ORIGINAL RESEARCH

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Role of Titin Missense Variants in Dilated Cardiomyopathy

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Background—The titin gene (*TTN*) encodes the largest human protein, which plays a central role in sarcomere organization and passive myocyte stiffness. *TTN* truncating mutations cause dilated cardiomyopathy (DCM); however, the role of *TTN* missense variants in DCM has been difficult to elucidate because of the presence of background *TTN* variation.

Methods and Results—A cohort of 147 DCM index subjects underwent DNA sequencing for 313 *TTN* exons covering the N2B and N2BA cardiac isoforms of *TTN*. Of the 348 missense variants, we identified 44 "severe" rare variants by using a bioinformatic filtering process in 37 probands. Of these, 5 probands were double heterozygotes (additional variant in another DCM gene) and 7 were compound heterozygotes (2 *TTN* "severe" variants). Segregation analysis allowed the classification of the "severe" variants into 5 "likely" (cosegregating), 5 "unlikely" (noncosegregating), and 34 "possibly" (where family structure precluded segregation analysis) disease-causing variants. Patients with DCM carrying "likely" or "possibly" pathogenic *TTN* "severe" variants did not show a different outcome compared with "unlikely" and noncarriers of a "severe" *TTN* variant. However, the "likely" and "possibly" disease-causing variants were overrepresented in the C-zone of the A-band region of the sarcomere.

Conclusions—*TTN* missense variants are common and present a challenge for bioinformatic classification, especially when informative families are not available. Although DCM patients carrying bioinformatically "severe" *TTN* variants do not appear to have a worse clinical course than noncarriers, the nonrandom distribution of "likely" and "possibly" disease-causing variants suggests a potential biological role for some *TTN* missense variants. **(*J Am Heart Assoc*. 2015;4:e002645 doi: 10.1161/JAHA.115.002645)**

Key Words: cardiomyopathy • cardiovascular genetics • dilated cardiomyopathy • heart failure • missense variants

Familial dilated cardiomyopathy (DCM) is a genetic heart muscle disease caused by mutations in genes encoding structural proteins of the cytoskeleton and sarcomere.[1] Recent investigations have focused attention on the titin gene (*TTN*), which encodes the giant sarcomeric protein titin, the largest known human protein and key contributor to passive myocyte stiffness.[2] *TTN* mutations were first associated with tibial muscular dystrophy, but truncation mutations of *TTN* have recently been implicated as the most frequent identifiable cause of DCM.[3] Titin is composed of >33 000 amino acid residues, and >60 000 missense variants are reported in the 1000 Genomes Project.[3,4] This frequency is far above the expected frequency of disease-causing mutations for the *TTN* gene. Indeed, Golbus et al analyzed the data of the 1000 Genomes Project and found a high frequency of predicted pathogenic protein altering variation in *TTN*.[5] They suggested that many of these variants could be either benign or insufficient on their own to cause disease but could act as modifiers in genetically susceptible hosts. Additional evidence supporting a modifier role for *TTN* comes from Roncarati et al, who performed whole-exome sequencing in a large family with DCM, and found a subset of relatives with both *TTN* missense and Lamin A/C (*LMNA*) variants showing a more severe early-onset phenotype.[6] The high prevalence of missense variants in *TTN* and potential modifier roles complicate interpretation of detected *TTN* missense variants in both research and clinical settings.

To further address the role of *TTN* missense variants, we sequenced *TTN* in a large cohort of 147 DCM index patients and sorted the variants by using a rigorous bioinformatic

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Accompanying Figure S1 and Tables S1 through S3 are available at http://jaha.ahajournals.org/content/4/11/e002645/suppl/DC1

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pipeline followed by segregation analysis to identify *TTN* rare missense variants likely to be pathogenic. We further investigated if carriers of *TTN* putative disease-causing missense mutations display a distinct phenotype and survival compared with noncarriers.

Methods

Patient Population

Probands from 147 DCM families were selected from the International Familial Cardiomyopathy Registry, a multicenter, 3-decade–long ongoing project studying human hereditary cardiomyopathies. The diagnosis of DCM was created on the basis of the 1999 consensus criteria of the Guidelines for Familial Dilated Cardiomyopathy (FDC) and all available living subjects were evaluated by the investigators.[7] Clinical data collected included family history, physical examination, laboratory investigations, electrocardiogram, and echocardiogram. Medical records from deceased subjects were reviewed when available. Criteria for the diagnosis of DCM were the presence of left ventricular fractional shortening <25% and/or an ejection fraction <45%, and left ventricular end-diastolic diameter >117% of the predicted value provided by the Henry formula.[7] Exclusion criteria included any of the following conditions: blood pressure >160/110 mm Hg, obstruction >50% of a major coronary artery branch, alcohol intake >100 g/d, persistent high-rate supraventricular arrhythmia, systemic diseases, pericardial diseases, congenital heart diseases, cor pulmonale, and myocarditis.[7] Informed consent was obtained from living subjects, and local institutional review boards approved the protocol.

