A 'second truncation' in *TTN* causes early onset recessive muscular dystrophy

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Abstract

Mutations in the gene encoding the giant skeletal muscle protein titin are associated with a variety of muscle disorders, including recessive congenital myopathies ±cardiomyopathy, limb girdle muscular dystrophy (LGMD) and late onset dominant distal myopathy. Heterozygous truncating mutations have also been linked to dilated cardiomyopathy. The phenotypic spectrum of titinopathies is emerging and expanding, as next generation sequencing techniques make this large gene amenable to sequencing. We undertook whole exome sequencing in four individuals with LGMD. An essential splice site mutation, previously reported in dilated cardiomyopathy, was identified in all families in combination with a second truncating mutation. Affected individuals presented with childhood onset proximal weakness associated with joint contractures and elevated CK. Cardiac dysfunction was present in two individuals. Muscle biopsy showed increased internal nuclei and immunoblotting identified reduction or absence of calpain-3 and demonstrated a marked reduction of C-terminal titin fragments. We confirm the co-occurrence of cardiac and skeletal myopathies associated with recessive truncating titin mutations. Compound heterozygosity of a truncating mutation previously associated with dilated cardiomyopathy and a 'second truncation' in *TTN* was identified as causative in our skeletal myopathy patients. These findings add to the complexity of interpretation and genetic counselling for titin mutations.

1. Introduction

Limb girdle muscular dystrophies (LGMD) are a clinically and genetically highly variable group of disorders [1,2] which show considerable clinical overlap with other genetic muscle disorders such as congenital myopathies, myofibrillar myopathies (MFM) and Emery–Dreifuss Muscular Dystrophy (EDMD) and as such obtaining a genetic diagnosis is often difficult. The application of next generation sequencing (NGS) techniques has enabled the genetic basis of several of these diseases to be elucidated, and it is now apparent that mutations in a single gene can produce a range of phenotypes. Titin provides a florid example of this, with dominant mutations in its C-terminus associated with Tibial Muscular Dystrophy (TMD) [3], other mutations in the C-terminus responsible for recessive distal titinopathy [4], mutations in the A-band causing Hereditary Myopathy with Early Respiratory failure (HMERF), a form of MFM with characteristic cytoplasmic bodies [5]; and recessive mutations attributed as the cause of LGMD2J [3,6], congenital myopathies [7,8], adult onset LGMD [9], and EDMD-like phenotype without cardiac involvement [10]. The full spectrum

of titin phenotypes is yet to be defined as reports of patients with atypical presentations illustrate [6,9,11].

Through the application of whole exome sequencing (WES) we have been able to identify recessive mutations in titin in four individuals with a muscle disorder, which was classified as LGMD but with notable similarities to EDMD. All four individuals share a common essential splice site mutation, which has also been reported in a heterozygous state in a patient with Dilated Cardiomyopathy (DCM). However, the heterozygote parent carriers in these families were healthy demonstrating the complexity of titinopathies in their genetic aetiology and highlighting the need for a comprehensive approach to diagnostic evaluation, counselling and cardiac surveillance in individuals with pathogenic titin mutations.

2. Materials and methods

2.1. Patient selection

Four affected individuals from three families with genetically undiagnosed limb girdle muscular dystrophy were clinically assessed within the Newcastle University John Walton Muscular Dystrophy Research Centre, and had already undergone extensive diagnostic investigations. Informed consent for research was obtained for all patients under the protocol for Newcastle MRC Centre Biobank for Neuromuscular Diseases (REC reference: 08/H0906/28 + 5). Clinical data were extracted from medical records.

2.2. Genetic analysis

Exome sequencing was performed at the Broad Institute (Boston, USA), with Agilent Sure-Select Human All Exon v2.0 (44 Mb) on Illumina HiSeqXs platform (Illumina, Inc. San Diego, CA), and sequence alignment, variant calling and functional annotation was performed with Burrows Wheel Aligner, Genome Analysis Tool Kit and Variant Effect Predictor (VEP). Variants were filtered using The Broad Institute's proprietary software, XBrowse to include only those that were rare (with a variant frequency of <0.01 in reference datasets (in house control dataset at The Broad Institute, 1000 Genomes, and ExAC)), which were predicted to be moderately to severely damaging by VEP and which passed the XBrowse quality filter. Identified variants from a list of known muscle disease genes (supplemental Table S1) were reviewed manually. Given the high frequency of missense variants in *TTN* which are not of clinical significance we adopted a conservative approach and disregarded missense variants unless in a region where proven mutations had been previously reported. All truncating *TTN* variants (nonsense, frameshift, or essential splice site) mutations were confirmed by Sanger sequencing in the Northern Genetics Laboratory and segregated amongst available family members.

2.3. Muscle MRI and CT

Muscle MRI of lower limbs was performed in patients F1-II-1 and F2-II-2 and T1 weighted axial images were reviewed. Patient II-1 from family 3 was unable to undergo MRI due to having a PPM in situ and therefore CT of lower limbs was performed instead.

