Exome Sequencing and Genome-Wide Linkage Analysis in 17 Families Illustrates the Complex Contribution of TTN Truncating Variants to Dilated Cardiomyopathy

Running title: Norton et al.; Exome Sequence Analysis in TTN DCM

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Abstract:

**Background** - Familial dilated cardiomyopathy is a genetically heterogeneous disease with >30 known genes. *TTN* truncating variants were recently implicated in a candidate gene study to cause 25% of familial and 18% of sporadic dilated cardiomyopathy (DCM) cases.

**Methods and Results** - We used an unbiased genome-wide approach employing both linkage analysis and variant filtering across the exome sequences of 48 individuals affected with DCM from 17 families to identify genetic cause. Linkage analysis ranked the *TTN* region as falling under the second highest genome-wide multipoint linkage peak, MLOD 1.59. We identified six *TTN* truncating variants carried by affected with DCM in 7 of 17 DCM families (LOD 2.99); 2 of these 7 families also had novel missense variants segregated with disease. Two additional novel truncating *TTN* variants did not segregate with DCM. Nucleotide diversity at the *TTN* locus, including missense variants, was comparable to five other known DCM genes. The average number of missense variants in the exome sequences from the DCM cases or the ~5,400 cases from the Exome Sequencing Project was ~23 per individual. The average number of *TTN* truncating variants in the Exome Sequencing Project was 0.014 per individual. We also identified a region (chr9q21.11-q22.31) with no known DCM genes with a maximum heterogeneity LOD score of 1.74.

**Conclusions** - These data suggest that *TTN* truncating variants contribute to DCM cause. However, the lack of segregation of all identified *TTN* truncating variants illustrates the challenge of determining variant pathogenicity even with full exome sequencing.

**Key words:** genetics, human, genome-wide analysis, dilated cardiomyopathy, exome
Introduction

Whole exome sequencing technologies are rapidly enabling the identification of novel rare variants in patients with cardiomyopathy, but assigning pathogenicity remains challenging. Truncating variants in TTN were recently observed in 25% of familial dilated cardiomyopathy (DCM) cases.1 DCM is genetically heterogeneous with rare variants in over 30 disease genes, including TTN, previously indicated to cause DCM.2,3 Prior to the recent publication of TTN contributing to a major fraction of genetic DCM, the fraction of cases attributable to any single gene ranged from <0.5% to ~6% per disease gene.4

Discovery and incorporation into clinical tests of a single gene accounting for a large fraction of DCM cases could be helpful for presymptomatic diagnosis in at-risk family members, but the clinical translation of this finding is confounded by several factors. First, despite a significant excess of truncating variants in DCM cases, these variants also occur in ~3% of controls.1 This is not unusual in complex trait analysis, where common genetic variants occur more frequently but not exclusively in cases compared to controls, and increase disease risk or susceptibility. However, in the context of DCM, which has been categorized primarily as a rare-variant Mendelian disease with marked locus and allelic heterogeneity,5 it is essential to know which truncating variants are pathogenic. Second, with over 300 exons and >34,000 amino acids, TTN has the largest coding sequence in the genome, and the majority of the general population will have at least one rare (defined as a mean allele frequency <0.5%) missense or truncating variant at this locus. Next Generation Sequencing (NGS) now allows rapid variation analysis of the TTN gene despite its size. However, it relies on an economy of scale, and for a similar cost as sequencing TTN alone, NGS can be used to sequence the entire coding sequence of the genome. This allows DCM patients to be screened for sequence variants in TTN in parallel with all other
known DCM genes and the rest of the coding genome, leading to the third issue: the recent study of TTN truncating variants in DCM\(^1\) used a custom NGS panel specific for TTN, and therefore neither the role of genetic variants in other known DCM genes nor segregating variants in novel DCM genes could be assessed. Detailed examination of TTN truncating variants in the context of all coding variants in known and potentially novel DCM genes is needed to assess variant pathogenicity.

In this study we used exome sequence data from seventeen families, each with three or more members affected with DCM, in an effort to identify a genetic cause of DCM, as each family proband was negative for mutations in the coding regions of 16 DCM genes, as previously reported.\(^6-11\) From these exome sequences we identified several families with TTN truncating variants. To more carefully assess TTN variants as a cause of DCM, we constructed a linkage map of common informative single nucleotide variants (SNVs) from our exome data in all seventeen families and performed linkage analysis across the genome. We hypothesized that if TTN is causative of 25% of DCM, we would observe a significant combined LOD score across our 17 families at this locus compared to the rest of the genome. Second, we evaluated all other unbiased rare variation in the exome data to identify putative DCM causative variants in each family. We describe the variants identified in TTN in the context of other top ranking variants across the exome sequence of each family. Third, we examined the nucleotide diversity at the TTN locus in the 5,400 exome sequences available from the Exome Variant Server\(^12\) to determine if the large amount of variation within this gene is accounted for by size or if the TTN locus is more genetically diverse than other known DCM genes.
Materials and Methods

