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RESEARCH ARTICLE

Novel *TTN* mutations and muscle imaging characteristics in congenital titinopathy

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Abstract

Objective: We present clinical features, muscle imaging findings, and genetic characteristics of five unrelated Chinese patients with congenital titinopathy, emphasizing the diagnostic role of muscle MRI. **Methods**: Five patients who recessive titinopathies were recruited. All patients received muscle biopsies. Mutations were detected by panel massively parallel sequencing and confirmed by Sanger sequencing. Western blotting of muscle proteins was performed. Leg muscle MRIs were performed in four patients. **Results**: Four patients aged 1–4 years old showed delayed motor development from early infancy, while a 17-year-old boy showed only a 1-year history of exercise intolerance. Physical examination showed proximal weakness in three patients. Muscle biopsies demonstrated multiple myopathological changes, including increased internalized nuclei, multicores, central cores, and dystrophic changes. Genetic sequencing revealed compound heterozygous or homozygous novel *TTN* mutations, including six frameshift mutations, one nonsense mutation, two missense mutations, one splicing mutation, and one small nonframeshift deletion. Protein analyses revealed significant decrease of full-length titin in all patients. Thigh muscle MRIs in four patients showed prominent fatty infiltration in the upper portion of semitendinosus and the peripheral portion of gluteus medius, while the sartorius and gracilis were relatively preserved. **Interpretation**: These cases provided further evidence that *TTN* mutations are likely responsible for an increasing proportion of congenital myopathies than currently recognized. The novel mutations reported expand the mutation spectrum of the *TTN* gene. There is a characteristic pattern of muscle involvement in congenital titinopathy regardless of clinical or pathological phenotype, providing valuable clues for guiding a genetic diagnosis workup.

Introduction

Congenital myopathies are a heterogeneous group of disorders characterized by early-onset and distinctive myopathological changes on muscle biopsy, including central cores, multiminicores, nemaline rods and central nuclei.[1] The diagnosis of congenital myopathies mainly relies on clinical manifestations, muscle imaging, and histological features on muscle biopsy but is ultimately based on genetic data. Notably, muscle MRI has emerged as a useful tool for offering clinical clues in the differential diagnosis of congenital myopathy.[2] With the advent of massively parallel sequencing, an increasing number of mutations in more than 20 genes have been identified in patients with congenital myopathies.[3] Particularly, *TTN* gene has attracted considerable attention in recent years.

Titin, the largest known protein, is encoded by the *TTN* gene (OMIM #188840). This protein is expressed in both cardiac and skeletal muscles of many different species including humans, spanning the whole distance from the Z-disc to the M-band and interacting with various proteins.[4] The *TTN* gene consists of 364 exons (the first noncoding exon and 363 coding exons), and there are traditionally three main categories of titin isoforms

– N2A, N2B, and N2BA, based on the presence of the N2A and N2B elements in the I-band region.[5] The N2A isoforms (mainly expressed in the skeletal muscles) contain the N2A element but not the N2B element. The N2B isoforms only contain the N2B element, while the N2BA isoforms (mainly expressed in the heart) contain both the N2A and the N2B elements. There are also many other transcripts descripted (including Novex-1, Novex-2, Novex-3) and more yet to be identified. However, the inferred complete metatranscript (NM_001267550.1) has never been confirmed and specific exons included in this metatranscript are thought to be expressed only during embryonic development.[6]