2.4. cDNA analysis

Total RNA from patient F1-II-1 biopsy was extracted with RNeasy Fibrous Tissue Mini kit (Qiagen GmbH, Germany) according to manufacturer's instructions. cDNA was synthesised with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random hexanucleotides. Primers spanning exon–exon junctions were designed for cDNA with Primer3 software (TTN-cDNA-ex351-353-F [CTCCACTGGCCTTACTGAAG]; TTN-cDNA-ex351-353-R [GGGAATGGTTTTCCTTTGATT]; TTN-cDNA-ex361-364 [GATGCTCCAGCCTTCATCTC]; TTN-cDNA-ex361-364R [CATGTTACTTCTGGGGTAGGC]). Fragments were amplified with DreamTaq™ DNA Polymerase (Fermentas) separated in agarose gel, and optionally isolated from gel for sequencing. PCR products were sequenced on an ABI3730xl DNA Analyzer (Applied Biosystems), using the Big-Dye Terminator v3.1 kit and analysed with Sequencher 5.0 software (Gene Codes Corporation).

2.5. Muscle biopsy immunoanalysis

Muscle biopsy immunoanalysis was performed by the Muscle Immunoanalysis Unit, Newcastle UK, using a standard panel of dystrophy antibodies as part of routine investigations [12].

Muscle tissue from patient F1-II-1 underwent Western blot analysis of C-terminal titin fragments. Biopsies from a control individual free of muscular disease, FINmaj heterozygous TMD patient, and FINmaj homozygous LGMD2J patient served as controls. Frozen muscle biopsies were mechanically homogenised in Laemmli sample buffer. SDS-PAGE and western blotting were performed using standard protocols, and ECL-detection. Two different antibodies recognising the last Ig domain M10 of titin were used for detection of titin C-terminal fragments, the polyclonal M10-1 [13] and the monoclonal 11-4-3 [6]. Myosin heavy chain (MyHC) band was used as a loading control.

3. Results

3.1. Genetic results

In F1-II-1 WES data analysis identified four known muscle disease genes with rare variants that were predicted to be moderately to severely damaging (supplemental Table S2). Single heterozygous variants in *LAMA2, MSTN* and *SYNE1* were deemed unlikely to be pathogenic based on further examination of genetic data and correlation with phenotype in this patient. There were six rare variants in *TTN*, of which four were missense variants and two were predicted to be highly damaging by VEP: a nucleotide substitution in intron 362 (c.107377 + 1G > A) which is predicted to disrupt the consensus splice site, and a novel nonsense mutation in exon 352 (c.97863G > A p.(Trp32621*)).

WES data from patients F2-II-1 and II-2 were analysed together assuming an autosomal recessive model. The only known muscle disease gene with variants in was *TTN*: the same

essential splice site variant (c.107377 + 1G > A) identified in patient F1-II-1 in addition to a missense mutation. Removing quality filters identified a second truncating variant in *TTN*; a novel deletion leading to a frameshift in exon 353 (c.98603delT p.(Phe32868Serfs*11)) (supplemental Table S2).

In patient F3-II-1 WES data identified six known muscle disease genes with rare variants. Review of these variants and comparison to the observed phenotype enabled variants in *SIL1, SYNE2, PLEC*, and *ETFB* to be discounted (supplemental Table S2). There were six variants in *TTN*: three missense changes and two other variants predicted to be highly damaging. The first of these was the same essential splice site variant (c.107377 + 1G > A) also identified in families 1 and 2; and the second was a novel nonsense variant in exon 329 (c.87529A > T p.(Lys29177*)) (supplemental Table S2).

Compound heterozygous variants in *TTN* that are predicted to be highly damaging to protein function were identified in all four patients. The common c.107377 + 1G > A splice site mutation identified in all three families has been observed in a heterozygous state in only two people out of >12,000 alleles in the Exac database (allele frequency: 0.00001661; one European (non-Finnish) individual and one African individual), consistent with this being a rare recessive disease causing allele. Construction of alleles in families 1 and 2 demonstrated evidence of a shared haplotype (Fig. 1) suggesting that variant has a shared origin in these families. This variant was recently identified in a heterozygous state in an individual with dilated cardiomyopathy [14]. All identified truncating variants are expressed in both the N2-A and N2-B isoforms, which are the predominant isoforms in skeletal and cardiac muscle respectively. All variants were

confirmed by Sanger sequencing and segregation of these variants within all available family members was consistent with pathogenicity (Fig. 1).

3.2. Clinical results

Clinical findings for all patients are summarised in Table 1.

