ORIGINAL ARTICLE

Truncations of Titin Causing Dilated Cardiomyopathy

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ABSTRACT

BACKGROUND

Dilated cardiomyopathy and hypertrophic cardiomyopathy arise from mutations in many genes. *TTN*, the gene encoding the sarcomere protein titin, has been insufficiently analyzed for cardiomyopathy mutations because of its enormous size.

METHODS

We analyzed *TTN* in 312 subjects with dilated cardiomyopathy, 231 subjects with hypertrophic cardiomyopathy, and 249 controls by using next-generation or dideoxy sequencing. We evaluated deleterious variants for cosegregation in families and assessed clinical characteristics.

RESULTS

We identified 72 unique mutations (25 nonsense, 23 frameshift, 23 splicing, and 1 large tandem insertion) that altered full-length titin. Among subjects studied by means of next-generation sequencing, the frequency of *TTN* mutations was significantly higher among subjects with dilated cardiomyopathy (54 of 203 [27%]) than among subjects with hypertrophic cardiomyopathy (3 of 231 [1%], $P=3\times10^{-16}$) or controls (7 of 249 [3%], $P=9\times10^{-14}$). *TTN* mutations cosegregated with dilated cardiomyopathy in families (combined lod score, 11.1) with high (>95%) observed penetrance after the age of 40 years. Mutations associated with dilated cardiomyopathy were overrepresented in the titin A-band but were absent from the Z-disk and M-band regions of titin ($P\leq0.01$ for all comparisons). Overall, the rates of cardiac outcomes were similar in subjects with and those without *TTN* mutations, but adverse events occurred earlier in male mutation carriers than in female carriers ($P=4\times10^{-5}$).

CONCLUSIONS

TTN truncating mutations are a common cause of dilated cardiomyopathy, occurring in approximately 25% of familial cases of idiopathic dilated cardiomyopathy and in 18% of sporadic cases. Incorporation of sequencing approaches that detect *TTN* truncations into genetic testing for dilated cardiomyopathy should substantially increase test sensitivity, thereby allowing earlier diagnosis and therapeutic intervention for many patients with dilated cardiomyopathy. Defining the functional effects of *TTN* truncating mutations should improve our understanding of the pathophysiology of dilated cardiomyopathy. (Funded by the Howard Hughes Medical Institute and others.)

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GENE MUTATION IS AN IMPORTANT cause of cardiomyopathy. Mutations in eight sarcomere-protein genes cause hypertrophic cardiomyopathy, detected in 40 to 70% of patients.[1,2] Variations in more than 40 genes, most of which encode components of the sarcomere, the cytoskeleton, or the nuclear lamina, have been shown or posited to cause dilated cardiomyopathy.[3,4] Clinical evaluation identifies 30 to 50% of patients with dilated cardiomyopathy as having a relative who is affected or likely to be affected,[5-7] implicating a genetic cause. However, pathogenic mutations have been found in only 20 to 30% of patients.[8]

TTN, the gene encoding titin, has been implicated in cardiomyopathy but has been incompletely studied, owing to technical challenges posed by the large size of its coding sequence (approximately 100 kb). *TTN* mutations have been definitively linked to dilated cardiomyopathy in three families,[9-11] but not to hypertrophic cardiomyopathy. In addition, *TTN* mutations have been implicated in congenital myopathies involving cardiac and skeletal muscle, hereditary myopathy with early respiratory failure, tibial muscular dystrophy, and limb-girdle muscular dystrophy.[12-15]

Titin is the largest human protein (composed of approximately 33,000 amino acids) and the third most abundant striated-muscle protein.[16] Two titin molecules together span the sarcomere (about 2 μm in width) and are anchored at the Z-line and M-line (Fig. 1).[18] Titin is necessary for sarcomere assembly[19,20] and in striated muscle provides most of the passive force[21,22] and modulates the active contractile force.[23,24] There are many different isoforms of titin; those in the heart are predominantly N2B or N2BA (Fig. 1).[25] *TTN* also encodes a separate cardiac isoform, novex-3 titin, which is only 5600 amino acids in length, lacks the A-band and M-band segments of titin,[26] and is less abundant in cardiac tissue than full-length titin.

We undertook filter-based hybridization capture followed by next-generation sequencing[27] or traditional dideoxy sequencing to assess the contribution of *TTN* mutations to cardiomyopathies, analyzing DNA from 312 subjects with idiopathic dilated cardiomyopathy, 231 subjects with hypertrophic cardiomyopathy, and 249 control subjects.

