The Genetics of Dilated Cardiomyopathy: A Prioritized Candidate Gene Study of *LMNA*, *TNNT2*, *TCAP*, and *PLN*

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ABSTRACT

Background: Dilated cardiomyopathy (DCM), which is characterized by left ventricular enlargement and systolic dysfunction, is divided into cases with a clear predisposing condition (eg, hypothyroidism, chemotherapeutic agents, alcoholism, ischemia) and those of unknown cause (idiopathic DCM). Many cases (20%–35%) of DCM are familial, implicating a genetic contribution to the etiology. More than 30 genes have been identified, many involving "private" mutations not shared among families. Evidence suggests that nonfamilial cases also have a genetic predisposition, again involving many genes. The goal of this study was to identify mutations in genes associated with DCM in a Québec study sample including familial and nonfamilial DCM cases.

Hypothesis: A prioritized gene study conducted within a framework for the classification of identified genetic variants could yield etiological information even in the absence of family data.

Methods: We sequenced 4 previously identified genes: lamin A/C (*LMNA*), cardiac troponin T type 2 (*TNNT2*), titin-cap (*TCAP*), and phospholamban (*PLN*).

Results: We discovered a nonsense mutation in the *LMNA* gene and a frameshift mutation in the *TNNT2* gene, as well as other clinically significant variants that were not observed in publicly available databases or in Québec-based controls. *PLN* was sequenced to investigate a previously published promoter variant. However, our data confirm that this variant does not have a causal role in DCM.

Conclusions: Despite high locus and allele heterogeneity, we demonstrate that a prioritized gene study, combined with next-generation exome-sequencing data, can be fruitful for the identification of DCM mutations.

Introduction

Dilated cardiomyopathy (DCM) affects 1 in 2500 individuals.[1] It is characterized by left ventricular (LV) enlargement and systolic dysfunction, resulting in impaired heart function that causes significant mortality and morbidity, including heart failure (HF).[2–4] Idiopathic DCM (IDC) is diagnosed when detectable causes of DCM (eg, hypothyroidism, chemotherapeutic agents, alcoholism, ischemia) are excluded. Clinical screening of first-degree relatives reveals that 20% to 35% of idiopathic cases are familial DCM (FDC).[5] Familial occurrence of the disease implicates genetic transmission, and in fact, mutations in >30 genes are associated with this disease. Most families (90%) display an autosomal-dominant pattern of inheritance.[6] Clinical and genetic diagnoses are complex, as FDC displays variable and age-dependent penetrance, variable expression, and locus and allelic heterogeneity.[7,8] In addition, there is increasing evidence that disease-causing mutations can be present in nonfamilial cases.[9–11]

Genetic screening is recommended for patients and, if positive, family members as well, to identify those at risk. As DCM is a significant cause of morbidity and mortality, the purpose of this study was to identify specific disease-causing variants, which would contribute to a more complete understanding of the genetic etiology of DCM. This could help clinicians in the identification of at-risk family members and could also be beneficial for accurate genetic counseling.

This work was supported by a grant from The Research Institute of the McGill University Health Centre Pilot Project Competition.
The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Clin. Cardiol. 36, 10, 628–633 (2013)
Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22193

Received: February 1, 2013
Accepted with revision: June 19, 2013

Classification of disease-causing variants is not always straightforward. Recent work has attempted to classify the pathogenicity of genetic variants using metrics such as family segregation, evolutionary conservation, prior observations, functional studies, and in silico analysis of identified variants.[12] In light of the frequent lack of family data in many clinics, we endeavored to outline a strategy focused on extensive control data and computational analysis. We hope in this way to extend a framework for assessing genetic variants to patients without available family data.

Methods

The McGill University Health Centre (MUHC) Biomedical Research Ethics Board approved all study protocols, and participants provided informed consent. Investigations were conducted in accordance with the Declaration of Helsinki. For this study we used the first 96 cases presenting with DCM (left ventricular ejection fraction [LVEF] <50%) to the Heart Failure Clinic of the MUHC between April 2008 and August 2009. All patients underwent an angiogram to rule out coronary artery disease, and diagnosis of ischemic cardiomyopathy was a basis for exclusion. Data on ethnicity and clinical factors (previous medical history, family history of DCM, age of onset, sex, and LVEF) were collected for all cases (Table 1). The Québec-based controls were 184 patients from the Division of Cardiology without DCM (LVEF >50%), and thus they represent the same catchment area as the study population. On average, controls were older than cases (60.2 ± 11.0 years compared with 53.3 ± 11.6 years) and predominantly male (65.21%), which is similar to cases (68.75%).

