The importance of an integrated genotype-phenotype strategy to unravel the molecular bases of titinopathies
Aurélien Perrin, Raul Juntas Morales, Francois Rivier, Claude Cances, Ulrike Walther-Louvier, Charles van Goethem, Corinne Thèze, Delphine Lacourt, Henri Pégeot, Reda Zenagui, et al.

To cite this version:
Aurélien Perrin, Raul Juntas Morales, Francois Rivier, Claude Cances, Ulrike Walther-Louvier, et al.. The importance of an integrated genotype-phenotype strategy to unravel the molecular bases of titinopathies. Neuromuscular Disorders, Elsevier, 2020, 10.1016/j.nmd.2020.09.032 . hal-02985653

HAL Id: hal-02985653
https://hal.archives-ouvertes.fr/hal-02985653
Submitted on 11 Nov 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
The importance of an integrated genotype-phenotype strategy to unravel the molecular bases of titinopathies

Aurélien Perrin\textsuperscript{a,1}, Raul Juntas Morales\textsuperscript{a,1}, François Rivier\textsuperscript{c}, Claude Cances\textsuperscript{d}, Ulrike Walther-Louvier\textsuperscript{c}, Charles Van Goethem\textsuperscript{a}, Corinne Thèze\textsuperscript{a}, Delphine Lacourt\textsuperscript{a}, Henri Pégeot\textsuperscript{a}, Reda Zenagui\textsuperscript{a}, Emmanuelle Uro-Coste\textsuperscript{e}, Nicolas Leboucq\textsuperscript{f}, Edoardo Malfatti\textsuperscript{g,h}, Constance Delaby\textsuperscript{i}, Sylvain Lehmann\textsuperscript{i}, Valérie Rigaut\textsuperscript{j,k}, Michel Koenig\textsuperscript{a}, Mireille Cossée\textsuperscript{a,}\textsuperscript{s}

\textsuperscript{a}Laboratoire de Génétique Moléculaire, Centre Hospitalier Universitaire de Montpellier, LGMR, Université de Montpellier, France
\textsuperscript{b}Service de Neurologie, Centre de référence des Maladies Neuromusculaires AOC (Atlantique-Occitanie-Caraïbe) Centre Hospitalier Universitaire de Montpellier, France
\textsuperscript{c}Département de Neurologie Pédiatrique and Centre de Référence Maladies Neuromusculaires AOC (Atlantique-Occitanie-Caraïbe), CHU Montpellier, PhysMedExp, Université de Montpellier, INSERM, CNRS, Montpellier, France
\textsuperscript{d}Service de Neuropédiatrie, Centre Hospitalier Universitaire de Toulouse, Centre de référence des Maladies Neuromusculaires AOC (Atlantique-Occitanie-Caraïbe), France
\textsuperscript{e}Département d’anatomie et cytologie pathologiques, CHU Toulouse, France
\textsuperscript{f}Neuroradiologie, CHU de Montpellier, 34090 Montpellier, France
\textsuperscript{g}Service Neurologie Médicale, Centre de Référence Maladies Neuromusculaires Nord-Est-Ile-de-France, CHU Raymond-Poincaré, Garches, France
\textsuperscript{h}Univ Montpellier, INSERM U1179 UVSQ-INSERM Handicap Neuromusculaire: Physiologie, Biothérapie et Pharmacologie appliquées, UFR des sciences de la santé Simone Veil, Université Versailles-Saint-Quentin-en-Yvelines, France
\textsuperscript{i}Laboratoire de Biochimie et Protéomique Clinique-IRMB, CHU Montpellier, Unité Montpellier, INSERM U1183 Montpellier, France
\textsuperscript{j}AOC (Atlantique-Occitanie-Caraïbe) Reference Center for Neuromuscular Disorders, Aquitaine, France
\textsuperscript{k}Department of Pathology, Centre Hospitalier Universitaire Montpellier, Montpellier, France

Abstract

Next generation sequencing (NGS) has allowed the titin gene (TTN) to be identified as a major contributor to neuromuscular disorders, with high clinical heterogeneity. The mechanisms underlying the phenotypic variability and the dominant or recessive pattern of inheritance are unclear. Titin is involved in the formation and stability of the sarcomeres. The effects of the different TTN variants can be harmless or pathogenic (recessive or dominant) but the interpretation is tricky because the current bioinformatics tools can not predict their functional impact. Moreover, TTN variants are very frequent in the general population. The combination of deep phenotyping associated with RNA molecular analyses, western blot (WB) and functional studies is often essential for the interpretation of genetic variants in patients suspected of titinopathy. In line with the current guidelines and suggestions, we implemented for patients with skeletal myopathy and with potentially disease causing TTN variant(s) an integrated genotype-transcripts-protein-phenotype approach, associated with phenotype and variants segregation studies in relatives and confrontation with published data on titinopathies to evaluate pathogenic effects of TTN variants (even truncating ones) on titin transcripts, amount, size and functionality. We illustrate this integrated approach in four patients with recessive congenital myopathy.

