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

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Background—Familial dilated cardiomyopathy (DCM) 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 DCM cases.

Methods and Results—We used an unbiased genome-wide approach using 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, multipoint logarithm of odds, 1.59. We identified 6 *TTN* truncating variants carried by individuals affected with DCM in 7 of 17 DCM families (logarithm of odds, 2.99); 2 of these 7 families also had novel missense variants that 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 with 5 other known DCM genes. The average number of missense variants in the exome sequences from the DCM cases or the ≈5400 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 logarithm of odds 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. **(*Circ Cardiovasc Genet*. 2013;6:144-153.)**

Key Words: dilated cardiomyopathy ■ exome ■ genetics ■ genome-wide analysis ■ human

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 >30 disease genes, including *TTN*, previously indicated to cause DCM.[2,3] Before 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]

Clinical Perspective on p 153

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 with 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 >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 1 rare (defined as a mean allele frequency <0.5%) missense or truncating variant

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at this locus. Next-generation sequencing now allows rapid variation analysis of the *TTN* gene despite its size. However, it relies on an economy of scale, and at a cost similar to that of sequencing *TTN* alone, next-generation sequencing 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 next-generation sequencing 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 17 families, each with ≥3 members affected with DCM, in an effort to identify a genetic cause of DCM, because 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 17 families and performed linkage analysis across the genome. We hypothesized that if *TTN* variants were causative of 25% of DCM, we would observe a significant combined logarithm of odds [LOD] score across our 17 families at this locus compared with the rest of the genome. Second, we evaluated all other unbiased rare variations 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 5400 exome sequences available from the Exome Variant Server[12] to determine whether the large amount of variation within this gene is accounted for by size or whether 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 and Science University and the University of Miami approved the study. The investigation included 17 families, each with ≥3 members affected with DCM and with each proband already known to be point mutation negative for 16 known DCM genes.[6–11] Genomic DNA was extracted from whole blood according to a standard salting-out procedure, as previously reported.[6–11]

Linkage Analysis

Two-point and multipoint parametric linkage analyses were performed with the Merlin software[13] 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 heterogeneity LOD score resulting from a test of linkage in the presence of genetic heterogeneity was calculated. A genome-wide linkage map of common informative markers was constructed by identifying all SNVs present from the exome data that overlap with known SNVs in 60 unrelated Europeans from the International HapMap. SNVs with minor allele frequency <1% or mendelian errors within the HapMap were excluded. The remaining markers were then pruned with the PLINK software[14] using pairwise r^2<0.1 in sliding windows of 50 SNVs, moving in intervals of 5 SNVs. This resulted in a final exome-wide marker set of 4601 SNVs. 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=3499 individuals) or African American ancestry (n=1864 individuals).

Exome Sequencing and Analysis

Exome sequencing was performed at the University of Washington Genome Science Center across 17 families (48 individuals) with NimbleGen V2 in solution capture and Illumina HiSeq. Sequences were aligned with the Burrows-Wheeler Aligner,[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 4 unrelated individuals, 2 with DCM and 2 unaffected individuals, as previously published.[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: variants with read depth ≥5 and quality scores ≥40; variants that were missense, nonsense, splice site, or a coding insertion or deletion; variants shared across all affected members of a family; variants with a frequency <0.5% in 5400 exomes from the Exome Variant Server (EVS); variants that have either a Phastcons[16] score >0.4 or a Genomic Evolutionary Rate Profiling[17] score >2; and variants with expression in our heart transcriptome data set with Reads Per Kilobase per Million mapped reads >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.[18] We also excluded filtered variants that were present in all 48 exomes and variants occurring in >1 family that did not segregate with disease status in at least 1 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/).[19] A total of 200 non-DCM exomes and 48 DCM exomes were used. Singular value decomposition transformation was used to remove systematic bias, removing 8 components. The final singular value decomposition -ZRPKM signal was then smoothed, and the duplication/deletion break points were found using a threshold of ±1.5 singular value decomposition -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.[11] Primer sequences are shown in Table I in the online-only Data Supplement. 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, θ, as a measure of nucleotide diversity across the 5379 exomes available from the Exome Variant Server. θ was calculated as described by Cargill et al[20] for all coding sequence and separately for both missense and truncating variants.

