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Novel Missense *CAPN3* Mutation Responsible for Adult-Onset Limb Girdle Muscular Dystrophy with Calves Hypertrophy

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Abstract

CAPN3 gene encodes for calpain-3; this protein is a calcium-dependent intracellular protease. Deficiency of this enzyme leads to weakness of the proximal limb muscles and pelvic and shoulder girdles, the so-called limb-girdle muscular dystrophy type 2A (LGMD2A). Here, we reported the case of a Tunisian patient with LGMD2A associated with a novel missense mutation (c.T1681C/p.Y561H). A 61-year-old man, with consanguineous parents, was referred for gait difficulties and slowly progressive proximal weakness of the four limbs associated with moderate hypertrophy of the calves but his facial muscles were unaffected. Electromyography showed that the profile was myopathic pattern and creatine kinase (CK) level was high. Muscle biopsy processing included routine histological, immunohistochemical, and Western Blot reactions, using a panel of antibodies directed against dystrophin, dysferlin, calpain-3, sarcoglycan α, β, γ, and δ. For mutation analysis, we designed an NGS-based screening. Immunological analyses demonstrated a total deficiency in calpain-3 and δ-sarcoglycan, and a reduced expression of dysferlin. The genetic study yielded a homozygous missense mutation (c.T1681C) of the 13th exon of the *CAPN3* gene. The mutation found in our patient (c.T1681C/p.Y561H) has not been previously reported. It is responsible for complete calpain-3 and δ-sarcoglycan deficiency and reduced dysferlin expression. The genetic study is mandatory in such cases with multiple-protein deficiency and ambiguous results of immune-histology and Western Blot studies.

Keywords Muscular dystrophies · Immunohistochemistry · Western Blot · CAPN3 protein · DNA sequencing · LGMD2A

Introduction

Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of inherited diseases characterized by progressive weakness of pelvic and shoulder girdles muscles and a highly variable clinical course (Straub et al. 2018). Till now, LGMDs involve nine autosomal dominant forms (LGMD1) and 25 autosomal recessive ones (LGMD2) (Kaplan et al. 2016). The Limb-girdle muscular dystrophy type 2A (LGMD2A), also referred to as "calpainopathy", is the most frequent form of LGMD worldwide (Urtasun et al. 1998; Fanin et al. 2005). Its clinical picture is similar to other LGMDs with symmetrical and progressive weakness and wasting of proximal limb and girdle muscles (Angelini et al. 2018). Calf muscle hypertrophy is rare and always discrete; the face and the heart are usually not involved and there is no mental retardation (Fanin and Angelini 2015). There is great variability of age at onset between patients and even between affected persons in the same family (Hadj Salem et al. 2011). In the majority of cases, the first symptoms of LGMD2A appear between the ages of 5 and 18 years (range from 2 to 40 years) (Angelini et al. 2010).

The LGMD2A is caused by mutations in the human *CAPN3* gene located in chromosome 15 (15q15.1–q21.1) (Kaplan et al. 2016; Ye et al. 2018). Up to date, more than 500 different mutations of this gene have been reported (Ye et al. 2018). The *CAPN3* gene contains 24 exons and covers a genomic region of 50 kb. It codes for a 94 kDa protein, known as "calpain-3" (calcium-activated neutral protease-3) (Nigro

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and Savarese 2014). This enzyme is a multi-domain protein: domain I has a regulatory role, domain II is the proteolytic module, domain III has a C2-like domain, and domain IV, Penta EF-hand, binds Ca2+ ions. In addition, it contains three unique sequence inserts NS, IS1, and IS2 (Sorimachi et al. 2011; Park et al. 2016). Calpain-3 is a muscle-specific enzyme involved in many physiological processes, such as homeostasis of the muscle sarcomere, muscle remodeling, and regulation of the myocyte cytoskeleton (Kramerova et al. 2005; Duguez et al. 2006; Fanin and Angelini 2015). From these suggested roles of calpain-3, it can be postulated that deregulation of sarcomere remodeling would constitute the origin of LGMD2A pathogenesis (Piluso et al. 2005; Park et al. 2016).

In this study, we reported the case of a Tunisian patient presenting late onset and slowly progressive muscular dystrophy with calpain-3, dysferlin, and δ-sarcoglycan deficiency, related to a novel missense mutation in the *CAPN3* gene.

