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Novel dominant distal titinopathy phenotype associated with copy number variation

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Abstract

The aim of this study was to analyze patients from two distinct families with a novel distal titinopathy phenotype associated with exactly the same CNV in the *TTN* gene. We used an integrated strategy combining deep phenotyping and complete molecular analyses in patients. The CNV is the most proximal out-of-frame *TTN* variant reported and leads to aberrant splicing transcripts leading to a frameshift. In this case, the dominant effect would be due to dominant-negative and/or haploinsufficiency. Few CNV in *TTN* have been reported to date. Our data represent a novel phenotype–genotype association and provides hypotheses for its dominant effects.

Introduction

Titin is the largest known protein and is encoded by the *TTN* gene of 363 exons. Introduction of NGS in clinical practice allowed to increase the number of genotype–phenotype associations of titinopathies.[1] The mode of inheritance is either autosomal dominant or recessive. The combination of deep phenotyping associated with RNA molecular analyses, western blot (WB), and functional studies is often essential for the interpretation of *TTN* variants.[2, 3, 4, 5] Autosomal dominant *TTN* mutations cause two skeletal muscle disorders. Tibial muscular dystrophy (TMD), a late-onset distal myopathy exclusively affecting the anterior compartment of the lower leg muscles,[6] is the most frequent adult-onset myopathy in Finland. Patients are heterozygous for the FINmaj mutation, an 11-bp insertion/deletion in the last exon of the gene.[6] Hereditary myopathy with early respiratory failure (HMERF)[7] is characterized by proximal and distal weakness of the four limbs associated with early onset respiratory involvement. Most HMERF pathogenic variants are missense mutations, always localized in *TTN* exon 344.[7]

Few copy number variations (CNV) in *TTN* have been reported to date, which is surprising, given the large size of the gene. This may be explained by the variability of capture technologies, bioinformatic pipelines for CNV detection.[8, 9] Here we described patients from two distinct families with a novel distal phenotype of titinopathy, in whom we identified by NGS an identical CNV.

Materials and Methods

Patient consents

Informed consent signed by patients were obtained for all samples. The study was approved by the ethical guidelines issued by our institutions for clinical studies in compliance with the Helsinki Declaration. Patients or parents gave informed consent for the genetic analysis according to the French legislation (Comité de Protection des Personnes OUEST 6-CPP1128HPS3 IDRCB-2018-AO2287-48).

Molecular analyses

The *TTN* gene (NM_001267550.1) CNV was previously identified in patients from families 1 and 2 by NGS targeted on 185 genes[8] and 54 genes,[3] respectively. The pathogenicity of the identified *TTN* variant was assessed through a set of criteria previously reported,[8] according to the American College of Medical Genetics and Genomics (ACMG) guidelines.[10]

Single-nucleotide polymorphisms (SNP) within and surrounding the TTN locus were analyzed by NGS and the corresponding genotypes were compared between the three patients.

Morphological analyses of muscle and immunohistochemistry

Open muscle biopsies were performed, frozen, and fixed following previously described standardized methods and analyzed with histochemical, histoenzymatic, techniques with light, and electron microscopy.[11], [12]

Transcripts analysis

mRNAs extraction from muscle biopsy, RT-PCR and Sanger sequencing, and RNAseq experiments were performed as previously described.[2] Primers used for cDNA sequencing are indicated in Table S1.

Western blot analysis

Proteins solubilization from muscle biopsies and WB analyses on agarose and acrylamide gel were performed as described in [2]. Primary antibodies, anti-titin specific for C-terminal part M10-1 (Rabbit; 1:1000; kindly provided by Dr Isabelle Richard[13]), and anti-titin N-terminal (Mouse; 1:1000; Sigma SAB1400284, St-Louis, MO) were incubated overnight at 4°C in Odyssey® blocking buffer in PBS.