METHODS

SUBJECTS

Studies were performed according to institutional guidelines and the U.K. Human Tissue Act guidelines and with the approval of local ethics committees. The 312 subjects with idiopathic dilated cardiomyopathy were from three cohorts (see Table 3 in the Supplementary Appendix, available with the full text of this article at NEJM.org): 92 subjects recruited at Brigham and Women's Hospital (group A), 71 subjects recruited during evaluation for cardiac transplantation at the Royal Brompton and Harefield National Health Service Trust (group B), and 149 subjects prospectively recruited in Colorado or Italy into a Familial Dilated Cardiomyopathy Registry (group C). Groups A and C had a high frequency of familial disease. The 231 subjects with hypertrophic cardiomyopathy were recruited at Brigham and Women's Hospital or the Mayo Clinic. Dilated cardiomyopathy and hypertrophic cardiomyopathy were diagnosed on the basis of published criteria.[28,29] The 249 control subjects without cardiomyopathy were recruited from multiple sites. No subject within a cohort had a known familial relationship with any other subject in the cohort. For more information on the cohorts, see the Supplementary Appendix.

DNA SEQUENCING AND GENOTYPING

Genomic DNA from each subject was used to construct DNA libraries. The DNA libraries were enriched for *TTN* by means of filter-based hybridization capture[27] with minor modifications (Tables 1 and 2 in the Supplementary Appendix) and were studied with the use of single-end or paired-end sequencing (with the Illumina Genome Analyzer II or HiSeq).[30] The *TTN* sequence was assessed in group C by means of traditional Sanger dideoxy sequencing performed at the Department of Genome Sciences, University of Washington.

SEQUENCE ANALYSES

Next-generation sequence data were analyzed by means of a custom pipeline that integrated existing tools, including Novoalign (www.novocraft.com) and the Genome Analysis Toolkit,[31] as well as Perl (with the use of Bio-Samtools) and R[32] scripts. Primary analyses of *TTN* variations were performed among subjects studied by the same ap-
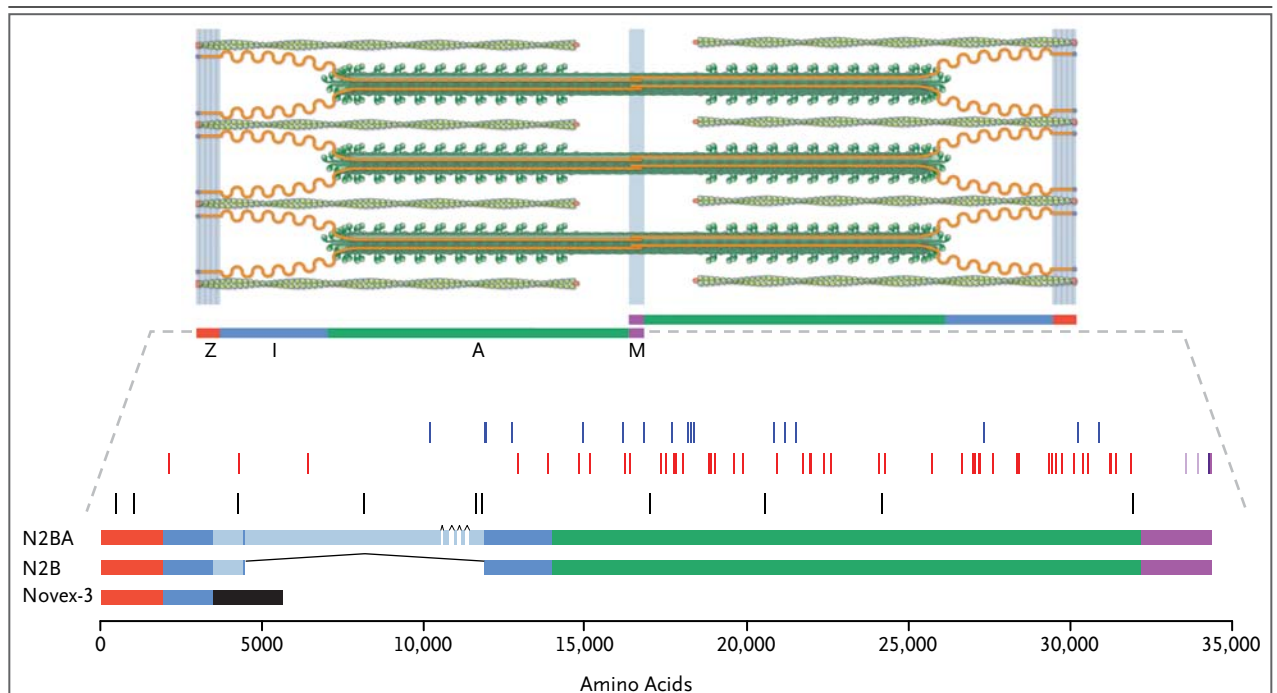
Figure 1. Spatial Distribution of Truncating Mutations in Titin.