Subjects

Written, informed consent was obtained from all subjects, and the Institutional Review Boards at the Oregon Health & Science University and the University of Miami approved the study. The investigation included 17 families, each with three or more members affected with DCM, and with each proband already known to be point mutation negative for 16 known DCM genes. Genomic DNA was extracted from whole blood according to a standard salting out procedure, as previously reported. Two-point and multipoint parametric linkage analysis were performed with the Merlin software program. We assumed an affected only model with a disease allele frequency of 0.0001 and penetrance of 0.9. In addition to traditional LOD scores, a HETLOD score resulting from a test of linkage in the presence of genetic heterogeneity was also calculated. A genome-wide linkage map of common informative markers was constructed by identifying all SNV’s present from the exome data that overlap with known SNV’s in 60 unrelated Europeans from the International HapMap. SNV’s with minor allele frequency <1% and/or Mendelian errors within the HapMap were excluded. The remaining markers were then pruned using the PLINK software using pairwise r² <0.1 in sliding windows of 50 SNV’s, moving in intervals of 5 SNV’s. This resulted in a final exome-wide marker set of 4,601 SNV’s. Marker allele frequencies for linkage analysis were determined by the frequency of each SNV in the Exome Sequencing Project (ESP) in the relevant ethnically matched population (either European (n=3,499 individuals) or African American ancestry (n=1,864 individuals).
Exome sequencing and analysis

Exome sequencing was performed at the University of Washington Genome Science Center across seventeen families (48 individuals) with NimbleGen V2 in solution capture and Illumina HiSeq. Sequences were aligned with BWA\textsuperscript{15} and realignment and single nucleotide and insertion-deletion variants were called with GATK version 1.4, at the Hussman Institute for Human Genomics. Vcf files were then imported into an in-house database, Genomes Management Application (GEMapp) to facilitate storage, variant annotation, querying and analysis. In addition to our exome data, GEMapp was used to store transcriptome data from the left ventricle of four unrelated individuals, two with DCM and two unaffected individuals, as previously published.\textsuperscript{11} This allowed us to filter variants mapped to genes expressed in heart tissue.

Using GEMapp, we queried each family to determine putative disease-causing variants that met the following criteria: read depth $\geq 5$ and quality scores $\geq 40$; variants that were either missense, nonsense, splice site, or a coding insertion or deletion, shared across all affected members of a family; frequency $<0.5\%$ in 5,400 exomes from the Exome Variation Server (EVS); have either a Phastcons\textsuperscript{16} score $>0.4$ or a GERP\textsuperscript{17} score $>2$; expression in our heart transcriptome dataset with Reads Per Kilobase per Million mapped reads, (RPKM) $>3$. These criteria were defined from our previous work on 197 variants in known DCM genes published as disease-causing and that was analyzed by our group.\textsuperscript{18} We also excluded filtered variants that were present in all 48 exomes and variants occurring in more than one family that did not segregate with disease status in at least one other family.

Copy number variation

Copy number variation in 48 DCM exomes was also assessed by the Structural Variant Working
Group at the University of Washington, using CoNIFER (Copy Number Inference From Exome Reads, http://conifer.sourceforge.net/).\(^1\)\(^9\) A total of 200 non-DCM exomes and 48 DCM exomes were used. Singular Value Decomposition (SVD) transformation was used to remove systematic bias, removing 8 components. The final SVD-ZRPKM signal was then smoothed and the duplication/deletion breakpoints found using a threshold of ±1.5 SVD-ZRPKM.

**Sanger sequencing validation**

All variants passing filter criteria and occurring within \(TTN\) were validated with Sanger sequencing and run on a 3130xl as previously published.\(^1\)\(^1\) Primer sequences are shown (Supplementary Table 1). Any additional DNA samples (n=29) from affected and unaffected family members were also sequenced for these variants.

**Nucleotide diversity**

A total of 316 exons in \(TTN\) were targeted in our exome sequence. To assess the genetic variation at this locus, accounting for the large amount of coding sequence, we used the normalized number of variant sites, \(\theta\), as a measure of nucleotide diversity across the 5,379 exomes available from the Exome Variant Server. \(\theta\) was calculated as described in Cargill et al for all coding sequence and also separately for both missense and truncating variants.\(^2\)\(^0\)

**Results**

**Exome-wide linkage analysis across seventeen families with DCM**

The maximum LOD score across the genome and the LOD score at the \(TTN\) locus are shown for each family (Table 1). In those families with \(TTN\) truncating variants identified by exome sequencing analysis, the maximum LOD score achieved across the genome for each family was comparable to the LOD score at the \(TTN\) locus (Table 1). The highest multipoint peak within the genome fell in the region spanning chromosome 9q21.11-q22.31 (hg19:71,862,987-95,840,256),
producing a heterogeneity LOD score of 1.74. Overall, in the seventeen families, the TTN locus was the second highest (HLOD=1.59; Table 2).

**Exome sequence analysis from seventeen families with DCM**

Our criteria for putative DCM variants identified in the exome sequences was based on defined criteria (Methods) and as previously described.11 The number of shared variants meeting these criteria present in each family ranged from 1-80, (average 28.1, median 24). We had previously reported that of the 197 variants already published as causative of DCM, 16% were present in 2,400 exomes from the Exome Sequencing Project, and of those with functional data (and therefore presumed to be pathogenic variants of very low frequency), the median frequency in the Exome Sequencing Project population was 0.04%.18 Applying this maximum 0.04% frequency criterion to the current exome analysis, the number of shared filtered variants per family ranged from 1-49, (average 16.8, median 15).

CNV analysis using exome data did not identify any shared rare variants (frequency < 1% in the ESP dataset) across these families.

A total of six TTN truncating variants (two frameshift, three nonsense and 1 splice variant that occurred in two DCM families) were identified among the filtered candidates in seven of our 17 families (41%) (Table 3). Our approach to identifying which of these truncating variants were likely disease-causing within these families was to genotype them in additional DNA samples in the extended families where possible to assess segregation of the variant with disease, and we also consider them in the context of the additional shared variants in our filtered lists for each family. We further observed from our linkage data that those families with highly negative LOD scores at the TTN locus had no TTN truncating variants that passed our exome analysis filtering criteria. In addition, we screened against presence in the 1000 genomes data (which is
independent of the EVS dataset). None of the six truncating variants were present in this dataset.