Results

Exome-wide Linkage Analysis Across 17 Families With DCM

The maximum LOD score across the genome and the LOD score at the *TTN* locus are shown for each family in Table

Table 1. Genome-wide Linkage and Rare Variant Exome Sequence Analysis Summary by Family

Family	MLOD	hg19 Region	*TTN* MLOD	Filtered Exome Variants (*TTN* Variants), n
Pedigrees with segregating *TTN* truncating variants				
A	1.178	chr19:57802806-58058739	0.722	1 (*TTN* Arg13527stop)
B	0.899	chr11:1782594-5625847	0.725	6 (*TTN* Ser19378stop, Ile2686Val)
C	0.902	chr6:35477032-42666164	0.034	12 (*TTN* IVS 275+2 T>A)
D	0.602	chr18:6890434-7017322	0.444	34 (*TTN* IVS 275+2 T>A, Gly29127Arg)
E	0.301	N/A*	0.283	28 (*TTN* Val28259SerfsX22)
F	0.301	N/A*	0.236	24 (*TTN* Lys28880AsnfsX8)
G	0.601	chr11:290816-6891605	0.544	24 (*TTN* Arg31175stop)
Other pedigrees without segregating *TTN* truncating variants				
Family 8	0.901	chr10:62863518-64597506	−2.173	10
Family 9	0.474	chr5:137754695-137426447	N/A	16
Family 10	0.601	chr11:62863518-64597506	N/A	18
Family 11	0.301	N/A*	0.296	22
Family 12	0.602	chr22:29456733-35660875	N/A	29
Family 13	0.301	N/A*	−0.347	36
Family 14	0.301	N/A*	−0.305	43
Family 15	1.242	chr10:99504630-100219374	N/A	43
Family 16	0.301	N/A*	−1.238	51
Family 17	0.301	N/A*	−0.66	80

MLOD indicates multipoint logarithm of odds (LOD) score across genome; N/A, not applicable because single-nucleotide variants at *TTN* locus were uninformative for linkage; and *TTN* MLOD, multipoint LOD score at *TTN* locus, number filtered exome variants refers to the number of top-ranking variants from exome pipeline with annotation of all top-ranking truncating and missense variants in *TTN*. Pedigrees A–G and 8–14 had 3 and pedigrees 15–17 had 2 affected family members, who underwent exome sequencing.

*Not applicable in that >1 region had the same MLOD score.

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 17 families, the *TTN* locus was the second highest (heterogeneity LOD=1.59; Table 2).

Exome Sequence Analysis From 17 Families With DCM

Our criteria for putative DCM variants identified in the exome sequences were 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 to 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 2400 exomes from the ESP, and of those with functional data (and therefore presumed to be pathogenic variants of very low frequency), the median frequency in the ESP population was 0.04%.[18] When this maximum 0.04% frequency criterion was applied to the present exome analysis, the number of shared filtered variants per family ranged from 1 to 49 (average, 16.8; median, 15). Copy number variant analysis using exome data did not identify any shared rare variants (frequency <1% in the ESP data set) across these families.

A total of 6 *TTN* truncating variants (2 frameshift, 3 nonsense, and 1 splice variant that occurred in 2 DCM families) were identified among the filtered candidates in 7 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 when possible to assess segregation of the variant with disease; 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 Genome data (which are independent of the EVS data set). None of the 6 truncating variants were present in this data set.

