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Recessive mutations in proximal I-band of *TTN* gene cause severe congenital multi-minicore disease without cardiac involvement

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Abstract

Titin, encoded by the gene *TTN*, is one of the main sarcomere components. It is involved in not only maintaining the structure of cardiac and skeletal muscles, but also in their development, extensibility, elasticity, and signaling events. Congenital titinopathy increasingly appears an important and common form of axial predominant congenital myopathy. The pathophysiological role of *TTN* in congenital titinopathy and pediatric heart diseases is yet to be explored. Here, we delineate the phenotype of two female siblings who developed severe congenital multi-minicore disease without cardiac involvement. Genetic investigation by whole exome sequencing demonstrated compound heterozygous *TTN* mutations (c.15496+1G>*A*, p.5166_5258del; c.18597_18598insC, p.Thr6200Hisfs*15), corresponding to the Ig domain of the proximal I-band. Aberrant splicing causing exon skipping was verified by *in vitro* minigene analysis. Our results suggest that *TTN* mutations affecting the Ig domain of the proximal I-band may be a cause of severe congenital defect in skeletal muscles without severe cardiac involvement, thereby providing evidence for the hypothesis that congenital titinopathy patients carrying biallelic N2BA only mutations are at lower cardiac risk than those with other combinations of mutations. Meanwhile, this study confirm the hypothesis on recessive truncating variants of *TTN* experimentally and thus support earlier reported genotype-phenotype correlations.
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Keywords: Congenital titinopathy; Electron microscopy study; Histochemical staining; Minigene; Multi-minicore disease; *TTN*.

1. Introduction

Titin, encoded by the 364-exon *TTN* gene [OMIM #188840], is the largest protein in nature [1]. It acts as one of the main sarcomere components, each titin molecule bridges half of the entire sarcomere to form a continuous elastic myofilament. These myofilaments provide a scaffold for sarcomere assembly during muscle development, maintain sarcomeric structural integrity, generate passive tension, and serve as key mechanosensing and signaling hubs [2,3].

Titin comprises four main parts: an amino-terminal Z-disc region, middle I-band and A-band regions, and a carboxyl-terminal part spanning the M-band. Based on the presence of the N2A and N2B elements in the I-band region [4], the *TTN* gene generates numerous alternative splicing variants resulting in various isoforms (http://cardiodb.org/titin/titin_transcripts.php), with variable expression in skeletal and cardiac muscles and across different developmental and physiological states [5].

Autosomal recessive *TTN* mutations have been reported in patients with prenatal or infant onset forms of titinopathy, which termed as "congenital titinopathy" [3]. Cases were described as early onset myopathy with fatal cardiomyopathy [6], centronuclear myopathy [7], core myopathy with heart disease [8], and arthrogryposis multiplex congenita with myopathy [9]. Owing to the large size of the protein, diagnosis by identification of underlying *TTN* variants and their clinical interpretation represented a challenge.

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We report here a congenital titinopathy, manifesting as an early-onset multi-minicore disease (MmD) without cardiac involvement. This is the first experimental description of compound heterozygous *TTN* mutations affecting the Ig domain of the proximal I-band. Our study: (1) provide evidence for the hypothesis that congenital titinopathy patients carrying biallelic N2BA only mutations are at lower cardiac risk than those with other combinations of mutations. (2) confirm the hypothesis on recessive truncating variants of *TTN* experimentally and thus support earlier reported genotype-phenotype correlations [3,10].

2. Subjects and methods

2.1. Ethical approval and consent

The study protocol (2015[916]) was approved by the Ethics Committee of Peking University First Hospital (Beijing, China). Written informed consent for research participation and use of non-obscured facial photographs was obtained from the parents of the two patients. All experiments were performed in accordance with the relevant guidelines and regulations.

2.2. Morphological studies

Open biopsy of the musculus biceps brachii of P1 was performed. Samples were frozen or fixed and processed for standard histological, histochemical staining, and electron microscopy-based studies.

2.3. Genetic investigation, confirmation, and annotation

Whole exome sequencing was used to identify causative mutations in P1 and P2. Bioinformatics analysis pipelines focused on compound heterozygous or homozygous variants within known neuromuscular disease genes that were likely to be pathogenic. Candidate variants were validated by Sanger sequencing and co-segregation analysis in the family. Mutations were reported according to Human Genome Variation Society recommendations (http://varnomen.hgvs.org/) using the inferred complete *TTN* metatranscript as reference (NM_001267550.1; LRG391_t1). Exons were numbered 1 to 364 according to the LRG schema. The Leiden Muscular Dystrophy (http://www.dmd.nl/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and Cardiodb mutation databases (http://cardiodb.org/titin/) were interrogated to identify previously reported mutations. The Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/) was used to determine the frequency of each mutation in the general population. The Cardiodb database (http://cardiodb.org/titin) was also interrogated to determine which isoforms the mutations were predicted to alter.