Both dominant and recessive *TTN* mutations have been reported to cause a wide spectrum of cardiac and skeletal muscle diseases. Dominant titinopathies include hereditary myopathy with early respiratory failure (HMERF) caused by mutations in exon 344, and late-onset tibial muscular dystrophy (TMD).[7,8] Recessive titinopathies include limb-girdle muscular dystrophy 2J, young- or early-adult-onset distal titinopathy, Emery-Dreifuss-like myopathy without cardiomyopathy, and congenital myopathy with or without heart disease.[9–14] The term "congenital titinopathy" has been suggested for patients with prenatal- or infant-onset form of titinopathy, including early-onset myopathy with fatal cardiomyopathy, centronuclear myopathy, core myopathy with heart diseases, and arthrogryposis multiplex congenita with myopathy.[14] As the largest known protein, many mutations of the *TTN* gene have been detected by massively parallel sequencing, and meanwhile the spectrum of phenotypes of titinopathies is expanding. There was only one report of congenital titinopathy in Chinese with multiminicore disease recently.[15] In addition, the muscle MRI features have only been reported in several patients with congenital titinopathies.[14] In this study, we describe five patients with infant-onset motor related symptoms or myopathological changes in accordance with congenital myopathy, and also summarize the muscle MRI features in these patients in order to further broaden our understanding of congenital titinopathy.

Methods

Patients recruitment

We retrospectively reviewed all of the patients who visited our neuromuscular clinic and received muscle biopsies. Patients with myopathological findings suggesting genetic diseases received genetic analyses with consent. Genomic DNA was extracted from peripheral blood samples of patients and their parents. A neuromuscular disease panel (Agilent, USA) was designed to capture the 420 genes known to be associated with known inherited neuromuscular disorders, as we have previously described. Samples were sequenced on an Illumina HiSeq 2500 Sequencer (Illumina, San Diego, CA) and the resulting data were analyzed using the same protocol as we have previously described.[16] Variants with suspected pathogenicity were confirmed by Sanger sequencing. In addition, these variants were also tested in their parents by Sanger sequencing to confirm the segregation.

Five patients with myopathological changes suggesting congenital myopathies and genetic results showing recessive *TTN* mutations were enrolled for further analyses. Clinical information was collected, including natural histories of clinical symptoms, distributions of muscle weakness, evaluations of cardiac and pulmonary functions, as well as serum creatine kinase (CK) levels and electromyogram (EMG) findings. All patients received muscle biopsies from the biceps, and serial frozen sections of muscle tissues were stained by routine histological and histochemical methods. Variants in *TTN* gene are described referring to transcript variant-IC (NM_001267550.1). This study was approved by the Human Research Ethics Committee of Peking University First Hospital, and all participants provided written informed consent.

Western blot analysis

Western blotting (WB) of muscle biopsy samples were performed in all five patients. The detailed protocol was the same as previously reported,[17] with titin N-terminal (Sigma SAB1400284) antibodies.

Muscle MRI

Muscle MRI using 1.5-T or 3.0-T magnetic resonance scanners (GE) of the thigh was performed in two patients, and of both the thigh and calf was performed in two patients. Axial scanning in conventional T1-weighted and short T1 inversion recovery (STIR) sequences was carried out and additional images were acquired in the coronal plane when necessary. The detailed protocol for the scanning was the same as our previous study.[18] The MRI findings were independently interpreted by an experienced radiologist and a neurologist and any disagreements were resolved by consensus.

Results

Clinical and myopathological features

Clinical data from the five unrelated patients with recessive titinopathies are summarized in Table 1. There were three males and two females, aged between 1 and 17 years

old. All of the cases were sporadic, and the patients were born from nonconsanguineous parents. The ages of onset were infancy in four patients and 16 years old in patient 2. The main clinical symptoms of four patients (patients 1, 3, 4, and 5) were delayed early motor development, and of patient 2 were chest pain after intensive exercise accompanied by exercise intolerance, which worsened with time. Physical examinations showed neck flexor and proximal limb muscle weakness in patients 1, 4, and 5, while in patients 2 and 3 muscle strength was almost normal. High-arched palate, rigid spine, and contracture of the Achilles tendon were observed in patients 1, 4, and 5. No evidence of cardiac or respiratory involvement was found except for patient 4, who had congenital atrial septal aneurysm, which led to an atrial septal defect. The serum CK levels were normal in patients 1 and 5 and were mildly to moderately elevated in patients 2, 3, and 4 (200–2800 IU/L). Four patients received EMGs, and all showed myopathic changes.