Table 2. Top 5 Positive Linkage Regions in 17 Families With Dilated Cardiomyopathy

chr	hg19 Start	hg19 End	Maximum HLOD
9	71 862 987	95 840 256	1.743
2	174 128 513	215 820 013	1.588
6	311 938	6 318 795	1.304
3	336 508	16 268 974	1.11
5	94 994 339	138 456 815	1.072

HLOD indicates heterogeneity logarithm of odds score.
TTN locus is the second row of the table.

Table 3. *TTN* Filtered Variants Identified in Exome Analysis and Validated With Sanger Sequencing That Segregated in Families With Dilated Cardiomyopathy

Hg19 Position	Family Identification	Variant Function Class	PhastCons	GERP	Grantham	Additional Filtered Variants in Family	MAF % (EA)	MAF % (AA)	cDNA	Protein
Truncating										
chr2:179481235	A: F128	Stop-gained	1.00	3.90	N/A	0 (0)	0	0.03	c.40579C>T	Arg13527stop
chr2:179447693	B: F533	Stop-gained	1.00	5.02	N/A	4 (2)	0	0	c.58133C>G	Ser19378stop
chr2:179424036	C: F27	Splice-5	0.99	5.61	N/A	11 (8)	0	0	N/A	IVS 275+2 T>A
chr2:179424036	D: F40	Splice-5	0.99	5.61	N/A	32 (17)	0	0	N/A	IVS 275+2 T>A
chr2:179413874	E: F2B	Frameshift	N/A	N/A	N/A	27 (14)	0	0	c.84774insT	Val28259SerfsX22
chr2:179411904	F: F19	Frameshift	N/A	N/A	N/A	23 (18)	0	0	c.86640delAGAA	Lys28880AsnfsX8
chr2:179400115	G: F35	Stop-gained	1.00	4.64	N/A	23 (10)	0	0	c.93523C>T	Arg31175stop
Missense										
chr2:179635998	B: F533	Missense	0.98	2.44	29	4 (2)	0	0	c.8056A>G	Ile2686Val
chr2:179410975	D: F40	Missense	1.00	5.66	125	32 (17)	0	0.06	c.87379G>A	Gly29127Arg

MAF% indicates percent minor allele frequency from 5379 exomes in the Exome Variant Server (EVS), given in European American (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; the number of variants under more stringent filtering criteria of <0.05% in the EVS is given in parentheses. Conservation scores are given as PhastCons and GERP.

Clinical Characteristics Families With DCM and Segregating *TTN* Variants

The pedigree structures are shown (Figure) 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 3 additional members. Sanger sequencing showed that all 6 family members were heterozygous for the truncating variant. Subject III.3, a woman who carried the *TTN* variant, died at 69 years of age with mild systolic dysfunction (ejection fraction, 42%) but without left ventricular enlargement, having suffered a myocardial infarction in her 50s, 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 carriers in their 20s, had no evidence of DCM.

Family B

In addition to the 3 samples that had exome sequencing, family B had DNA samples available from 6 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 76 years of age without a cardiovascular history. Another female obligate carrier (II.5) had no cardiovascular history at 70 years of age. A male who carried the *TTN* variant at 69 years of age (II.6) had only borderline systolic dysfunction without left ventricular enlargement.