To assess copy number variants, array-based comparative hybridization was performed using the Agilent Human Genome G3 SurePrint 8*60 K Microarray (Agilent Technologies, USA). DNA labeling, array hybridization, scanning and data analysis were carried out as described previously [11].

2.4. Minigene construction, site-directed mutagenesis and splicing analysis

An *in vitro* hybrid minigene splicing assay was used to evaluate the transcriptional consequences of the splice site change. Minigene constructs containing a genomic fragment spanning from the exon 52 to the exon 55 of *TTN* were synthesized and cloned into the pcDNA 3.1 plasmid. Mutation c.15496+1G>*A* was introduced with the QuickChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Wild-type and mutant minigene constructs were transiently transfected into HEK-293 cells using Lipofectamine (Invitrogen, USA). Cells were then incubated for 72 h before isolation of total RNA using Tiangen Reagent (Tiangen, Beijing, China). cDNA was synthesized using 2 μg of RNA with the M-MLV reverse transcriptase kit (Promega, Madison, WI) and amplified with specific primers including primer upstream in exon 52 (forward 5′-ATGACGTCGGCAGTGATAGC-3′) and primer downstream in exon 55 (reverse 5′-TGGATCAGTTCTGCTCGCTC-3′).

3. Results

3.1. Clinical features

The two female twins (P1 and P2) were one year and five months of age at the time of first clinical visit. They were born to a healthy non-consanguineous couple of Chinese ancestry. An elder brother was healthy (Supplementary Figure S1). There was no previous family history of neuromuscular disorders. At birth, the twins presented with hypotonia and feeding difficulties following cesarean delivery at term. The two children showed delayed motor milestones; they raised their head at 7 months and could sit independently only at 15 months of age. They were unable to stand without support until 30 months of age, and acquired unstable independent gait for 20 m at the age of 3 years. Global motor performances exhibited slow improvement. Language, social, and cognitive developments were normal. Clinical examination in infancy and early childhood demonstrated symmetric and generalized muscle weakness that involved predominantly proximal and upper limbs, suggesting distal arthrogryposis. There was marked axial weakness on neck and trunk flexors, in addition to scoliosis that developed after 12 months (Fig. 1). Facial muscles were also affected, and a high arched palate and mild ptosis were observed. Proximal contractures were not present, and the deep tendon reflexes were diminished. No ophthalmoplegia was observed. Routine laboratory investigations revealed that the levels of serum creatine kinase, urinary organic acid, and blood amino acid and acylcarnitine were normal. Electrocardiography was

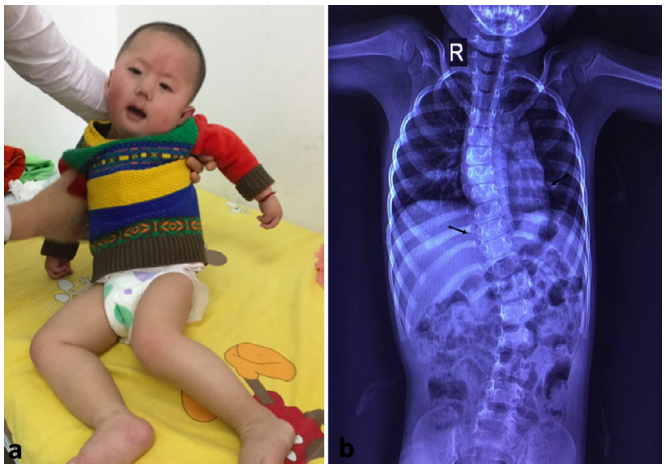

Fig. 1. Clinical characteristics and chest X-ray findings of the two patients. (a) P1 presented with hypotonia and myopathic facial features, cannot sit firmly until 18 months old. (b) Chest X-ray of P1 at 3years. Scoliosis developed from childhood without dilated cardiomyopathy.

normal (Supplementary Fig. S2) and ultrasonic cardiogram (UCG) showed no structural or functional abnormalities on the latest follow-up (at the age of three years and ten months) (Supplementary Fig. S3). Brain MRI and evoked potentials were normal. T1 weighted muscle MRI-imaging showed symmetrical fatty infiltration of the caput longum musculi bicipitis femoris along with the semimembranosus and semitendinosus, whereas other muscle groups within the thigh and the lower legs were spared (Fig. 2). Of note, although the fatty infiltration was disproportionately involved within the thigh, the same muscle was relatively spared on the level of the pelvis. In addition, bilateral diffused atrophy in thigh muscles was observed.