**Clinical characteristics families with DCM and segregating TTN variants**

The pedigree structures are shown (Figure 1) and the clinical characteristics of relevant family members are provided (Table 4) of the DCM families with segregating TTN variants (Table 1).

**Family A.** Family A had DNA samples available for three additional members. Sanger sequencing showed that all six family members were heterozygous for the truncating variant. Subject III.3, a female who carried the TTN variant, died at age 69 with mild systolic dysfunction (ejection fraction of 42%) but without left ventricular enlargement (LVE), having suffered an myocardial infarction in her 50’s, and thus confounding assessment of whether the TTN variant, the myocardial infarction, or both contributed to her systolic dysfunction. Two subjects (IV.3, V.1), both mutation carrier’s in their 20’s, had no evidence of DCM.

**Family B.** In addition to the three samples that had exome sequencing, Family B had DNA samples available from six other family members. Sanger sequencing confirmed the nonsense variant as present in all affected family members. A female obligate carrier (II.2) died of cancer at age 76 without a cardiovascular history. Another female obligate carrier (II.5) had no cardiovascular history at age 70. A male who carried the TTN variant at age 69 (II.6) had only borderline systolic dysfunction without LVE.

**Family C.** Only three DNA samples were available for this family and were used for exome sequencing. Subject III.3, who died of DCM, was an identical twin by family history and thus may have been an obligate carrier. Another obligate carrier (III.3) had no known DCM but by death certificate died of ventricular tachycardia and coronary artery disease. None of the additional twelve variants passing filtering criteria (eight under more stringent filtering of population frequency <0.05% in the ESP exome dataset) occurred in known DCM or other
cardiomyopathy associated genes.

**Family D.** This family of European ancestry carried the same *TTN* splice variant identified in Family C. Of the additional 32 variants also identified as putative disease-causing in this family, only one occurred in a known DCM gene, a missense variant in *TTN*, chr2:179,410,975, NM_133378.4, Gly29127Arg.

**Family E.** This family carried a single base insertion in *TTN* resulting in a frameshift mutation. Of the additional 26 segregating variants also identified in this family, the only variant in a gene with a reported association with hypertrophic cardiomyopathy occurred in *SOS1.*21 One of the affected children (II.2) carried a rare variant in *MYBPC3* inherited from his mother (I.2), previously reported by us as likely disease-causing9 but suggested to be of unknown significance based on a subsequent study;22 we also note that this variant is present at a frequency 0.12% in the EVS, making it more common than many DCM rare variants.18

**Family F.** A four bp deletion in *TTN* resulted in a frameshift mutation. DNA was available from one additional affected member and three unaffected family members, and the *TTN* variant was confirmed to be present in all four affected members by Sanger sequencing and was not present in the three unaffected members. None of the other 23 segregating variants identified occurred in known cardiomyopathy-associated genes.

**Family G.** No DNA samples beyond those used for exome sequencing were available to assess segregation in this family. Of the additional 23 variants that segregated, none occurred in other cardiomyopathy-associated genes.

**Segregating missense variants at the TTN locus**

We also considered the implication of segregating *TTN* missense variants passing our exome filtering pipeline as a class of variants that were not discussed in the recent *TTN* paper.1
Determination of pathogenicity of these variants will be extremely challenging because of the large number of coding exons. In the 5,400 ESP exome datasets, the average number of TTN missense variants per individual was 23.3, ranging 6 to 55. These were comparable results to those observed in the exome sequences of our DCM families, with the average number of TTN missense variants per DCM individual at 22.75, (ranging 11 to 43). Application of our DCM filtering criteria to missense variants in the 5,400 ESP exomes (frequency <0.5% and either a PhastCons score >0.4 OR a GERP score of >2) resulted in an average of 1.91 missense variants per individual (ranging 0 to 23). Five TTN missense variants passed our exome filtering approach (that segregated with all individuals affected with DCM in a family): one each in two DCM families who also had segregating truncating variants (Table 3), and the others in two families, each with high quality candidates in known cardiomyopathy genes so they were not further prioritized. The average number of TTN missense variants without regard to sharing, that is, an analysis of only one individual from each of the 17 families, a less stringent approach and similar to the analysis of all missense TTN variants conducted for the EVS, was 1.88.

Non-segregating, truncating variants

Given the observed excess of TTN truncating variants in both familial and in sporadic DCM versus controls, we thought it also relevant to report the number of non-segregating truncating variants in TTN identified in the exome sequences of these 48 individuals with DCM, as these may be potential susceptibility variants. We observed two non-segregating TTN truncating variants that were validated with Sanger sequencing. First, a C insertion at hg19 chr2:179,426,992 generating a frameshift in one of three family members with DCM who underwent exome sequencing (Family 14, Table 1), and a nonsense variant at hg19 chr2:179,605,218, NM_003319.4 Gln3885stop in two of three family members (Family 17,
Table 1). Neither variant was observed in the 5,400 ESP exome sequences or in the 1000 Genomes data, making them potential susceptibility variants. In the case of the frameshift variant, this family had already been shown to segregate a variant published as disease-causing accompanied by functional data and in Family 14 with the nonsense variant, a total of 43 segregating variants were identified by our exome filtering pipeline (Table 1), none of which were in previously published cardiomyopathy genes.