The cardiac sarcomere (top) consists of titin (orange), the third major filament, in addition to the thick filaments (green rods with globular heads) and thin filaments (green coiled ovals). Regions of the sarcomere are demarcated as follows: Z-disk (red), I-band (blue), A-band (green), and M-band (purple). The sarcomere depiction is adapted from Granzier and Labeit.[17] *TTN* mutations are shown as thin vertical bars (middle), with overlapping mutations appearing as thicker bars. Splicing and copy-number mutations (blue) and nonsense and frameshift mutations (red) in subjects with dilated cardiomyopathy are shown, including two frameshift mutations previously reported to be linked to dilated cardiomyopathy (Table 14 in the Supplementary Appendix). All types of truncating mutations in controls and subjects with hypertrophic cardiomyopathy (black) are also indicated, as are truncating mutations previously identified in patients with congenital myopathy (light purple) or limb-girdle muscular dystrophy (dark purple). Titin isoforms and sequence variants are shown relative to the titin sequence (UniProt sequence Q8WZ42, www.uniprot.org) (bottom). Analysis of the N2BA class of cardiac titin isoforms excluded exons (black carets) with little evidence for cardiac expression (see Table 15 in the Supplementary Appendix). In cardiac tissue, *TTN* isoforms N2BA and N2B span the sarcomere, whereas novex-3 titin is shorter and less abundant (the exon specific to novex-3 titin is shown as a black bar).

proach (Table 1) in order to control for differences in variant detection. The amino acid positions of titin variants were identified with the use of the UniProt titin sequence (Q8WZ42), and mutations were reported with the use of Human Genome Variation Society nomenclature (Table 4 in the Supplementary Appendix). Variant confirmation and genotyping were performed by means of one or more of these methods: polymerase-chain-reaction amplification followed by dideoxy sequencing; restriction digestion and gel electrophoresis[26]; and RNA sequencing of cardiac tissue.[33]

STATISTICAL ANALYSIS

Association, cross-cohort, and cross-group analyses were performed with the use of Fisher's exact test, exact conditional tests of independence, or goodness-of-fit tests, unless otherwise specified. The uniformity of the spatial distribution of mutations was assessed by means of a chi-square goodness-of-fit test incorporating the size of each region. The clinical characteristics of each group in the dilated cardiomyopathy cohort were compared with the use of two-tailed, unpaired t-tests. Kaplan–Meier curves were computed by means of the "survfit" function in R and were compared by means of the "coxph" function in R.[32]

We calculated two-point lod scores for 19 families with dilated cardiomyopathy (Fig. 2 and Table 13 in the Supplementary Appendix) by using FASTLINK software, computed with the settings $\theta = 0$, phenocopy rate $= 0.005$,[34] and indicated dis-

Table 1. Subjects with *TTN* Truncating Variants, According to Cohort.

Type of Mutation	Subjects with Dilated Cardiomyopathy (N=312)	Subjects with Hypertrophic Cardiomyopathy (N=231)	Controls (N=249)	P Value*	
				All Subjects	Selected Subjects
		number of subjects			
Nonsense†	28	0	0	1×10^{-10}	5×10^{-11}
Frameshift	19	2	2	0.0004	1×10^{-5}
Splicing†	19	1	5	0.001	4×10^{-6}
Copy number	1	0	0	NA	NA
Any mutation	67	3	7	$<2\times10^{-16}$	$<2\times10^{-16}$

* P values were calculated on the basis of either all subjects or selected subjects (those studied by means of next-generation sequencing). Forty subjects with dilated cardiomyopathy who were excluded from *TTN* sequencing were included in these comparisons, for a total of 352 subjects with dilated cardiomyopathy. The one copy-number mutation was excluded from the calculations.

† Three nonsense and three splicing mutations each occurred in two subjects with dilated cardiomyopathy. An additional subject with dilated cardiomyopathy had two different splicing mutations.

ease penetrances.[35] An indeterminate status was assigned to family members 40 years of age or younger who did not meet clinical criteria for dilated cardiomyopathy[28] and to family members with confounding cardiac diagnoses.

RESULTS

STUDY SUBJECTS

Idiopathic dilated cardiomyopathy was diagnosed in the 312 subjects from the three independent groups: group A, group B, and group C (Tables 9, 10, and 11, respectively, in the Supplementary Appendix). Hypertrophic cardiomyopathy was diagnosed in 231 subjects; 249 subjects without known cardiomyopathy served as controls. Summary characteristics of each group and cohort are presented in Table 3 in the Supplementary Appendix.

TTN GENETIC VARIATION

DNA Sequencing

Using genomic DNA isolated from subjects with dilated cardiomyopathy in groups A and B and from the subjects with hypertrophic cardiomyopathy and controls, we captured and performed next-generation sequencing of 145 kb of the *TTN* gene, including all annotated exons and splice sites. For each subject, more than 97% of the targeted bases were sequenced at least 20 times (data not shown). From genomic DNA isolated from subjects with dilated cardiomyopathy, we determined *TTN* sequences by means of traditional dideoxy sequencing.

