Figure 3A. Freedom from depressed left ventricular ejection fraction (LVEF); C-terminal domain.... 19

Figure 3B. Freedom from depressed left ventricular ejection fraction (LVEF); actin-binding domain and cysteine-rich domain ................................................................. 20

Conflicts of interests ................................................................................................................................... 21
**Abbreviations**

ABD1 = N-terminal actin-binding domain  
BMD = Becker muscular dystrophy  
CMR = cardiac magnetic resonance  
CRD = cysteine-rich domain  
CTD = C-terminal domain  
DBMD = Duchenne and Becker muscular dystrophy  
DMD = Duchenne muscular dystrophy  
LGE = late gadolinium enhancement  
LVEF = left ventricular ejection fraction  
RR = relative risk
Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are caused by dystrophin (DMD) gene mutations\(^1,2\) and comprise part of the clinical spectrum of disease often referred to as dystrophinopathies.\(^3-5\) The dystrophinopathies have variable clinical manifestations. By definition, DMD is characterized by a more severe skeletal muscle phenotype while BMD has a less severe skeletal muscle phenotype.\(^6\) In terms of the cardiac phenotype, DMD and BMD patients commonly exhibit left ventricular dilation and develop depressed left ventricular ejection fraction (LVEF) in the second decade of life,\(^7-9\) but the onset and progression of cardiac impairment are very variable.\(^5\) Large studies have seen no significant difference in the variation of the cardiac phenotype between DMD and BMD,\(^9,10\) allowing us to consider them as a combined dystrophinopathic group (DBMD). Given the wide variation in cardiac phenotype within the DBMD population, and the fact that cardiac dysfunction is a leading cause of morbidity and mortality in DBMD patients,\(^11,12\) identification of predictors and modifiers of the cardiac phenotype is paramount. The mechanisms underlying cardiac dysfunction in DBMD are unclear, but the development of myocardial fibrosis may play a central role.\(^13-15\)

Dystrophin genotype-skeletal muscle phenotype correlations have been studied extensively. One theory to explain skeletal muscle phenotype variation, the reading-frame rule, proposes that a semifunctional dystrophin protein, coded for in patients with in-frame mutations, leads to a less severe skeletal muscle phenotype (i.e. BMD) than a nonfunctional protein coded for by frameshift or early truncating mutations (i.e. DMD).\(^16\) However, there are exceptions to this theory, e.g. DMD patients with in-frame mutations and BMD patients with out-of-frame mutations, suggesting that dystrophin genotype-skeletal muscle phenotype correlations are incomplete.\(^2,17-19\) Viral gene therapy studies using mini- and microdystrophins in mice suggest that the N-terminal actin-binding domain (ABD1) and the cysteine-rich domain (CRD) are necessary for restoring skeletal muscle function.\(^20-22\)

Dystrophin genotype-cardiac phenotype correlations have been less thoroughly studied. Previous investigations in patients with DMD, BMD, and X-linked dilated cardiomyopathy have implicated
dystrophin exon 45,23,24 exons 48-49,7,25 exons 51-52,10 and the N-terminal actin-binding,21 rod,10,26,27 hinge-III,17,23,25 cysteine-rich, and C-terminal domains (CTD).28 Specifically in DBMD patients, studies have suggested that mutations in exon 45 lead to an earlier onset of cardiomyopathy in BMD patients;23 that mutations in exons 51 and 52 were protective from cardiac involvement in DBMD patients;10 that mutations in the N-terminal actin-binding domain lead to an earlier onset of cardiomyopathy in BMD patients;23 that mutations in the rod domain that change the phase of spectrin repeats lead to an earlier onset of cardiomyopathy in BMD patients;23 and that mutations in the hinge-III region were protective from onset of cardiomyopathy in BMD patients.23 However, not all dystrophin genotype-cardiac phenotype studies have found a correlation.29,30

In general, genotype and phenotype categorization schemes have been inconsistent among studies. Specifically, many studies incorporating mutation information have focused on the location (whether exonic or intronic) of the mutation or the mutation type (e.g. deletion or duplication) without regard to the predicted effects of the mutation on dystrophin structure. Our aim in this study was to take into account the potential effects of mutations on dystrophin protein domains by using patient-specific mutation data to predict the presence or absence of previously identified critical regions and functional domains within the dystrophic protein.