Results

Clinical and imaging findings

Family 1 Patient 1 (P1) and patient 2 (P2), her daughter, were affected with proximodistal lower limb predominant myopathy. P1 has 68 years old and developed, from the age of 40 years old, difficulty for climbing stairs and walking on sloping ground. Few years later, she developed a stepping walking. Neurologic examination showed atrophy of hamstrings, pseudohypertrophic aspect of the calves (Fig. 1 B1–3), and proximodistal weakness of the lower limbs. Ankle dorsiflexion was affected as was plantar flexion (4/5). CK levels were mildly elevated between 200 and 500 UI/L. P2, her daughter, 47 years old, developed from 2013 progressive left calf atrophy and weakness for plantar flexion. At the end of 2013, neurological examination showed mild bilateral posterior thigh atrophy, with marked asymmetric bilateral calf and left tibialis anterior atrophy (Fig. 1B6,7). Muscular testing did not show proximal weakness. Left dorsiflexor was at 4/5 and left plantar flexor was at 3/5. Upper limbs were normal. CK levels were normal and electromyography showed a myogenic pattern. Cardiac echography and electrocardiogram were normal. Whole-body muscular MRI showed involvement with fatty infiltration of gracilis, left tibialis anterior, and medialis gastrocnemius (Fig. 1B8). P2 sisters, P3 aged 52 years, and P4 aged 51 years currently had no muscular complaints. However, neurological examination showed for both, a discrete amyotrophy of the unilateral right anterior leg, more marked for P3, at the limit of perception for P4. P4 has a thin face with masseter amyotrophy. Muscle testing was normal in all four limbs, CK levels were normal, and cardiological tests (including echography) were normal. P3 muscle scan showed discrete fatty infiltration in the calf (Fig. S1A).

Figure 1

Morphological, histopathological, and ultrastructure (EM) characteristics of patients. (A) Pedigree of the family 1. Patients heterozygous for the CNV are marked "Del<11–18>/wt". P3 and P4 are colored in grey as there are paucisymptomatic. (B1,2) Calf pseudohypertrophy and posterior thigh atrophy in P1. (B3) P1 T1 weighted whole-body muscular MRI: Bilateral posterior thigh, tibialis anterior, and gastrocnemius fatty replacement. (B8) Right tibialis anterior and bilateral calf atrophy in P2. (B4,9) Biopsy of tibialis anterior for P1 and gastrocnemius for P2. HE staining of patients P1 and P2: multiple muscular fibers with internal and centralized nuclei (B5,10) NADH staining show multifocal areas with reduction of staining compatible with minicores in P2. (C) Pedigree of the family 2. P6 is colored in grey as she is paucisymptomatic. (D1) Atrophy of lower limbs in patient 5 (D2) T1 weighted muscular MRI in P5. Diffuse fatty replacement of muscles in thigh posterior compartment and of tibial anterior. (D3–6) Muscle biopsy of left tibialis anterior in P5, (D3,4) HE staining: atrophic fibers, intracytoplasmic vacuoles (D3), eosinophilic aggregate (D4), which appears not fuschinophilic on Gomori Trichrome (D5), NADH staining (D6), showing the absence of reactivity in multiple areas.