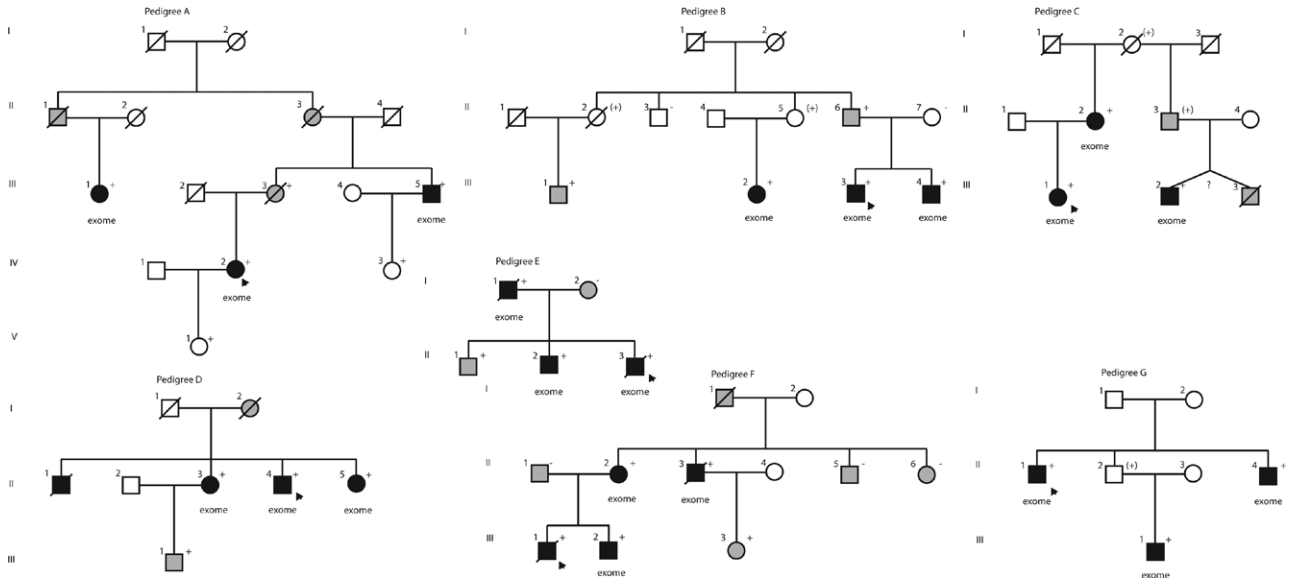

Figure. Pedigrees of families with dilated cardiomyopathy (DCM). Squares represent male subjects; circles represent female subjects. Diagonal lines mark deceased individuals. Solid symbols denote DCM. Gray symbols represent any cardiovascular abnormality. Open symbols represent unaffected individuals 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 as (+) and unknown zygosity as (?). Individuals who underwent exome sequencing are denoted as (exome).

Table 4. Clinical Characteristics

Subject	Age at Diagnosis, Screening or Family History Information, y	Sex	DCM	ECG/Arrhythmia	LVEDD, mm (*Z* score)	LV Septum, Posterior Wall Thickness, mm	EF or FS, %	TTN Truncating Mutation Present	Other Mutation? (Genotype)	Comment
Pedigree A: Arg13527X										
IV-2	42	F	Yes	PVCs	56, NA	NA	20	Yes		
III-1	43	F	Yes		54 (2.44)	NA, 11	40	Yes		
III-3	69	F	No	Anterior MI	54, NA	11, 10	42	Yes		CAD diagnosis at age 69
III-5	51	M	Yes	PVCs, AF	67 (4.04)	NA	35	Yes		
IV-3	27	F	No					Yes		history: paroxysmal atrial tachycardia and syncope
V-1	23	F	No					Yes		records documenting normal echocardiogram
Pedigree B: Ser19378X										
III-3	29	M	Yes	PVCs	56 (1.54)	10, 10	47	Yes	TTN Ile2640Val	
II-2	NA	F	No					Obligate carrier	obligate carrier of TTN Ile2640Val	history: died at 76 from cancer
II-3	66	M	No					No		history: normal cardiac screening
II-5	70	F						Obligate carrier	obligate 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										
III-1	37	F	Yes	NSSTT	68 (5.6)	NA	40	Yes		Right deltoid muscle biopsy attributable to history of muscle weakness revealed congenital type I fiber predominance or chronic neurogenic atrophy with reinnervation.
I-2	NA	F						Obligate carrier		History: died at 65 from cancer
II-2	52	F	Yes	NSSTT	57 (3.29)	11, 11	22	Yes		
II-3	59	M	No					Obligate carrier		Death certificate: VT, CAD
III-2	39	M	Yes	PVCs, LAE, NSSTT	73 (4.69)	10, 10	32	Yes		
Pedigree D: IVS 275+2 T>A										
II-4	39	M	Yes	AF, cardioversion	66 (3.58)	11, 11	39	Yes	TTN Gly29127Arg	
II-3	49	F	Yes	PVCs, PACs, MI	58 (3.56)	10, 10	25 (FS)	Yes	TTN Gly29127Arg	
II-5	46	F	Yes	NSSTT	53 (2.26)	8, 8	46	Yes	TTN Gly29127Arg	
III-1	32	M	No	RBBB, MI	51 (-0.07)	8, 8	41	Yes		