3.2. Muscle morphological pattern

Skeletal muscle biopsies demonstrated a recognizable and distinct morphological change. In infancy, two muscle samples taken from P1 and P2 at 10 months of age (Fig. 3a, b) showed type 1 fiber atrophy and type 2 fiber compensatory hypertrophy. These structural changes coexisted with centrally located nuclei (CLN). No endomysial fibrosis or necrosis/regeneration lesions were present. Local doctors suggested a histopathological diagnosis of centronuclear myopathy (CNM). However, on review of their biopsy, the appearance was less suggestive of typical CNM; because of the uncertain diagnosis, muscle biopsy was repeated. Muscle biopsy performed on P2 at 16 months (Fig. 3c–h) showed increased variability in fiber size and an increase in internal nuclei. There was predominance of type 1 fibers and multi-minicore lesions (foci of mitochondria depletion and sarcomere disorganization). Similarly, no endomysial fibrosis or necrosis/regeneration lesions were present.

Skeletal muscle ultrastructural studies in P1 and P2 confirmed the presence of multiple foci of sarcomere disruption and mitochondria depletion (Fig. 4). Noticeably, some M-lines, in contrast to the relatively preserved Z-lines, appeared to be pulled apart. This finding was different from the typical minicores wherein Z-line streaming is often more apparent than in disintegrated sarcomere center [12]. These minicores involved up to six adjacent myofibrils and spanned generally three to six contiguous sarcomeres along the longitudinal fiber axis.

Immunohistochemical studies on skeletal muscle from P1 showed normal expression and distribution of dystrophin; α-, β-, and γ-sarcoglycan; dysferin; desmin; MHC-1; and C5b-9.

3.3. Genetic findings and splice site mutation analysis

Whole exome sequencing, performed on P1 and P2, yielded on average 11.55 gigabases (Gb) of high-quality sequence data (sequence of all exons and 100 bp flanking region), with 98.06% target coverage and 131.87X mean coverage depth. The coverage of the target region that was sequenced at least 10 times (depth > 10X) was 95.01%. We verified that all exons of *TTN* were adequately covered. Considering the functional impact of the variants and the recessive inheritance in the pedigree, two pathogenic *TTN* sequence changes were identified (Fig. 5a), and neither was observed in the general population. A reported G-to-A change at the first nucleotide (+1) of titin intron 53 (NM_001267550.1: c.15496+1G>*A*) [3], inherited from the healthy mother, disrupted the conserved GT dinucleotides at the splice donor site and was predicted to result in exon skipping. An additional novel one-base-pair insertion was identified in exon 65 (NM_001267550.1: c.18597_18598insC, p.Thr6200Hisfs*15), inherited from the healthy father. This mutation was predicted to result in protein truncation after the addition of 15 novel amino acids. Both the mutations affected the proximal Ig-domain of the titin I-band (Fig. 6), within the inferred complete *TTN* metatranscript but not within exons that encode the N2B mature cardiac isoform. Chromosome karyotype was normal, and no pathogenic copy number variation was detected.

A minigene splicing assay was applied to verify the transcriptional consequences of the splice site change (Fig. 5b). An electropherogram of PCR products revealed that the wildtype control had the predicted size 640-bp band, while the mutated had a smaller size 361-bp band (Fig. 5c). Sequencing of the cDNA revealed altered splicing and an in-frame skipping of exon 53 was occurred (Fig. 5d).