DNA Sequence Analysis

The University of Washington, Department of Genome Science, completed DNA resequencing of *TTN* in 2008 under National Heart, Lung, and Blood Institute (NHLBI) grant N01-HV-48194.[3,8] Exons and periexonic regions of titin isoform N2A (NM_133378) along with additional exons unique to the principal cardiac isoform N2B (NM_003319) were amplified from genomic DNA by using polymerase chain reaction (PCR). This covered 312 exons (311 expressed as titin protein) and the complete 3′-untranslated region. The PCR primers were Tm matched and designed from a masked reference sequence. Each primer pair was "tailed" with a universal M13 forward and reverse sequencing primer for subsequent sequencing. Once the regions were amplified, each PCR product was sequenced from the forward and reverse direction to provide double-stranded coverage. Sequencing was carried out with Sanger Big-Dye Terminator sequencing on capillary-based machines (AB 3730; Applied Biosystems).

Bioinformatic Filter Criteria

The single nucleotide polymorphisms (SNPs) identified from the sequence were annotated using by ANNOVAR[9] and dbNSFP,[10] the database for nonsynonymous SNP functional predictions, which provides scores from the widely used algorithms SIFT, PolyPhen2, LRT, Mutation Taster, and GERP. Variants were then scored as "severe" based on the following criteria (Table S1): SIFT score of zero,[11] Polyphen2 HDVAR "possibly damaging" or "damaging",[12] GERP score >4.2,[13] absence in the 1000 Genomes Project cohort,[14] and an allele frequency of ≤0.04% in the NHLBI Exome-Sequencing Project dataset (Figure S1 and Table S2).[15] Variants were excluded and considered "nonsevere" if they (1) failed to meet at least 1 of these criteria or (2) they were nonsynonymous or intronic mutations predicted to have no effect on splicing. In families where samples from >1 affected family member were available, segregation analysis was performed by using Sanger sequencing (Table S3). In accordance with the scheme proposed by Hershberger et al, variants that showed concordant or discordant segregation among affected and unaffected relatives were classified as "likely" or "unlikely" to be disease causing, respectively; variants where no segregation analysis was achievable were classified as "possibly" disease causing; and probands with no bioinformatically "severe" variants were classified as "noncarriers" (Figure 1).[16]

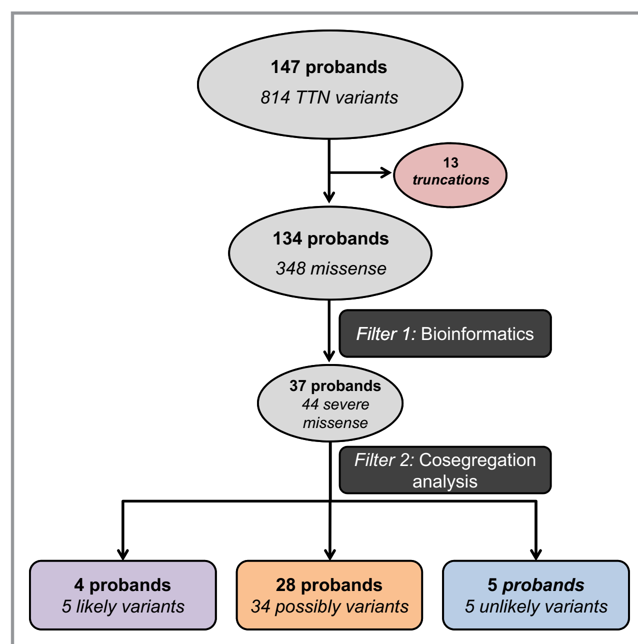


Figure 1. Bioinformatic and segregation analysis in 134 DCM Probands. *TTN* variants were filtered with bioinformatic algorithms as described and classified into "severe" or "nonsevere" categories. Mutations initially identified as "severe" were studied by segregation analysis when samples were available and further classified as "likely," "possibly," or "unlikely" to be disease causing. DCM indicates dilated cardiomyopathy; *TTN*, titin gene.

Statistical Analysis

To estimate the effect of *TTN* rare missense variants on the phenotype and natural history of DCM, we compared the clinical data and long-term death/heart transplantation event-free survival of the index DCM subjects among the different *TTN* variant groups and with the noncarriers. Summary statistics of clinical and instrumental variables at enrollment were expressed as median and IQR values or as counts and percentages, as appropriate. Comparison between rare variant carriers and noncarriers for continuous variables were analyzed by using ANOVA, with the Brown–Forsythe statistic when the assumption of equal variances did not hold, and the χ^2 or Fisher exact test was used for discrete variables. Event-free survival curves for death/heart transplantation were estimated and plotted by using the Kaplan–Meier method, and the log-rank test was applied to investigate differences in long-term survival. Statistical analyses were performed by using the IBM SPSS Statistical Package version 19.0.