(*Continued*)

Table 4. (Continued)

Pedigree E: Val28259SerfsX22										
II-3	14	M	yes		75 (6.30)	5, 8	13 (FS)	yes		Heart transplantation
I-1	44	M	yes	LAHB, MI, NSSTT	62 (3.06)	10, 9	25	yes		
I-2	42	F	no	Normal	55 (2.32)	8, 9	60	no	MYBPC3 Ala833Thr	
II-1	20	M	no	Normal	60 (2.28)	10, 9	60	yes		
II-2	22	M	yes	AF, ICD	63.5 (3.01)	10, 7	20	yes	MYBPC3 Ala833Thr	Heart transplantation
Pedigree F: Lys28880AsnfsX8										
III-1	16	M	yes	AF, NSSTT			21	yes		Cardiomegaly (CXR); heart transplantation. Died post-transplant, age 21.
II-1	47	M	no	Normal	52 (0.28)	12, 9	83	no		
II-2	45	F	yes	NSSTT	51 (1.78)	NA	48	yes		
II-3	52	M	yes	AF, NSCD, cardioversion	50 (NA)	8, 9	35	yes		Death from noncardiovascular cause
II-5	52	M	no	1AVB	50 (-0.22)	9, 10	77	no		
II-6	41	F	no	MI, NSSTT	55 (2.97)	6, 8	68	no		
III-2	20	M	yes	ICD, tachycardia	60 (2.55)	8, 6	38	yes		Heart transplantation
III-3	25	F	no	Normal	51 (0.53)	7, 7	47	yes		
Pedigree G: Arg31175X										
II-1	52	M	yes		56 (NA)	NA	10	yes		
II-2		M						obligate carrier		By history: no known heart problems
II-4	49	M	yes	LAE, tachycardia, NSSTT	70 (5.04)	12, -	FS 17	yes		
III-1	35	M	yes	LAE, tachycardia, NSSTT	69 (5.21)	10, 9	20	yes		

1AVB indicates first-degree atrioventricular block; AF, atrial fibrillation; DCM, dilated cardiomyopathy; 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, nonspecific conduction delay; NSR, normal sinus rhythm; NSSTT, nonspecific ST-T changes; PAC, premature atrial contraction; PVC, premature ventricular contraction; and RBBB, right bundle-branch block.

Family C

Only 3 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 according to the death certificate died of ventricular tachycardia and coronary artery disease. None of the additional 12 variants passing filtering criteria (8 under more stringent filtering of population frequency <0.05% in the ESP exome data set) 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 1 variant 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 causing[9] but suggested to be of unknown significance on the basis of a subsequent study[22]; we also note that this variant is present at a frequency of 0.12% in the EVS, making it more common than many DCM rare variants.[18]

Family F

A 4-bp deletion in *TTN* resulted in a frameshift mutation. DNA was available from 1 additional affected member and 3 unaffected family members, and the *TTN* variant was confirmed to be present in all 4 affected members by Sanger sequencing and was not present in the 3 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* study.[1] Determination of pathogenicity of these variants will be extremely challenging because of the large number of coding exons. In the 5400 ESP exome data sets, the average number of *TTN* missense variants per individual was 23.3, ranging 6 to 55. These results were comparable 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 (range, 11–43). Application of our DCM filtering criteria to missense variants in the 5400 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 (range, 0–23). Five *TTN* missense variants passed our exome-filtering approach (that segregated with all individuals affected with DCM in a family): 1 each in 2 DCM families who also had segregating truncating variants (Table 3) and the others in 2 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 1 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.