**Nucleotide diversity of TTN**

The NimbleGen V2 in solution capture target included 315 discrete exons from six TTN transcripts (NM_001256850.1, NM_133432.3, NM_133378.4, NM_003319.4, NM_133437.3 and NM_133379.3), totaling 110,459 bp coding sequence. Our exome pipeline identified TTN truncating variants in seven DCM families. This could simply be a result of the large number of exons. Hence, we investigated the nucleotide diversity at the TTN locus in a non-DCM population. The Exome Variant Server (EVS) contains annotated exome sequence from 5,379 individuals at the TTN locus totaling 2,425 and 25 discrete missense and nonsense variants, respectively. Across 5,379 EVS individuals there are a total of 125,575 missense alleles and 77 nonsense alleles, averaging 23 and 0.014 per individual, respectively. We calculated the normalized number of variant sites, $\theta^{20}$, accounting for sample size and the number of coding bases in the EVS individuals at the TTN locus to be $2.23 \times 10^{-3}$ and $2.3 \times 10^{-5}$ for missense and nonsense variants respectively. The same calculation across five other known DCM genes (MYBPC3, TNNC1, TNNI3, MYH6 and TPM1) in the EVS data gave comparable results to those observed in TTN, (for missense variants, $\theta$ ranged from $6.9 \times 10^{-4}$ at TPM1 to $1.05 \times 10^{-3}$ at TNNC1 and for truncating variants, $\theta$ at TNNC1 and TNNI3 to $1.29 \times 10^{-4}$ for MYBPC3), suggesting that
the excess of shared truncating variants in our DCM families is not due to the large number of exons alone and that nucleotide diversity at TTN is comparable to other known DCM genes.

Discussion

This is the first independent replication study of TTN truncating variants as frequently involved in the pathogenesis of familial DCM. Herman et al recently identified TTN truncating mutations in 25% of familial DCM and 18% of sporadic DCM, a significant excess compared to 3% of controls.1 The authors concluded that truncating mutations in TTN are a frequent cause of DCM, as all prior reports of unselected patients with DCM of unknown cause ranged from <1% to 5-8%.4 However, 3% of controls in the Herman et al publication1 also were observed to have TTN truncating variants, suggesting that the interpretation of specific TTN truncating variant pathogenicity would be challenging, especially in simplex cases. Analysis across nineteen DCM families segregating rare TTN truncating variants in the Herman et al study1 yielded a combined LOD score of 11.1, providing strong evidence that the truncating variants in those families were pathogenic. However, in that study TTN was sequenced in isolation so that the relevance of the linkage evidence in the TTN region could not be compared to the rest of the genome.

We hypothesized that if rare variants in TTN indeed account for one-quarter of familial DCM, this locus should also be detected using an unbiased genome-wide linkage approach across our 17 DCM families, as they should be enriched for causative variants at this locus, especially since the DCM families in this study were selected for exome sequencing because they were already known to be point mutation negative for 16 other known DCM genes6-11 (with the exception of one family segregating a previously described variant6 in a gene attributing ~0.5% of DCM). Genome-wide linkage analysis yielded the second most significant evidence of
linkage at the *TTN* locus compared to other regions in the genome, which we interpret as evidence of the *TTN* locus in DCM pathogenesis.

Next we identified those nonsense, missense, splice and frameshift variants in the exome sequences, meeting our filtering approach that included conservation and myocardial expression, which segregated with DCM affection status in each family. Seven of 17 families (41%) had segregating *TTN* truncating variants identified in their filtered exome variants. Using common informative SNV’s within the exome sequences, the combined LOD score at the *TTN* locus for these seven families was 2.99, and in each family the maximum LOD score at the *TTN* locus was either the maximum LOD score achieved in that family across the whole exome or comparable to the maximum observed LOD score at any other locus. We interpret these data as replication of *TTN* truncating variants as frequently linked with DCM.

Despite the previous evidence1 and our findings presented here, all which collectively support the concept that *TTN* truncating variants are highly relevant for DCM pathogenesis, determining the pathogenicity of any specific variant remains extremely challenging. We interpret the 2 truncating variants not shared by all affected family members in two families (Families 14 and 17) as unlikely to be causative of DCM. We also note that in the 7 families where all those affected with DCM carried a truncating variant, some unaffected members at older ages also carried the same truncating variant. This observation confounds pedigree analysis even though it is consistent with reduced penetrance, which is commonly observed with familial DCM. Further, the plethora of *TTN* missense variants observed in all individuals, whether from control or DCM cohorts, further complicates *TTN* variant interpretation. The available evidence from the ESP data has shown that most individuals will carry numerous *TTN* missense variants,
some even very rare, and even if such variants segregate with DCM in a family, this may occur as a play of chance. This concept may also apply to truncating variants.

These issues raise two central questions of \textit{TTN} biology in DCM. Which specific variants, whether truncating or missense, play a role in DCM pathogenesis? Do \textit{TTN} variants include causative as well as risk alleles? Titin splicing and titin biology are exceedingly complex,\textsuperscript{23, 24} and penetrance is well known to be incomplete and expressivity variable in familial DCM,\textsuperscript{4, 5} so it is possible if not likely that some \textit{TTN} variants, whether missense or truncating, may also modulate penetrance and expressivity in DCM. Addressing these questions will require much larger DCM cohorts with detailed phenotypic data, ideally with knowledge of extended family structure (including presymptomatic DCM), genome-wide sequence data, and comprehensive, insightful analysis of the pathophysiological effects of \textit{TTN} variants.

Though linkage analysis has been less frequently utilized in the GWAS era, our study highlights the importance of coupling linkage information with sequence data. This provides us both a measure of evidence for a cumulative effect of rare variants, since linkage is not compromised by allelic heterogeneity (i.e., multiple rare disease variants within a gene), and an assessment of the evidence in the context of the rest of the genome. Together, linkage analysis and sequencing provide complementary evidence that can improve the efficiency of gene discovery in sequencing studies.