Blood samples were collected from the 96 DCM patients and 184 controls. DNA was isolated from ~0.3 mL of buffy coat, using the FlexiGene DNA kit (Qiagen Inc., Toronto, ON) according to the manufacturer's instructions. Sanger sequencing was performed using Big Dye Terminator chemistry (Applied Biosystems/Life Technologies, Carlsbad, CA) and sequencing reactions were analyzed on a 3730*xl* DNA analyzer (Applied Biosystems/Life Technologies). Sequences were analyzed using the Phred/Phrap/Consed package (Genome Sciences Department, University of Washington, Seattle, WA). Based on a previously developed and published prioritization strategy,[13] 3 previously identified genes (lamin A/C [*LMNA*], cardiac troponin T [*TNNT2*], and titin-cap [*TCAP*]) were selected for sequencing of all coding exons. The strategy prioritizes genes according to an empirical estimation of mutation frequency in each gene coupled with the number of exons in the gene to maximize the chances that the sequencing identifies a causative mutation. In addition, to investigate a previously published promoter variant 36 bp upstream of the phospholamban (*PLN*) gene,[14] exon primers for that gene were designed that also covered that variant.

Classification of Variant Pathogenicity

Discovered variants were classified by novelty and predicted functional effect using the software programs Sorting Intolerant From Tolerant (SIFT),[15] PolyPhen2,[16] and Protein Variant Effect Analyzer (PROVEAN).[17] Novel variants are those not found in either the National Heart, Lung, and Blood Institute Exome Sequencing Project (ESP) or 1000 Genomes Project databases, or in the Québec-based controls. Following the terminology of Lakdawala et al,[12] variants were considered clinically significant variants (CSV) if they resulted in novel protein-altering changes that led to either deletions or truncations or deleterious amino-acid changes, as assessed by ≥1 predictive software. Variants were considered variants of unknown significance (VUS) if they were amino acid–changing mutations that were not novel or not predicted to have an effect on protein

Table 1. Characteristics of the 96 DCM Cases

Age, y[a]	53.3 ± 11.6
Range	26–81 y
Sex, n (%)	
M	66 (68.8)
F	30 (31.3)
LVEF, %	19.1 ± 9.8
Range	5–44
Patients with (n, %):	
Pacemaker and/or ICD	62 (64.6)
Hypertension	40 (41.7)
DM	28 (29.2)
Heart transplant	27 (28.1)
Thyroid condition	16 (16.7)
Excessive alcohol consumption	9 (9.4)
Stimulant use	5 (5.2)
Ethnicity, n (%)	
French Canadian	26 (27.0)
Other European	46 (47.9)
Middle Eastern	6 (6.3)
Mixed and other	18 (18.8)
Family history of DCM, n (%)	
Y	26 (27.1)
N	55 (57.3)
Possible[b]	10 (10.4)
Unknown[c]	5 (4.2)

Abbreviations: DCM, dilated cardiomyopathy; DM, diabetes mellitus; F, female; ICD, implantable cardioverter-defibrillator; LVEF, left ventricular ejection fraction; M, male; MUHC, McGill University Health Centre; N, no; Y, yes.
[a]Age is either age at diagnosis or age at first visit to the MUHC heart failure clinic. [b]Self-reported history of heart disease, with DCM unconfirmed. [c]Patient either adopted or has no knowledge of their family.

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22193

function, structure, or conservation based on software prediction (see above). Variants were presumed benign (PB) if they were neither novel nor predicted to be deleterious nor overrepresented in our cases (in the case of the noncoding variant).

Control Populations

We sequenced 184 controls presenting with other cardiac diseases, but in addition to these local controls, we queried the ESP's publicly available database (Exome Variant Server Release version v.0.0.14 from June 20, 2012), which totals 6503 individuals from the United States (4300 European Americans and 2203 African Americans), as well as the 1000 Genomes database (Phase 1 release, v3, November 23, 2010), which comprises 26 different populations from around the globe. Because our study cohort is predominantly but not exclusively of European descent, we report the data for the total population from these databases, as well as European subpopulation data (referred to as "All" and "Eur" in Table 2).