In terms of evaluation of the cardiac phenotype, the only markers of cardiac phenotype that have been evaluated in previous studies are the age of onset of depressed LVEF/shortening fraction and the presence of left ventricular dilation.10,23,24,26,27,29 Cardiac magnetic resonance (CMR) is a superior method of evaluating the DBMD cardiac phenotype because it provides more accurate and reproducible volumetric data compared to echocardiography and allows the ability to perform late gadolinium enhancement (LGE) imaging, a CMR marker for myocardial fibrosis.31 To date, LGE has not been used as a cardiac phenotype endpoint in DBMD cardiomyopathy studies, nor has CMR been used as the primary modality of phenotype evaluation. We aimed to perform dystrophin genotype-cardiac phenotype correlations for a large cohort of DBMD patients using our direct translation model to categorize patients based on
predicted presence or absence of critical functional protein domains, and using both LGE status and depressed LVEF (as determined by CMR) for cardiac phenotyping.

**Methods**

**Study population**

All boys with a dystrophinopathy who underwent clinical CMR studies at Cincinnati Children’s Hospital Medical Center (CCHMC) between January 2005 and January 2013 were identified by querying the CMR database. We included only those who had dystrophin mutations confirmed by genetic testing (described below). For the evaluation of dystrophin genotype-cardiac phenotype correlations, we limited the known DBMD patients to those with whole-exon deletion, indel, and nonsense mutations known or predicted to be disease-causing (described below). The Institutional Review Board approved the study.

**Genotype characterization**

For each DBMD patient who had undergone a CMR study, we reviewed clinically-obtained dystrophin mutation analysis. Several clinical diagnostic laboratories were used during the study period and methods for molecular analysis varied by lab; methods used included Southern blot, PCR, single condition amplification/internal primer (SCAIP), comparative genomic hybridization (CGH), and/or multiplex ligation-dependent probe amplification (MLPA). For classification of the mutation data for the cohort, each clinical diagnostic test result was checked using the Leiden reading-frame checker ([http://www.humgen.nl/scripts/DMD_frame.php](http://www.humgen.nl/scripts/DMD_frame.php)) and then analyzed against the Leiden whole exon change database, the Leiden point mutation database, or the UMD database as appropriate for the specific mutation. Mutations previously described as disease-causing, or mutations expected to change the coding sequence of dystrophin, were considered pathogenic. Mutations were defined as nonsense if a base pair change created a premature stop codon at the mutation site. All mutations defined as indel were the result of insertion or deletion of 1-4 nucleotides and all resulted in incorporation of a premature stop codon. Mutations were defined as splicing if they occurred at a predicted splice site as reported by the
diagnostic lab. Mutations were defined as intronic if they occurred outside both the coding and splice site sequences, as defined by the diagnostic lab.

In order to predict the presence or absence of critical functional protein domains, we first determined which base pairs were predicted to be present for each patient based on their specific mutation. A direct translation model was then used for whole-exon deletion, indel, and nonsense mutation types. These mutational mechanisms are predicted to result in a truncated protein and/or protein missing specific domains. The model assumed that the mRNA resulting from the patient-specific mutations was stable and not subject to nonsense mediated decay. The predicted mRNA was then translated to determine the predicted presence or absence of each critical functional protein domain. Exon boundaries were extracted from GenBank (accession NM_004006.2) and protein domain boundaries were extracted from the eDystrophin project and GenBank (accession NM_004006.2). For out-of-frame whole-exon deletion mutations, we predicted that exons and protein domains encoded entirely 5’ to the deletion start site would be present. For in-frame whole-exon deletion mutations, we predicted that exons and protein domains coded entirely either 5’ or 3’ of the deleted segment would be present. For indel and nonsense mutations, we predicted that exons and protein domains encoded entirely 5’ to the mutation site would be present. The creation of new 3’ protein domains in patients with out of frame mutations was not incorporated into the model. For each patient, we then determined whether each exon and protein domain of interest was predicted to be present or absent.