The observation that 7 of 17, or 41%, of families with \textit{TTN} truncating variants that segregated with DCM is higher than the 25% frequency observed in families with DCM the Herman et al study.\textsuperscript{1} This most likely resulted from a sample bias in our study because our families were already known to be point mutation negative for 16 other known DCM genes, and thus were likely enriched for \textit{TTN} variants.
We also examined TTN missense variants in our seventeen families using an unbiased approach to exome analysis and additional data from >300 exomes with neurological phenotypes collated in our in-house database, GEMapp. Our calculation of nucleotide diversity in the Exome Sequencing Project dataset for this gene suggested that diversity at the TTN locus was comparable to other known DCM genes relative to the number of coding nucleotides. However, the >300 TTN exons resulted in a very large number of missense variants identified in both the exome sequence from individuals with DCM and individuals in the ESP dataset. Two TTN missense variants from two DCM families, both also carrying TTN truncating variants, passed our exome filtering criteria. We considered these two missense variants (Gly29127Arg and Ile2685Val) of unknown significance, as each met our stringent exome filtering criteria and were not present in over 300 other exomes in GEMapp but occurred in Families B and D that also had TTN truncating variants.

We also note that the most highly linked region in this study on chromosome 9q21.1-q22.31 did not contain any known DCM genes and that none of our filtered genes mapped to this region. The positive linkage at this region could be a chance finding. Alternatively, this could represent a region of the genome containing a novel DCM gene missed by our exome pipeline for two possible reasons. Our rare variant exome analysis approach is based on assumptions, based on our previous work.\(^1\)\(^8\) Firstly, we assumed that causative rare variants were missense, nonsense, splice or frameshift and the allele frequency of these variants would be <0.5%. We note here that the majority of known (published) DCM variants are significantly less frequent than 0.5% in the general population\(^1\)\(^8\) but there are examples of known DCM variants with convincing functional data where the variant frequency is very close to this cut-point (CSRP3 Trp4Arg variant\(^2\)\(^5\) has a frequency of 0.35% in European ancestry EVS dataset). Whilst this
variant would have been identified in our pipeline, it is possible that other pathogenic variants
have frequencies slightly greater than this. Secondly, our genome-wide linkage approach could
also have identified regions containing common susceptibility or modifying variants, again that
would not have been detected by our exome analysis approach. We note recent prior evidence of
linkage to congenital heart defects and low atrial rhythm to this region, suggesting the
possibility of cardiovascular modifying variants located here.

In conclusion, our data show that TTN was the only gene with implicated rare variants
that occurred in multiple DCM families, and hence, have replicated the prior finding\(^1\) that TTN
truncating variants do contribute frequently to DCM pathogenesis. We reiterate that TTN
analysis for DCM causation should be considered within the context of the genome. While
interpreting individual TTN truncating or missense variants will remain challenging due to the
complexity of TTN biology, the availability of sequencing data from known DCM genes and
variants at other exomic loci will assist in categorizing the pathogenicity of these variants.

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Conflict of Interest Disclosures: None.
References:


**Table 1:** Genome-wide linkage and rare variant exome sequence analysis summary by family.

<table>
<thead>
<tr>
<th>Pedigrees with segregating <em>TTN</em> truncating variants</th>
<th>Family</th>
<th>mlod</th>
<th>hg19 region</th>
<th><em>TTN</em> mlod</th>
<th>number filtered exome variants (TTN variants)</th>
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<td>Other pedigrees without segregating <em>TTN</em> truncating variants</td>
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<tr>
<td>Family 9</td>
<td>0.474</td>
<td>chr5:137754695-137426447</td>
<td>N/A</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Family 10</td>
<td>0.601</td>
<td>chr11:62863518-64597506</td>
<td>-N/A</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Family 11</td>
<td>0.301</td>
<td>N/A*</td>
<td>0.296</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Family 12</td>
<td>0.602</td>
<td>chr22:29456733-35660875</td>
<td>N/A</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Family 13</td>
<td>0.301</td>
<td>N/A*</td>
<td>-0.347</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Family 14</td>
<td>0.301</td>
<td>N/A*</td>
<td>-0.305</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Family 15</td>
<td>1.242</td>
<td>chr10:99504630-100219374</td>
<td>N/A</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Family 16</td>
<td>0.301</td>
<td>N/A*</td>
<td>-1.238</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Family 17</td>
<td>0.301</td>
<td>N/A*</td>
<td>-0.66</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

mlod, multipoint lod score across genome; *TTN* mlod, multipoint lod score at *TTN* locus; number filtered exome variants refers to the number top ranking variants from exome pipeline with annotation of all top ranking truncating and missense variants in *TTN*; N/A, not applicable because SNV’s at *TTN* locus were uninformative for linkage. N/A* is not applicable, in that more than one region had the same mlod score. Pedigrees A-G and 8-14 had three and pedigrees 15-17 had two affected family members respectively, who underwent exome sequencing.
Table 2: Top five positive linkage regions in seventeen DCM families

<table>
<thead>
<tr>
<th>chr</th>
<th>hg19 start</th>
<th>hg19 end</th>
<th>maximum HLOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>71,862,987</td>
<td>95,840,256</td>
<td>1.743</td>
</tr>
<tr>
<td>2</td>
<td>174,128,513</td>
<td>215,820,013</td>
<td>1.588</td>
</tr>
<tr>
<td>6</td>
<td>311,938</td>
<td>6,318,795</td>
<td>1.304</td>
</tr>
<tr>
<td>3</td>
<td>336,508</td>
<td>16,268,974</td>
<td>1.11</td>
</tr>
<tr>
<td>5</td>
<td>94,994,339</td>
<td>138,456,815</td>
<td>1.072</td>
</tr>
</tbody>
</table>

HLOD, heterogeneity LOD score. \textit{TNN} locus in bold.
Table 3: TTN filtered variants identified in exome analysis and validated with Sanger sequencing that segregated in families with DCM.