Results

Clinical characteristics and demographic data on the 96 DCM patients are summarized in Table 1. Comorbid conditions included hypertension, diabetes, thyroid conditions, excess alcohol consumption, and stimulant abuse.

We sequenced the 4 selected DCM genes in these 96 patients. Variants were considered novel when not encountered in the ESP or the 1000 Genomes databases, or in the Québec-based controls. These variants are described in Table 2 along with estimates of their deleterious effect and their frequencies in cases and controls, and in the ESP and 1000 Genomes samples.

We identified 11 amino acid–changing variants, of which 7 were novel, in 12/96 probands (12.5%), as 2 mutations were seen twice and 1 proband carried 2 mutations. Furthermore, 12 probands carry the previously published *PLN* promoter variant, and 3 of these also carry another protein-altering mutation.

Clinically Significant Variants

Of the novel variants identified in this study, 2 are very disruptive to the gene product (Table 2), and thus have a high probability of being disease causing. One patient has a nonsense mutation in the *LMNA* protein (E384X). This mutation would truncate approximately half the protein, causing a severe, if not complete, loss of function. Sequencing also revealed a frameshift mutation present in the *TNNT2* gene of a patient at amino acid 141 that results in a stop codon at position 180, and thus the complete loss of the protein downstream, again truncating slightly more than half the protein. These variants are considered CSVs.

A third patient had a 3bp deletion in the *TCAP* gene (codon 13), resulting in the deletion of a glutamic acid, and PROVEAN predicts this to be deleterious. Interestingly, the *TCAP* E13Del is a previously published variant, initially identified in patients with hypertrophic cardiomyopathy[18] but later in unaffected individuals as well.[19,20] This suggests that the E13Del variant may be a rare nonsynonymous polymorphism that does not contribute to the etiology of DCM. Our analysis yielded 3 further CSVs: *LMNA* T150I, *TCAP* R158C, and *TNNT2* G186V. These amino acid–changing mutations probably have a negative impact on either the structure or conservation of the gene product, based on both the SIFT and PolyPhen2 predictive tools (Table 2). Furthermore, these variants are novel, as they were not in the ESP, 1000 Genomes, or our Québec control datasets.

Variants of Unknown Significance

The mutation E39K in the *TNNT2* gene, although novel, does not yield predictive scores suggesting a deleterious effect. Of the remaining protein-altering variants identified, 4 are previously discovered variants with dbSNP ID numbers: *TCAP* E105K (rs146906267), R106C (rs45578741), and A118V (rs143233087); and *LMNA* R614C (rs142000963). In addition, the *TCAP* R106C variant was observed in the Québec control samples. Thus, although not novel, based on SIFT and PolyPhen2 predictions of damaging effects, these are VUS (Table 2). Available family members (n = 6) of those patients presenting with protein-altering variants were sequenced. None of these family members were affected with DCM, nor did they carry the protein-altering variants (data not shown).

Variants Presumed Benign

Finally, analysis of the previously published common promoter variant in *PLN* reveals that its minor allele frequency (MAF) is consistent between the Québec cases (MAF = 6.25%) and controls (MAF = 5.71%; $P = 0.16$) (Table 3). This MAF is also consistent with 1000 Genomes data for this variant (MAF = 7.00%).

Discussion

It is well documented that genetic variants are associated with some cases of DCM, and the Heart Rhythm Society/European Heart Rhythm Association expert consensus statement recommends genetic testing for patients with DCM and cardiac conduction disease. It further states that it can be useful for patients presenting with FDC to identify family members at risk to facilitate counseling.[2] There is also long-standing evidence indicating that prophylactic treatment of asymptomatic individuals with depressed cardiac function (established by LVEF) is beneficial.[21] Thus, early treatment of asymptomatic patients with FDC may improve their prognosis, and disease-causing variants in other family members should be identified, especially in families with histories of sudden death or conduction disease. However, a full catalogue of all DCM-causing mutations is far from being achieved, and studies such as the present one are needed to increase our knowledge of disease-causing variants.

Our study demonstrated that the prioritized candidate gene approach is useful in detecting CSVs and VUSs through sequencing even a small number of genes. The high yield of CSVs from this study was probably due to the use of a novel candidate gene strategy[13] that takes into account the frequency of previously discovered variants and the number of exons in a gene, resulting in an economical approach.