3.2.1. Family 1, patient 1

Patient F1-II-1 presented with elevated serum creatine kinase (CK) levels of ~5700 iu/L at age 9 years as an incidental finding as part of investigations for raised liver transaminase levels. At that time he developed a rash that was subsequently diagnosed as eczema, but went on to have a muscle biopsy, which excluded a diagnosis of dermatomyositis. He developed mild progressive proximal weakness with contractures of ankles and elbows, a rigid spine (Fig. 2), and a mild cardiomyopathy requiring treatment from age 9 years old. At last cardiac assessment, at age 19 years old, he was on beta blocker and ACE inhibitor therapy and had stable mild left ventricular impairment with an ejection fraction of 45% (lower limit of normal is 55%) and fractional shortening in the normal range. Respiratory function was preserved. Genetic testing of several known genes was negative (*CAPN3, DMD, FKRP, ANO5, LMNA/C, FHL1, DES, MYOT, CRYAB, ZASP, BAG3*). Haplotype analysis at known autosomal recessive LGMD loci demonstrated possible linkage at *DYSF, SGCG, SGCA* and *TTN* loci.

3.2.2. Family 2, patient II-1

Patient F2-II-1 walked at 13 months but found running and jumping difficult from childhood. At 12 years old he was noted
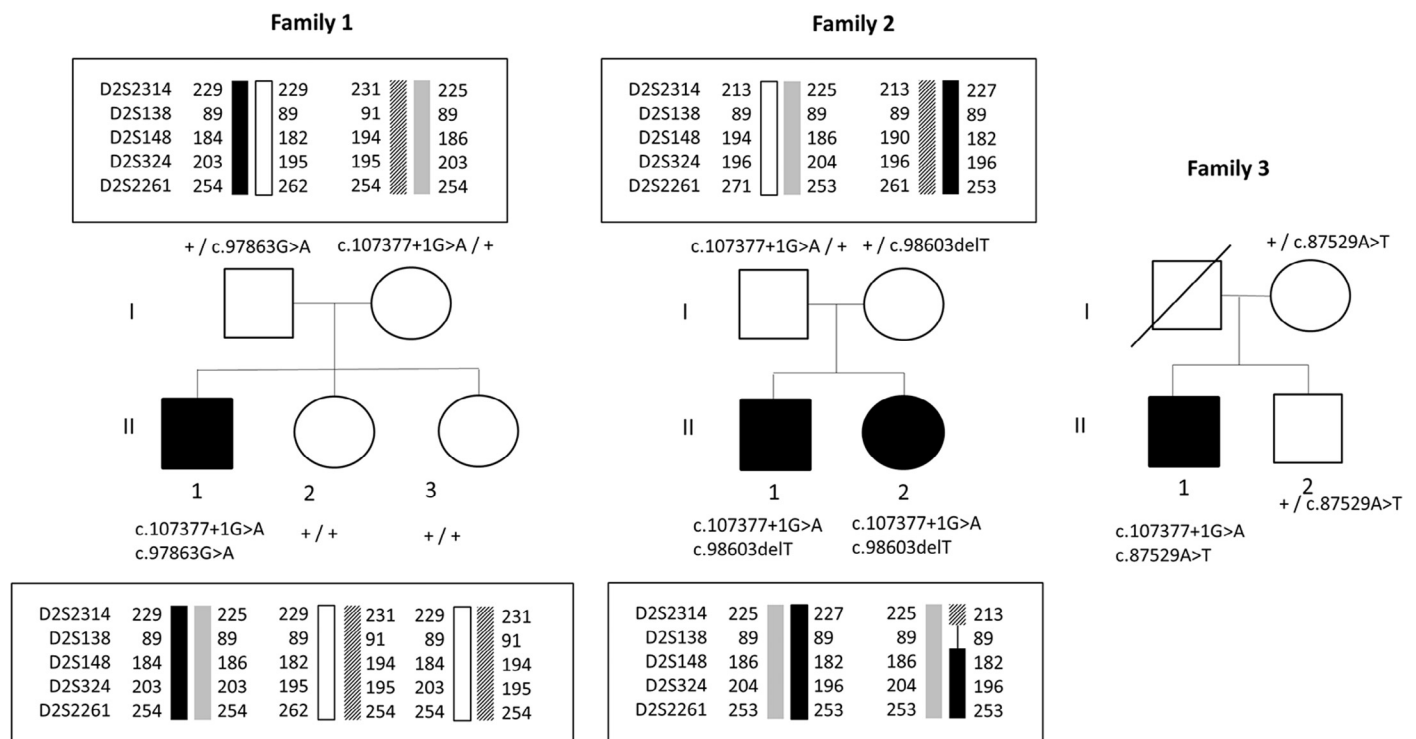

Fig. 1. Pedigrees and segregation of truncating mutations in TTN (NM_001267550.1).

Table 1
Summary of clinical features in patients with recessive truncating TTN mutations.

Family /patient	F1-II-1	F2-II-1	F2-II-2	F3-II-1
Sex/age onset	M/9 y	M/childhood	F/5 y	M/20 y
Age last assessment	17 y	32 y	30 y	41 y
Pattern of weakness	Proximal UL and LL	Proximal UL and LL	Proximal UL and LL	Proximal > distal LL
Ambulation status	Ambulant	Ambulant	Ambulant	Ambulant
Contractures	Ankles, elbows	Ankles, elbows	Elbows, wrists, fingers, hips, ankles	Elbows, ankles
Scapular winging	No	Yes	Yes	No
Cardiac impairment (age onset)	Mild LV systolic impairment (12 y)	−	−	Atrial fibrillation age (20 y), PPM
Respiratory impairment	−	−	+	−
Rigid spine	+	−	+	+
CK (U/L)	3378	'Markedly elevated'	4000–5000	2700

UL, upper limbs, LL, lower limbs, LV, left ventricular, PPM, permanent pacemaker.