After excluding *TTN* variants with frequencies of 0.01 or greater in the 1000 Genomes Project (www.1000genomes.org/data) or present in all 792 study subjects, we identified 951 rare missense (minor allele frequency <0.01), nonsense, frameshift, or splicing or copy-number *TTN* variants that are predicted to change the titin amino acid sequence (Tables 4 and 5 in the Supplementary Appendix). Every study subject had approximately 1 rare missense variant (range of averages per group or cohort, 0.91 to 1.45 per subject).

Since the *TTN* transcript novex-3 (Fig. 1) is thought not to interact with the sarcomere M-band, and its expression in the heart is approximately one twentieth that of full-length titin isoforms (unpublished data), we did not study variants that exclusively altered novex-3 transcripts. We prioritized for analysis four types of variants — i.e., nonsense, frameshift, splicing, and copy-number — that (unlike most missense variants) are predicted to have a profound effect on the structure of full-length titin polypeptides (Fig. 1). We classify these as *TTN* truncating variants.

TTN Variants in Subjects with Hypertrophic Cardiomyopathy and in Controls

In the subjects with hypertrophic cardiomyopathy, we found three *TTN* truncating variants: two frameshift variants and one splicing variant (Table 1, and Tables 6 and 7 in the Supplementary Appendix). In each of the three affected subjects, concurrent analyses revealed a pathogenic mutation in the well-established hypertrophic cardiomyopathy genes

encoding β-myosin heavy chain (*MYH7*) or myosin binding protein C (*MYBPC3*) (data not shown). No family members were available for segregation analyses. In control subjects, we identified two frameshift and five splicing variants (Table 1, and Tables 6 and 7 in the Supplementary Appendix). The frequency of *TTN* truncating variants did not differ significantly between subjects with hypertrophic cardiomyopathy and controls (1% and 3%, respectively; P=0.34).

Subjects with Dilated Cardiomyopathy

Nonsense and Frameshift Variants

We identified 44 unique nonsense or frameshift variants that altered full-length titin in 47 subjects with dilated cardiomyopathy (Table 1, and Table 6 in the Supplementary Appendix). In the cohorts and groups studied by means of next-generation sequencing, the proportion of subjects with these variants was higher among the subjects with dilated cardiomyopathy in groups A and B (21 of 92 subjects [23%] and 14 of 71 [20%], respectively) than among the subjects with hypertrophic cardiomyopathy (2 of 231 [1%], $P=2\times10^{-12}$) or the control subjects (2 of 249 [1%], $P=3\times10^{-13}$). Twelve of the 149 subjects (8%) with dilated cardiomyopathy in group C, studied by means of traditional dideoxy sequencing, had such variants. We observed strong cosegregation (lod score, 9.3) of nonsense and frameshift variants with clinical status among 60 members of 16 families affected by dilated cardiomyopathy (Fig. 2 and Tables 12 and 13 in the Supplementary Appendix), indicating an odds of approximately 1 in 10^9 that the segregation of these *TTN* variants occurred by chance. One subject who presented with dilated cardiomyopathy at 17 years of age had one rare variant on each *TTN* allele: a previously described pathogenic missense mutation[9] and a nonsense variant inherited from her mother (Fig. 2 in the Supplementary Appendix). In all other families, there was coinheritance of dilated cardiomyopathy and a single *TTN* frameshift or nonsense variant. Among the 32 family members who were more than 40 years of age and for whom data were available, the penetrance of *TTN* truncating mutations was more than 95%.

Splicing Variants

We identified 17 *TTN* variants in 19 subjects with dilated cardiomyopathy that are predicted to alter RNA splicing, including 11 that altered absolutely conserved splice-site nucleotides (Table 1, and Table 7 in the Supplementary Appendix). RNA sequencing of cardiac tissue specimens from two subjects with splicing variants (one an adult child from a consanguineous marriage with a homozygous splicing variant [data not shown], the other described in Fig. 3 in the Supplementary Appendix) confirmed aberrant *TTN* splicing. Among subjects studied with the use of next-generation sequencing, we observed significant enrichment in splicing variants in the subjects with dilated cardiomyopathy in group A (with 15 of 92 subjects [16%] affected) and group B (with 3 of 71 subjects [4%] affected) as compared with the subjects with hypertrophic cardiomyopathy (with only 1 of 231 subjects [<1%] affected, $P=7\times10^{-8}$) and the control subjects (with 5 of 249 subjects [2%] affected, $P=9\times10^{-6}$). We found complete cosegregation of splicing variants and dilated cardiomyopathy (lod score, 1.8, indicating an association that was 60 times as likely as that due to chance) among 11 members of three families (Fig. 2 and Table 13 in the Supplementary Appendix).