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22193

Table 2. Variants Found in the DCM Study Sample

a.a. Change (nt Change)	Grade	rs# Chromosomal Location[a]	SIFT Prediction (Score)	PolyPhen2 Prediction (Score)	Times Seen in Cases, n = 96	MAF in ESP, All(Eur)	MAF in 1000K, All(Eur), n = 2184	Presence in Québec Controls
LMNA, lamin A/C								
T150I (A > T)	CSV	Novel chr1:156100500	Damaging (0.01)	Possibly damaging (0.94)	1	—	—	—
E384X (G > T)	CSV	Novel chr1:156105905	NA	NA	1	—	—	—
R614C (C > T)	VUS	rs142000963, chr1:156108510	Tolerated (0.06)	Probably damaging (0.998)	1	0.0011 (0.0011), n = 12 992	—	—
TCAP, titin-cap or telethonin								
E13Del (GAGdel)	CSV	Novel chr17:37821649	Deleterious (−1.278)[b]	NA	1	–	—	—
E105K (G > A)	VUS	rs146906267, chr17:37822171	Tolerated (0.13)	Possibly damaging (0.801)	1	0.0005[c] (0.0006), n = 12 998	0.0009 (–)	—
R106C (C > T)	VUS	rs45578741, chr17:37822174	Damaging (0)	Probably damaging (1)	2	0.0036 (0.0042), n = 12 955	0.0100 (0.0026)	+
A118V (C > T)	VUS	rs143233087, chr17:37822211	Tolerated (0.08)	Probably damaging (0.998)	1	0.0008 (–), n = 12 992	0.0009 (–)	—
R158C (C > T)	CSV	Novel chr17:37822330	Damaging (0)	Probably damaging (1)	1	—	—	—
TNNT2, cardiac troponin T type 2								
E39K (G > A)	VUS	Novel chr1:201337308	Tolerated (0.14)	Benign (0.079)	1	—	—	—
R141-FS G > del	CSV	Novel chr1:201333464	NA	NA	1	—	—	—
G186V G > T	CSV	Novel chr1:201332437	Damaging (0)	Probably damaging (0.997)	2	—	—	—
PLN, phospholamban								
Promoter variant A > C	PB	rs77186188, Chr6:118869423	NA	NA	MAF = 6.25%	NA	3% (7%)	5.71%

Abbreviations: 1000K, 1000 Genomes Project; a.a., amino acid; All(Eur), all populations combined (European population only); CSV, clinically significant variant; DCM, dilated cardiomyopathy; ESP, Exome Sequencing Project; FS, frameshift; MAF, minor allele frequency; n, number of sampled chromosomes; N, patient sample size; NA, not applicable; NCBI, National Center for Biotechnology Information; nt, nucleotide; PB, presumed benign; PROVEAN, Protein Variant Effect Analyzer; rs#, reference single nucleotide polymorphism identification number; SIFT, Sorting Intolerant From Tolerant; VUS, variant of unknown significance.
[a] Chromosomal location refers to location in NCBI build 37. [b] Prediction from PROVEAN, because of its ability to predict the pathogenicity of indels (http://provean.jcvi.org/index.php). PROVEAN classifications for the all other a.a.-changing variants were not included in the table, because the predictions were consistent with SIFT. [c] This locus is triallelic. MAF here represents the other minor allele (C) resulting in E105Q. The A allele was not found in any other population.

Table 3. *PLN* Promoter Minor Allele Frequency in Cases vs Controls

Origin of Study Sample	Cases	Controls
Greece[a]	3.67%	0.17%
Ohio[a]	1.84%	
Québec	6.25%	5.71%
1000 Genomes	—	7.00%
Brazil[b]	3.06%	2.50%

Abbreviation: *PLN*, phospholamban.
[a]See Haghighi et al[14] for details. [b] See Santos et al[23] for details.

Sequencing only 4 genes in 96 probands yielded 11 protein-altering variants in 12 of 96 patients (12.5%), 6 of which are CSVs (6.25%). The prevalence of *TCAP* mutations in our case populations is 6.25%, whereas that of both the *LMNA* and *TNNT2* genes is 3.13%. These identified mutations still underestimate the genetic etiology, as we only sequenced a fraction of the >30 genes that have been implicated thus far.