Keywords: Titinopathy; Congenital myopathy; Deep phenotyping; Titin transcripts; Titin western-blots.

\textsuperscript{a} Corresponding author.
\textsuperscript{E-mail address: mireille.cossée@inserm.fr (M. Cossée).}
\textsuperscript{1} These authors contribute equally to this work.
1. Introduction

TTN encodes titin, a giant sarcomeric protein [1] that plays important functional and structural roles in the sarcomere. Mutations in TTN are associated with a broad range of skeletal and/or cardiac phenotypes with autosomal dominant or recessive inheritance. Since the widespread use of Next Generation Sequencing (NGS), the exhaustive analysis of the 364 coding exons of TTN revealed an increasing number of reported TTN variants. Nevertheless, assessing the pathogenicity of the identified variants, in particular missense ones, is often difficult, due to the clinical heterogeneity of the pathology, the large size of the gene, the frequency of TTN variants in the general population (including 0, 36% of truncating variants affecting all transcripts [2]) and the complexity of gene expression (many different isoforms are expressed over time and tissue location) [3]. Different types of mutation have been reported as pathogenic, but their consequences on transcripts and protein, in particular loss or gain of titin function, are not fully elucidated. In myopathic titinopathies, several studies demonstrated that TTN truncating mutations are associated with loss function leading to recessive titinopathies, including congenital titinopathies. Interpretation of non-truncating (mainly missense) variants remains more difficult. Their localization in important functional domains (such as the titin kinase domain) and interaction domains (with proteins such as calpain-3, actin, myosin, nebulin, or one of the numerous interacting proteins (for review, see [4])) may suggest a potential pathogenicity. Specific variants in the last exon (including missense variants) and all the truncating variants causing the loss of the C-terminal portion (i.e. M-band truncating variants) causes the loss of the interaction with calpain-3 and its reduction at western blot, may contribute to disease pathogenesis, raising the possibility that the decrease of calpain-3 level may contribute to disease pathogenesis [5–9].

Expert laboratories have worked on the establishment of specific recommendations for TTN variants interpretation [10], based on familial and genetic data (number and type of variant(s)), histopathology [11], magnetic resonance imaging (MRI) [12] or clinical features [13]. In line with the current guidelines and suggestions [2,3,14] the combination of deep phenotyping associated with RNA molecular analyses, western blot (WB) and functional studies is often essential for the interpretation of genetic variants in patients suspected of titinopathy. Namely, pattern featuring a prominent semitendinous muscle involvement on MRI, prominent nuclear internalization, peculiar alteration of oxidative reactions (such as cores, minicores and inverted cores) and presence of cytoplasmic bodies on muscle biopsy are suggestive and common, although non-specific, features found in all titinopathies.

For these reasons, we implemented a workflow based on deep phenotyping, comprehensive TTN variants assessment including bioinformatic predictions and evaluation of their effects on skeletal muscle titin transcripts and protein. We hereby describe our strategy for TTN variant interpretation in four patients with recessive congenital myopathy.

2. Methods

2.1. Patients

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 French legislation (Comité de Protection des Personnes OUEST 6-CPP1128HPS3 IDRCB-2018-AO2287-48).

2.2. Bioinformatic evaluation of TTN Variants Pathogenicity

TTN (NM_001267550.1) variants were identified by NGS targeted on exons and exon-intron junctions of 185 genes, as previously reported [15] and Juntas Morales et al, submitted.

The pathogenicity of variants was assessed through a set of criteria reported by Zenegui et al.[15], according to the American College of Medical Genetics and Genomics (ACMG) guidelines [16]. In the present work, additional tools recently described were used: the MPA software [17] that aggregates bioinformatic prediction tools and provides a 0–10 prioritization score, accessible from https://mobidetails. iurc.montp.inserm.fr/, and the TITINdb, a database that takes into account several statistical and structural criteria for the interpretation of TTN variants (http://fraternalilab.kcl.ac. uk/TITINdb/) [18]. In addition, to take into account the complexity of skeletal transcripts of TTN, in particular the fact that some exons are only expressed during fetal development ("metatranscript-only exons"), we systematically referred to the recent study reported by Savarese et al. [19]. All variants presented in this study have been submitted in Leiden Open Variation Database (LOVD) https://databases.lovd.nl/shared/genes/TTN.