Muscle biopsies revealed myopathic changes in all five patients with different features. The major myopathological changes were many fibers with multiple cores (multicores) in patient 1, a few fibers with central cores with ambiguous margins in patient 2, dystrophic changes with endomysial fibrosis and scattered regenerating fibers in patient 3, and increased internal nuclei in patients 4 and 5. In addition, other accompanying myopathological changes were also observed, including increased fiber size variation in all five patients, increased internal nuclei in patient 1 (seen in approximately 12% of muscle fibers) and patient 3 (seen in approximately 6% of muscle fibers), fiber type disproportion in patients 1 and 4, mild

Table 1. Summary of clinical features, muscle MRI manifestations, and myopathological changes

Patient	1	2	3	4	5
Gender/age at biopsy (years)	M/5	M/17	F/1	M/3	F/5
Age at onset (years)	Infant	16	Infant	Infant	Infant
Symptoms	Delayed early motor development, unable to run or jump	Chest pain after exercise, with exercise intolerance	Delayed early motor development, difficulty in sucking	Delayed early motor development, difficulty in sucking	Delayed early motor development, exercise intolerance
Neck flexion	2	5	5	3	2
Shoulder abduction	4	5	5	5	5
Elbow flexion	4	5	5	5-	4
Elbow extension	4	5-	5	5-	4
Gripping	5	5	5	5	5
Hip flexion	5	5	5	5	4
Knee flexion	4-	5	5	5	4
Knee extension	5	5	5	5	5
Ankle dorsiflexion	4	5	5	5-	5
Ankle extension	5	5	5	5	5
Rigid spine	Yes	No	No	Yes	Yes
Contractures	Achilles	No	No	Achilles	Achilles
High-arched palate	Yes	No	No	Yes	Yes
Cardiac/respiratory involvement	No/No	No/No	No/No	Atrial septal aneurysm, atrial septal defect/No	No/No
Serum CK level (IU/L)	Normal	200–2000	400–600	300–2800	Normal
EMG	Myopathic	Myopathic	NA	Myopathic	Myopathic
Muscle fatty infiltration on MRI	ST, RF, SM, BF (thigh) SOL, GC, TA, TP (calf)	ST, BF, SM, VI	NA	ST	ST, BF, SM, RF (thigh) TP (calf)
Muscle pathology	IFSV, increased internalized nuclei, multicores, FTD, subsarcolemmal mitochondrial accumulations	IFSV, center cores, fiber splitting, subsarcolemmal mitochondrial accumulations	IFSV, increased internalized nuclei, endomysial fibrosis, regenerating fibers, whorled fibers, MHC-I positive fibers	IFSV, increased internalized nuclei, FTD, regenerating fibers	IFSV, increased internalized nuclei, subsarcolemmal mitochondrial accumulations

Abbreviation: MRI, magnetic resonance imaging; CK, creatine kinase; EMG, electromyography; NA, not available; ST, semitendinosus; RF, rectus femoris; SM, semimembranosus; BF, biceps femoris; SOL, soleus; GC, gastrocnemius; TA, tibialis anterior; TP, tibialis posterior; VI, vastus intermedius; MHC-I, major histocompatibility complex class I; FTD, fiber type disproportion; IFSV, increased fiber size variation.

subsarcolemmal mitochondrial accumulations in patients 1, 2, and 5, splitting fibers in patient 2, whorled fibers in patient 3, and major histocompatibility complex class I (MHC-I) positive fibers in patient 3. The characteristic myopathological changes are presented in Figure 1.