4. Discussion

With the recent progress in understanding titin function and structural organization, congenital titinopathy has emerged as an important cause of early onset myopathy. The distinct clinical features, muscle histopathology, radiological findings on muscle MRI, and inheritance pattern indicate that the patients described in this study might have a recessive titinopathy, manifesting as a severe congenital multi-minicore disease without cardiac involvement. However, since the

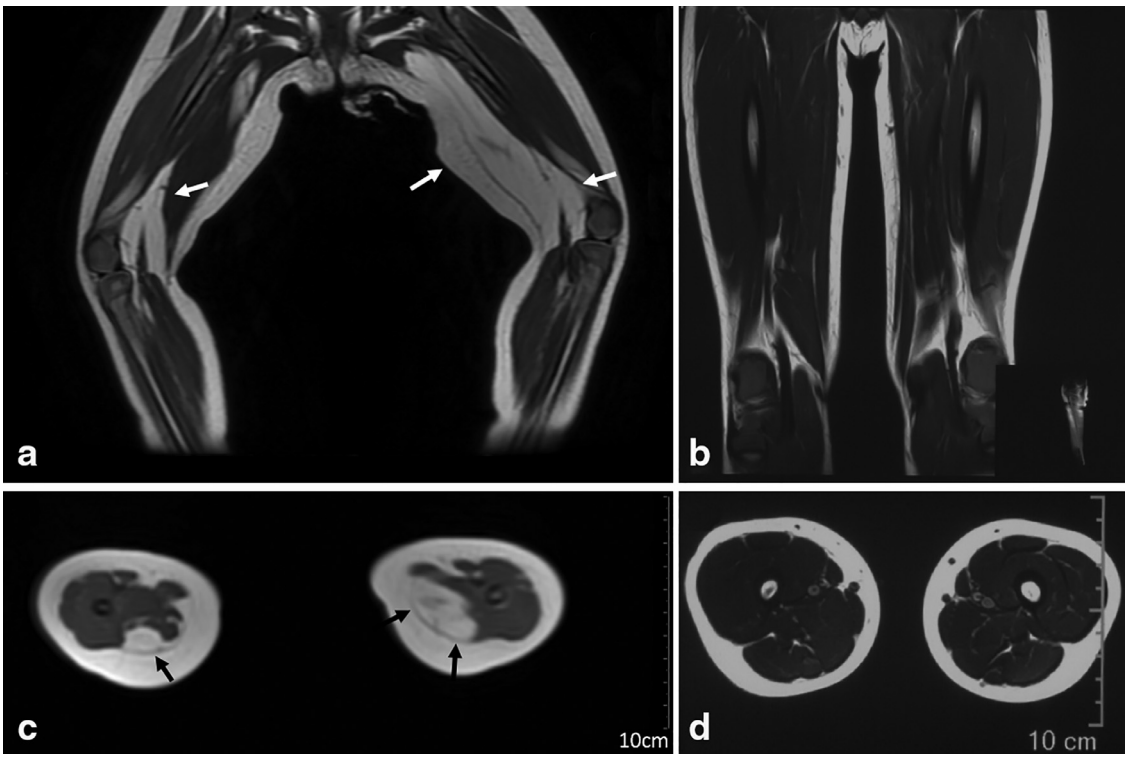

Fig. 2. Muscle magnetic resonance imaging (MRI). (a, c) P1 at 2y5m, T1-weighted images, longitudinal and transverse sections from the thigh. Symmetric increase in signal intensity within the caput longum musculi bicipitis femoris, semimembranosus and semitendinosus were present, different from classic *RYR1*- and *SEPN1*- related myopathies. (b, d) Normal control at 3y, T1-weighted images, longitudinal and transverse sections from the thigh.