<table>
<thead>
<tr>
<th>hg19 position</th>
<th>Family ID</th>
<th>Variant function class</th>
<th>Phast Cons</th>
<th>GERP</th>
<th>Grantham</th>
<th>additional filtered variants in family</th>
<th>MAF % (EA)</th>
<th>MAF % (AA)</th>
<th>cDNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr2:179481235</td>
<td>A: F128</td>
<td>stop-gained</td>
<td>1.00</td>
<td>3.90</td>
<td>N/A</td>
<td>0 (0)</td>
<td>0</td>
<td>0.03</td>
<td>c.40579C&gt;T</td>
<td>Arg13527stop</td>
</tr>
<tr>
<td>chr2:179447693</td>
<td>B: F533</td>
<td>stop-gained</td>
<td>1.00</td>
<td>5.02</td>
<td>N/A</td>
<td>4 (2)</td>
<td>0</td>
<td>0</td>
<td>c.58133C&gt;G</td>
<td>Ser19378stop</td>
</tr>
<tr>
<td>chr2:179424036</td>
<td>C: F27</td>
<td>splice-5</td>
<td>0.99</td>
<td>5.61</td>
<td>N/A</td>
<td>11 (8)</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>IVS 275+2 T&gt;A</td>
</tr>
<tr>
<td>chr2:179424036</td>
<td>D: F40</td>
<td>splice-5</td>
<td>0.99</td>
<td>5.61</td>
<td>N/A</td>
<td>32 (17)</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>IVS 275+2 T&gt;A</td>
</tr>
<tr>
<td>chr2:179413874</td>
<td>E: F2B</td>
<td>frameshift</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>27 (14)</td>
<td>0</td>
<td>0</td>
<td>c.84774insT</td>
<td>Val28259SerfsX22</td>
</tr>
<tr>
<td>chr2:179411904</td>
<td>F: F19</td>
<td>frameshift</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>23 (18)</td>
<td>0</td>
<td>0</td>
<td>c.86640delAGAA</td>
<td>Lys28880AsnfsX8</td>
</tr>
<tr>
<td>chr2:179400115</td>
<td>G: F35</td>
<td>stop-gained</td>
<td>1.00</td>
<td>4.64</td>
<td>N/A</td>
<td>23 (10)</td>
<td>0</td>
<td>0</td>
<td>c.93523C&gt;T</td>
<td>Arg31175stop</td>
</tr>
</tbody>
</table>

Conservation scores are given as PhastCons and GERP. MAF %, % minor allele frequency from 5,379 exomes in Exome Variant Server (EVS), given in European (EA) and African American (AA) populations. The number of additional filtered variants in other genes identified in each family with a TTN variant are given, and in parentheses, number of variants under more stringent filtering criteria of <0.05% in the EVS.
Table 4: Clinical Characteristics

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age at diagnosis, screening or family history information, years</th>
<th>Gender</th>
<th>DCM</th>
<th>ECG/Arrhythmia</th>
<th>LVEDD, mm (Z-score)</th>
<th>LV septum, posterior wall thickness, mm</th>
<th>EF or FS, %</th>
<th>TTN truncating mutation present (yes, no)</th>
<th>Other mutation? (genotype)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pedigree A: Arg13527X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV-2</td>
<td>42</td>
<td>F</td>
<td>yes</td>
<td>PVCs</td>
<td>56, NA</td>
<td>NA</td>
<td>20</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-1</td>
<td>43</td>
<td>F</td>
<td>yes</td>
<td></td>
<td>54 (2.44)</td>
<td>NA, 11</td>
<td>40</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-3</td>
<td>69</td>
<td>F</td>
<td>no</td>
<td>anterior MI</td>
<td>54, NA</td>
<td>11, 10</td>
<td>42</td>
<td>yes</td>
<td>CAD diagnosis at age 69</td>
<td></td>
</tr>
<tr>
<td>III-5</td>
<td>51</td>
<td>M</td>
<td>yes</td>
<td>PVCs, AF</td>
<td>67 (4.04)</td>
<td>NA</td>
<td>35</td>
<td>yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV-3</td>
<td>27</td>
<td>F</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>yes</td>
<td>history: paroxysmal atrial tachycardia and syncope</td>
<td></td>
</tr>
<tr>
<td>V-1</td>
<td>23</td>
<td>F</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>yes</td>
<td>records documenting normal echocardiogram</td>
<td></td>
</tr>
</tbody>
</table>