Mapping the TTN Variants to Protein Domains

Severe variants in "likely" and "possibly" disease-causing categories were mapped to their respective protein domains starting from their exon location by using the Ensembl database (http://uswest.ensembl.org) with the identifier Q8WZ42-12 to locate the variants within the cDNA sequence, followed by mapping to amino acid position by referencing the Uniprot database (http://www.uniprot.org) to identify titin domains. Pearson's χ^2 goodness-of-fit tests were used to test the random distribution of the "likely" and "possibly" disease-causing variants, incorporating the size in amino acids of each protein region tested as one group and the remainder of titin protein as a second group (df=1).[17]

Results

Genetic Analysis of TTN Missense Variants

TTN was sequenced in 147 DCM probands. Truncating *TTN* variants were found in 13 of 147 and have previously been reported.[3] Among the remaining 134 probands, we found 814 rare variants (≤0.04% in NHLBI Exome-Sequencing Project dataset); of those, 466 variants were either synonymous or intronic and were not further studied. Of the remaining 348 missense variants, 44 were bioinformatically classified as "severe" and were present in 37 probands (Table 1). In 9 of 37 families, samples from other affected and unaffected family members were available to test for segregation analysis. Segregation of the rare *TTN* variants was confirmed by the presence in an affected or absence in an unaffected relative within the family. Among those 9 families, confirmation by Sanger sequencing revealed there were 5 *TTN* rare missense variants among 4 families.

Two variants (*TTN*-251996 and *TTN*-247572) in 2 different families (DNFDC014 and DNFDC131, respectively), 2 *TTN* variants (*TTN*-178217 and *TTN*-254772) in the same family TSFDC044, and 1 variant (*TTN*-172724) in family DNFDC074 segregated with the DCM phenotype and were classified as "likely" disease-causing (Figure 2 and Table 2). The remaining 5 tested variants failed to segregate with the DCM phenotype and were classified as "unlikely" disease causing. The 34 variants present in 28 families that could not be examined for segregation because of lack of additional samples for testing were classified as "possibly" disease causing.[18] Seven probands harbored compound heterozygous "severe" *TTN* variants. Among the 4 families with "likely" *TTN* variants, 1 also harbored an *LMNA* mutation (Table 2).

TTN Variant Association With Functional Domains

The "likely" and "possibly" disease-causing *TTN* variants were nonrandomly distributed across the titin protein with overrepresentation of variants found in the C-zone repeats of titin (P<0.01) (Figures 3 and 4) with a bias toward variants in the terminal 3 fibronectin type III (Fn-III) domains.

Genotype–Phenotype Analysis

No phenotypic differences were observed comparing the 32 probands of "likely" (n=4) or "possibly" (n=28) disease-causing *TTN* variants with the 102 noncarriers (97 "noncarriers" plus 5 probands with "severe" variants subsequently classified as "unlikely" by segregation analysis) (Table 3). Moreover, during a mean follow-up of 8 years (Figure 5), event free-survival from cardiovascular death/transplant was not different between the 2 groups. Survival rates free from cardiovascular death/heart transplant at 40, 50, and 60 years of age were 96%, 77%, and 29% versus 87%, 77%, and 48% in carriers and noncarriers, respectively (P=NS). Including data from the 13 patients with truncating *TTN* variants previously reported[3] did not identify any clinical or prognostic differences with respect to carriers of *TTN* "severe" missense or truncation variants compared with other DCM patients (Table 3 and Figure 5). No significant difference in clinical phenotype was noted between the group of subjects carrying a single *TTN* variant and the smaller groups carrying double or compound *TTN* variants. In addition, among the 5 "unlikely" families, the affected individuals who carried a specific *TTN* variant did not express a more severe phenotype than did affected individuals who did not carry the *TTN* variant, as measured by left ventricular ejection fraction, left ventricular end-diastolic diameter, and New York Heart Association