Patients and Methods

Case Presentation

A 61-year-old man was referred to our department for walking difficulties. In fact, since the age of 35, he developed slowly progressive weakness of lower limbs with a waddling gait, frequent falls, difficulty in climbing stairs, and rising from the floor. He became wheelchair-bound at the age of 54. Currently, after more than 25 years of disease progression, he is completely bedridden. His parents are consanguineous but there are no similar cases in the family.

Neurological examination revealed severe muscle weakness and wasting, affecting predominantly the proximal segments of the four limbs, as well as the pelvic and shoulder girdles. We noticed marked atrophy of the biceps and the shoulder muscles with scapular winging. He also had severe atrophy of hip adductors and the posterior thigh muscles. Calves were moderately hypertrophied. According to the Medical Research Council scale, muscle strength was grade 2 in the proximal part of limbs and grade 3 in the extremities. Oculomotor and facial muscles were unaffected, and cranial nerve examination was normal. Deep tendon reflexes were present and normal. Sensory examination was unremarkable.

Laboratory investigations disclosed a slight increase of creatine kinase at 713 U/L (normal < 232 U/L). Thyroid tests were in the normal range. Electromyography showed chronic myopathic features with low amplitude and polyphasic motor units, early recruitment, and full interference pattern. Nerve conduction study was normal. Electrocardiogram and echocardiogram were unremarkable and the pulmonary function test (PFT) was normal. The above clinical presentation and electromyographic findings were suggestive of progressive muscular dystrophy.

Immunohistochemical Examination and Western-Blot Analysis of the Muscle

Biopsy of the left deltoid muscle was performed. Frozen sections of muscle specimens were processed according to protocol: histological technics included routine staining and histochemical reactions (hematoxylin /eosin (H&E), Periodic Acid Schiff (PAS), Oil red O, modified Gomori trichome, and NADH-TR) and immuno-histological reactions using a panel of antibodies directed against dystrophin, α, β, γ, and δ-sarcoglycans (Novocastra-A, B, G, and D-SARC-CE).

For Western Blot analysis, we prepared a lysate. Frozen muscle tissue specimen was homogenized in lysis buffer (RIPA Buffer) with the addition of protease inhibitors (Pierce™ Protease and Phosphatase Inhibitor). Samples of this lysate were electrophoretically separated on NuPAGE™ 10% Bis-Tris Midi Protein Gels. After blocking with Fish Gelatin Blocking buffer and washing with TBST, the membrane was incubated with a monoclonal antibody and washed with TBST. Then, the secondary antibody was applied, and detection by chemiluminescent reagent ECL (SuperSignal™ West Femto Maximum Sensitivity Substrate) was performed. The membranes were incubated with the following primary monoclonal antibodies:

- NCL-CALP-2C4 (Novocastra) that reacts with the 94 and 30 kDa domains of calpain-3
- NCL-CALP-12A2 (Novocastra) that reacts with the 94 and 60 kDa domains of calpain-3
- NCL-Hamlet (Novocastra) that reacts with dysferlin
- D-SARC-CE (Novocastra) that reacts with δ-sarcoglycan

Next Generation Sequencing (NGS) Target Gene Panel

Informed consent was obtained from the patient to perform blood sampling and DNA extraction for genetic study. Our study was approved by our institutional ethics committee. We designed an NGS-based screening of six currently most prevalent LGMD2 genes: *CAPN3*, *DYSF*, *SGCA*, *SGCB*, *SGCG*, and *SGCD*. The indexed paired-end libraries of genomic DNA were prepared using the protocol of "Illumina® TruSeq® Custom Amplicon Low Input Kit". Captured libraries were sequenced on the Illumina MiSeq system using Protocol A: Standard Normalization Method. MiSeq Reporter Software was used for sequence analysis and detection of the different variants in the 6 LGMD2 genes. Human reference sequence GRCh37/hg19 assembly was used for sequence alignment.

The mean coverage was 686× (range 92–1083×). Targeted exons with coverage less than 100 reads were screened subsequently by Sanger sequencing. Variants were prioritized based on their frequencies (MAF < 1%) in public databases

(1000Genomes/ExAC/TOPMED), nucleotide, and amino-acid conservation and predicted pathogenicity.

All reported variants of interest were verified by bidirectional Sanger sequencing using primers designed with Primer3 on an ABI 3730 automated sequencer (Life Technologies).