Nonsegregating, Truncating Variants

Given the observed excess of *TTN* truncating variants in both familial and in sporadic DCM versus controls,[1] we thought it also relevant to report the number of nonsegregating truncating variants in *TTN* identified in the exome sequences of these 48 individuals with DCM, because they may be potential susceptibility variants. We observed 2 nonsegregating *TTN* truncating variants that were validated with Sanger sequencing. First, a C insertion at hg19 chr2:179,426,992, generating a frameshift in 1 of 3 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 2 of 3 family members (family 17; Table 1). Neither variant was observed in the 5400 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,[6] 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 6 *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 7 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 EVS contains annotated exome sequence from 5379 individuals at the *TTN* locus, totaling 2425 and 25 discrete missense and nonsense variants, respectively. Across 5379 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, θ,[20] accounting for sample size and the number of coding bases in the EVS individuals at the *TTN* locus to be 2.23×10^{-3} and 2.3×10^{-5} for missense and nonsense variants, respectively. The same calculation across 5 other known DCM genes (*MYBPC3*, *TNNC1*, *TNNI3*, *MYH6,* and *TPM1*) in the EVS data gave results comparable to those observed in *TTN* (for missense variants, θ

ranged from 6.9×10^{-4} at *TPM1* to 1.05×10^{-3} at *TNNC1* and for truncating variants, from 0 at *TNNC1* and *TNNI3* to 1.29×10^{-4} for *MYBPC3*), suggesting that the excess of shared truncating variants in our DCM families is not attributable to the large number of exons alone and that nucleotide diversity at *TTN* is comparable to that of 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[1] recently identified *TTN* truncating mutations in 25% of familial DCM and 18% of sporadic DCM, a significant excess compared with 3% of controls. The authors concluded that truncating mutations in *TTN* are a frequent cause of DCM because all previous reports of unselected patients with DCM of unknown cause ranged from <<1% to 5% to 8%.[4] However, 3% of controls in the Herman et al[1] study 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 19 DCM families, segregating rare *TTN* truncating variants in the Herman et al[1] study, 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 the relevance of the linkage evidence in the *TTN* region could not be compared with 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 because they should be enriched for causative variants at this locus, especially because 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 genes[6–11] (except for 1 family segregating a previously described variant[6] 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 with 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 SNVs within the exome sequences, we obtained a combined LOD score at the *TTN* locus for these 7 families of 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 evidence[1] and our findings presented here, all of 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 2 families (families 14 and 17) as unlikely to be causative of DCM. We also note that in the 7 families in whom 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, although it is consistent with reduced penetrance, which is commonly observed with familial DCM. Furthermore, 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, it may occur as a play of chance. This concept may also apply to truncating variants.

These issues raise 2 central questions of *TTN* biology in DCM. Which specific variants, whether truncating or missense, play a role in DCM pathogenesis? Do *TTN* variants include causative and risk alleles? Titin splicing and titin biology are exceedingly complex,[23,24] and penetrance is well known to be an incomplete and expressivity variable in familial DCM,[4,5] so it is possible if not likely that some *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 *TTN* variants.

Although linkage analysis has been used less frequently in the genome-wide association study 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, because linkage is not compromised by allelic heterogeneity (ie, 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.

We observed that 41% (7/17) of families carried *TTN* variants that segregated with DCM, which is higher than the 25% frequency observed in the Herman et al[1] study. 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 *TTN* variants.