Table 1. *TTN* Rare Variants Classified as "Severe" by Bioinformatic Analyses

DCM Gene Mutation	Disease Associated	AA Change (NP_001254479)	Exon (per Bang, et al[17])	Domain (per Bang, et al[17])	Nucleotide Change (NM_001267550)	RS Number	Non-*TTN* Gene Mutation	LVEF%
Z-disk								
TTN	Possibly	Glu222Lys	5	Unique sequence	c.664 G>A	72647844	—	30
TTN	Possibly	Glu1039Gly	19	Unique sequence	c.3116 A>G	72647867	MYH6: Pro830Leu	49
I-Band								
*TTN**	Possibly	Ala2258Val	29	I2 Ig	c.6773 C>T	72647881	—	17[a]
*TTN**	Possibly	Ser7726Leu	81	I59 Ig	c.23177 C>T	17452588	—	22[b]
*TTN**	Possibly	Gly8886Arg	93	I71 Ig	c.26656 G>C	72648991	—	
TTN	Likely	Ser13702Pro	225	I84 Ig	c.41104 T>C	72650078	—	35
TTN	Possibly	Thr14183Ala	231	I89 Ig	c.42547 A>G	72650081	—	45
*TTN**	Likely	Arg14640Cys	237	I94 Ig	c.43918 C>T	72650088	—	43[c]
*TTN**	Possibly	Ile15254Phe	247	I101 Ig	c.45760A>T	72677226	—	25[f]
TTN	Possibly	Arg15484Met	250	I104 Ig	c. 46451 G>T	72677229	—	59
I-Band/A-Band Junction								
TTN	Possibly	Leu16469His	263	I114 FN3	c.49406 T>A	72677245	—	32
TTN	Possibly	Glu16938Gln	269	I118 FN3	c.50812 G>C	72677250	—	45
A-Band (D-Zone)								
TTN	Unlikely	Arg17086His	271	A2 FN3	c.51257 G>A	72632860	SCN5A: Arg222Gln	37
TTN	Possibly	Arg19705Cys	300	A28 FN3	c.59113 C>T	72646839	—	25
TTN†	Possibly	Ser20273Tyr	304	A34 FN3	c.60818 C>T	72646844	—	7[g]
	Possibly	Ser20273Tyr	304	A34 FN3	c.60818 C>T	72646844	—	10
A-Band (C-Zone)								
TTN	Possibly	Leu21176Ser	306	A43 Ig	c.63527 T>C	72646854	—	50
TTN	Possibly	Pro21563Ala	310	A47 FN3	c.64687 C>G	72646860	—	44
TTN	Unlikely	Arg22029His	314	A51 FN3	c.66086 G>A	72646868	—	40
TTN	Unlikely	Phe22653Leu	320	A58 FN3	c.67959 T>A	72646877	LMNA: Arg89Leu	NA
TTN	Possibly	Glu23217Gly	325	A63 FN3	c.69650 A>G	72646884	—	30
TTN	Possibly	Tyr23494His	326	A66 FN3	c.70480 T>C	72646888	—	30
*TTN**	Possibly	Ala24343Thr	326	A75 FN3	c.73027 G>A	72646895	—	17[a]
*TTN**	Possibly	Val24516Ile	326	A76 Ig	c.73546 G>A	72646897	—	25[d]
TTN	Possibly	Pro25207Arg	326	A84 FN3	c.75620 C>G	72646900	—	29
*TTN**	Possibly	Tyr27008Asp	326	A102 FN3	c.81022 T>G	72648211	—	55[e]
*TTN**	Possibly	Arg27563Cys	326	A107 FN3	c. 82687 C>T	72648214	—	55[e]
TTN	Possibly	Ser27585Tyr	326	A108 FN3	c.82754 C>A	72648215	—	15
TTN	Likely	Arg28118His	326	A113 FN3	c.84353 G>A	72648220	—	23
TTN	Likely	Ser29303Gly	329	A125 FN3	c.87907 A>G	72648231	LMNA: c.936 G>A	17
TTN	Possibly	Leu29499Arg	331	A127 Ig	c. 88496 T>G	72648234	—	25
*TTN**	Possibly	Gly29562Asp	332	A128 FN3	c.88685 G>A	72648235	—	25[f]
*TTN**	Likely	Glu29590Gln	332	A128 FN3	c.88768 G>C	72648236	—	43[c]
TTN	Possibly	Gly30358Glu	335	A136 FN3	c.91073 G>A	72648243	—	38
TTN	Possibly	Trp30667Arg	338	A139 FN3	c.91999 T>A	72648246	—	29
TTN†	Possibly	Ile31757Thr	343	A150 FN3	c.95270 T>C	72648259	—	25[d]