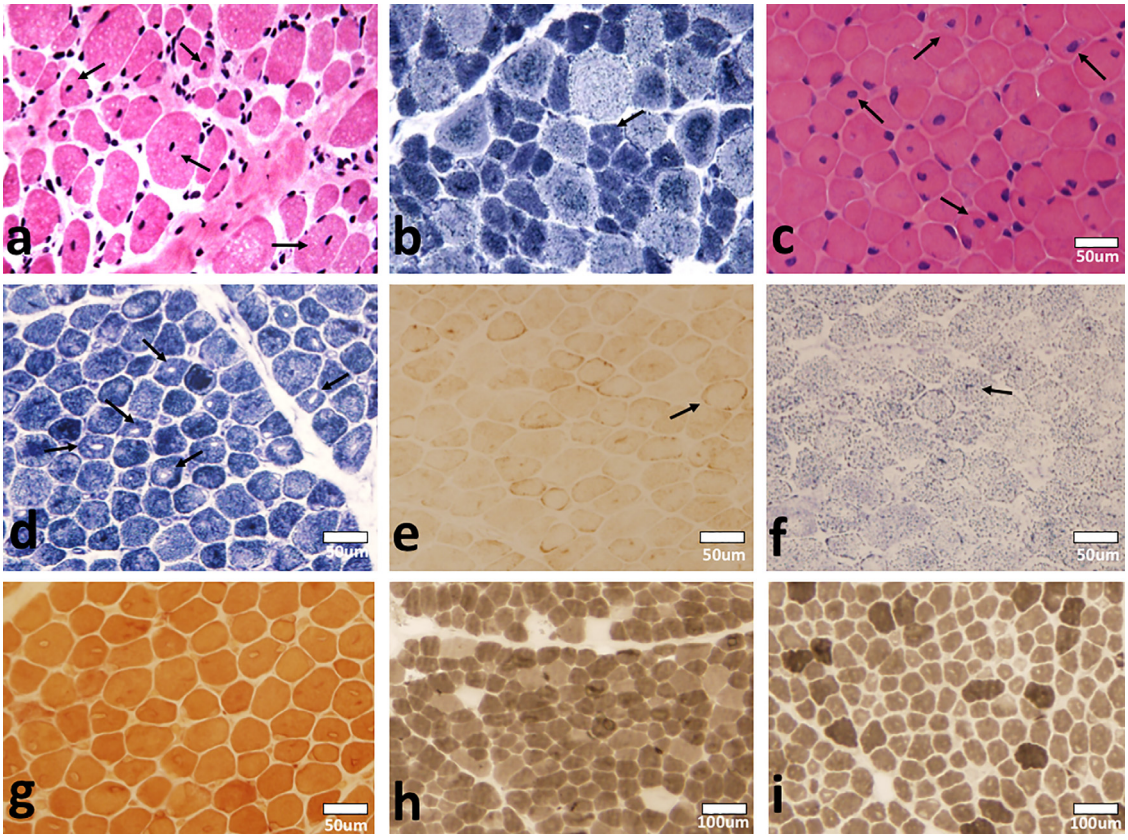

Fig. 3. Skeletal muscle histopathological pattern. Muscle biopsies from P2 at 10 months (a, b) and 16 months (c–i). Fiber size variability and type 1 fiber predominance (h, i). Slight (a) or moderate (c) number of nuclear centralizations. Minicores were visible as small light foci of mitochondria depletion (b, d). Transverse cryosections stained with HE (a, c), NADH-TR (b, d), Cox (e), SDH (f), NSE (g), ATPase 4.6 (h), ATPase 10.6 (i) ($40 \times$ c–g; $20 \times$ h–i).

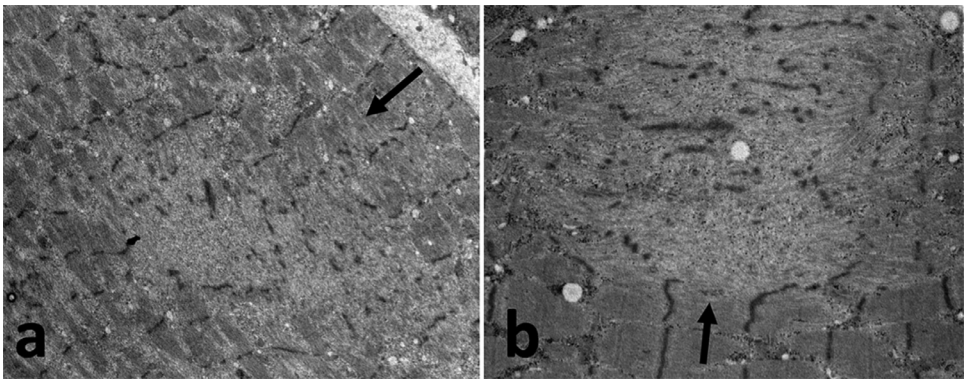

Fig. 4. Ultrastructural lesions in skeletal muscle. Longitudinal electron microscope sections of biopsies from P1 (a) and P2 (b). Focal areas of severe myofibrillar disorganization; the disrupted sarcomeres look pulled apart, curving away the contiguous ones. (a) Some of the sarcomeres show disintegration of the M-line, which contrasts with relative preservation of the flanking Z-lines. (b) a typical minicore structure with no myofibrillar arrangement with decreased mitochondria.

twins are young, the possibility of cardiac dysfunction needs to be followed up, further cardiac screening including echocardiograph of the two patients should be required in future.