2.3. Muscle morphological analyses 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 [11,20].

2.4. Transcripts analysis

mRNAs were extracted from 20 mg of muscle biopsy and prepared as described in [15]. 2 μg of RNA was reverse-transcribed with Invitrogen Superscript II reverse transcriptase. For conventional analysis, we performed overlapping RT-PCR reactions covering regions with TTN variants (supplementary table 1). For RNAseq analyses, sequencing was performed by Integragen SA (Evry, France) using the same protocol with poly-A selection of mRNA (Illumina (San Diego, CA, USA) TruSeq® Stranded Total
RNA Library Prep) used in the GTEx sequencing project [21]. Paired-end 75-bp sequencing was performed on Illumina NovaSeq6000® platform as described in [21]. 88 Millions of reads were generated by RNAseq, including 687000 reads for *TTN* transcripts. RNA sequences alignment was performed using Ultrafast universal RNA-seq aligner (STAR) software [22] and analyzed using Integrative Genomics Viewer (IGV) [23] targeted on titin transcripts. The mean depth of RNA sequencing was 131 reads for titin transcripts NM_133378 corresponding to N2A isoform. Four exons had coverage under 30×: exon 11 (7×), exon 149 (14×), exon 150 (20×), and exon 151 (15×). These exons were described in [19] to be constitutively splice out for exon 11 and included in 35%, 34% and 40% of transcripts for exons 149, 150, 151 respectively. Exon 1 had a depth of sequencing of 157×, confirming that the 5’ extremity was well covered.

2.5. Western blot analysis (WB)

Protein solubilization from muscle biopsies was prepared as previously described [24]. For full length titin WB, 5 µl of protein extract were loaded on a SDS-Agarose 0.8% gel prepared as previously described [25,26] and ran for 3h20 at 15mA. Protein transfer on PVDF membrane was performed using wet transfer Trans blot® cell Bio-Rad (Bio-Rad, Hercules, CA, USA) at 400mA for 3h. Membranes were subsequently incubated in Odyssey® blocking buffer, washed and incubated overnight at 4 °C in Odyssey® blocking buffer in PBS specific primary antibodies: anti-titin specific for the C-terminal part M10-1 (Rabbit; 1:1000; kindly provided by Dr Isabelle Richard [7]) or N-terminal part (Mouse; 1:1000; Sigma SAB1400284). Membranes were washed and incubated for one hour with donkey anti-mouse IR Dye® 800CW (1:30,000) or donkey anti-rabbit IR Dye®680RD (1:30,000). Membranes were then washed, dried and scanned with the Odyssey® Infrared Imaging system.

For C-terminal part of titin WB, SDS-acrylamide 10% gel was used as previously described [7]. Anti-titin antibody M10-1, specific for the C-terminal part, was used at 1/500 followed by horseradish peroxidase–conjugated secondary antibodies (goat anti-rabbit sc2004, 1/20000, Santa Cruz) and enhanced chemiluminescence detection using the Substrat HRP Immobilon Western (Millipore-WBKLS0500).

3. Results

3.1. Clinical and imaging findings

Clinical features of all the patients are summarized in Table 1.

Patients 1 and 2 (P1, P2) were twin sisters born to a non-consanguineous family. Pregnancy and delivery were normal, no neonatal hypotonia was reported. At the age of six months, they developed axial hypotonia. The motor development was delayed, and they were not able to walk before the age of 29 months. Neurological examination at the age of 4 years showed facial weakness, high arched palate, and mild proximal and axial weakness with Gowers’s sign. There was no ptosis or ophthalmaparesis. Cardiological test at 9 years showed for P2 an ejection fraction at the lower limit of the normal range. P1 and P2 progressively developed hip, ankle, elbow and cervical spine contractures. Complementary exams were performed for patient 1 and results were as follow: Creatin kinase (CK) level was normal and electromyography showed myopathic pattern. In patient 1, WB studies with an antibody against calpain-3 revealed reduced protein level (data not shown). *CAPN3* screening was normal. Brain MRI disclosed the presence of moderate periventricular white matter hyperintensity in P2 and P3. (C) Axial T1 weighted signal, bilateral atrophy and fatty replacement in soleus and gastrocnemius muscles, right tibialis anterior and peroneus. Tight muscles were not involved.