Continued

Table 1. Continued

DCM Gene Mutation	Disease Associated	AA Change (NP_001254479)	Exon	Domain	Nucleotide Change (NM_001267550)	RS Number	Non-*TTN* Gene Mutation	LVEF%
			per Bang, et al[17]					
	Possibly	Ile31757Thr	343	A150 FN3	c.95270 T>C	72648259	—	37
TTN	Possibly	Arg31856Gly	344	A151 FN3	c.95566 C>G	72648261	—	28
*TTN**	Possibly	Arg33052His	354	A163 FN3	c.99155 G>A	72648276	—	10g
C-Zone/M-Band Junction								
TTN	Unlikely	Gly33319Arg	356	A166 FN3	c.99955 G>A	72648279	—	28
M-Band								
TTN	Possibly	Arg33903Leu	358	Kinase	c.101708 G>T	72629782	—	50
TTN	Possibly	Lys34293Glu	358	M2 Ig	c.102877 A>G	72629783	—	15
TTN	Possibly	Ile34411Asn	358	M3 Ig	c.103232 T>A	72629784	—	25
TTN	Unlikely	Arg34653Leu	358	is2 between M3 and M4	c.103958 G>T	72629786	LMNA: Arg166Pro	25

Variants classified by bioinformatic analysis as "severe" are shown along with categorization as "likely," "possibly," or "unlikely" disease causing based on whether segregation with DCM was confirmed. DCM indicates dilated cardiomyopathy; LMNA, Lamin A/C; LVEF, left ventricular ejection fraction; NA, not available; *TTN*, titin gene.
*Compound heterozygous *TTN* variants: a, b, c, d, e, f, g.
†*TTN* variant shared by 2 nuclear families.

functional classifications (data not shown). These results would imply that there is no modifier effect within families.

Interestingly, there was a trend toward a lower left ventricular ejection fraction for variants located farther from the Z-disk: the mean left ventricular ejection fraction was 40% in Z-disk, 35% in I-band, and 29% in A-band variants. We also noted that "likely" and "possibly" carriers of *TTN* variants with double or compound heterozygous mutations (Table 1) had lower left ventricular ejection fractions (29±16%) compared with those with single missense variants (34±14%), although because of the small number of observations the difference did not reach statistical significance.

Discussion

The *TTN* gene, encoding the giant protein titin, harbors a large array of amino acid sequence variation, rendering genetic analysis for cardiomyopathy diagnosis challenging. Our bioinformatic filtering process identified variants bioinformatically classified as "severe" in 12.6% (44/348) of *TTN* missense variants and 27.6% (37/134) of DCM subjects. The variant analysis used stringent filtering criteria as currently recommended in research and clinical settings.[15,19] Despite the filtering process, 5 of 9 families with *TTN* variants classified as "severe" demonstrated incompatible segregation with the

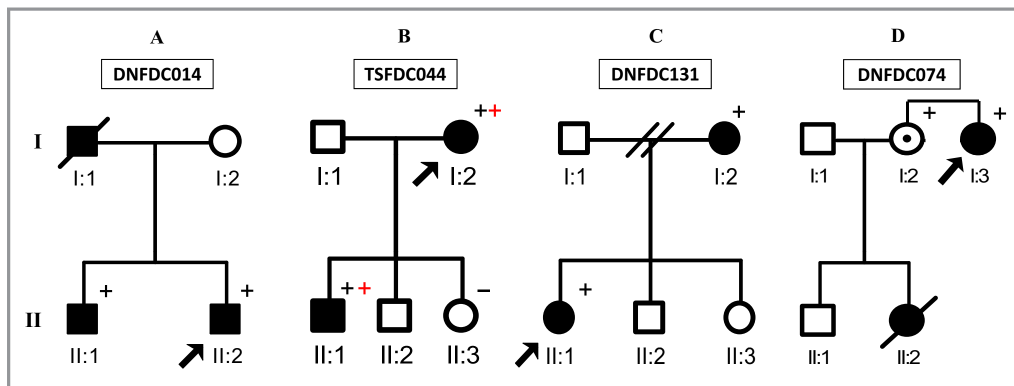


Figure 2. Pedigrees for "likely" *TTN* families harboring disease-causing pathogenic variants. Squares, circles, plus signs, and arrows indicate males, females, positive *TTN* variant status, and probands, respectively. An obligate carrier is indicated by a dotted circle. Black shading indicates individuals affected with DCM. Double plus symbols mean double heterozygous variants. DNFDC014 has variant rs72648231, TSFDC044 has variants rs72648236 and rs72650088, DNFDC131 has variant rs72648220, and DNFDC074 has variant rs72650078. DCM indicates dilated cardiomyopathy; *TTN*, titin gene.

Table 2. Clinical Characteristics of "Likely" *TTN* Rare Variant Carriers

Family	DNFDC014		DNFDC131		DNFDC074		TSFDC044	
TTN variant	Ser29303Gly		Arg28118His		Ser13702Pro		Arg14640Cys Glu29590Gln	
Chr. 2; Exon(s)	2; 329		2; 326		2; 225		2;	237 332
Individual	II-1	II-2	II-1	I-2	I:2*	I:3	I-2	II-1
Sex	M	M	F	F	F	F	F	M
NYHA	3	1.5	2	3	1	1	1	1
LVEDD, mm	50.3	53	64.2	50	45	58	60	56
LVEF, %	17	46	23	40	62	35	42	43
Non-*TTN* gene mutation	LMNA c.936 G>A	LMNA c.936 G>A						
Age of onset, y	60	40	49	39	54	48	42	26
Transplantation	Yes	No	No	No	No	No	No	No

LMNA indicates Lamin A/C; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association functional classification; *TTN*, titin gene.