Bioinformatic Analysis

The prediction of the potential pathogenetic effect of exonic variation at the protein level was performed using multiple in-silico tools. The multiple alignments of the calpain-3 peptide sequences were performed using the ClustalW program. Sequences from the species were obtained from the NCBI database. The pathogenicity and the effect on the protein structure of the variation were predicted using Provean, Poly-phen2, SIFT, SNPs&GO, PhD-SNP, and Mutpred.

To better understand the effect of predicted deleterious variations at the structural level, we performed a 3D modeling study using DeepView/Swiss PDB Viewer 3.7. The generation of both normal and mutated models was ensured by RaptorX. All 3D models were constructed based on the template 1kfuL crystal Structure of Human m-Calpain Form II.

Results

Histological, Immunohistochemical, and Western Blot Analysis

Histological study showed a dystrophic pattern with great variability of muscle fibers size and presence of necrotic and regenerative fibers. We observed a slight proliferation of connective tissue but there was no inflammatory infiltrate (Fig. 1). Immunohistochemical reactions demonstrated that muscle fibers were positive for dystrophin, α, β, and γ-sarcoglycans but negative for δ-sarcoglycan.

Immunoblotting analysis disclosed a reduced expression of dysferlin and absence of the δ-sarcoglycan band. For calpain-3, the three bands (Calp94, Calp60, and Calp30) were absent, suggesting a total deficiency in calpain-3 (Fig. 2).

Molecular Analysis

Molecular analysis revealed a novel variation at the homozygous state in the *CAPN3* gene (NM-000070/P20807): c.T1681C (Fig. 3). No other rare deleterious variation at the homozygous or the heterozygous state was identified in all other studied genes. This result demonstrates that our patient presented LGMD2A. His parents died some time ago, and no biological material from the other members of the family was available.

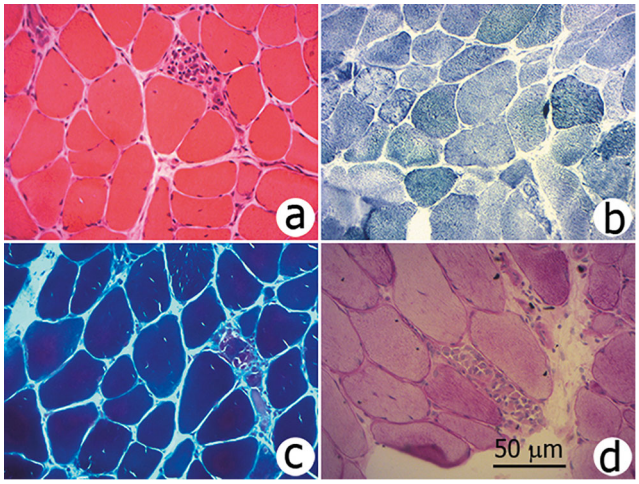

Fig. 1 Histological examination of muscle biopsy showing myopathic changes with great variability of fibers size, necrotic fiber, and macrophagic resorption (**a** hematoxylin-eosin staining). There is no type grouping (**b** NADH-TR reaction), mitochondrial accumulation (**c** modified Gomori trichrome), nor glycogen deposit (**d** PAS staining)

The c.T1681C variation had not been previously reported in any database. This variation substitutes a highly conserved hydrophobic amino acid (tyrosine) at position 561 with a basic hydrophilic amino acid (histidine) in domain III (Fig. 4a).

We used the most common in-silico tools to know the effect of this variation; all of them predicted that this variation is deleterious (Table 1). Furthermore, the 3D modeling study was used to predict the effect of the p.Y561H variation on the protein structure. It showed that substitution leads to the destruction of the hydrogen bond SER559-PRO558 and the appearance of two new hydrogen bonds: Glu562-Thr560 and Ser559-Thr560 (Fig. 4b). The root mean square value (RMS) calculated between backbone atoms of both wild-type and mutant protein models was very significant (28.31 Å). This variant was not found in 100 Tunisian healthy control individuals, supporting its pathogenic nature; thus, it could be considered a disease-causing mutation.