![Fig. 1. Clinical and magnetic resonance imaging (MRI) findings. (A) and (B) Flair weighted signal: Mild periventricular white matter hyperintensity in P2 and P3. (C) Axial T1 weighted signal, bilateral atrophy and fatty replacement in soleus and gastrocnemius muscles, right tibialis anterior and peroneus. Tight muscles were not involved.](image-url)
Table 1
Summary of clinical findings of the patients.

|                | P1–P2                                  | P3                                               | P4                                               |
|----------------|----------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Age            | Twin sisters, 7 years old              | 18 year                                          | 55 year                                          |
| Genetic variations | c.105036C>A p.(Tyr35012*)              | c.26503 A>T p.(Lys8835*)                         | c.-14+5G>C c.68029A>G; p.(Thr22677Ala)           |
|                | c.106531G>C p.(Ala35511Pro)            | c.51437-4_51444delTCAGAACCCTCC                   | c.65575+2T>G                                    |
| Age of onset   | <1 year                                 | 3 year                                           | 2 year                                           |
| Neonatal features | Not                                    | Unknown (adopted child)                          | Not                                              |
| Early motor development | Axial hypotonia (6 months)              | Normal                                           | Delayed walking (24 months)                      |
| Muscle weakness distribution | Moderate proximal, distal and axial weakness | Mild proximal weakness                         | Initially, mild proximal weakness, with slow progression: the patient was wheelchair-bound in theforties with severe proximal, axial and distal weakness of four limbs |
| Axial features  | Lumbar hyperlordosis Mild rigid spine  | Mild bilateral scapular winging                   | Lumbar hyperlordosis                             |
| Contractures   | Hip, elbow and ankle contractures (15°) | Not                                              | Ankle                                            |
| Foot deformities | Not                                    | Pes cavus                                        | Pes cavus                                        |
| Ophthalmoceleia | Not                                    | Not                                              | Not                                              |
| Posis          | Not                                    | Not                                              | Not                                              |
| Facial weakness | Yes                                    | Yes                                              | Yes                                              |
| High arched palate | Yes                                  | Not                                              | Not                                              |
| Ocicular involvement | Not                               | Not                                              | Not                                              |
| Age of last cardiac assessment | 9 year                               | 14 year                                          | 55 year                                          |
| Cardiac involvement | P1 Not                             | Not                                              | Mild late onset dilated cardiopathy (FE 45%)      |
|                | P2 Lower limit of normal (FE 55%)      |                                                  |                                                  |
| Forced vital capacity | P1: 64%                  | 33.2%                                            | 58%                                              |
|                | P2: 62%                                 |                                                  |                                                  |
| Creatin Kinase | P1: 337 UI/l                           | Normal                                           | Normal                                           |
| Muscular biopsy |                                      |                                                  |                                                  |
| Optic microscopy | Excessive internal nuclei 10% Minicores | Excessive internal nuclei 8% Minicores             | Excessive internal nuclei 95% Minicores             |
| Electronic microscopy | N.D.                           | Minicores                                        | Cap structure                                     |
| Electromyogram | N.D.                                    | Myopathic                                        | Myopathic                                        |
| Muscular MRI   | N.D.                                    | Bilateral soleus and gastrocnemius Right lateral peroneus and tibialis anterior | N.D.                                             |
| Brain MRI      | P1: periventricular hyperintensity     | Periventricular hyperintensity                   |                                                   |
|                | P2: N.D.                                |                                                  |                                                   |

N.D: Not Done.

Facial weakness, moderate *scapula alata* and mild proximal weakness. There were no contractures or skeletal deformities. Cardiac assessment at 14 years was normal. There was a severe restrictive respiratory syndrome with Forced Vital Capacity (FVC) at 1.2L (33.2% of predicted). As for P1, brain MRI revealed the presence of moderate periventricular white matter hyperintensity on Flair weighted signal (Fig. 1(B)).

Patient 4 (P4) is a sporadic case. The girl developed, from the age of 4 years, slowly progressive lower limb weakness. The patient progressively lost ambulation and was wheelchair bound at 17 years. Neurological examination at 45 years showed bilateral facial weakness, severe axial and proximal weakness of four limbs without contractures. Mild distal weakness was also present. There was restrictive respiratory syndrome with an FVC at 58%. At 55 years, a mild dilated cardiomyopathy with ejection fraction of 45% was revealed by cardiac ultrasonography. Muscle MRI of lower limbs showed bilateral soleus, lateral peroneus and tibial anterior involvement.

### 3.2. Muscle morphological data

A skeletal muscle biopsy was obtained from all patients except P2. There was an important percentage of centralized nuclei, from 9% in P1 to 95% in P4 (Fig. 2(A) and (B)) and minicone-like lesions with NADH oxidative reaction (Fig. 2(C)–(E)). P1, P2, P3 and P4 were diagnosed as having a centronuclear myopathy. Ultrastructural studies in P3 and P4 revealed the presence of multifocal areas of sarcomeric disruption. In P4, we observed well delimited areas containing filamentous material and cellular debris corresponding to ‘Cap’ structures were visualized (Fig. 2(F)–(H)).
As a whole, excessive internal nuclei, minicore-like and cap structures were consistent with TTN related muscle morphological presentation.