*DNFDC074, individual I:2 is an obligate carrier.

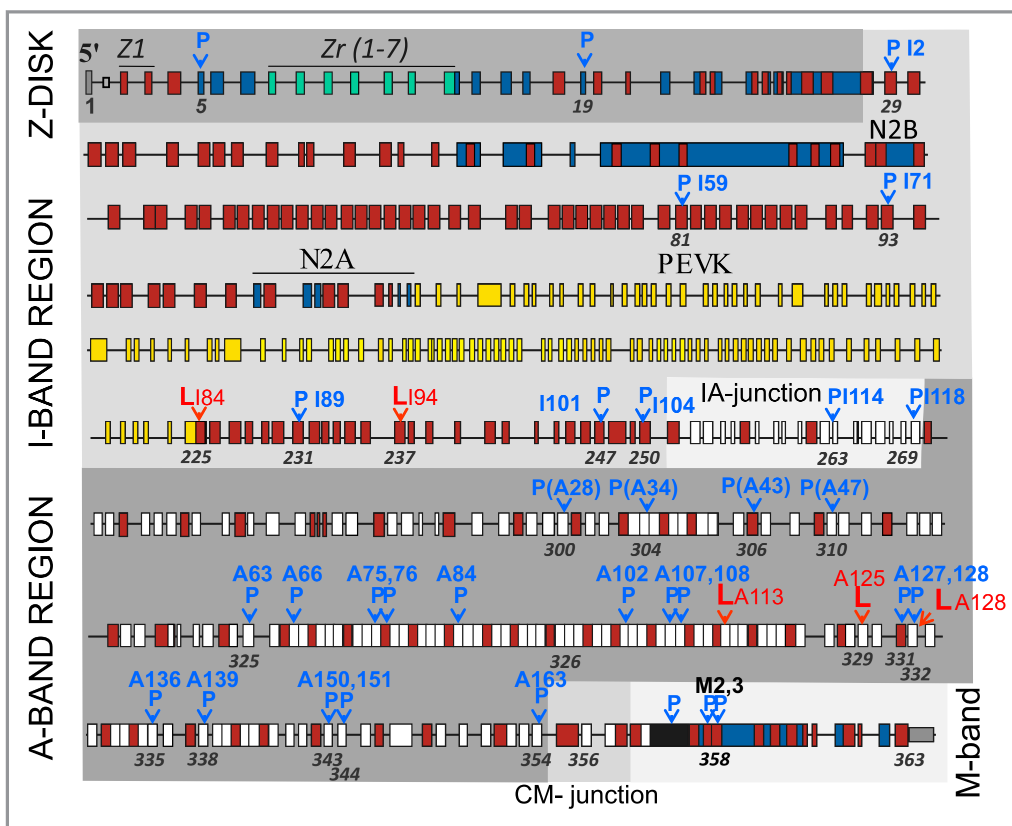
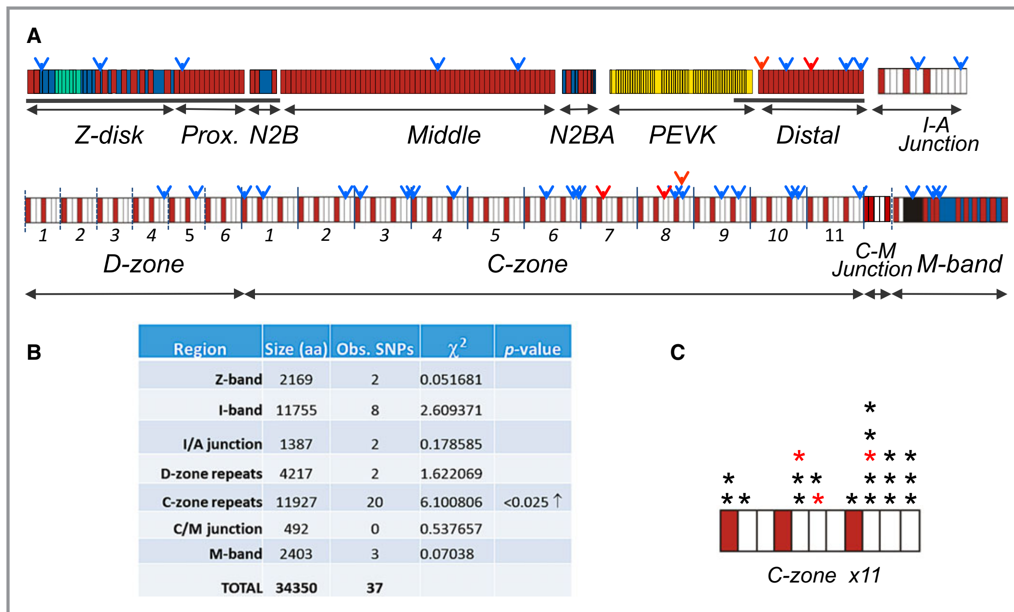