Fig. 2. Clinical photographs and muscle MRI findings. Clinical photos of patient F1-II-1 demonstrating rigid spine (A) and limited range of movement at wrist (B); patient F2-II-1 demonstrating range of movement at wrist (C) and elbow contractures (D); patient F2-II-2 demonstrating elbow contractures and rigid spine age at 19 years old (E) and in same patient aged 30 years old demonstrating finger flexion and wrist contractures (F).

to have some proximal muscle weakness, a stiff spine and tight ankles and elbows. He began to mobilise with a wheelchair at 26 years old. At his last assessment he was 32 years old and was able to stand and walk sideways holding onto furniture and to transfer independently. On examination he had symmetrical scapular winging and prominent contractures of ankles and elbows (Fig. 2). Weakness was predominantly proximal in both upper and lower limbs, with shoulder movements MRC power grade 3 and hip movements MRC power grades 2 to 3−. Cardiac and respiratory assessments were normal, with FVC 4.57L (88% predicted) in sitting and 4.46L (85% predicted) in lying.

3.2.3. Family 2, patient II-2

Patient F2-II-2 presented at age 5 years old with toe walking and complaining of stiffness. She was found to have elevated CK levels at >5000 iu/L. At 10 years old she was noted to have limb girdle weakness but was able to walk and participate in physical activities in school. On examination at 20 years old

she walked on her toes with bent elbows and was noted to have a rigid spine, hyperlordosis and prominent contractures of upper and lower limbs. At last assessment she was 30 years old and usually mobilised using a walking stick or scooter. On examination she had a rigid spine, scoliosis, and mild asymmetric scapular winging. She had severe contractures of both upper and lower limbs affecting heels, hips, knees, elbows and long finger flexors (Fig. 2). There was weakness of proximal upper limbs (MRC power grade 3− to 4+) with distal preservation of strength. In lower limbs weakness was predominantly proximal, with hip extension weakest (MRC power grade 2). Cardiac assessment was normal. There was respiratory insufficiency with FVC 1.78L (58% predicted), but no postural drop in FVC. A recent sleep study was satisfactory. The patient reported mild dysphagia to some foods.

Genetic testing of several known genes was negative in the siblings in family 2 (*SGCA, SGCB, SGCG, SGCD, FKRP, CALPN3, FHL1, BAG3, COL6A1, COL6A2, COL6A3, LMNA/C*).

Haplotype analysis at known autosomal recessive LGMD loci demonstrated possible linkage to *TTN* as well as *DYSF, SGCD, SGCA, TTN* and *TCAP*.

3.2.4. Family 3, patient II-1

Patient F3-II-1 presented with toe walking and atrial fibrillation in his early 20s. A pacemaker was inserted at this time. At most recent cardiac assessment there had been no episodes of atrial fibrillation. The patient experienced frequent palpitations, which were attributed to ventricular premature beats. There was normal left ventricular function on recent echocardiogram. On examination he showed predominantly proximal lower limb weakness with ankle and elbow contractures and rigid spine, and was ambulant at age 41 years. CK was 2600 iu/L at age 35 years old.

3.3. Muscle imaging results

Muscle MRI in patient F1-II-1 at 17 years old did not demonstrate any evidence of muscle pathology (Fig. 3). Muscle MRI in patient F2-II-2 at age 28 years old demonstrated atrophy and diffuse fatty replacement in all muscle groups. The most

severely affected muscles, the obturatorius and rectus femoris, showed some asymmetry. In the lower legs the peroneus longus, soleus and tibialis anterior muscles were more affected distally. CT of the lower limbs performed in patient F3-II-1 at age 48 years demonstrated in the thigh muscles that the semitendinosus was most affected with relative sparing of the medial vastus. In the distal lower limbs there were severe fatty replacement in calf muscles and asymmetric fatty replacement of muscle tissue in the tibialis anterior muscle in the left but not the right leg.

3.4. Muscle biopsy results

The muscle biopsy in patient F1-II-1 showed mild myopathic changes. Most fibres showed internal nuclei that tended to be located in a central position. NADH staining showed some central speckled areas of increased staining (supplemental Fig. S1). All proteins analysed showed normal expression with the exception of calpain-3, which was undetectable on immunoblot (Fig. 4).