**Cardiac phenotype**

Image acquisition for our dystrophinopathy cohort has been described previously. The CMR studies were conducted on clinical 3T or 1.5T scanners, whichever was available for each study, without regard to patient status or the scanner in which the patient had previously been imaged. At our institution we routinely perform CMR on every DBMD patient annually; only patients who refused or could not tolerate lying in the scanner did not undergo the study, and an annual CMR study was recommended regardless of previous refusal or inability to undergo CMR.
Ventricular function imaging

Routine cardiac functional imaging was performed as described previously with a retrospectively vectorcardiographic-gated, segmented steady state free precession (SSFP) technique after localized shimming and/or frequency adjusting when available. Subjects were imaged with breath-held technique as tolerated; for those subjects who could not adequately breath-hold, a free breathing technique with multiple signal averaging was used. No anesthesia or sedation was used for these studies. Standard functional imaging included a short axis stack of cine SSFP images from cardiac base to apex; the short axis was prescribed as the perpendicular plane to the left ventricular long axis in 2 and 4 chamber views, as per previously published protocols. Typical scan parameters were as follows: slice thickness = 5 mm, in-plane resolution = 1.5 mm. A minimum of 12 slices were performed with 30 phases/slices. The typical temporal resolution of the cine SSFP images was 30–40 ms. The LVEF was assessed using standard planimetry techniques (QMASS MR, version 7.5, Medis Medical Imaging Systems) by an expert reader (RF, KNH, JJS, MDT). The LVEF was calculated for each subject and then exported to a spreadsheet file. The LVEF was defined as depressed if it was <55%. Our interobserver variability was ~4.0% and intraobserver variability was ~2% for LVEF (unpublished data).

Late gadolinium enhancement imaging

LGE imaging was performed via a standard inversion sequence recovery protocol 8 minutes after 0.2 mmol/kg gadolinium diethylenetriamine penta-acetic acid (Gd-DTPA) injection. LGE was considered positive if any left ventricular segment showed sub-epicardial or mid-myocardial hyperenhancement.

Data analysis

For each region of interest, we compared patients in the predicted present group against those in the predicted absent group. For overall risk of development of LGE and depressed LVEF we used chi-square analyses; for age of development of LGE and depressed LVEF we used t-tests, and for freedom from LGE and depressed LVEF we used Kaplan-Meier log-rank analyses (SAS version 9.3, SAS Institute, Cary, NC). All tests were 2-sided, and a p-value of <0.05 was considered statistically significant.
Results

Study cohort

The DBMD cohort contained 322 patients for whom genotype data were available. The mutation distribution was similar to other reported populations:18,19 212 (65.8%) whole-exon deletions, (30 (9.3%) in-frame, 182 (56.5%) out-of-frame); 39 (12.1%) whole exon duplications; 39 (12.1%) nonsense mutations; 23 (7.1%) indel mutations (19 (5.9%) deletion, 4 (1.2%) insertion); 7 (2.2%) splicing mutations; and 2 (0.6%) intronic mutations. Patients ranged in age from 4.9 years to 29.7 years at time of CMR.

Dystrophin genotype-cardiac phenotype correlations

We analyzed the dystrophin mutations and cardiac phenotype of 274 DBMD patients who met the inclusion criteria for genotype analysis. Patients were dichotomized to either the predicted present or predicted absent group for each region of interest based on the process detailed above. The subset of patients with in-frame deletions overlapped exactly with patients predicted to have the C-terminal domain present (Figure 1).