Molecular genetics

DNA sequencing and family co-segregation analysis proved compound heterozygous mutations or homozygous mutations of the *TTN* gene in all five patients (Table 2). None of the mutations were detected in various population databases or have been reported in the Human Gene Mutation Database (HGMD). The most common type of mutation was a frameshift mutation, which appeared in 6/11 of the mutated alleles. Other types of mutations included nonsense mutations (1/11), missense mutations (2/11), splicing mutations (1/11) and small nonframeshift deletions (1/11). None of the mutations have been reported, and are novel. Three mutations were located in exon 358, while the other mutations were located in different exons. When referring to different transcripts, all mutations were predicted to impact both N2BA and N2B cardiac isoforms except for three mutations. The c.25165_25176del mutation carried by patient 1 and c.29708_29809insTGAT mutation carried by patient 2 were predicted to impact only the N2BA cardiac isoform and the N2A skeletal muscle isoform but not to impact the N2B cardiac isoform. Meanwhile, the c.35730delA mutation carried by patient 2 was predicted to be a metatransrcipt-only mutation, which was not included in either the N2A skeletal muscle isoform or the N2B cardiac isoform.

Protein analyses

The expression of titin proteins was analyzed by using biopsy muscle samples from all five patients (Fig. 2). WB

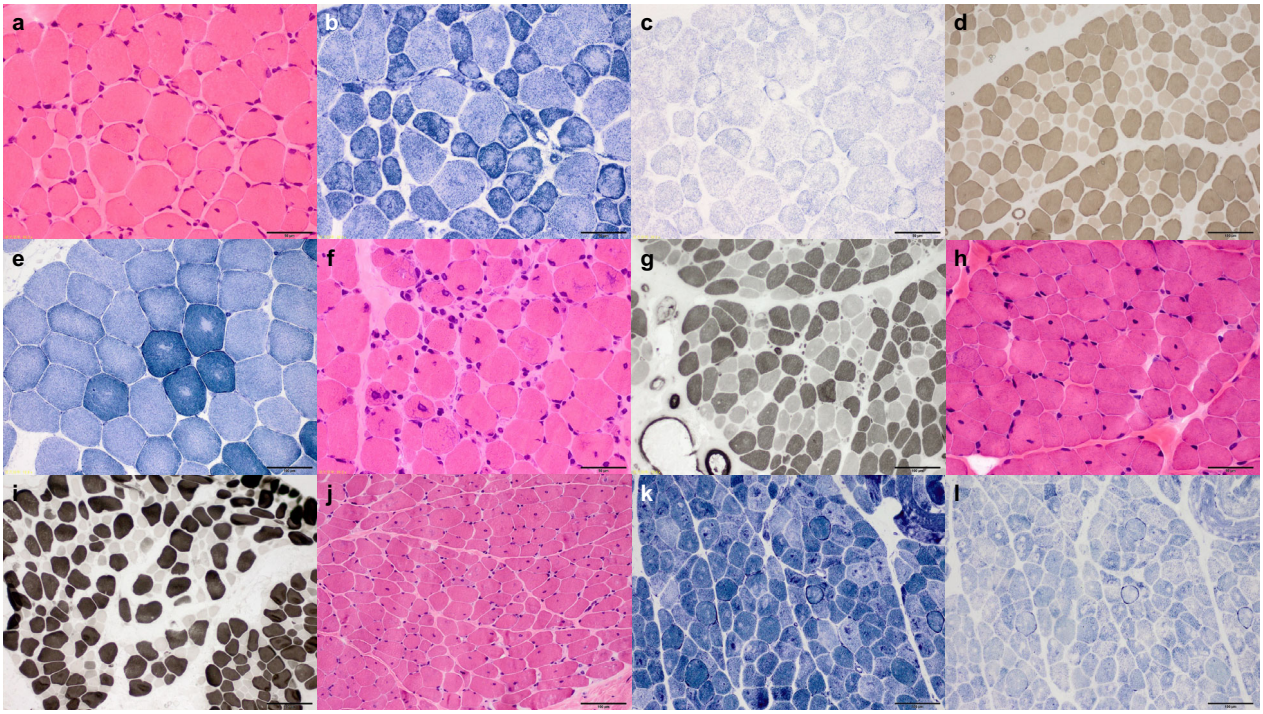

Figure 1. Myopathological and ultrastructural changes in this group of patients with congenital titinopathies. A–D belonging to patient 1 show very few centralized nuclei and peripheral basophilic deposits on hematoxylin and eosin (H&E) staining (A), multicores as well as peripheral dark deposits on reduced nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) staining (B) and succinate dehydrogenase (SDH) staining (C), and type 2 fibers significantly larger than type 1 fibers on ATPase pH 10.85 staining (D). E belonging to patient 2 shows a few center cores on NADH-TR staining. F and G belonging to patient 3 show abundant of small fibers as well as necrotic and regenerated fibers on H&E staining (F), and extremely small fibers of both types confirmed on ATPase pH 10.6 staining (G). H and I belonging to patient 4 shows centralized nuclei on H&E staining (H), and type 2 fibers significantly larger than type 1 fibers on ATPase pH 10.6 staining (I). J–L belonging to patient 5 show centralized nuclei and peripheral basophilic deposits on H&E staining (J), which are dark on NADH-TR staining (K) and SDH staining (L). Scar bars of A–C, F, H are 50 μm, of D, E, G, I–L are 100 μm