Copy-Number Variants

We assessed copy number across *TTN* in the subjects with dilated cardiomyopathy in groups A and B by comparing the distribution of sequence reads among subjects (Fig. 4 in the Supplementary Appendix). The traditional dideoxy sequencing used for samples from the subjects with dilated cardiomyopathy in group C did not permit these analyses. One copy-number variant was identified in a subject with dilated cardiomyopathy. This tandem insertion of 28 kb (spanning introns 71 to 124) was predicted to incorporate a 13% internal duplication of a portion of titin; its presence was confirmed in the proband, and it was absent in two healthy relatives (Fig. 5 in the Supplementary Appendix). No *TTN* copy-number variations were observed among the subjects with hypertrophic cardiomyopathy or the control subjects (480 in all).

CONSEQUENCES OF *TTN* TRUNCATING VARIATIONS

Because *TTN* nonsense, frameshift, splicing, and copy-number variants that are predicted to substantially alter titin structure were significantly more frequent among subjects with dilated cardiomyopathy than among subjects with hypertrophic cardiomyopathy ($P=3\times10^{-16}$) or controls ($P=9\times10^{-14}$) and were coinherited with dilated cardiomyopathy in families (combined lod score, 11.1), we concluded that these variants cause dilated cardiomyopathy. Six *TTN* mutations were each

present in two subjects; results of analyses of one subject pair were consistent with a shared haplotype (data not shown).

To conservatively estimate the frequency of truncating *TTN* mutations in subjects with dilated cardiomyopathy, we increased our population of subjects with dilated cardiomyopathy in group A by adding 40 subjects who were recruited concurrently with the other subjects in the group and whose *TTN* sequences were not analyzed because studies revealed a pathogenic mutation in another dilated cardiomyopathy gene. After this addition, the frequencies of *TTN* truncating mutations among the subjects with dilated cardiomyopathy in groups A and B were 28% and 24%, respectively (P=0.74). Subjects in group C, whose samples were studied with the use of a different DNA-sequencing platform, had significantly fewer *TTN* mutations (9%, P<0.001). The frequency of *TTN* mutations did not differ significantly between subjects with and those without a family history of dilated cardiomyopathy (P=0.36) (Table 3 in the Supplementary Appendix).

TTN truncating mutations found in subjects with dilated cardiomyopathy were nonrandomly distributed within titin (Fig. 1): they were overrepresented in the A-band region as compared with either the remainder of N2BA (P=0.0004) or N2B (P=0.01) and were absent from the Z-disk and

M-band regions (P=0.006 and P=0.001 for the comparison with N2BA and with N2B, respectively). The spatial distribution of the 10 variants found in the subjects with hypertrophic cardiomyopathy and the control subjects (Fig. 1) was distinct from that of the variants found in the subjects with dilated cardiomyopathy (P=0.001). The variants in the subjects with hypertrophic cardiomyopathy and the control subjects were less enriched for the A-band region of titin (40% vs. 84% for mutations in the subjects with dilated cardiomyopathy, P=0.006) and included Z-band variants (20% vs. 0%, P=0.01).

CLINICAL CHARACTERISTICS

There were no significant differences between subjects with and those without *TTN* truncating mutations with respect to the age at diagnosis, left ventricular end-diastolic dimensions, ejection fraction, or rates of cardiac transplantation, implantation of a left ventricular assist device, and death from cardiac causes (Table 2 and Fig. 2A and 2B).

The diverse clinical manifestations of dilated cardiomyopathy were reflected in subjects in groups A, B, and C. Subjects in group A were more likely to have familial disease than subjects in group B (P=6×10⁻¹³) or group C (P=0.005). Subjects in group B (whose clinical features were ascertained during evaluation for cardiac transplan-

Table 2. Characteristics of Subjects with Dilated Cardiomyopathy, According to the Presence or Absence of *TTN* Mutations.