Patients predicted to have exons 45, 48-49, 51-52, or 1-45 present did not show a significant difference in the risk of, age of onset of, or freedom from LGE compared to the respective predicted absent groups (Table 1). Patients predicted to have the N-terminal actin-binding domain, hinge-III region, or rod domains present also did not show a significant difference in risk of, age of onset of, or freedom from LGE compared to the respective predicted absent groups. Patients predicted to have the cysteine-rich and C-terminal domains present did show a decreased risk of LGE (relative risk (RR) 0.40, 95% confidence interval 0.18-0.92, p= 0.012 and RR 0.37, 0.15-0.92, p=0.012 respectively), but there was no significant difference in age of onset of LGE compared to the predicted absent groups. There was a trend toward greater freedom from LGE for patients who were predicted to have the cysteine-rich domain present (25% time-to-event 14.3 vs. 13.0 years, overall log-rank p=0.060, Figure 2A) and the C-terminal domain present (25% time-to-event 13.6 vs. 13.0 years, overall log-rank p=0.054, Figure 2B). We then considered
patients who were predicted to have both the N-terminal actin-binding domain and cysteine-rich domain present (ABD1+CRD), as suggested by mouse models mentioned above. Patients predicted to have both the N-terminal actin-binding and cysteine-rich domains present had a lower risk of developing LGE (RR 0.27, 0.07-1.00, p=0.014) and greater freedom from LGE (p=0.025, Figure 2C) compared to those without both predicted to be present. There was no significant difference in age of onset of LGE. Patients predicted to have exons 45, 48-49, 51-52, or 1-45 present did not show a significant difference in the risk of, age of onset of, or freedom from depressed LVEF (Table 1) compared to the respective predicted absent groups. Patients predicted to have the N-terminal actin-binding domain, hinge-III region, rod domain, or cysteine-rich domains present also did not show a significant difference in the risk of, age of onset of, or freedom from depressed LVEF compared to the respective predicted absent groups. Patients predicted to have the C-terminal domain present (same as those with in-frame mutations) trended toward a decreased risk of depressed LVEF (RR 0.21, 0.03-1.46, p=0.095) and greater freedom from depressed LVEF (overall log-rank p=0.090, Figure 3A). There was no significant difference in age of onset of depressed LVEF. Patients predicted to have both the N-terminal actin-binding domain and cysteine-rich domain present trended toward decreased risk of depressed LVEF (RR 0.31, 0.05-2.14, p=0.331) and greater freedom from depressed LVEF (p=0.132, Figure 2B). There was no significant difference in the age of onset of depressed LVEF.

**Discussion**

**Dystrophin genotype-cardiac phenotype correlations**

Our data suggest that dystrophin mutations may correlate with the severity of the cardiac phenotype in DBMD patients. Specifically, patients predicted to have the cysteine-rich domain, C-terminal domain, and both the N-terminal actin-binding and cysteine-rich domains present, and those with in-frame mutations, trended toward milder cardiac phenotypes in our study; in our cohort, patients with in-frame deletions were likely to have both the cysteine-rich (n=34) and C-terminal domains (n=30) predicted present. These results suggest that the reading-frame rule for skeletal muscle may also be applicable in human cardiac
disease, and that this may be due to predicted present cysteine-rich domain or C-terminal domain. There is evidence that maintaining the C-terminal domain can lead to milder skeletal muscle phenotypes in humans;\textsuperscript{40} our data showing a trend in freedom from depressed LVEF in patients predicted to have the C-terminal domain present may be analogous to this. Mouse rescue studies suggest that both the N-terminal actin-binding domain and cysteine-rich domain are important\textsuperscript{35,41,42} in restoring skeletal muscle function; our results suggest that this may also be true in human cardiac disease, given the trends toward freedom from depressed LVEF seen in patients predicted to have both the N-terminal actin-binding domain and cysteine-rich domain present.

Our use of the development of LGE as a cardiac phenotype is novel. LGE, a marker for myocardial fibrosis,\textsuperscript{31} precedes the development of depressed LVEF in dystrophinopathic patients.\textsuperscript{14,15} We hypothesize that the patterns seen overall for patients with predicted present cysteine-rich domain, C-terminal domain, and both the N-terminal actin-binding and cysteine-rich domain in the development of LGE will be reflected in the development of depressed LVEF as well, as LGE is an earlier marker of the cardiac phenotype than depressed LVEF. Studies with a larger cohort and longer longitudinal follow-up will be required to test this hypothesis.