3.3. Molecular and protein results

Localization of variants on corresponding titin domains is shown on Fig. 3. The exon numbering is reported following the numbering used in LOVD. No other pathogenic variants than the TTN ones were identified in the reported patients.

3.3.1. P1 and P2 patients

P1 and P2 have two variants, a nonsense variant in exon 359, c.105036C>A p.(Tyr35012*) inherited from the mother and a missense variant in exon 360, c.106531G>C p.(Ala35511Pro) from the father, both absent from gnomAD population database. Patients do not have other siblings to enlarge the segregation study. The variants are both localized in the genomic region encoding the M-band domain of titin (Fig. 3). The missense variant in exon 360 affects the last nucleotide of the exon and is predicted to have an impact on exon 360 RNA splicing (HSF score shift at -46.1% and MaxEnt score at-29%). To confirm this prediction, we investigated the region of transcripts containing the variant (exons 359-362) by RT-PCR and Sanger sequencing (Fig. 4(A)). cDNA agarose gel revealed the presence of two bands of intensity difference, and sequencing of RT-PCR products identified two populations of transcripts, a major one with aberrant splicing (exon 360 skipping) and a minor one with normal splicing of exon 360. This minor transcript with normal exon 360 splicing do not carry the paternal c.106531G>C variant in exon 360, indicating that the paternal
variant results in 100% of the transcripts with the exon 360 skipping. This experiment also suggests that the transcripts with the maternal nonsense variant have a reduced level, probably due to RNA degradation by Nonsense Mediated RNA Decay (NMD). Exon 360 skipping disrupts the open reading, leading to a Stop codon 6 nucleotides downstream from the frameshift. Overall, both TTN mutated alleles in P1 and P2 are predicted to produce a M-band truncated protein (Fig. 5(A)). WB analysis of the C-terminal part of titin showed the loss of the C-term p45 band in P1 and, thereby, the loss of titin C-terminal portion (Fig. 5(B)). The total absence of the C-terminal fragments is a further argument suggesting that no normal transcripts are produced from the paternal allele with the variant affecting the last nucleotide of the exon.

3.3.3. P4 patient

P4 has three TTN variants, c.-14+5G>C in exon 1 and c.68029A>G; p.(Thr22677Ala) in exon 321, both absent in her mother (no sample was provided for the father) and c.65575+2T>G in intron 313 inherited from the mother. There was no proband’s siblings to complete the segregation study. All these variants were absent from gnomAD population database. Exon 1 is reported to be expressed but not translated into amino acids. Presumably on the same allele, the nonsense mutation in exon 321, p.(Thr22677Ala), is localized in A band, Fibronectin type-II 64 domain. It has an MPAC score at 6.7/10 and is predicted to destabilize the protein structure, according to titindb. cDNA analyses of the region containing the c.68029A>G; p.(Thr22677Ala) variant showed normal splicing and normal allelic balance with the maternal allele, not supportive of a potential reduction of TTN expression caused by the exon 1 variant presumably in cis. On the other allele, the c.65575+2T>G variant is predicted to have an impact on exon 313 splicing (HSF and MaxEnt prediction scores: 100%). This was confirmed by RT-PCR sequencing and RNAseq that revealed a complex effect with two abnormal populations of transcripts (Fig. 4(C) and (D)). A full exon 313 skipping (306 nucleotides) was found in one of the transcript population, leading to the synthesis of an internally deleted protein lacking 102 residues in A-band, in a myosin interaction domain [27]. This deletion is extremely small compared to the size of the protein and is therefore not detectable in WB. In the second population, transcripts are deleted of the 163 last nucleotides of exon 313, due to the use of a cryptic donor splice site (GTACGG) within this exon. This transcript population induces a shift in the reading frame and a Stop codon 12 nucleotides downstream from the shift. If not degraded by nonsense mediated RNA decay (NMD), this allele is predicted to produce a truncated protein with an approximate molecular weight of 2.2MDa (Fig. 5(C)), lacking a large part of the A-band and the M-band. RNA-seq analyses allowed to exclude elusive variants such as deep intronic variants affecting splicing. WB analysis of muscle biopsy showed the presence of normal N2A protein.
Fig. 4. Titin transcripts analyses. (A) gDNA and cDNA analyses of P1 c.106531G>C variant. gDNA Sanger sequence of the c.106531G>C in exon 360 is shown. cDNA analyses showed an impact on splicing. Agarose gel of cDNA shows two bands, a faint one at the normal size (3.8MDa) and a second smaller molecular weight one corresponding to the exon 360 skipping allele that disrupt the open reading frame. (B) gDNA and cDNA analyses schemes for P3 titin transcripts. The exon 273 c.51437-4_51444delTCAGAACCCCT variant deletes the natural acceptor splice site and leads to the use of a cryptic acceptor splice site 21 bases downstream, represented by a blue arrow. This deleted transcript is in frame and is predicted to result to the lack of seven amino acids at the beginning of exon 274 in frame. (C) cDNA study by RT-PCR sequencing of P4 titin transcripts containing the c.65575+2T>G mutation. Three populations of transcripts were visible on agarose gel in P4. Black arrow corresponds to the WT allele, green arrow to the complete skipping of exon 313 (306 nucleotides loss), red arrow to the out of frame deletion of 163bp in exon 313 resulting to the use of a cryptic donor splice site in this exon (red GTACGG sequence in the scheme of exon 313). (D) P4 RINseq transcripts pattern from exons 311–315. In gray are represented the coverage of each sequence. The crescents represent the splice junctions identified in the control (blue crescents) and the P4 patient (red crescents) transcripts, with the number of reads indicated above. In P4, the same three transcript populations identified by RT-PCR Sanger sequencing were also detected: one normal splice pattern between exons 312 and 314 (similar to the healthy control), a transcript with complete skip of exon 313 and another one with the use of the cryptic donor splice site in exon 313.
(3.8MDa) and one additional band (although at a low amount) at an approximate molecular weight of 2.2MDa, with no associated background noise (Fig. 5(D)). This band could correspond to the truncated protein due to the premature stop codon in transcripts induced by the splicing defect in exon 313.