In patient F2-II-2 muscle biopsy at age 11 years old was dystrophic. A small sample was available for limited immunoanalysis but no specific abnormalities were identified
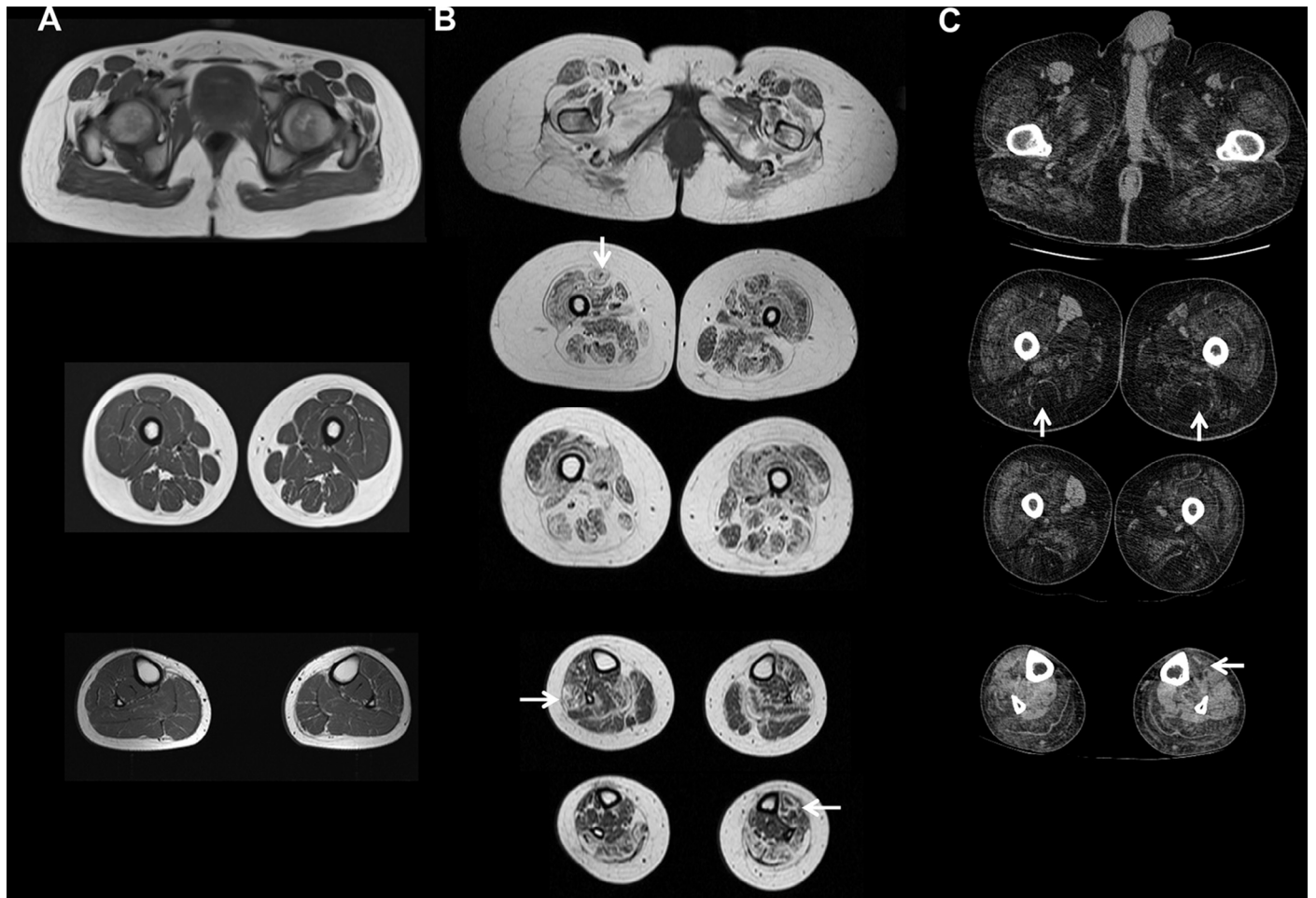