| Characteristic	Group A		Group B		Group C	
	Mutation (N=37)	No Mutation (N=55)	Mutation (N=17)	No Mutation (N=54)	Mutation (N=13)	No Mutation (N=136)
Female sex — no. (%)	11 (30)	17 (31)	2 (12)	14 (26)	4 (31)	56 (41)
Familial dilated cardiomyopathy — %	92	79	21	24	85	66
Age — yr	45.9±13.6	46.7±14.3	48.4±11.0	51.7±12.8	49.2±12.8	49.7±14.0
Age at diagnosis — yr	37.1±13.2	37.3±14.6	38.5±9.8	41.3±13.4	37.6±14.7	39.6±12.3
LVEF — %	26.4±10.8	30.7±11.6	24.6±11.0	24.8±11.7	29.3±8.5	31.4±11.8
Fractional shortening — %	19.6±9.9	19.6±10.2	NA	NA	13.5±3.4	16.6±6.9
LVEDD						
Absolute diameter — mm	63.7±10.9	61.4±9.7	70.8±8.1	74.2±10.2	65.5±9.2	65.6±10.4
Relative diameter — mm/cm² of body-surface area	33.0±5.1	NA	37.6±6.0	NA	34.0±7.1	NA
NYHA III or IV — %	62	41	94	91	46	43
ICD — no. (%)	22 (59)	30 (55)	1 (6)	2 (4)	NA	NA
Cardiac transplantation, implantation of VAD, or death from cardiac causes — no. (%)	14 (38)	17 (31)	17 (100)	47 (87)	1 (8)	31 (23)

* Plus–minus values are means ±SD. The values for left ventricular ejection fraction (LVEF), fractional shortening, and left ventricular end-diastolic diameter (LVEDD) are based on echocardiographic measurements. ICD denotes implantable cardioverter–defibrillator, NA not available, NYHA New York Heart Association functional class, and VAD ventricular assist device.

tation) were more likely to have severe dilated cardiomyopathy than subjects in groups A and C, with significantly increased left ventricular end-diastolic diameter (P=1×10^{-9} and P=3×10^{-6}, respectively) and a lower left ventricular ejection fraction (P=0.03 and P=0.0001, respectively) (Table 2, and Fig. 1 in the Supplementary Appendix).

Rates of cardiac transplantation, implantation of a left ventricular assist device, and death from cardiac causes among subjects and family members with *TTN* mutations did not appear to be influenced by mutation type but were influenced by the sex of the subjects (Fig. 2C and 2D). The mean (±SD) age at the time of these adverse events in
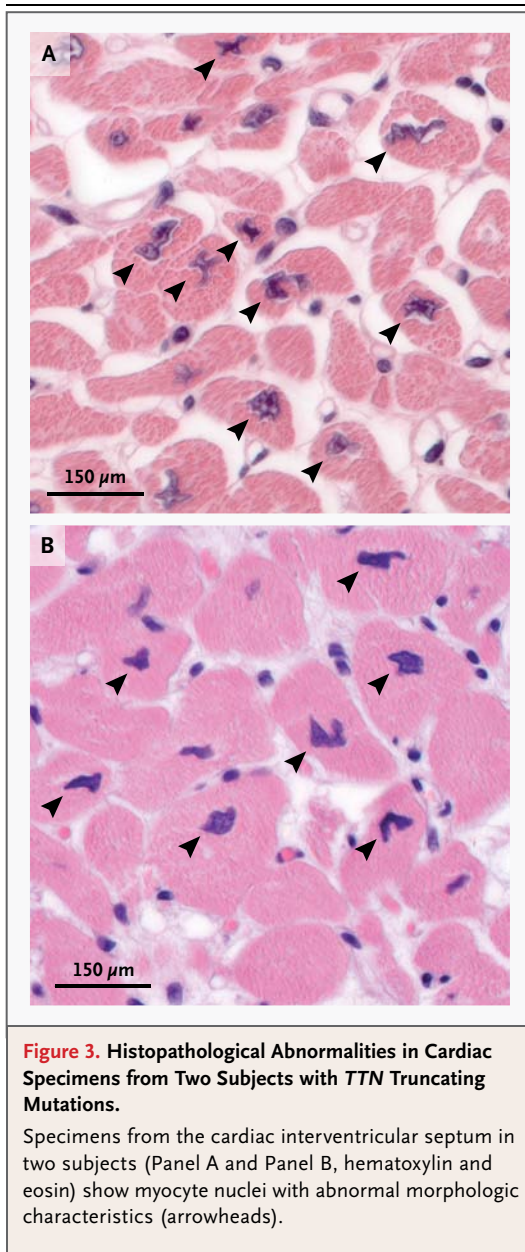
94 mutation carriers within 19 families was 68±5 years for the 33 women and 56±3 years for the 61 men (P=4×10^{-5}) (Fig. 2 in the Supplementary Appendix).