4. Discussion

Due to the widespread use of NGS, TTN is emerging as a major causative gene of neuromuscular disorders. Interpretation of the pathogenic effect of variants is particularly challenging. In this study we present an
exhaustive workflow in order to confirm the pathogenicity of TTN variants in four patients with recessive congenital myopathy with centralized nuclei, minicores and Cap structures.

With respect to the phenotype of the patients, the presence of mild diffuse weakness, facial weakness and respiratory insufficiency was concordant with titinopathies phenotypic spectrum [5,14]. In a recent series of 30 titin patients, most of them had mild to moderate weakness (81%), facial weakness (70%) and respiratory insufficiency (63%) [14]. Although not specific of titinopathies, some signs were previously reported on congenital myopathy patients suspected of titinopathies [14]. In Oates et al., study (2018), 19 of 26 patients had high arched palate, one of 22 had pes cavus and 3 of 12 patients had minor structural abnormalities of unknown significance on brain MRI. This sign could be better explored in the future.

Cardiomyopathy was also a frequent feature found in 46% of cases [14]. Of note, P4 developed dilated cardiomyopathy at 50 years and P2 had ejection fraction at the lower limit of the normal range. Our study confirms that a strict cardiac workup should be performed in all congenital titinopathy patients as subtle cardiac involvement might be present in infancy and an overt cardiomyopathy might develop later in life.

From a myopathologic standpoint, all patients presented centralized nuclei and minicore-like lesions. Our findings are in line with data reported by us and others [13,14], where the presence of prominent nuclear centralizations and alterations in the intermyofibrillar network are prominent features of congenital titinopathies.

With respect to the interpretation of genetic variants in TTN, Savarese and colleagues have proposed that identifying two TTN truncating variants in trans plus a suggestive phenotype results in a diagnosis of titinopathy, but a complete molecular characterization of variants affecting the canonical or noncanonical splice sites by cDNA or protein studies was suggested. [3]. In our cohort, P1 and P2 have a nonsense in the first M-band exon (359) on the maternal allele and a variant affecting the last nucleotide of the following exon (360) on the paternal allele leading to an out-of-frame exon 360 skipping in 100% of the transcripts. Both TTN variants lead to a M-band truncation. In line with the current understanding, and considering that the total absence of titin is most probably incompatible with life [28,29], we expect both the mutated alleles to result in a truncated protein. Of interest, WB showed calpain-3 clear reduction consistent with previous reports of calpain 3 deficiency secondary to truncation of the titin C-terminal part [7,8].

P3 has a nonsense variant in exon 93 in the I-band domain and a splicing variant resulting in a small internal intrame deletion in exon 273. Considering the current understanding, the nonsense variant in the I-band most probably causes a NMD and is unlikely to result in a truncated protein. The aberrant transcripts resulting from the splicing variant lacks the 21 first nucleotides of exon 273, leading to the loss of seven residues within a titin-myosin interaction domain. Since phasing with the nonsense variant is impossible, and the effect of the splicing variant is not an out-of-phase truncation, this splice variant is then considered as probably pathogenic and P3 is most probably a titinopathy case.