Family 2 Patient 5 (P5) developed difficulty for walking and when 50 years old developed difficulty in climbing stairs. When 65, he presented with a stepping walking and used a cane 4 years later. When 71, he presented weakness in the right upper limb and often fell and used a wheelchair soon after. The last neurologic examination, when he was 80, showed considerable proximal (in the posterior compartment of thighs) and distal atrophy of lower limbs, distal atrophy of upper limbs, proximodistal weakness of lower limbs with predominance in distal segments, and distal weakness of upper limbs (Fig. 1D1). CK levels were mildly elevated (300 UI/l). Cardiac echography showed a slight left ventricular hypertrophy. Muscular MRI showed fatty infiltration of posterior thigh muscles, gracilis, adductors, asymmetric involvement of lateralis vasti and right fatty infiltration of left intermedius vastus, fatty infiltration of tibialis anterior muscles, and right gastrocnemius medialis (Fig. 1D2). P6, his daughter, 56 years old, initially reported as asymptomatic, has very discrete clinical abnormalities: anterior leg deficit predominantly on the right and childhood Achilles' tendon retraction (has never been able to squat with feet on the ground) with toe retraction, predominantly on the right (Fig. S2B). CK levels were mildly elevated (575 UI/L). Muscle scan showed bilateral tibialis anterior atrophy and fatty degeneration. P7, the mother of P5, developed when 50-year-old difficulty for walking with frequent falls. Neurologic examination when she was 80, showed marked bilateral tibial anterior, gastrocnemius, and glutei atrophy. Upper limbs were spared. CK levels were slightly elevated (242 UI/L). Cardiac evaluation was normal. Muscle MRI showed bilateral posterior compartment of thigh muscles and tibial anterior fatty replacement. Maternal aunt (P8) and grandmother (P9) were reported to be affected with the same presentation but were not evaluated by a neurologist. No family link with Family 1 was reported, but both families were from the northwest of France.

Muscle morphological analysis

Analysis of the muscles biopsies of family 1 patients revealed multiple muscular fibers with internal and centralized nuclei (Fig. 1B4,9) and the presence of multifocal areas with reduction of staining compatible with minicores with NADH staining (Fig. 1B5,10). The muscle biopsy of left tibialis anterior of patient 5 (Fig. 1D3,4) shows atrophic fibers, intracytoplasmic vacuoles (Fig. 1D3) H&E staining, eosinophilic aggregate (Fig. 1D4), which appeared not fuschinophilic on Gomori Trichrome (Fig. 1D5). NADH staining (Fig. 1D6), showed the absence of reactivity in multiple areas, with minicores-like aspects.

Molecular results

NGS analyses identified in P1, P2, P3, P4 from family 1 and in P5, P6, P7 from family 2, a heterozygous deletion of *TTN* exons 11 to 18, with deletion breakpoints in introns 10 and very closed to exon–intron junctions. Genomic Sanger sequencing confirmed the precise breakpoints, c.1662+15_3101-3del (Fig. 2A),[8] resulting in a tiny 16 nucleotides long recombinant intron between exons 10 and 19. This CNV is not reported in the database of genomic variants (http://dgv.tcag.ca/dgv/app/home). NGS analyses did not detect other *TTN* variants *in trans* no*r* other shared variants of interest among both families.

Figure 2

Genomic characteristics of the CNV, transcripts, and protein analyses. (A) cDNA sequencing of the titin c.1662+15_3101-3del allele using primers in exons 10 and 21 detected the deleted transcripts and showed retention of the first 14 bases of intron 10 and the last two bases of intron 18. This leads to a frameshift and a stop codon occurrence 11 codons downstream from the shift, in exon 19. (B) RNAseq titin transcripts pattern of P1 and a control muscle sample. Upper panel: control (blue) and P1 (red) sashimi plots from IGV. Lower panel: focus on the reading depth at the splice junctions for the control and P1 muscles. Read depth was specified in black numbering. P1 shows abnormal splicing from intron 10 to intron 18 (represented in a green dashed line), corresponding to the CNV. In red, all other junctions were similar to those of the control sample. Intronic retention of part of intron 13 was detected in the normal allele, but this was also present in the control sample. Sequencing depth of intron retention was 112 reads in intron 10 compared to 294 total reads in adjacent exon 10 (38%), 103 reads in intron 18 compared to 216 total reads in exon 19 (48%), (C) WB showing normal titin N2A isoform band at 3,8MDa from P1 and P2 biopsies. “I703” is a positive control with C-term homozygous truncated titin (c.106139dupA, p.(Ser35381Glufs*4)) and “Ctrl” is a negative control from a healthy patient (Ctrl) (biopsy from Myobank-AFM).