Fig. 3. Muscle imaging results. (A) MRI of patient F1-II-1 does not show any muscle pathology; (B) MRI of patient F2-II-2 demonstrating widespread fatty replacement of muscle tissue, particularly affecting the rectus femoris muscle (downward arrow), the peroneus longus (rightward arrow) muscle, and the tibialis anterior muscle, which is more affected distally (leftward arrow); (C) CT of patient F3-II-1 demonstrating prominent fatty replacement of the semitendinosus muscle (upward arrow), and the left tibialis anterior muscle (leftward arrow).
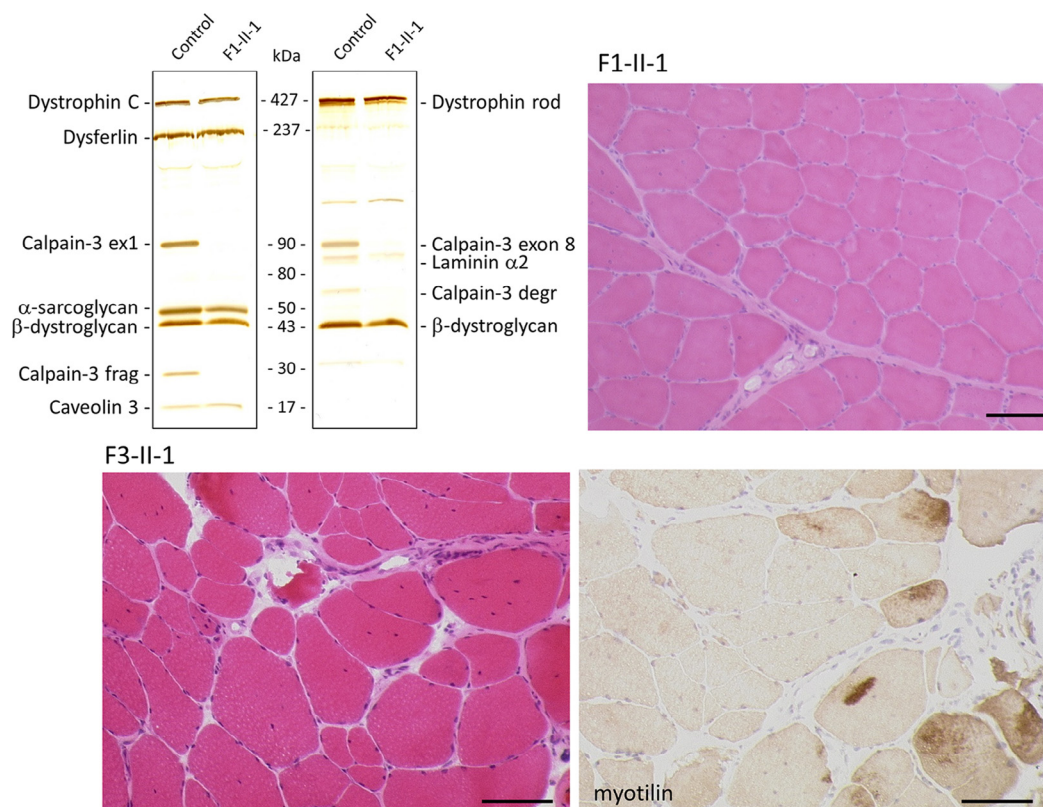
Fig. 4. Muscle biopsy analysis: Immunoblot of patient F1-II-1 showing absence of calpain-3 with two antibodies. Haematoxylin and eosin (H&E) staining shows mild myopathic features and internal nuclei. H&E staining of patient F3-II-1 shows a dystrophic muscle with protein aggregates of myofibrillar proteins (i.e. myotilin). Scale bars 100 µm.

on immunohistochemistry. Immunoblotting showed a reduction in calpain-3 using two different antibodies, with only very faint bands at 94 kDa and lower molecular weight bands not visible (not shown).

In patient F3-II-1 the muscle biopsy showed dystrophic changes with significant fibre size variation and multiple internally placed nuclei. Immunohistochemistry showed some non-specific cytoplasmic accumulation of myofibrillar proteins such as desmin and myotilin (Fig. 4). NADH staining did not show any pathological abnormality (supplemental Fig. S1). Z line streaming was seen on electron microscopy (not shown). There was insufficient tissue for Western blotting to assess calpain-3.

3.5. cDNA analysis results

cDNA analysis of patient F1-II-1 muscle biopsy was performed in order to find out the effects of the mutations at mRNA level. The mutation in TTN exon 352 (c.97863G > A p.(Trp32621*)) creates a premature termination codon, which is believed to cause nonsense-mediated decay (NMD). This is suggested by the lower peak of the mutated G>A allele as seen on the cDNA sequencing graph (Fig. 5A). A smaller amount of transcript escaping from NMD can be seen. However, cDNA sequencing is not a quantitative proof of NMD. The mutation in intron 362 (c.107377 + 1G > A) results in three different transcripts (Fig. 5B): (1) normal; (2) TTN missing the last 7 nt of Mex4 mRNA, causing frameshift and premature termination

codon; again some residual expression of this truncated product is observed; and (3) TTN missing the last 69 nt of Mex4 mRNA, causing an in-frame deletion, and replacement of 24 amino acids by an alanine residue.

3.6. Western blotting results

In order to confirm pathogenicity of the shared essential splice site mutation at the protein level, Western blot analysis of C-terminal fragments of titin was performed on muscle tissue from patient F1-II-1. This demonstrated a loss of small (<20 kD) C-terminal fragments, and reduction of higher molecular weight M10 domain-containing bands (Fig. 6), consistent with truncation of the protein on both alleles as predicted by the genetic findings and cDNA analysis. The C-terminal titin fragment resulting from the transcript lacking the last 69 nt of Mex4 was not observed, probably due to its low abundance.