Discussion

In the present study, we report a sporadic case of myopathy. The clinical presentation, consanguinity in this family and, course of, the disease are suggestive of autosomal recessive limb-girdle muscular dystrophy type 2. The genetic analysis revealed a novel missense mutation (c.T1681C/ p.Y561H) at the homozygous state in exon 13 of the *CAPN3* gene. Indeed, it is well known that the missense mutations are the most frequent type of mutations (60–70%) and frequently occur in the domain III of calpain-3 (Ono et al. 2016; Fadaee et al. 2016). This mutation, located in the C2-like domain (domain III), leads to a significant change in the 3D structure of the calpain-3 protein. Therefore, this may disrupt the conformation of the domain III, compromising proper

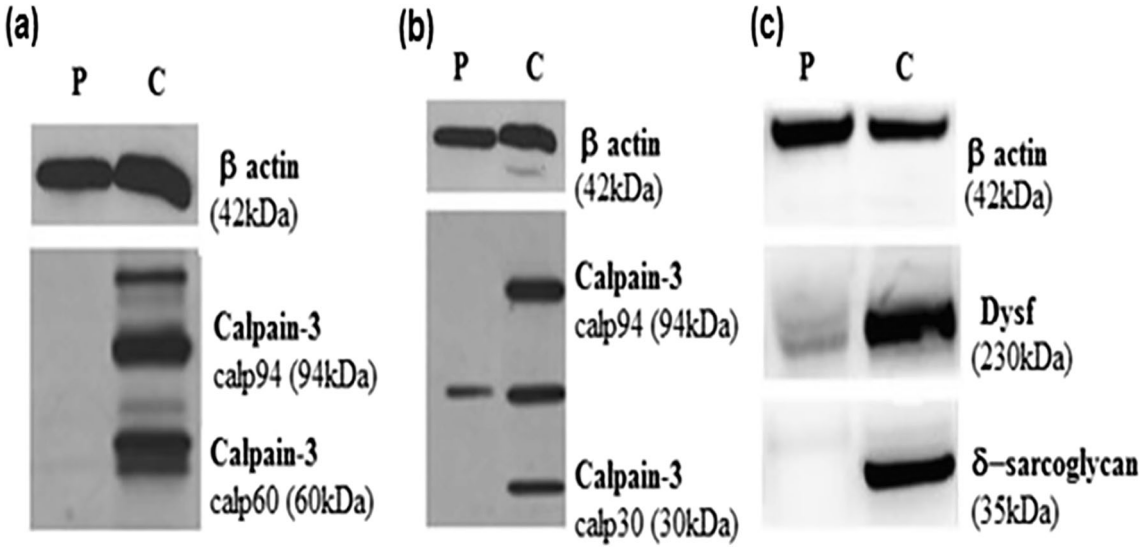

Fig. 2 Immunoblot analysis showing a complete absence of calpain-3 (**a**, **b**), absence of δ-sarcoglycan, and reduced expression of Dysferlin (**c**). P patient, C control

substrate recognition of calpain-3. In fact, domain III of this protein is known to interact with substrate aars (aminoacyl-tRNA synthetase), an important class of enzymes crucial for maintaining accuracy during translation of the genetic code (Srujana et al. 2012). Therefore, these mutations alter the regulation of its activity (Huang et al. 2005; Ono et al. 2016). On the other hand, it has been demonstrated that missense mutations in this domain can affect the enzyme activity and the ability of CAPN3 to bind to titin (Ono et al. 1998; Kramerova et al. 2004). Titin (also called connectin) is a giant cytoskeletal protein important for both sarcomere assembly and function (Gregorio et al. 1999). Also, titin serves to stabilize CAPN3 from autoproteolytic degradation. Hence, loss of titin anchorage and disruption of proteolytic activity are the two ways in which CAPN3 mutations might be pathogenic (Kramerova et al. 2007). A large number of published studies suggest that calpain-mediated proteolysis may be early rate-limiting steps in

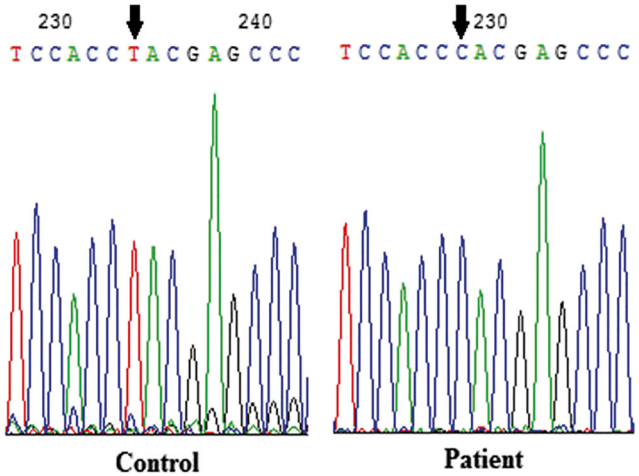

Fig. 3 Electropherograms of exon 13 of *CAPN3* gene: note the transition of thymine by cytosine

myofibrillar protein degradation during muscle atrophy. So, we suggest that the mutation identified in our case favored this step of rapid protein degradation and the development of muscle atrophy (Jackman et al. 2004; Shenkman et al. 2015).