Figure 3. Exon structure of human *TTN* gene with identified DCM missense mutations. P indicates "possible" disease-causing mutation; L, "likely" disease-causing mutation. Indicated are also titin domain numbers (top) and exon numbers (bottom). Red rectangle represents immunoglobulin-like domain; white, fibronectin type 3 domain; blue, unique sequence; green, z-repeat domain; yellow, PEVK domain; black, titin kinase domain (gene structure based on Bang et al[17]). DCM indicates dilated cardiomyopathy; *TTN*, titin gene.



Region	Size (aa)	Obs. SNPs	χ^2	*p*-value
Z-band	2169	2	0.051681	
I-band	11755	8	2.609371	
I/A junction	1387	2	0.178585	
D-zone repeats	4217	2	1.622069	
C-zone repeats	11927	20	6.100806	<0.025 ↑
C/M junction	492	0	0.537657	
M-band	2403	3	0.07038	
TOTAL	34350	37		

Figure 4. Missense variants mapped on *TTN* gene. A, Protein domain structure of largest full-length titin isoform (UniProt entry Q8WZ42-12, which includes all exons except for Novex-3; this is the same as Ensembl transcript Ttn-018Ensembl) with indicated locations of "possibly" (blue arrowheads) and "likely" (red arrowheads) SNPs. Top: Z-disk and I-band region of titin with tandem immunoglobulin segments (proximal [prox.], middle and distal subsegments), N2B and N2BA elements, and PEVK segment of I-band indicated separately and I/A junction. Bottom: A-band and M-band regions of titin with D-zone (6 super-repeats each containing 7 domains) and C-zone (11 super-repeats with 11 domains each), C-M junction, and M-band regions indicated. I-band region of titin expressed in N2B cardiac titin (the dominant isoform in the LV) is shown by the gray underline (the A-band region is constitutively expressed in all full-length isoforms). B, Pearson's χ^2 goodness-of-fit tests were used to test if the distribution of the "likely" and "possibly" categorized nonsynonymous missense SNPs were random or not, incorporating the size in amino acids of each protein region tested as one group and the remainder of titin protein as a second group (df=1). Mutations in the C-zone repeats are overrepresented (indicated by direction of arrow). C, The SNPs in the C-zone are not randomly distributed over the 11 domains of the super-repeats but appear to be biased toward the last 3 Fn-III domains (the 11 super-repeats were aligned and the number of SNPs in each of the 11 domains summed). LV indicates left ventricle; SNPs, single nucleotide polymorphisms; TTN, titin gene.

affected phenotype, implying a significant false-positive rate from the bioinformatics analysis alone. Four families harbored 5 "severe" *TTN* variants that segregated with the DCM phenotype, and 28 probands had "severe" variants that could not be assessed by segregation. Thus, in contrast to *TTN* truncation variants that account for up to 25% of DCM,[3]

Table 3. Genotype–Phenotype Analysis by Variant Category

	Truncations	Likely+Possibly	Unlikely+Noncarriers	*P* Value
Subjects, n	13	32	102	
Women, n	4 (31%)	13 (41%)	40 (39%)	0.817
Age of onset, y	40±14	44±11	41±14	0.555
NYHA, median [IQR]	1 [1;2]	2 [1;2.5]	2 [1;2.5]	0.251
LVEDD, mm	64±11	64±9	64±11	0.997
LVEF, %	32±11	32±15	33±13	0.934
Follow-up (y), median [IQR]	8 [1; 16]	7 [4; 12]	8 [2; 13]	0.758
Heart transplant, n	3 (23%)	5 (16%)	21 (21%)	0.787
Cardiovascular death, n	3 (23%)	6 (19%)	19 (19%)	0.928

LVEDD indicates left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association functional classification.