4. Discussion

Classification of TTN variants identified by WES is problematic due to the large number of variants in this gene as a consequence of its size, and the prevalence of heterozygous truncating variants in up to 2% of the normal population [14–16]. We adopted a pragmatic approach, requiring two truncating variants per patient in order to consider them as putatively pathogenic. We describe four new individuals from three families with muscular dystrophy and contractures in association with compound heterozygous truncating mutations in TTN. All four individuals shared a
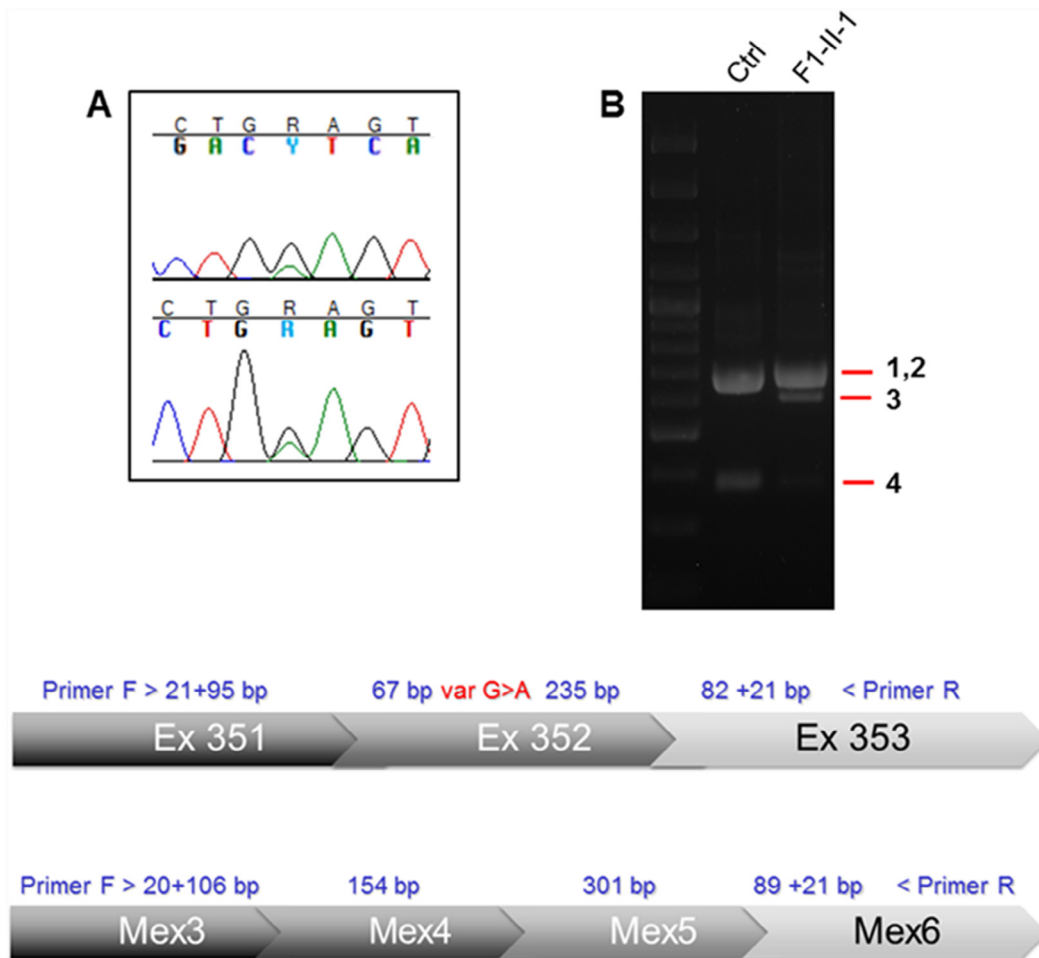
Fig. 5. Patient F1-II-1 muscle biopsy cDNA analysis of *TTN* exons ex351–353 (A) and ex361–364 (Mex3–6) (B). The sequencing graph in (A) shows the mutation in *TTN* exon 352 (c.97863G > A p.(Trp32621*)). The graph suggests unequal expression of the *TTN* alleles with a lower peak of the *TTN* allele with the variant G > A. The gel picture in (B) shows the mRNA species resulting from the consensus splice site mutation in intron 362 (c.107377 + 1G > A). The large band consists of two fragments, the normal transcript (1), and another lacking the last 7 nt of Mex4 (2), causing frameshift. The band (3) represents the transcript lacking the last 69 nt of Mex4, and the band (4) is the (normal) Mex5− isoform, weaker in the patient. The exon intron arrangements for the exons 351–353 and the exons Mex3–Mex6 are indicated.

common essential splice site mutation in the M-band of *TTN*, and in addition had a second truncating mutation (frameshift or nonsense in the A-band). Segregation amongst family members, correlation with muscle biopsy pathology, and Western blot for C-terminal titin fragments and calpain-3 are all in keeping with pathogenicity of the identified variants.

All four patients showed a common phenotype, namely onset in childhood or young adulthood of proximal muscle weakness, joint contractures and significantly elevated CK. There were several similarities between our patients and those previously reported with skeletal myopathies due to recessive *TTN* mutations. The biopsy findings in patient F1-II-1 demonstrated multiple internalised and central nuclei, similar to that observed in patients described as centronuclear titin myopathy [8]. The absence of calpain-3 on WB has been documented in association with C-terminal titin mutations as well [10,17], due to the loss of calpain-3 binding site in the titin C-terminus. Since calpain-3 has no expression in heart muscle, this is not the cause of cardiomyopathy, but rather the loss of C-terminus

itself or other ligands. The presence of prominent contractures in our patients and others has led to some patients with titinopathies being compared to EDMD [10], and a limb girdle pattern of weakness classified as LGMD2J is well documented [17]. The pattern of muscle involvement observed on muscle MRI in patient F3-II-1 showed some features also reported in other titinopathies.