The results of the immunohistological study and immunoblotting analysis were misleading. In addition to the calpain-3 deficit, the immunoblotting analysis showed the absence of the δ-sarcoglycan band with reduced expression of dysferlin. Immunohistological test confirmed complete absence of δ-sarcoglycan.

The deficit of calpain-3 with reduced expression of dysferlin was already reported by several authors. They described a secondary decrease of sarcolemmal dysferlin staining in some patients with LGMD2A (Chrobáková et al. 2004; Groen et al. 2007) (Table 2). On the other hand, the reduction of calpain-3 was found in patients with dysferlinopathy (LGMD2B) (Anderson et al. 2000; Lennon et al. 2003; Fanin and Angelini 2015). Furthermore, in a recent study using co-immunoprecipitation assays, a biochemical interaction between dysferlin and calpain-3 was demonstrated (Lennon et al. 2003). Thus, the reciprocal loss of calpain-3 and dysferlin in LGMD2A and LGMD2B could be explained. It is suggested that the biochemical interaction between these two proteins is necessary for the homeostasis of the myocyte. Dysferlin functions to stabilize Calpain-3 at the membrane and vice versa (Lennon et al. 2003). Alternatively, calpain-3 may play a role in dysferlin-mediated plasma membrane repair. Other studies had hypothesized that calpain-3 might function in membrane repair by regulating annexins A1 and A2, proteins that interact with dysferlin (Lennon et al. 2003).

On the other hand, our patient also presented δ-sarcoglycan deficiency. Usually, patients with calpainopathy have normal expression of the different types of sarcoglycans (Guyon et al. 2003) (Table 2). However, an in vitro study performed by