Our results provided evidence for the hypothesis that congenital titinopathy patients carrying biallelic N2BA only mutations (i.e., 2 mutations that alter N2BA but spare N2B) are at lower cardiac risk than those with other combinations of mutations. Based on the presence of the N2A and N2B elements and tandem lengths of PEVK regions in the I-band, alternative titin isoforms can be divided into six main classes [13] (Fig. 6). Skeletal muscles predominantly express the N2A isoform (NP_596869.4; 3680 kDa), which is characterized by the exclusion of the cardiac-specific N2B element and inclusion of the N2A element. Cardiac muscles express both N2BA and N2B isoforms. N2BA (NP_001243779; 3780 kDa) includes both the N2B and N2A elements along with the PEVK region, whereas N2B (NP_003319.4; 2960 kDa) is a cardiac-specific isoform and lacks both the N2A elements and some PEVK-encoding exons. The cardiac isoform novex-1 (NP_597676.3; 2980 kDa) and the cardiac and skeletal

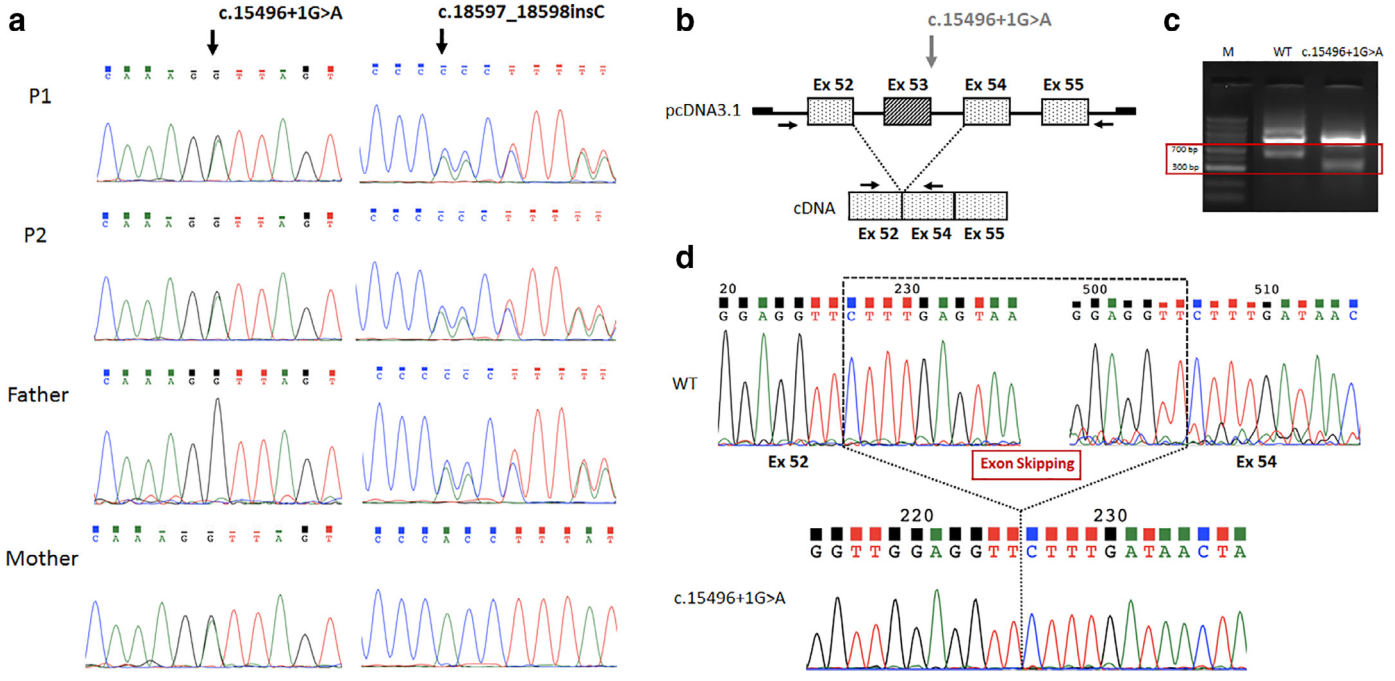

Fig. 5. *TTN* variants and minigene studies of mRNA splicing following transient expression in HEK-293 cells. (a) Results for genomic sequencing. **(b)** Schematic representation of the minigene vectors used for the *in vitro* splicing assay. **(c)** Electropherogram of PCR products. The wildtype control had the predicted size 640-bp band, while the mutated had a smaller size 361-bp band. **(d)** Direct sequencing of the amplified minigene cDNA PCR products confirmed skipping of exon 53.