Pedigree B: Ser19378X

| III-3   | 29                                                          | M      | yes | PVCs            | 56 (1.54)           | 10, 10                                | 47         | yes                                      | TTN Ile2640Val             |         |
| II-2    | NA                                                          | F      | no  |                 |                     |                                        |            | oblige carrier of TTN Ile2640Val          | history: died at 76 from cancer |         |
| II-3    | 66                                                          | M      | no  |                 |                     |                                        |            | no                                       | history: normal cardiac screening |         |
| II-5    | 70                                                          | F      |     |                 |                     |                                        |            | oblige carrier of TTN Ile2640Val          | history: heart attack at age 50-60. |         |
| II-6    | 64                                                          | M      | no  |                 | 56 (1.42)           | 9, 8                                  | 49.5       | yes                                      | TTN Ile2640Val             | Borderline systolic dysfunction |
| II-7    | 62                                                          | F      | no  |                 | 42 (-1.15)          | 10, 10                                | 39 (FS)    | no                                       |                             |         |
| III-1   | 43                                                          | M      | no  | ICD             |                     |                                        |            | yes                                      | TTN Ile2640Val             | CAD diagnosis at age 43      |
| III-2   | 47                                                          | F      | yes | PVCs, bigeminy  | 64 (5.21)           | 8, 6.8                                | 21         | yes                                      | TTN Ile2640Val             |         |
| III-4   | 36                                                          | M      | yes | ICD, PVCs       | 64 (3.43)           | 9, 10                                 | 24.5       | yes                                      | TTN Ile2640Val             |         |
### Pedigree C: IVS 275+2 T>A

<table>
<thead>
<tr>
<th>Member</th>
<th>Age</th>
<th>Sex</th>
<th>Maternal Status</th>
<th>Clinical Findings</th>
<th>Height</th>
<th>Weight</th>
<th>Years</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-1</td>
<td>37</td>
<td>F</td>
<td>yes</td>
<td>NSSTT</td>
<td>68 (5.6)</td>
<td>NA</td>
<td>40</td>
<td>yes</td>
</tr>
<tr>
<td>I-2</td>
<td>NA</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-2</td>
<td>52</td>
<td>F</td>
<td>yes</td>
<td>NSSTT</td>
<td>57 (3.29)</td>
<td>11, 11</td>
<td>22</td>
<td>yes</td>
</tr>
<tr>
<td>II-3</td>
<td>59</td>
<td>M</td>
<td>no</td>
<td>PVCs, LAE, NSSST</td>
<td>73 (4.69)</td>
<td>10, 10</td>
<td>32</td>
<td>yes</td>
</tr>
<tr>
<td>III-2</td>
<td>39</td>
<td>M</td>
<td>yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Right deltoid muscle biopsy due to history of muscle weakness revealed congenital type I fiber predominance or chronic neurogenic atrophy with reinnervation.**
- **Obligate carrier history: died at 65 from cancer.**

### Pedigree D: IVS 275+2 T>A

<table>
<thead>
<tr>
<th>Member</th>
<th>Age</th>
<th>Sex</th>
<th>Maternal Status</th>
<th>Clinical Findings</th>
<th>Height</th>
<th>Weight</th>
<th>Years</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-4</td>
<td>39</td>
<td>M</td>
<td>yes</td>
<td>AF, cardioversion</td>
<td>66 (3.58)</td>
<td>11, 11</td>
<td>39</td>
<td>TTN Gly29127Arg</td>
</tr>
<tr>
<td>II-3</td>
<td>49</td>
<td>F</td>
<td>yes</td>
<td>PVCs, PACs, MI</td>
<td>58 (3.56)</td>
<td>10, 10</td>
<td>25</td>
<td>TTN Gly29127Arg</td>
</tr>
<tr>
<td>II-5</td>
<td>46</td>
<td>F</td>
<td>yes</td>
<td>NSSTT</td>
<td>53 (2.26)</td>
<td>8, 8</td>
<td>46</td>
<td>TTN Gly29127Arg</td>
</tr>
<tr>
<td>III-1</td>
<td>32</td>
<td>M</td>
<td>no</td>
<td>RBBB, MI</td>
<td>51 (-0.07)</td>
<td>8, 8</td>
<td>41</td>
<td>yes</td>
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</table>

### Pedigree E: Val28259SerfsX22

<table>
<thead>
<tr>
<th>Member</th>
<th>Age</th>
<th>Sex</th>
<th>Maternal Status</th>
<th>Clinical Findings</th>
<th>Height</th>
<th>Weight</th>
<th>Years</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-3</td>
<td>14</td>
<td>M</td>
<td>yes</td>
<td></td>
<td>75 (6.30)</td>
<td>5, 8</td>
<td>13 (FS)</td>
<td>yes</td>
</tr>
<tr>
<td>I-1</td>
<td>44</td>
<td>M</td>
<td>yes</td>
<td>LAHB, MI, NSSTT</td>
<td>62 (3.06)</td>
<td>10, 9</td>
<td>25</td>
<td>MYBPC3 Ala833Thr</td>
</tr>
<tr>
<td>I-2</td>
<td>42</td>
<td>F</td>
<td>no</td>
<td>normal</td>
<td>55 (2.32)</td>
<td>8, 9</td>
<td>60</td>
<td>no</td>
</tr>
<tr>
<td>II-1</td>
<td>20</td>
<td>M</td>
<td>no</td>
<td>normal</td>
<td>60 (2.28)</td>
<td>10, 9</td>
<td>60</td>
<td>yes</td>
</tr>
<tr>
<td>II-2</td>
<td>22</td>
<td>M</td>
<td>yes</td>
<td>AF, ICD</td>
<td>63.5 (3.01)</td>
<td>10, 7</td>
<td>20</td>
<td>MYBPC3 Ala833Thr</td>
</tr>
</tbody>
</table>