Subjects with *TTN* truncating mutations had dilated cardiomyopathy that was usually unaccompanied by conduction system or skeletal-muscle disease (Tables 9, 10, and 11 in the Supplementary Appendix), although overt skeletal myopathy occurred in one subject with a homozygous *TTN* splicing mutation. Histopathological characteristics of cardiac specimens from subjects with *TTN* truncating mutations were typical of idiopathic dilated cardiomyopathy. Some sections revealed foci of myocytes with bizarre, stellate nuclear mor-
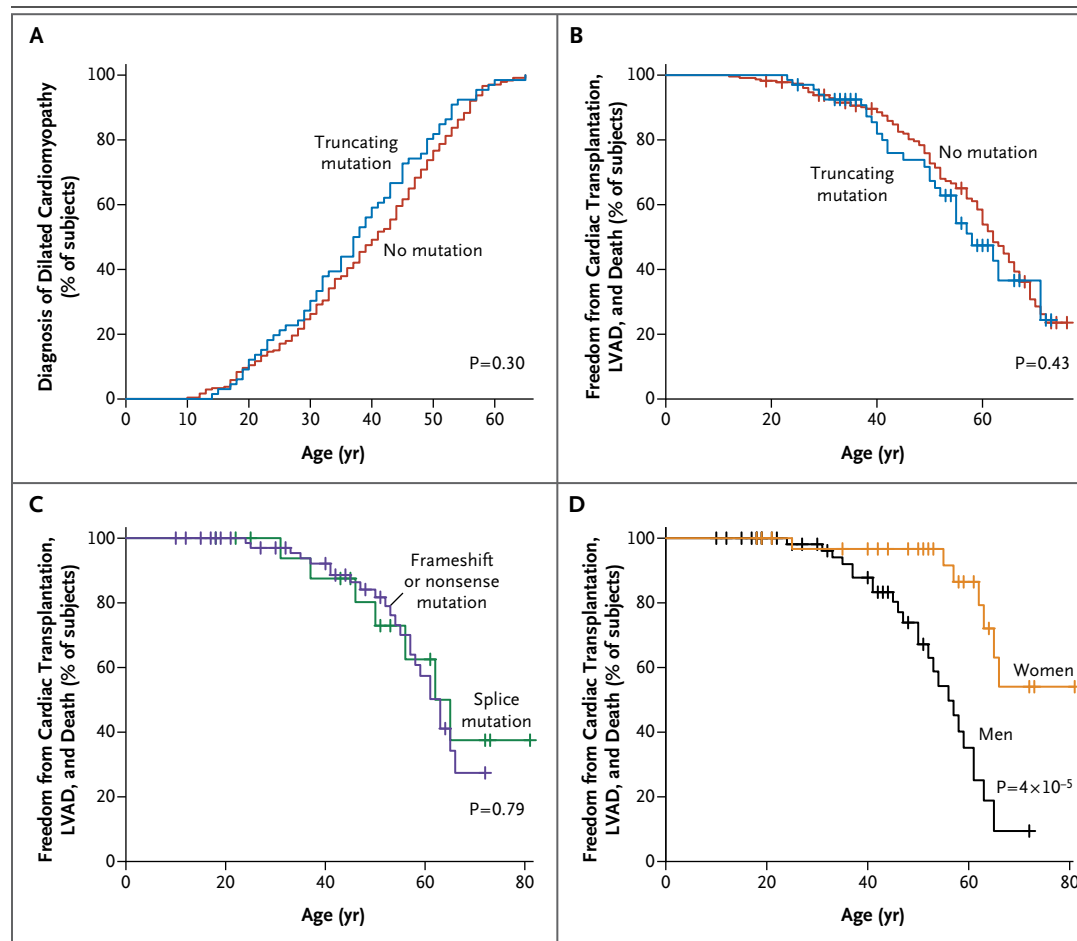
Figure 2. Kaplan–Meier Estimates of Age at Diagnosis and Clinical Progression in Subjects with Dilated Cardiomyopathy Caused by *TTN* Mutations.

Panel A shows data for the time of diagnosis, and Panels B, C, and D show freedom from cardiac transplantation, implantation of left ventricular assist device (LVAD), and death from cardiac causes. Panels A and B show data for the 67 subjects who had mutations and the 228 who did not. Panels C and D show data for the 94 subjects (from 19 families) who had truncating mutations (Fig. 2 in the Supplementary Appendix; also see the Supplementary Appendix for a description of the effect of sex). Hatch marks indicate subjects with censored data.

Figure 3. Histopathological Abnormalities in Cardiac Specimens from Two Subjects with *TTN* Truncating Mutations.

Specimens from the cardiac interventricular septum in two subjects (Panel A and Panel B, hematoxylin and eosin) show myocyte nuclei with abnormal morphologic characteristics (arrowheads).

phology, best appreciated in cross section (Fig. 3). Electron-microscopical examination of one specimen obtained at autopsy showed intact sarcomeric structures (Fig. 6 in the Supplementary Appendix).

DISCUSSION

We identified *TTN* truncating variants in 67 subjects with dilated cardiomyopathy, 3 subjects with hypertrophic cardiomyopathy, and 7 controls (Tables 1 and 2, and Tables 3 through 7 in the Supplementary Appendix). *TTN* variants included

nonsense and frameshift mutations predicted to cause protein truncation, variants of splice donor or acceptor sites predicted to cause exon skipping or to include intronic sequence or delete exonic sequence, and a large tandem insertion. We posit that these mutant alleles produce shortened titin with abnormal properties that cause dilated cardiomyopathy.