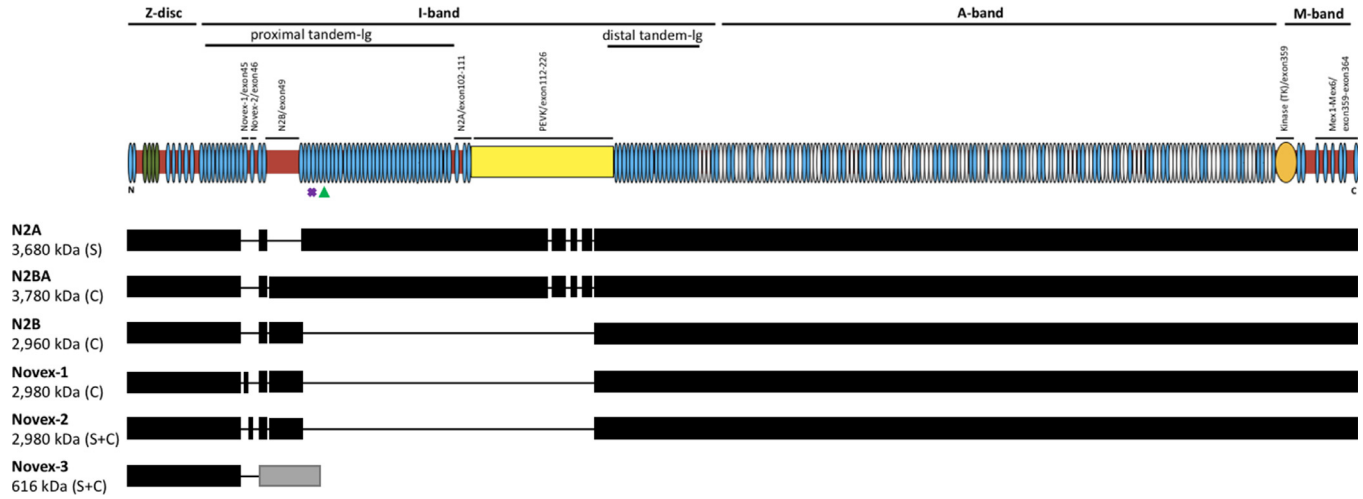

Fig. 6. The modular structure of the theoretical titin protein expressed in different muscle types. Top: Titin is mostly comprised of repeated immunoglobulin-like (Ig; blue) and fibronectin type 3-like (FN3; white) domains. Selected elements are labeled above the diagram with the classical titin nomenclature. Structural features are also indicated: the Novex-1 and Novex-2 exons, the N2B and N2A elements, the PEVK (proline/glutamate/valine/lysine-rich) region, and the alternatively spliced M-band region, followed by the corresponding exon number. Bottom: Schematic representation of the exons encoding the main TTN isoforms in NCBI RefSeq database. The bars align to the protein diagram of the meta isoform above, except for the alternative C-terminal exon of the Novex-3 isoform (gray). Sizes and tissue expression (S, skeletal muscle; C, cardiac muscle) of the corresponding encoded proteins are indicated below each isoform. Symbols below the diagram depict splicing and frameshift variants of the two patients in the I-band, respectively.

muscle isoform novex-2 (NP_597681.3; 2980 kDa) are nearly identical to N2B *TTN*, differing only in the incorporation of amino acid stretches in the I-band region. Finally, novex-3 is present in all striated muscles, and is the only isoform expressing the 8-kb-novex-3 exon that introduces an alternative terminal coding exon. It has recently been shown that heterozygous *TTN* truncating and essential splice site mutations that alter both N2BA and N2B, the 2 longest and most abundant adult cardiac isoforms, increase the risk of adult onset dilated cardiomyopathy (DCM) [14]. Truncating mutations that alter fetal and/or the smaller novex cardiac isoforms, or that impact only the N2BA isoform (N2BA-only mutations) are not associated with DCM in the heterozygous state [15]. This is presumably because the predominant adult cardiac isoform (N2B) can still be transcribed from the truncating allele. The heterozygous mutations found in our patients affect the Ig domain of the proximal I-band, downstream to the N2B element, which is presumably transcribed in the N2A isoform expressed in skeletal muscles, but not in the N2B isoform. This could be a probable explanation for the occurrence of the congenital muscle condition without severe cardiac involvement.