- **Heart transplantation.**
### Pedigree F: Lys28880AsnfsX8

<table>
<thead>
<tr>
<th>III-1</th>
<th>16</th>
<th>M</th>
<th>yes</th>
<th>AF, NSSTT</th>
<th>52 (0.28)</th>
<th>12, 9</th>
<th>21</th>
<th>yes</th>
<th>cardiomegaly (CXR); heart transplantation. Died post transplant, age 21.</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1</td>
<td>47</td>
<td>M</td>
<td>no</td>
<td>normal</td>
<td>50 (0.28)</td>
<td>12, 9</td>
<td>21</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>II-2</td>
<td>45</td>
<td>F</td>
<td>yes</td>
<td>NSSTT</td>
<td>51 (1.78)</td>
<td>NA</td>
<td>48</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>II-3</td>
<td>52</td>
<td>M</td>
<td>yes</td>
<td>AF, NSCD, cardioversion</td>
<td>50 (NA)</td>
<td>8, 9</td>
<td>35</td>
<td>yes</td>
<td>Death from non-cardiovascular cause</td>
</tr>
<tr>
<td>II-5</td>
<td>52</td>
<td>M</td>
<td>no</td>
<td>1AVB</td>
<td>50 (-0.22)</td>
<td>9, 10</td>
<td>77</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>II-6</td>
<td>41</td>
<td>F</td>
<td>no</td>
<td>MI, NSSTT</td>
<td>55 (2.97)</td>
<td>6, 8</td>
<td>68</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>III-2</td>
<td>20</td>
<td>M</td>
<td>yes</td>
<td>ICD, tachycardia</td>
<td>60 (2.55)</td>
<td>6, 6</td>
<td>38</td>
<td>yes</td>
<td>heart transplantation</td>
</tr>
<tr>
<td>III-3</td>
<td>25</td>
<td>F</td>
<td>no</td>
<td>normal</td>
<td>51 (0.53)</td>
<td>7, 7</td>
<td>47</td>
<td>yes</td>
<td></td>
</tr>
</tbody>
</table>

### Pedigree G: Arg31175X

<table>
<thead>
<tr>
<th>II-1</th>
<th>52</th>
<th>M</th>
<th>yes</th>
<th>56 (NA)</th>
<th>NA</th>
<th>10</th>
<th>yes</th>
<th>obligate carrier</th>
<th>By history: no known heart problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-2</td>
<td></td>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-4</td>
<td>49</td>
<td>M</td>
<td>yes</td>
<td>LAE, tachycardia, NSSTT</td>
<td>70 (5.04)</td>
<td>12,</td>
<td>FS</td>
<td>17</td>
<td>yes</td>
</tr>
<tr>
<td>III-1</td>
<td>35</td>
<td>M</td>
<td>yes</td>
<td>LAE, tachycardia, NSSTT</td>
<td>69 (5.21)</td>
<td>10,</td>
<td>9</td>
<td>20</td>
<td>yes</td>
</tr>
</tbody>
</table>

1AVB, first degree atrioventricular block; AF, atrial fibrillation; DCM, dilated cardiomyopathy; ECG, electrocardiogram; EF, ejection fraction; FS, fractional shortening; ICD, implantable cardiac defibrillator; LAE, left atrial enlargement; LAHB, left anterior hemiblock; LV, left ventricle; LVEDD, left ventricular end-diastolic dimension; MI, myocardial infarction pattern; NA, not available; NSCD, non-specific conduction delay; NSR, normal sinus rhythm; NSSTT, non-specific ST-T changes; PAC’s, premature atrial contractions; PVC’s, premature ventricular contractions; RBBB, right bundle branch block.
Figure Legend:

**Figure 1.** Pedigrees of families with DCM. Squares represent males, circles females. Diagonal lines mark deceased individuals. Solid symbols denote DCM. Gray symbols represent any cardiovascular abnormality. Open symbols represent unaffected individuals or individuals with no data available for analysis. The presence or absence of the family’s *TTN* truncating variant is indicated by a + or - symbol, respectively; obligate carriers are noted in parenthesis, (+), unknown zygosity, (?). Individuals who underwent exome sequencing are denoted (exome).
Exome Sequencing and Genome-Wide Linkage Analysis in 17 Families Illustrates the Complex Contribution of TTN Truncating Variants to Dilated Cardiomyopathy

Nadine Norton, Duanxiang Li, Evadnie Rampersaud, Ana Morales, Eden R. Martin, Stephan Zuchner, Shengru Guo, Michael Gonzalez, Dale J. Hedges, Peggy D. Robertson, Niklas Krumm, Deborah A. Nickerson and Ray E. Hershberger

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Norton et al, Circulation Cardiovascular Genetics 2013: Exome sequencing and genome-wide linkage analysis in 17 families illustrates the complex contribution of TTN truncating variants to dilated cardiomyopathy.

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ESP Groups

1Anthropometry Project Team, 2Blood Count/Hematology Project Team, 3Blood Pressure Project Team, 4Data Flow Working Group, 5Early MI Project Team, 6ELSI Working Group, 7Executive Committee, 8Family Study Project Team, 9Lipids Project Team.
Lung Project Team, Personal Genomics Project Team, Phenotype and Harmonization Working Group, Population Genetics and Statistical Analysis Working Group, Publications and Presentations Working Group, Quantitative Analysis Ad Hoc Task Group, Sequencing and Genotyping Working Group, Steering Committee, Stroke Project Team, Structural Variation Working Group, Subclinical/Quantitative Project Team

ESP Cohorts

Acute Lung Injury (ALI), Atherosclerosis Risk in Communities (ARIC), Cardiovascular Health Study (CHS), Chronic Obstructive Pulmonary Disease (COPDGene), Coronary Artery Risk Development in Young Adults (CARDIA), Cystic Fibrosis (CF), Early Pseudomonas Infection Control (EPIC), Framingham Heart Study (FHS), Jackson Heart Study (JHS), Lung Health Study (LHS), Multi-Ethnic Study of Atherosclerosis (MESA), Pulmonary Arterial Hypertension (PAH), Severe Asthma Research Program (SARP), Women’s Health Initiative (WHI)