Comparison of SNPs within and surrounding the TTN locus showed a common haplotype of at least 42 SNP (271 kb) between both families, suggesting a common ancestor shared by the two families (data not shown). However, the exact limits of the commonly linked region were not defined with the NGS analyses performed.

Titin cDNA analysis from the P1 muscle sample revealed that two nucleotides in intron 18 and 14 nucleotides in intron 10 are retained in the deleted transcripts. This intron retention results in frameshift and a stop codon occurrence 11 codons downstream from the shift, in exon 19 (Fig. 2A). RNAseq analysis confirmed the retention of the 16 nucleotides recombinant intron in the mutant allele (Fig. 2B). Sequencing depth of intron retention showed a proportion of deleted transcripts slightly lower than 50%, suggesting that nonsense-mediated RNA decay (NMD) of the deleted allele is probably absent or minimal. No additional aberrant transcript was detected. The expression of the mutated allele was evaluated with an expression ratio of four SNPs located downstream of the deletion. We found expression levels of the alleles associated with the CNV of 39% to 45% (Fig. S2), highlighting that nonsense-mediated decay of the deleted allele is probably absent or minimal. RNAseq analyses excluded the presence of elusive variants *in trans* not identified at the DNA level.

If not degraded by the NMD, the deleted transcripts are predicted to produce a truncated protein with an approximate molecular weight of 60 kDa. P1 and P2 muscle samples analyses by WB showed a titin band of normal size with C-term and N-term antibodies corresponding to the normal allele (Fig. 2C). To detect the predicted truncated protein, we performed WB analysis on acrylamide 12% gel, using an N-terminal anti-titin antibody against the 110 starting amino acids of titin (encoded by exon 1 to part of exon 4 of the *TTN* gene) that are located upstream of the deletion. But it was not possible to conclude on the absence or presence of the truncated 60 kDa band, due to the presence of several bands in this size range corresponding to shorter N-term physiological or degradation forms of titin (Fig. S3).

Discussion

We present a novel phenotype of dominant titinopathy due to a truncating CNV in the *TTN* gene. The patients presented a very slowly progressive proximodistal muscular involvement of late onset (between fourth and fifth decades) and predominantly affecting the lower limbs. The older patient (P5, 68 years old) developed also distal upper limb involvement in the eighties. Interestingly, the younger patient (P2, 47 years old) developed a very clear asymmetric calf atrophy followed by left tibialis anterior atrophy as the first symptoms. This particular phenotype with initially asymmetric calf involvement is not a typical feature associated with titinopathies. The presence of internal nuclei and minicores on histological sections and predominant semitendinosus involvement in whole-body muscular MRI at thigh level was supplemental arguments for diagnosis of titinopathy. Interestingly, familial segregation studies revealed that three relatives aged 51, 52, and 56 years, in the same age range as the younger (P2) patient, had milder phenotypes with mildly elevated CK and asymmetric anterior legs muscle injury.

We found in patients from both families a heterozygous deletion of exons 11 to 18 and the most of flanking introns in *TTN* with identical genomic breakpoints. There could be a link between the two families since we identified a commonly linked region of at least 271 kb, suggesting a common ancestor. Both families were from the northwest of France, but without being able to identify a common small village or island. The size of the common haplotype suggests a very ancient mutation event. Given the variable expression of the CNV reported in the study, and the possible technical difficulties in detecting it, we speculate that it may be relatively frequent but not detected in patients.

Segregation studies identified the CNV in the three relatives with a milder phenotype. As for many dominant diseases, the molecular basis of this variable expressivity is not deciphered; it could be due to modifier genes, environmental and/or epigenetic factors. Furthermore, the complexity of titin alternative splicing in the region of the CNV, probably variable according to patients' age and muscles,14 is not considered since RNAseq provides a snapshot of the transcriptome in the analyzed muscle (i.e., that specific anatomic muscle at that specific age).