Table 2. Variants of the patients, allele frequencies in various population databases and in silico analysis of novel candidate variants

Patient	Nucleotide change	Protein change	Allele	Exon	Global allele frequency				Mutation Taster	PolyPhen-2 HumVar	SIFT	Isoforms involved		
					gnomAD	ESP6500	TGP	ExAC				N2A	N2B	N2BA
1	c.25165_25176del	p.8389_8392del	M	87	absent	absent	absent	absent	Disease causing	–	–	+	–	+
	c.51504G>A	p.W17168*	P	272	absent	absent	absent	absent	Disease causing	–	–	+	+	+
2	c.35730delA	p.G11911Afs*59	M	162	absent	absent	absent	absent	Disease causing	–	–	–	–	–
	c.29708_29709insTGAT	p.K9904Dfs*5	P	105	absent	absent	absent	absent	Disease causing	–	–	+	–	+
3	c.106233delA	p.A35412 Lfs*10	M + P	358	absent	absent	absent	absent	Disease causing	–	–	+	+	+
4	c.105800_105801delCA	p.T35267Rfs*31	M	358	absent	absent	absent	absent	Disease causing	–	–	+	+	+
	c.3880_3884delGATTC	p.D1294Kfs*6	P	23	absent	absent	absent	absent	Disease causing	–	–	+	+	+
5	c.91916T>C	p.L30639P	M	338	absent	absent	absent	absent	Disease causing	Damaging	Deleterious	+	+	+
	c.44282-2A>G	splicing	P	240	absent	absent	absent	absent	-	–	–	+	+	+
	c.59864A>T	p.N19955I	P	302	absent	absent	absent	absent	Disease causing	Damaging	Deleterious	+	+	+

Abbreviations: M, maternal; P, paternal; gnomAD, Genome Aggregation Database; ESP6500, NHLBI Exome Sequencing Project (ESP6500) Exome Variant Server; TGP, 1000 Genomes Project; ExAC, Exome Aggregation Consortium Browser.

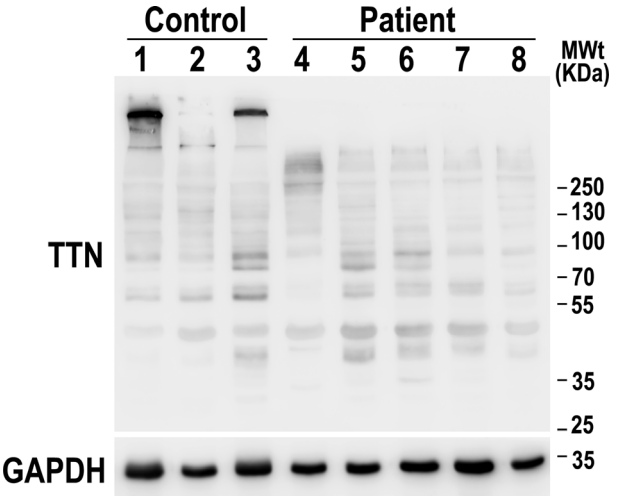

Figure 2. Western blot analyses for titin protein. Western blotting using antibody against the N-terminal of titin. Control 1–3 belong to muscle samples of healthy controls of similar ages to patients, and patient 4–8 belong to Patient 4, 5, 1, 3 and 2 respectively. All five patients present with significant decrease of full-length titin compared with controls. The splicing isoforms of titin show no obvious difference between controls and patients

results showed significant decrease of full-length titin proteins in patients compared with healthy controls. The splicing isoforms of titin proteins did not show any difference between patients and healthy controls.