For P4, the interpretation is still challenging. He harbors a missense in exon 321 and a splice variant in intron 313. Transcript analyses showed complex consequences of the splice variant in intron 313, with a population of transcripts harboring an in-frame complete exon 313 skipping associated with another population of out-of-frame transcripts resulting from activation of a cryptic splice site. If not degraded by NMD, the latter is predicted to lead to a truncated protein of an approximate molecular weight of 2.2MDa lacking a large part of the A-band and the M-band. One additional band of 2.2 Mb was detected by WB analysis on the P4 muscle. Even if the correspondence of this band with the truncated protein due to the characterized splicing defect is not certain, this observation suggests that the transcript with the premature stop codon in exon 313 would not be degraded by nonsense mediated decay. Missense variants interpretation is a real challenge and need the association of deep clinical, histopathological and radiological phenotyping, complete molecular analyses including large segregation studies, and functional studies. Our study has limitation since the presence of clinical and histological features consistent with a congenital titinopathy cannot be a stand-alone proof. We could not perform segregation studies nor functional analyses for the missense variant identified in P4, that is then considered as uncertain significance according to ACMG guidelines. With the confirmation of paternal allele inheritance, the missense variant for P4 would have been classified as “likely pathogenic”, underlining the importance of segregation studies. In their proposed workflow to interpret monoallelic TTN variants pathogenicity in congenital myopathies, Savarese et al. [10] also recommend to search for the presence of a further elusive truncating variant. No such variant was identified in P4 by RNAseq analysis. Even if the phenotype is in agreement, and the consequences of the splicing variant at the cDNA and protein levels have been demonstrated, P4 should be considered as a “possible titinopathy case”. Functional studies and/or identification of other cases with the same variant would be important to confirm pathogenic effects of the missense variant. This challenging case highlights the importance of sharing data using curated databases such as LOVD and publication reports using the same comprehensive approach [14,30]."
cases in publications using this approach. The next steps will include the study of protein dysfunction at the structural or interacting levels, since published studies suggest that some titin variants could affect the stability of the protein and its interaction with direct binding partners [3,5,31].

Acknowledgments

The authors thank Myobank-AFM for providing control muscle biopsies, Isabelle Richard for titin antibody provided and the French Titin Consortium for all the discussion about patient suspected of titinopathies.

Study funding

This work was funded by AFM 21384 grant (The French Muscular Dystrophy Association (AFM-Téléthon)), the Délégation à la Recherche Clinique et à l’Innovation du Groupement de Coopération Sanitaire de la Mission d’Enseignement, de Recherche, de Référence et d’Innovation (DRCI-GCS-MERRI) de Montpellier-Nîmes and the French Foundation for Rare Diseases (FFRD) under the program « High throughput screening and rare diseases ».