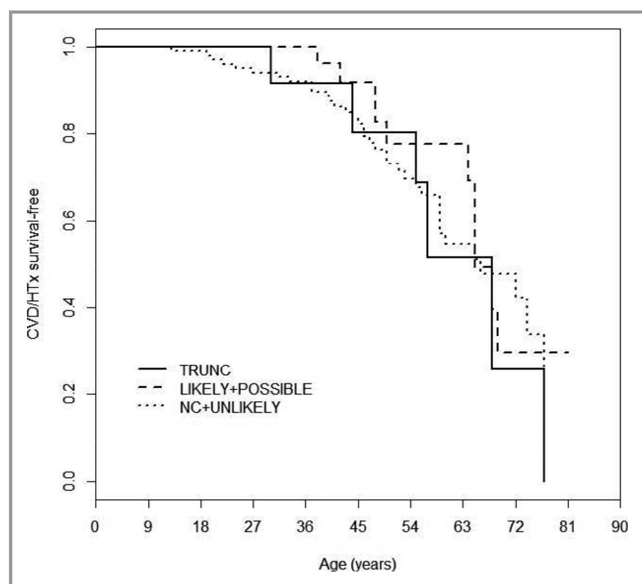


Figure 5. Long-term survival curves in *TTN* variant carriers. Kaplan–Meier event-free survival for CVD or HTx based on *TTN* variant categories: TRUNC, "likely" and "possibly"; NC, "unlikely." CVD indicates cardiovascular death; HTx, heart transplant; NC, noncarrier; TRUNC, truncations; *TTN* indicates titin gene.

pathogenic *TTN* missense variants are not easily resolved and likely contribute to a smaller fraction of DCM.

Similar to prior data on *TTN* truncations,[3] the presence of a *TTN* missense variant did not correlate with clinical measures of disease severity or progression, indicating that the DCM phenotype because of *TTN* missense variants is indistinguishable from other forms of DCM. However, although many variants could not be assessed by segregation analysis, the clustering of severe "possibly" variants in the A-band regions is suggestive of a biological consequence of A-band variation and aligns with previously noted overrepresentation of *TTN* truncating variants within this region.[3] We also observed a progression of lower ejection fractions with greater distance from the Z-disc; this trend toward lower ejection fractions in A-band variants is consistent with prior observations that A-band truncation variants have worse clinical outcomes.[20] The reason for these outcomes is unclear, but we speculate the likelihood that the mutant protein incorporates in the sarcomere exerts in a dominant negative effect that varies with the location of the mutation.

Interestingly, the distribution of bioinformatically "severe" *TTN* missense variants across titin domains was nonrandom and similar to what has been shown previously with *TTN* truncation variants. Indeed, *TTN* variants were overrepresented in the A-band region of titin,[3] specifically in the C-zone of the A-band that consists of a super-repeat of 11 immunoglobulin-like domains and Fn-III domains, that are organized in a consistent pattern repeated 11 times (Figure 4B and 4C). Interestingly, the Fn-III domains of the C-zone are overrepresented (Figure 4C). These Fn-III domains have previously been shown to bind myosin subfragment-1 and to be essential for the length dependency of force development (they depress calcium sensitivity at short sarcomere length).[21] Although the biological explanation for the clustering of A-band of variants and whether any modifying effects exist remain to be critically evaluated in future studies, we currently speculate that some A-band missense mutations may have a functional detrimental effect on contractility.

Our study was limited by lack of functional assays performed on the large number of *TTN* variants detected and by the possibility that some variants that did not meet criteria for "severe" could have effects on DCM risk. Cardiomyopathy gene mutations were not fully characterized in this cohort because samples were collected over 2 decades and gene screening was done throughout this period; therefore, some samples were not screened for all possible genes. Small family sizes and lack of additional DNA samples from affected relatives limited our ability to test for segregation in all cases. Given that instances of nonsegregation of "severe" variants did occur in our study, the interpretation of bioinformatically "severe" *TTN* variants in isolated patients remains challenging. Finally, this study considered only index patients in the outcome analysis.

In conclusion, *TTN* missense variants are common and the interpretation of *TTN* resequencing findings is challenging even when stringent bioinformatic and segregation criteria are used.[5,19] Using such criteria, missense variants bioinformatically classified as "severe" have a frequency comparable to that of *TTN* truncations in DCM. However, in contrast to truncation variants, our study did not demonstrate a strong association of missense variants with a risk of DCM, and when segregation analysis was possible, 13.5% of the "severe" variants were ultimately classified as "unlikely" because of incongruous segregation. These data argue that *TTN* missense mutations should not currently be interpreted as disease causing in most situations, urge caution in the interpretation of missense mutations in clinical practice, and support the need for the development of functional assays that better assess pathogenicity. Not surprisingly, the "severe" *TTN* missense variants did not associate with differences in clinical phenotypes in our DCM population, further arguing that many missense *TTN* variants are not disease-causing variants. Last, large-scale *TTN* sequencing and functional investigations on *TTN* variant domains will allow a better understanding of the role of *TTN* variants in DCM and pave the way to the development of diagnostic and therapeutic strategies.

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Disclosures

None.

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