Muscle MRI

In patient 1, a thigh muscle MRI showed fatty infiltration in the gluteus medius (mainly the peripheral part), rectus femoris, semitendinosus, semimembranosus, and biceps femoris. The upper part of semitendinosus was more severely involved compared to the lower part. The calf muscle MRI showed fatty infiltration of the soleus, gastrocnemius, tibialis anterior, and tibialis posterior. Edema was not observed in either the thigh or calf.

The thigh muscle MRI of patient 2 showed fatty infiltration of the semimembranosus, biceps femoris, vastus intermedius, and semitendinosus, which also showed a more severe distribution in the upper part. No edema was found in the thigh muscles.

The thigh muscle MRI of patient 4 showed fatty infiltration of the gluteus maximus, gluteus medius (mainly the peripheral part), gluteus minimus (mainly the peripheral part), and the upper part of the semitendinosus. Edema of muscles was observed in the semimembranosus, adductor magnus, and rectus femoris.

The thigh muscle MRI of patient 5 showed fatty infiltration of the gluteus medius (mainly the peripheral part),

semimembranosus, biceps femoris, and upper part of the semitendinosus, and edema of the quadriceps femoris. The calf muscle MRI showed only the tibialis posterior was affected with slight fatty infiltration.

The fatty infiltration patterns of the thigh muscles are summarized in Table 1 and presented in Figure 3.

Discussion

In this study, we reported five cases with congenital recessive titinopathies that were supported by clinical features, myopathological changes, and genetic data. Clinically, four patients younger than five years old presented with delayed motor development since early infancy. Except for the 1-year-old infant who had basically normal muscle strengths, neck flexion weakness was prominent in the three other patients, which was in keeping with previous reports.[10,14,19–21] Furthermore, rigid spine, contractures of Achilles, and high-arched palate were all observed in these three patients, showing high clinical consistency in clinical phenotypes with a higher prevalence than previous reports.[14] However, facial weakness was quite common in previous reports of patients with centronuclear myopathies caused by *TTN* mutations[10] but was not found in either of the two patients with centronuclear pathological changes in our study. It is noteworthy that the 17-year-