Notably, cardiac complications were observed in two of four patients reported here. Patient F1-II-1 developed cardiomyopathy in childhood and patient F3-II-1 had a history of atrial fibrillation requiring permanent pacemaker insertion. This was unsurprising given the central importance of titin in both cardiac and skeletal muscle tissue [18] and the fact that the identified mutations were present in both the predominant cardiac and skeletal muscle isoforms. Heterozygous truncating *TTN* variants have been reported to cause DCM [16] and co-existence of DCM and skeletal muscle disease in individuals with two truncating *TTN* variants is well known [7,19]. Interestingly the essential splice site mutation (c.107377 + 1G > A)
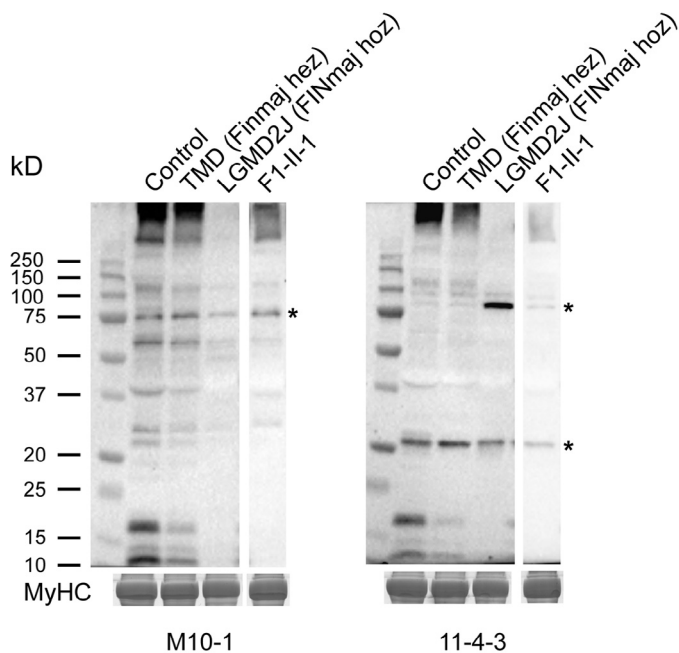
Fig. 6. Western blot for C-terminal titin fragments using two different antibodies against the M10 domain. * indicates that these bands are unspecific. TMD, tibial muscular dystrophy due to heterozygous FINmaj *TTN* mutation, LGMD2J – LGMD due to homozygous FINmaj mutation. Patient F1-II-1 with the common essential splice site mutation shows reduction/absence of titin-specific bands. Notably, the small <20 kD bands are missing, whereas the high molecular weight band(s) are reduced, in contrast to the homozygous LGMD2J where there is complete loss.

shared by all four patients has been reported by Roberts et al. (2015) in association with isolated dilated cardiomyopathy [14]. Our findings, with no clinically overt cardiac phenotype known in the heterozygous parents with this mutation, some of them already at advanced age, suggest that this variant demonstrates incomplete penetrance. One individual (F3-I-II) required cardiac valve replacement because of childhood rheumatic fever, and all other heterozygous carriers of these mutations have however been referred for cardiac surveillance. The most likely explanation would be that this essential splice site mutation in the M-band causes a dilated cardiomyopathy in combination with another (undetermined) titin mutation in trans, and early onset muscular dystrophy with contractures with or without cardiomyopathy when combined with a second truncating mutation in the C-terminal part of A-band or in the M-band titin.

The pathogenic mechanism by which heterozygous truncating *TTN* variants cause DCM has been postulated to be a dominant negative effect due to the presence of a truncated protein [14]. In normal muscle C-terminal titin undergoes cleavage by calpain-3 [20] and the Finnish founder mutation FINmaj [21] responsible for LGMD2J when homozygous and tibial muscular dystrophy when heterozygous, has been shown to result in a secondary aberrant cleavage of C-terminal titin catalysed by other proteases in the absence of calpain-3 [20]. A similar effect from other M-band *TTN* truncating mutations, such as the c.107377 + 1G > A variant reported in this study, is

not excluded although the mutation itself already makes a primary truncated product, and in the presence of an additional truncated *TTN* transcript there may be further pathogenic mechanisms involved, due to primary loss of M-band titin and its interactions [22].

These findings highlight common features in patients with recessive truncating *TTN* mutations, and provide further examples of the co-existence of cardiac and skeletal muscle disease due to recessive titin mutations. The patients reported here also demonstrate the genetic complexity of titinopathies, with implications for diagnostic evaluation, genetic counselling and cardiac surveillance of unaffected family members carrying heterozygous truncating mutations.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.nmd.2017.06.013.

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