It has become clear that many rare coding variants can be tolerated, representing a challenge in linking genotypes to phenotypes. However, Pan et al note the low tolerance of cardiomyopathy-related genes to mutation.[22] The lack of tolerance was greatest for nonsense mutations, but was present for missense variation. Moreover, Pan et al. observed that SIFT and PolyPhen2 had the best discriminatory power of the bioinformatic prediction tools that they tested. The data of Parks et al suggest that the use of both prediction algorithms is warranted.[10] They found that all the missense variants found in DCM patients and confirmed by segregation data were predicted to be damaging by either SIFT or PolyPhen2, but not always by both. Thus, the use of both SIFT and PolyPhen2 can help to classify variants, especially in cardiomyopathy genes, which appear to be particularly intolerant to mutation.[22]

Variants in noncoding regions may also play a role in DCM, but they were not the primary focus of our study. However, we did investigate 1 promoter variant 36 bps upstream of *PLN*. Haghighi et al[14] found that this variant contributed to decreased contractility and increased deterioration in HF patients, although our results are consistent with later findings suggesting that this variant does not contribute to DCM or a worse prognosis in HF.[23] Recent genome-wide studies have identified additional loci associated with HF due to DCM. Although one study did identify a common variation that contributes to sporadic DCM,[24] it also identified rare coding mutations in a gene (*BAG3*), which was replicated.[25] In the future, if family members are unavailable, such studies will need even larger samples of unaffected individuals or will rely more on public databases as we have in the present work.

The probands of our study were subdivided into FDC and IDC (IDC includes those with possible and unknown family history [see Table 1 note]). The mutation frequencies we observed for IDC and FDC (11.11% and 7.7%, respectively) support other evidence that sporadic cases of DCM also have a genetic basis.[9–11] In addition, one of the patients in our study with a coding variant also had nongenetic risk factors: excessive alcohol consumption and stimulant use. Findings by Ito et al. demonstrated a high prevalence of stimulant abuse in a cardiomyopathy study population,[26] and stimulant abusers also had more severe DCM. This underscores the possible contribution of genes even in the presence of other etiological factors. Although the primary mode of transmission of FDC is autosomal dominant, varying degrees of penetrance are observed, which could be explained in part by an interaction between genetic variants and environmental variables and in turn explain why some variants may still contribute to DCM, despite being present in control samples. This is consistent with recent work that demonstrated that some sarcomeric genes in the 1000 Genomes database contain more pathogenic mutations than would be expected from the population prevalence of cardiomyopathies.[27]

Genetic association studies may be facilitated by the local recruitment of controls because most rare SNPs will be population-specific.[28] On the other hand, large sample sizes are required to associate rare variants with complex traits.[29] In our study, we examined locally recruited population-specific controls for the presence of observed variants and complemented this with larger public databases (ESP and 1000 Genomes).

Study Limitations

This study is limited by the lack of family data for most of our probands. Segregation analysis of the observed variants would improve the evidence for causality. Given that the variant classification based on functional analysis in this study is derived solely from bioinformatic predictions, true functional analysis of the variants could further delineate mutation severity. Our study was limited to the complete exonic sequence of 4 genes, but future studies should involve a larger number of candidate genes or exome sequencing.

Conclusion

Given the locus heterogeneity of DCM, to make genetic testing for DCM a more powerful tool, classification standards need to be established for interpreting the results of genetic tests, especially exome sequencing. Building on previous work that used family data and segregation analysis,[12] we have implemented a framework for classifying variants even in the absence of family data that can serve clinicians and researchers.

Despite the high locus and allele heterogeneity, the sequencing of prioritized DCM genes can be fruitful to identify disease-causing variants. The 11 coding variants we observed must account for only a small fraction of the genetic contribution to DCM in this study sample. Similar mutation frequencies between FDC and DCM support increasing evidence for a genetic etiology for sporadic cases of DCM. Large-scale studies with multigenerational pedigree analysis and functional analysis of protein variants are needed for a more complete understanding of DCM etiology.

Acknowledgments

The authors acknowledge the sequencing expertise of David Roquis and Sébastien Brunet at the McGill University and

Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22193

Génome Québec Innovation Centre, and Pierre Lepage, Sohail Hooda, and Lise Coderre for helpful discussions.

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Published online in Wiley Online Library (wileyonlinelibrary.com)
DOI:10.1002/clc.22193