We did not find the same patterns suggested by Kaspar et al.,\textsuperscript{23} Jefferies et al.,\textsuperscript{10} and others who found that mutations in the N-terminal actin-binding domain region, around exon 45-49, and around exons 51 and 52 were correlated with the cardiac phenotype. However, our genotype characterization model takes the predicted effects of the mutation on dystrophin structure into account, whereas previous groups did not. In addition, we use a more standardized and reproducible measurement of cardiac phenotype than previous studies. Our data are similar to others that suggest that large mutations in the rod domain may not significantly affect phenotype,\textsuperscript{43} though we did not take into account the exact cutpoints within the spectrin repeats as other studies have.\textsuperscript{23,35}

Our findings have implications in terms of future gene therapy trials and the cardiac phenotype. Similar to the mouse studies mentioned, therapies that ensure the presence of the N-terminal actin-binding domain and cysteine-rich domain, and the correct phasing of the rod domain, may lead to dystrophin proteins with
an appropriate 3D structure and can bind actin and appropriately form the dystrophin-glycoprotein complex.

Limitations

Our study has limitations, many of which are related to its retrospective nature. Genetic data were derived from clinical testing that varied by clinical diagnostic laboratory and evolved over the years covered by the study. Clinical genetic testing does not include evaluation of transcript or protein stability or nonsense-mediated decay, and predictions of present regions of interest made in this study assume stability of the message and protein. In terms of phenotypes, we were limited by having data only on those patients able to tolerate a CMR study, which may represent ascertainment bias. Longitudinal follow-up for patients was also not standardized. Due to the retrospective nature of our cohort, we were unable to take cardiac medication usage (e.g. ACE-inhibitors, beta-blockers) into account; the effects of these medications on cardiac function, however, are mixed. In terms of statistical analysis, even though the sample size is overall large, when looking at specific mutation groups the sample size became small at times, limiting power.

Conclusions

The cardiac phenotype of dystrophinopathic patients is complex in nature. Underlying mutations in dystrophin may play a role in the severity of the cardiac phenotype; patients predicted to have the cysteine-rich domain, C-terminal domain, and both the N-terminal actin-binding domain and cysteine-rich domain present trended towards having improved freedom from LGE and depressed LVEF. Larger, multi-centered, prospective studies of the role of dystrophin mutations on the cardiac phenotype should be undertaken, as these findings have implications for gene therapy in this population.
References


32 White SJ, den Dunnen JT. Copy number variation in the genome; the human DMD gene as an example. *Cytogenet Genome Res* 2006; **115**: 240–6.


Table 1. Relationship of cardiac phenotype markers to dystrophin genotypes.

<table>
<thead>
<tr>
<th>Exon/domain (n=274)</th>
<th>Predicted present (n)</th>
<th>Risk of LGE (95% CI†); p-value</th>
<th>Age of onset of LGE</th>
<th>Freedom from LGE; p-value</th>
<th>Risk of depressed LVEF (95% CI); p-value</th>
<th>Age of onset of depressed LVEF</th>
<th>Freedom from depressed LVEF; p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 45</td>
<td>120</td>
<td>NS#</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Exons 48-49</td>
<td>61</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Exons 51-52</td>
<td>44</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Exons 1-45</td>
<td>104</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ABD1*</td>
<td>224</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hinge-III</td>
<td>48</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rod domain</td>
<td>12</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CRD‡</td>
<td>34</td>
<td>RR** 0.40 (0.18, 0.92); p= 0.012</td>
<td>NS</td>
<td>NS; 25% time-to-event 14.3 vs. 13.0 years; p=0.060</td>
<td>NS</td>
<td>NS; RR 0.21 (0.03-1.46); p=0.090</td>
<td></td>
</tr>
<tr>
<td>CTD§/in-frame deletions</td>
<td>30</td>
<td>RR 0.37 (0.15, 0.92);</td>
<td>NS</td>
<td>NS; 25% time-to-event 14.3 vs. 13.0 years; p=0.060</td>
<td>NS</td>
<td>NS; RR 0.21 (0.03-1.46); p=0.090</td>
<td></td>
</tr>
</tbody>
</table>
A total of 274 patients were examined. The exon or domain examined is listed in the first column. The number of patients predicted to have that exon or domain present is in the second column. Comparisons are between patients predicted to have that exon or domain present vs. patients predicted to have that exon or domain absent. Relative risks were examined using chi-square analyses or Fisher’s exact test, age of onset via t-tests, and freedom using Kaplan-Meier log-rank survival analysis.