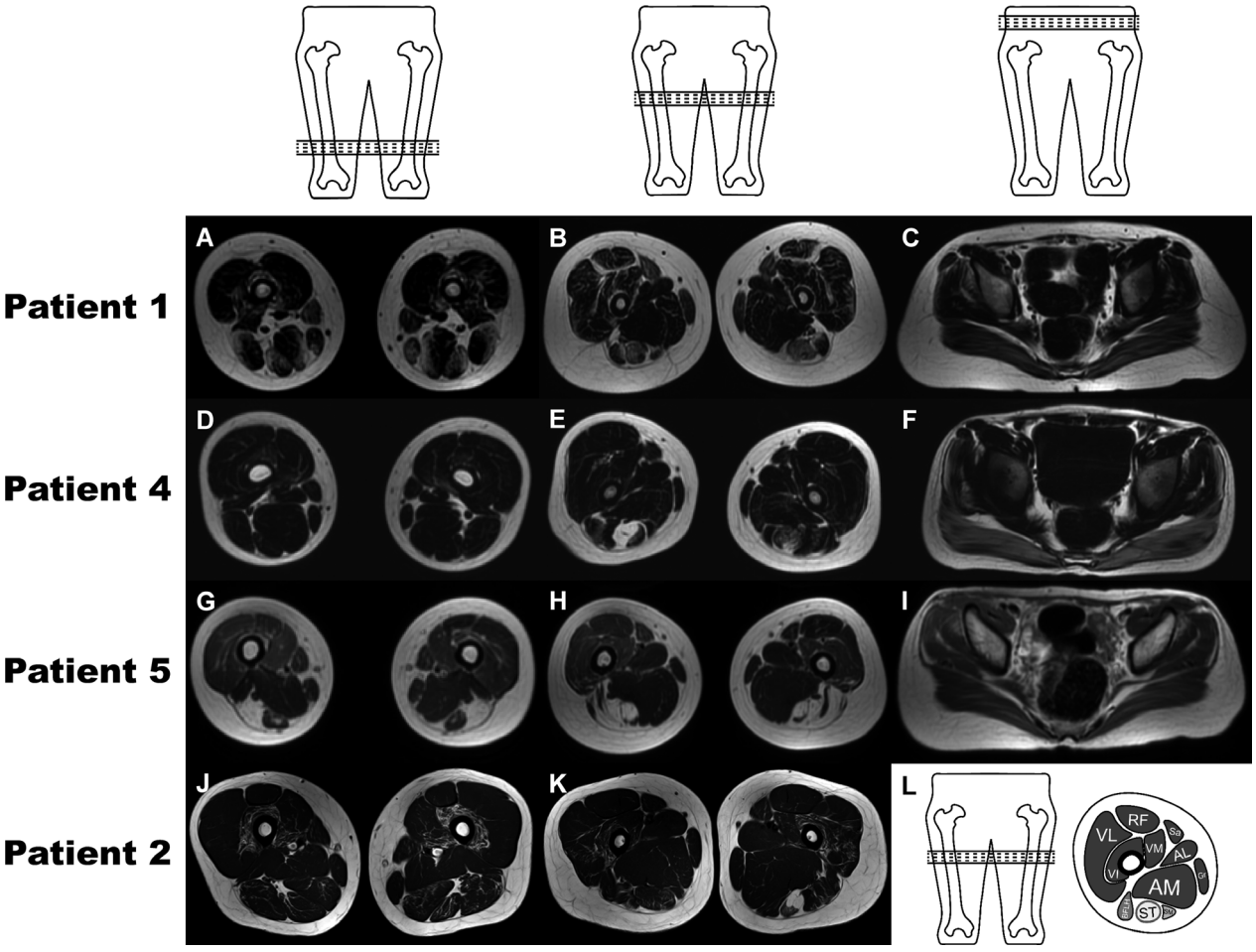

Figure 3. Muscle MRI features in patients with congenital titinopathies. A–K are T1-weighted muscle MR images, with transverse sections at the levels of lower-thigh, upper-thigh and pelvic separately. A–C of patient 1 show fatty infiltration of the peripheral part of gluteus medius (GMed), mainly the upper portion of the semitendinosus (ST), rectus femoris (RF), semimembranosus (SM), and biceps femoris (BF). D–F of patient 4 show fatty infiltration of the gluteus maximus (GMax), the peripheral part of the GMed, gluteus minimus (GMin), and mainly the upper portion of the ST. G–I of patient 5 show fatty infiltration of the peripheral part of GMed, mainly the upper portion of the ST, BF and SM. J–K of patient 2 show fatty infiltration of mainly the upper portion of the ST, BF, SM and vastus intermedius (VI), with an asymmetrical degree between both sides. L is a schematic representation of muscle infiltration at the level of upper thigh, showing predominant fatty infiltration of the ST

old patient in our cohort presented with only mild motor symptoms until his adolescence, and his muscle strengths were essentially normal with no specific physical signs. However, both the myopathological and genetic data from this patient support the diagnosis of congenital titinopathy, which further expand the phenotype of congenital titinopathy.

Cardiac or respiratory involvement was very rare in this group of patients, and only one patient was found to have an atrial septal defect caused by a congenital atrial septal aneurysm. However, only one mutation from patient 1 and one from patient 2 were predicted not to impact the N2B cardiac isoform, while the other mutation of patient 2 was predicted to be a metatransrcipt-only mutation. The relatively low prevalence of cardiac involvement and the inconsistency of isoform analysis, which is contrary to previous research,[14] might be partly due to the relatively young ages of the patients at the time of visit. Further close follow-ups to monitor cardiac function should be performed.