References

[1] Linke WA, Hamdani N. Gigantic business: titin properties and function through thick and thin. Circ Res 2014;114:1052–68.
[2] Akirinrade O, Koskenvuo JW, Alastalo TP. Prevalence of titin truncating variants in general population. PLoS One 2015;10:e0145284.
[3] Savarese M, Maggi L, Vihola A, Jonson PH, Tasca G, Ruggiero L, et al. Interpreting genetic variants in titin in patients with muscle disorders. JAMA Neurol 2018;75:557–65.
[4] Chauveau C, Rowell J, Ferreiro A. A rising titan: TTN review and mutation update. Hum Mutat 2014;35:1046–59.
[5] Ceyhan-Birsoy O, Agrawal PB, Hidalgo C, Schmitz-Abe K, Dechene ET, Swanson LC, et al. Recessive truncating titin gene, TTN, mutations presenting as centronuclear myopathy. Neurology 2013;81:1205–14.
[6] Chauveau C, Bonnemann CG, Julien C, Kho AL, Marks H, Talim B, et al. Recessive TTN truncating mutations define novel forms of core myopathy with heart disease. Hum Mol Genet 2014;23:980–91.
[7] Eviliä A, Vihola A, Sarparanta J, Raheem O, Palmo J, Sandell S, et al. Atypical phenotypes in titinopathies explained by second titin mutations. Ann Neurol 2014;75:230–40.
[8] De Cid R, Ben Yaou R, Roudaut C, Charton K, Baulande S, Leturcq F, et al. A new titinopathy: childhood-juvenile onset Emery-Dreifuss-like phenotype without cardiomyopathy. Neurology 2015;85:2126–35.
[9] Hackman P, Vihola A, Haravuori H, Marchand S, Sarparanta J, De Seze J, et al. Tibial muscular dystrophy is a titinopathy caused by mutations in TTN, the gene encoding the giant skeletal-muscle protein titin. Am J Hum Genet 2002;71:492–500.
[10] Savarese M, Johari M, Johnson K, Arumilli M, Torella A, Töpf A, et al. Improved criteria for the classification of titin variants in inherited skeletal myopathies. J Neuromuscul Dis 2020;7:153–66.
[11] Ávilá-Polo R, Malfatti E, Lornage X, Cheradu C, Nelson I, Nectoux J, et al. Loss of sarcomeric scaffolding as a common baseline histopathologic lesion in titin-related myopathies. J Neuropathol Exp Neurol 2018;77:1101–14.
[12] Carlier R-Y, Quijano-Roy S. Myoimaging in congenital myopathies. Semin Pediatr Neurol 2019;29:30–43.
[13] Hackman P, Udd B, Bönnemann CG, Ferreiro A, Udd B, Hackman P, et al. 219th ENMC International Workshop Titinopathies International database of titin mutations and phenotypes, Heemskerk, The Netherlands, 29 April–1 May 2016. Neuromuscul Disord 2017;27:396–407.
[14] Oates EC, Jones KJ, Donkervoort S, Chariton A, Brammah S, Smith JE, et al. Congenital titinopathy: comprehensive characterization and pathogenic insights. Ann Neurol 2018;83:1105–24.
[15] Zenagui R, Lacourt D, Pegeot H, Yauy K, Morales RJ, Theze C, et al. A reliable targeted next-generation sequencing strategy for diagnosis of myopathies and muscular dystrophies, especially for the giant titin and nebulin genes. J Mol Diagn 2018 Published Online First. doi:10.1016/j.jmoldx.2018.04.001.
[16] Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of medical genetics and genomics and the association for molecular pathology. Genet Med 2015;17:405–24.
[17] Yaay K, Baux D, Pegeot H, Van Goethem C, Mathieu C, Guignard T, et al. MoBiDC prioritization algorithm, a free, accessible, and efficient pipeline for single-nucleotide variant annotation and prioritization for next-generation sequencing routine molecular diagnosis. J Mol Diagnostics 2018;20:465–73.
[18] Laddach A, Gautel M, Fraternali F. TITINadb-a computational tool to assess titin’s role as a disease gene. Bioinformatics 2017;33:3482–85.
[19] Savarese M, Jonson PH, Huovinen S, Paulin L, Auvinen P, Udd B, et al. The complexity of titin splicing pattern in human adult skeletal muscles. Skelet Muscle 2018;8:11.
[20] Uro-Coste E, Fernandez C, Authier FJ, Bassez G, Butori C, Chapon F, et al. Management of muscle and nerve biopsies: expert guidelines from two French professional societies, Société française de neuropathologie and Société française de myologie and a patient-parent association, Association Française contre les myopathies, Rev Neurol (Paris) 2010;166:477–85.
[21] Cummings BB, Marshall JL, Tukiainen T, Lek M, Donkervoort S, Foley AR, et al. Improving genetic diagnosis in Mendelian disease with transcriptome sequencing. bioRxiv 2017;520907:074153.
[22] Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.
[23] Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 2013;14:178–92.
[24] Hudson B, Hidalgo C, Saripalli C, Granzer H. Hyperphosphorylation of mouse cardiac titin contributes to transverse aortic constriction-induced diastolic dysfunction. Circ Res 2011;109:858–66.
[25] Perrin A, Metay C, Villanova M, Carlier R, Pegoraro E, Inatsumura R, et al. A new congenital multicore titinopathy associated with fast myosin heavy chain deficiency. Ann Clin Transl Neurol 2020;7:1–9.
[26] Greaser ML, Warren CM. Electrophoretic separation of very large molecular weight proteins in SDS agarose. Methods Mol Biol 2019;1855:203–10.
[27] Kontrogianni-Konstantopoulos A, Ackermann MA, Bowman AL, Yap SV, Bloch RJ. Muscle giants: molecular scaffolds in sarcomereogenesis. Physiol Rev 2009;89:1217–67.
[28] Gerull B, Gramlich M, Atherton J, McNabb M, Trombitás K, Sasse-Klaassen S, et al. Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. Nat Genet 2002;30:201–4.
[29] Weinert S, Bergmann N, Luo X, Erdmann B, Gotthardt M. M line-deficient titin causes cardiac lethality through impaired maturation of the sarcomere. J Cell Biol 2006;173:559–70.

[30] Savarese M, Vihola A, Oates EC, Barresi R, Fiorillo C, Tasca G, et al. Genotype-phenotype correlations in recessive titinopathies. Genet Med 2020;0. doi:10.1038/s41436-020-0914-2.

[31] Rossi D, Palmio J, Evili A, Galli L, Barone V, Caldwell TA, et al. A novel FLNC frameshift and an OBSCN variant in a family with distal muscular dystrophy. PLoS One 2017;12:1–21.