Congenital or early onset cardiomyopathies are sometimes associated with muscle disorders caused by *MYH7*, *LMNA*, *FKRP*, *FKTN*, *SPEG*, *ACTA1* and *TAZ* mutations [16–19]; however, it is not typically associated with other forms of early onset muscle disease. Original MmD cases related to homozygous deletions in M-line titin described by Carmignac et al. involved both heart and skeletal muscles [6]. Most affected children present congenital forms of skeletal myopathy associated with various primary heart conditions, the most common being life-threatening DCM (also known as Salih myopathy or early-onset myopathy with fatal cardiomyopathy; OMIM 611705). Because of a severe DCM with disturbances in cardiac rhythm, a sudden premature death occurs before adulthood. The associated identified *TTN* variants are homozygous deletions in the exons of Mex1 and Mex3, and cause frameshifts leading to predicted premature termination of codons; this premature termination is experimentally confirmed with sarcomere disorganization and M-line disruption. Chauveau et al. identified seven novel homozygous or compound heterozygous *TTN* variants in five additional patients having MmD with heart disease (MmDHD), spanning from an Emery-Dreifuss-like form to a severe congenital muscle condition with neonatal cardiac failure, thereby expanding its clinical spectrum [8]. A newborn with multiple joint contractures, left ventricular non-compaction, and heart failure requiring heart transplantation at the age of 5 years represents the most severe and the first case of antenatal titinopathy reported to date [6]. All the affected patients carried either homozygous or heterozygous variants in the Mex1 and/or Mex 2 exons. Additionally, a recent study by Oates et al. reported a 30-member cohort of congenital titinopathy [3]. Fiber size variation, cores, and internalized nuclei, either alone, or in combination, were the most common histopathological abnormalities. Almost 50% of patients had congenital or early onset cardiac abnormalities. Until recently, due to the positional bias resulting from extensive scanning of M-band exons as compared to other *TTN* portions, only a limited amount of disease-causing *TTN* variants were identified. A wide range of phenotypes and low frequency of cases with pathogenically-convincing mutations have hitherto been major obstacles to interpret the association

of congenital MmD and pediatric heart disease with *TTN* mutations.

Another most striking finding was we confirmed the hypothesis on recessive truncating variants of *TTN* and thus supported earlier reported genotype-phenotype correlations [3,10]. Whole exome sequencing in the affected twins revealed compound heterozygous *TTN* variants: a splice-site variant in intron 53 (c.15496+1G>*A*, p.5166_5258del) and a single base-pair insertion in exon 65 (c.18597_18598insC, p.Thr6200Hisfs*15). A recent work by Savarese et al. [10] proposed a specific workflow for the clinical interpretation of genetic findings in titin: In the presence of monoallelic PTVs, a complete molecular characterization of variants affecting the canonical or noncanonical splice sites by cDNA or protein studies is suggested. To investigate the impact of the c.15496+1G>*A* variant, minigene constructs were generated and tested in transiently transfected cultured cells. Sequencing of the cDNA revealed altered splicing and an in-frame skipping of exon 53. This splice site mutation was shown to cause in-frame loss of a single exon, which should result in a near-normal sized protein product, the truncating mutation might also be responsible for minor amounts of full-length protein. This is the first experimental description of compound heterozygous *TTN* mutations affecting the Ig domain of the proximal I-band, and adds to the relatively small number of I-band variants identified as genetic causes of human neuromuscular diseases.

The histological pattern of congenital titinopathy is a pathological "chameleon" [3], presenting with a wide range of structural abnormalities. The muscle biopsy results of the present study showed typical minicores and fulfilled the diagnostic criteria of MmD, including marked type 1 fiber predominance and focal loss of striation. CLN were present, in accordance with other *TTN*-mutant patients [3,6,20]. There is in fact a molecular and morphological overlap between CNM and titinopathies, and this overlap suggests potential roles of titin in maintaining the normal subsarcolemmal nuclear positioning. The absence of ophthalmoplegia may be helpful in discriminating between congenital titinopathy and other CNM genetic subtypes. *RYR1*-associated MmD can show abundant internal nuclei but is typically without central basophilic lesions; both are usually absent in *SEPN1*-related myopathy. The ultrastructural change was also slightly different. Z-line streaming generally occurs earlier and is more apparent than M-line disintegration in *RYR1*-related cores [21].

Congenital titinopathy increasingly appears an important, common, and potentially severe form of axial predominant congenital myopathy [3]. Analysis of the clinical, histopathological and imaging findings of the twin patients expands our understandings of this disorder on the basis of experimental molecular diagnosis. We establish that compound heterozygous *TTN* mutations affecting the Ig domain of the proximal I-band may be a cause of MmD without severe cardiac involvement, which will facilitate the surveillance and management of affected individuals.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2019.03.007.

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