The frequency of *TTN* truncating variants identified by next-generation sequencing of samples from subjects with hypertrophic cardiomyopathy and controls was similar (1% and 3%, respectively; P=0.34). In addition, each subject with hypertrophic cardiomyopathy who had a *TTN* variant also had a pathogenic mutation in an established hypertrophic cardiomyopathy gene, suggesting that *TTN* truncations rarely, if ever, cause hypertrophic cardiomyopathy.

TTN truncating mutations were more common in subjects with dilated cardiomyopathy than in subjects with hypertrophic cardiomyopathy (P=3×10^{-16}) or controls (P=9×10^{-14}). Among the subjects with dilated cardiomyopathy, more *TTN* truncations were identified in those studied with the use of next-generation sequencing (groups A and B) than dideoxy sequencing (group C; P<0.001). Despite clinical differences (including more familial disease in group A and more severe dilated cardiomyopathy in group B) (Table 2, and Fig. 1 in the Supplementary Appendix), the frequency of *TTN* truncating mutations was similar in groups A and B (28% and 24%, respectively; P=0.74). The reduced frequency of mutations identified in the subjects in group C raises the possibility that the detection of mutations was better with the next-generation sequencing strategy than with dideoxy sequencing.

TTN truncating mutations and dilated cardiomyopathy were coinherited in families (combined lod score, 11.1) (Fig. 2 and Table 13 in the Supplementary Appendix). Segregation analyses of frameshift or nonsense mutations in 17 families (lod score, 9.3) and splice-site mutations in 3 families (lod score, 1.8) confirmed the coinheritance of each type of truncating mutation. Family studies also showed that the penetrance of *TTN* truncating mutations was more than 95% for the subjects who were more than 40 years of age (Fig. 2 and Table 12 in the Supplementary Appendix).

Among the subjects with dilated cardiomyopathy, those with and those without *TTN* truncating mutations had similar clinical manifestations and similar morbidity and mortality, but men with *TTN*

mutations had adverse events at significantly earlier ages than did women (P=4×10⁻⁵). Sex is reported to influence outcomes in heart failure of various causes.[36] However, the suggestion that sex would substantially influence an autosomal monogenic cause of heart failure is unexpected and warrants further study.

Mutations that substantially disrupt the structure of full-length titin might cause dilated cardiomyopathy by means of several mechanisms. RNA- and protein-surveillance pathways most likely degrade some truncated titin peptides.[37] Decreased titin levels could limit sarcomere formation and might cause cardiac dysfunction and remodeling. Yet this is not the case for previously reported *TTN* mutations that delete only part of the M-band portion of titin[14] (Fig. 1, and Table 14 in the Supplementary Appendix); immunohistochemical studies showed that some of these carboxy-terminal truncated titin proteins are integrated into the sarcomere and cause recessive, early-onset skeletal and cardiac myopathy, not dominant dilated cardiomyopathy. In addition, if more proximal *TTN* truncating mutations caused dilated cardiomyopathy through haploinsufficiency, the distribution of such mutations would be rather uniform across the susceptible portion of titin. In contrast, we observed a skewed mutation distribution in subjects with dilated cardiomyopathy, which was distinct from that observed in subjects without the disease (Fig. 1). This unequal mutation distribution may indicate that truncated titin proteins found in subjects with dilated cardiomyopathy, like previously studied carboxy-terminal titin truncations, are integrated into the sarcomere and cause dilated cardiomyopathy by means of a dominant negative mechanism.

If truncated titin proteins in subjects with dilated cardiomyopathy were incorporated into the sarcomere, they would most likely be anchored at the Z-line and would interact with the full complement of Z-disk factors (Fig. 1). However, these truncated titin proteins would not include the M-band residues that anchor titin to the middle of the sarcomere through myomesin, encode a kinase domain, and interact with many proteins.[38] The M-band portion of titin is implicated in sensing and modulating sarcomeric force.[12,39-41] We suggest that the loss of these interactions could lead to dilated cardiomyopathy through the disturbance of the normal regulation of sarcomeric force.

We conclude that *TTN* truncating mutations are the most common known genetic cause of dilated cardiomyopathy. Ongoing analyses of other classes of *TTN* variation (e.g., missense variants) may expand our understanding of the importance of *TTN* in the pathogenesis of dilated cardiomyopathy. Incorporation of next-generation sequencing analyses of *TTN* into clinical genetic screens should increase the detection of dilated cardiomyopathy mutations by approximately 50%, permitting earlier diagnosis and interventions to prevent disease progression. Further study of the functional consequences of *TTN* truncating mutations on myocardial physiological features and myocyte signaling is warranted.

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