*ABD1 = N-terminal actin-binding domain

†CI = confidence interval

‡CRD = cysteine-rich domain

§CTD = C-terminal domain

‖LGE = late gadolinium enhancement

¶LVEF = left ventricular ejection fraction

#NS = not significant

**RR = relative risk
**Figure 1. Graphical representation of predicted present base pairs.** The base pairs predicted present for each patient based on mutation data are represented with a horizontal black bar, aligned with the base pair number on the horizontal axis. Exon boundaries are marked in orange and protein domain boundaries are marked in purple and blue. A patient was predicted to have an exon or protein domain present if all the base pairs that code for that region of interest were predicted to be present.
Figure 2A. Freedom from late gadolinium enhancement (LGE); cysteine-rich domain.

Kaplan-Meier freedom from LGE for patients predicted to have the cysteine-rich domain present (n=34) versus those predicted to have the cysteine-rich domain absent. 25% time-to-event 14.3 vs. 13.0 years; log-rank p=0.060.
Figure 2B. Freedom from late gadolinium enhancement (LGE); C-terminal domain. Kaplan-Meier freedom from LGE for patients predicted to have the C-terminal domain present (n=30) versus those predicted to have the C-terminal domain absent. Patients predicted to have the C-terminal domain present were exactly those with in-frame deletions. 25% time-to-event 13.6 vs. 13.0 years; log-rank p=0.054.
Figure 2C. Freedom from late gadolinium enhancement (LGE); N-terminal actin-binding domain and cysteine-rich domain. Kaplan-Meier freedom from LGE for patients predicted to have both the N-terminal actin-binding and cysteine-rich domains present (ABD1+CRD) (n=21) versus those predicted to have at least one absent. Log-rank p=0.025.
Figure 3A. Freedom from depressed left ventricular ejection fraction (LVEF); C-terminal domain. Kaplan-Meier freedom from depressed LVEF (<55%) for patients predicted to have the C-terminal domain present (n=30) versus those predicted to have the C-terminal domain absent. Patients predicted to have the C-terminal domain present were exactly those with in-frame deletions. Log-rank $p=0.090$. 
Figure 3B. Freedom from depressed left ventricular ejection fraction (LVEF); actin-binding domain and cysteine-rich domain. Kaplan-Meier freedom from depressed LVEF (<55%) for predicted to have both the N-terminal actin-binding and cysteine-rich domains present (ABD1+CRD) (n=21) versus those predicted to have at least one absent. Log-rank p=0.132.
Contributors

Animesh Tandon, MD*; John L. Jefferies, MD*; Kan N. Hor, MD†; Brenda L. Wong, MD‡; Stephanie M. Ware, MD*, PhD; Zhiqian Gao, PhD*; Wojciech Mazur, MD§; Robert J. Fleck, MD‖; Joshua J. Sticka, MD*; D. Woodrow Benson, MD¶; Michael D. Taylor, MD, PhD*

*The Heart Institute, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA
†The Heart Center, Nationwide Children’s Hospital, Columbus, OH, USA
‡Department of Neurology, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA
§The Heart and Vascular Center at the Christ Hospital, Cincinnati, OH, USA
‖The Department of Radiology, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA
¶Herma Heart Center, Children’s Hospital of Wisconsin, Milwaukee, WI, USA

AT, JLJ, KNH, BLW, SMW, WM, DWB, and MDT all participated in the conception and design of the study. All authors participated in the analysis and interpretation of the data and in all stages of manuscript revision. All authors have read and approved all versions of the manuscript and its content.

Conflicts of interests

All authors declare no conflicts of interest.