We also examined *TTN* missense variants in our 17 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 ESP data set 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 data set. Two *TTN* missense variants from 2 DCM families, both also carrying *TTN* truncating variants, passed our exome-filtering criteria. We considered these 2 missense

variants (Gly29127Arg and Ile2685Val) of unknown significance because each met our stringent exome–filtering criteria and were not present in >300 other exomes in GEMapp but occurred in families B and D, who 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 2 possible reasons. Our rare variant exome analysis approach is based on assumptions based on our previous work.[18] First, we assumed that causative rare variants were missense, nonsense, splice, or frameshift and that 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,[18] but there are examples of known DCM variants with convincing functional data in which the variant frequency is very close to this cut point (*CSRP3* Trp4Arg variant[25] has a frequency of 0.35% in the European ancestry EVS data set). Although this variant would have been identified in our pipeline, it is possible that other pathogenic variants have frequencies slightly greater than this. Second, 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 evidence of linkage to congenital heart defects and low atrial rhythm to this region,[26] 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 previous finding[1] that *TTN* truncating variants contribute frequently to DCM pathogenesis. We reiterate that *TTN* analysis for DCM causation should be considered within the context of the genome. Whereas interpreting individual *TTN* truncating or missense variants will remain challenging because of 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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CLINICAL PERSPECTIVE

Rare variants that cause familial dilated cardiomyopathy (DCM) have been discovered in >30 genes but account for only 40% to 50% of cases. Exome sequencing, in which ≈19 000 coding genes in the human genome are sequenced simultaneously, provides a dramatically more powerful approach for DCM gene discovery. To this end, exome sequencing was undertaken in 3 or more affected members in 17 DCM families. Truncating rare variants in *TTN*, encoding the giant protein titin, were identified in 7 families and segregated with DCM and were not present in 5400 DNA exome sequences from the Exome Sequencing Project or the 1000 Genomes Project. A recent report suggested that rare *TTN* truncating variants cause up to 24% of familial dilated cardiomyopathy, but in that study, 3% of controls had *TTN* truncating variants, complicating assignment of pathogenicity to any specific *TTN* variant, and only *TTN* was sequenced (so in some cases mutations in other DCM genes that were not sequenced may have been relevant). The data from the present study strengthen the previous conclusion that *TTN* truncating variants are in general relevant for the pathogenesis of DCM: The genome-wide approach here excluded mutations in other known and novel DCM genes, and linkage analysis supported the pathogenicity of the *TTN* truncating variants in the 7 families. Nevertheless, 2 other *TTN* truncating variants did not segregate in 2 DCM families. Overall, our study suggests that *TTN* truncating variants contribute to the pathogenesis of DCM, but the lack of segregation of all identified *TTN* truncating variants illustrates the challenge of determining *TTN* variant pathogenicity even with exome sequencing.

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

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on behalf of the National Heart, Lung, and Blood Institute GO Exome Sequencing Project and the Exome Sequencing Project Family Studies Project Team

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NHLBI GO Exome Sequencing Project

BroadGO

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[10]Lung Project Team, [11]Personal Genomics Project Team, [12]Phenotype and Harmonization Working Group, [13]Population Genetics and Statistical Analysis Working Group, [14]Publications and Presentations Working Group, [15]Quantitative Analysis Ad Hoc Task Group, [16]Sequencing and Genotyping Working Group, [17]Steering Committee, [18]Stroke Project Team, [19]Structural Variation Working Group, [20]Subclinical/Quantitative Project Team

ESP Cohorts

[21]Acute Lung Injury (ALI), [22]Atherosclerosis Risk in Communities (ARIC), [23]Cardiovascular Health Study (CHS), [24]Chronic Obstructive Pulmonary Disease (COPDGene), [25]Coronary Artery Risk Development in Young Adults (CARDIA), [26]Cystic Fibrosis (CF), [27]Early Pseudomonas Infection Control (EPIC), [28]Framingham Heart Study (FHS), [29]Jackson Heart Study (JHS), [30]Lung Health Study (LHS), [31]Multi-Ethnic Study of Atherosclerosis (MESA), [32]Pulmonary Arterial Hypertension (PAH), [33]Severe Asthma Research Program (SARP), [34]Women's Health Initiative (WHI)