Despite the large size of the *TTN* gene, few CNVs have been reported, if compared to the *DMD* gene for example. In addition to technical reasons, this can also be explained by the low size of introns (since usually the breakpoints of CNVs are in intronic regions) and the fact that most of the *TTN* exons are symmetric (with a number of nucleotides multiple of 3). Probably some small CNVs result in a still functioning protein without any clinically relevant phenotype, as reported for deletions in exon 48 in *DMD*.15, 16 One published *TTN* CNV is a recessive large deletion of exons 34 to 41, associated *in trans* with a frameshift mutation p.(Lys3596Asnfs*), reported in a family with a severe phenotype (loss of ambulation before the age of 40 and marked hyperCKemia).17 The second CNV previously described is a deletion of exons 346 to 362 (A-band) predicted to cause frameshift with dominant inheritance9 with early onset skeletal muscle involvement and dilated cardiomyopathy developing in later decades. No WB data were reported to determine if the truncated titin was present or not.

In our report, muscle RNAseq studies detected, in addition to the exons 11–18 deletion, aberrant splicing with retention of the tiny 16 nucleotides recombinant intron, which is presumably too small to be recognized by splicing factors and leads to a stop 11 codons downstream from the frameshift. In the absence of detectable NMD, the deletion is predicted to lead to a titin protein of 60 kDa lacking a large part of I-band, all A-band, and M-band. This predicted truncated protein would preserve the insertion domains in the Z-disk, suggesting a possible dominant-negative effect. However, as WB studies could not confirm nor exclude the presence of the deleted protein, degradation of the highly truncated protein, due to its probable instability and non-functional conformation, cannot be excluded. This suggests a possible additional haploinsufficiency effect.

We thus report the identification of the first *TTN* CNV with skeletal involvement only and dominant inheritance. Although we have extensively tried to assess the effect of the deletion at the protein level using a comprehensive approach, the effect is still unclear. Additional functional studies would be useful to assess the effect of this deletion on skeletal muscle cells.

Author Contributions

A.P., R.J.M., C.M., and M.C. conceived and designed the study; A.P., R.J.M., F.C., C.T., D.L., H.P., E.U.C., D.G., N.L., M.M., E.L., V.R., K.G., P.R., M.K., C.M., and M.C. acquired and analyzed the data; A.P., R.J.M., and M.C. wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Supporting information

Table S1. Primers used for gDNA and cDNA analyses.

Figure S1. Additional phenotype data for P3 and P6. (A) P3 muscle scan showed discrete fatty infiltration in the calf (B) P6 clinical photography's, anterior leg deficit predominantly on the right and childhood Achilles' tendon retraction with toe retraction, predominantly on the right (C) P6 muscle scan showed bilateral tibialis anterior atrophy and fatty degeneration.

Figure S2. Evaluation of Nonsense-Mediated mRNA Decay (NMD) of the mutated allele using SNP expression levels. The four SNPs for which we could assign each allele to the mutated or wild-type transcript are represented on the top. For each of them, the percentage of expression of the allele on the mutated transcript and those on the wild-type transcript are indicated. They were obtained by performing a ratio based on the number of reads for each allele obtained by RNAseq in muscles from P1 and P2 patients. This analysis revealed on four locations of transcripts 39%, 45%, 44%, and 45% expression level of the mutated transcript, suggesting absence or minimal NMD.

Figure S3. Western blot analysis of P1 and P2 to detect potential titin truncated protein. Western blot analysis of P1 and P2 using SDS-12% Acrylamide gels, Coomassie coloration (A), and revelation by a titin N- terminal antibody (Sigma SAB1400284) (B). The predicted 60 kDa titin band corresponding to the truncated protein due to the c.1662+15_3101-3del variant is not detectable, due to the presence of several bands in this size range corresponding to shorter N-term physiological or degradation forms of titin.

Click here for additional data file. (518K, pdf)

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