All mutations in this study are novel, which might be due to the different ethnic background of the enrolled patients in our study. Truncating mutations were the most common type in this study. According to the algorithm for the clinical interpretation of genetic findings in titin proposed by Savarese et al.,[20] patients with biallelic truncating mutations can be directly diagnosed as titinopathy. In this way, four patients in our study belonged to this group. For patient 5, a single truncating mutation caused by splicing a mutation together with a missense mutation were detected on the paternal allele, while a different missense mutation was detected on the maternal allele. As the clinical and pathological features strongly supported the diagnosis of congenital titinopathy, the missense mutation c.91916T>C on the maternal allele was thought to be pathogenic. Further assessment with a computational tool called TITINdb showed mCSM score −1.424 and destabilizing, PolyPhen-2 score 0.982, and Condel score 0.563794569134, which was deleterious.[22] Besides, this variant was neither found in ExAC nor in 1000G, and the amino acid affected was extremely conserved among species, all suggesting its pathogenicity. However, the pathogenicity of the c.59864A>T variant is unclear and needs further confirmation. Western blotting demonstrated significant decrease of the full-length titin protein in all five patients, further proving the pathogenicity of the mutations. The mutations detected in our study were distributed nearly throughout the whole *TTN* gene, from the N-terminal to the C-terminal with a weak tendency of focus in the C-terminal region.

The muscle MRI results in our study showed that the upper portion of the semitendinosus and the peripheral portion of gluteus medius were the most commonly affected in all patients. Although the clinical phenotypes and myopathological changes varied between the different patients, the muscle MRI shared similar patterns of involvement. Muscle MRI studies of congenital titinopathies are limited, and in one previous study, the semitendinosus, together with gluteal and other hamstring muscles, was also involved in all three patients who received lower limb MRIs.[14] Other thigh muscles were involved to different degrees in different patients, while the adductors, sartorius, and gracilis were relatively preserved, which is also in accordance with previous studies.[14] In previous reports of patients with HMERF, the involvement of semitendinosus also showed high sensitivity, which varied between 93 and 100%.[7,23,24] However, involvement of the sartorius and gracilis in HMERF might be distinct from congenital titinopathy, but comparative studies in a larger cohort are needed.[24] In addition, in TMD patients, involvement of the semitendinosus was also observed in all 14 of 22 patients with both thigh and leg muscle involvement.[25] The relatively similar distribution pattern on the thigh muscle MRI of *TTN*-related myopathies indicates that common muscle MRI features might be shared independent of the clinical and pathological phenotypes.

On the other hand, congenital myopathies with the same myopathological changes caused by mutations of different genes also exhibited different patterns of muscle MRI features. For instance, in multiminicore disease, selective involvement of the vasti, sartorius, and adductor magnus and relative sparing of the rectus, gracilis and adductor longus was a specific feature in congenital myopathies caused by recessive *RYR1* mutations,[26] while in *SPEN1*-related multiminicore diseases, atrophy of the semimembranosus and fatty infiltration of adductor magus and the short head of biceps femoris were characteristic.[27] Although patients with congenital myopathies with different gene mutations might share common clinical features and similar myopathological changes, muscle MRI could assist in the differential diagnosis of different genotypes.[2]

Our findings provided further evidence that *TTN* mutations are likely responsible for a greater proportion of congenital myopathies than previously understood, including central core myopathy and central nuclei myopathy. The novel mutations reported here expand the mutational spectrum of the *TTN* gene. Fatty infiltration of the upper portion of semitendinosus and the peripheral portion of the gluteus medius on a thigh MRI is a characteristic pattern of muscle involvement in congenital titinopathy regardless of clinical or pathological phenotype, which could provide valuable clues for guiding genetic diagnostic workup.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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