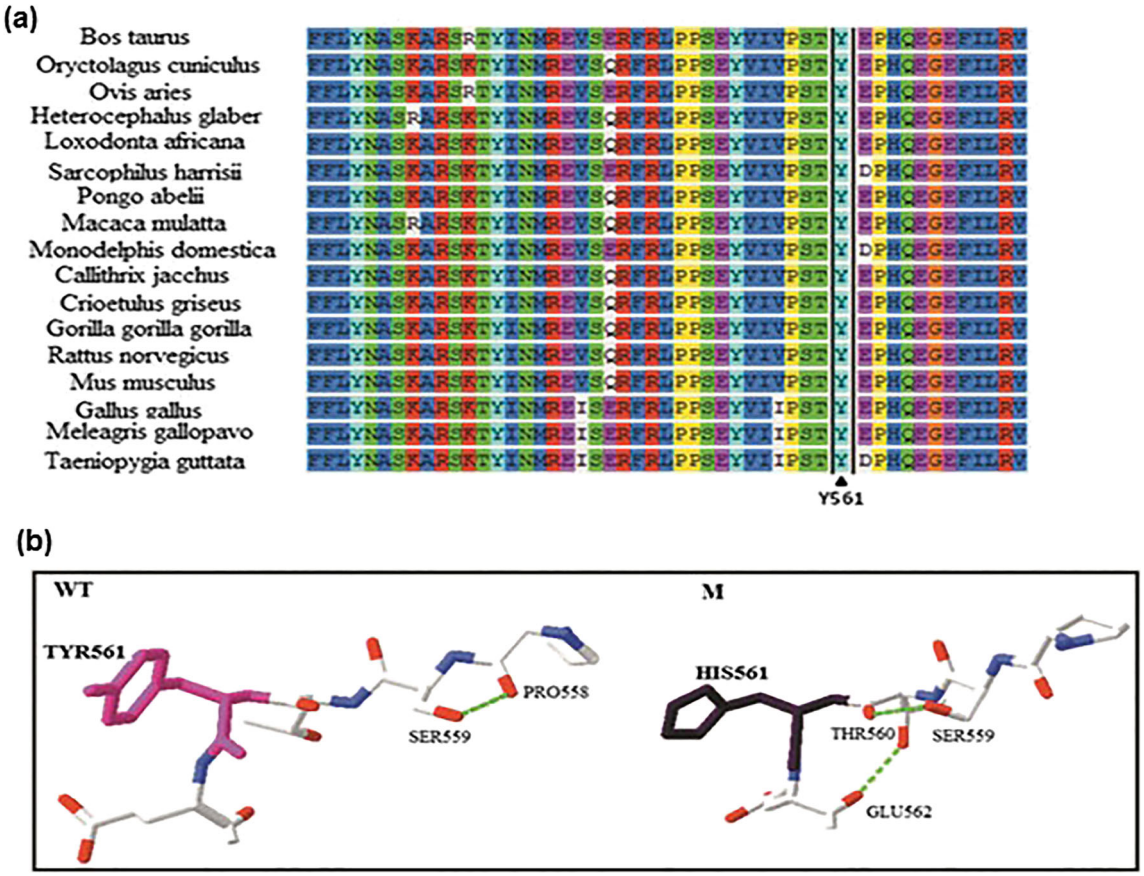

Fig. 4 **a** Multiple sequence alignment of CAPN3 protein in different species: note the conservation of the Tyrosine residue at position 561. **b** 3D modeling of the CAPN3 protein predicting the effect of the p.Y561H mutation on the spatial configuration of the protein

Jeffrey et al. demonstrated that the removal of the last 127 amino acids from FLNC (filamin C) abolishes the interaction of the C-terminus of FLNC with the cytoplasmic domains of both γ and δ-sarcoglycans. FLNC may be an in vivo substrate for calpain-3 and interferes with the functioning and regulation of protein-protein interactions with the sarcoglycans (Guyon et al. 2003). This could explain the absence of δ-sarcoglycan in our patient carrying the p.Y561H mutation. In fact, according to the 3D modeling study, this mutation causes a significant loss of the protein structure that may lead to severe distortions in domain III core, altering the shape of the FLNC binding site. Further studies are highly warranted to precise the effect of this c.T1681C mutation on the regulation and expression of δ-sarcoglycan.

Table 1 Pathogenicity prediction of the variation (c.T1681C/p.Y561H) using different in-silico tools

In silico tools	Prediction	Pathogenicity score
Provean	Deleterious	− 4.032
Poly-phen2	Probably damaging	0.998
SIFT	Damaging	–
SNPs&GO	Disease	0.745
PhD-SNP	Disease	–
Mutpred	• Loss of phosphorylation at Y561 (P value = 0.0278) • Gain of disorder (P value = 0.0332)	0.735

Our molecular results best correlate with the clinical features of our patient who developed LGMD2A at the age of 35. Interestingly, this missense mutation (c.T1681C/ p.Y561H) was associated with a milder phenotype of calpainopathy with a late onset of the disease, slow progression, and no evidence of respiratory or of cardiac failure. In general, patients with *CAPN3* gene homozygous missense mutations show a relatively milder clinical phenotype, whereas patients with null mutations show a more severe phenotype (de Paula et al. 2002) (Table 2). Our patient became wheelchair-bound after 19 years of disease evolution. According to the literature, loss of ambulation occurs about 10 to 30 years after the onset of weakness (Angelini et al. 2010). The patient presented moderate calf hypertrophy, which has rarely been reported in LGMD2A (Hadj Salem et al. 2011).

In conclusion, to the best of our knowledge, this is the first report of a Tunisian LGMD2A patient with a new missense mutation (c.T1681C/ p.Y561H) in domain III of calpain-3 protein. This mutation induced a total calpain-3 and δ-sarcoglycan deficiency and a reduction of the dysferlin protein.

Table 2 Comparative study between our patient and patients reported carrying homozygous missense mutations in the domain III. (*NA* not available)

Mutation	Exon	Age of the onset	Phenotype severity		Immunological patterns			Refs
					Calpain-3	*Dysferlin*	Other sarcolemmal proteins	
c.T1681C (p.Y561H)	Exon 13	35 years	Mild		Total deficiency	Partial deficiency	Total deficiency of the δ-sarcoglycan	Our study
c.C1309T (p.R437C)	Exon 10	60 years	Mild with early respiratory insufficiency		Important deficiency	Normal expression	Normal expression	(Martinez-Thompson et al. 2018)
c.C1381T (p.R461C)	Exon 11	7 years	Mild		Total deficiency	Partial deficiency	Normal expression	(Chae et al. 2001; Izumi et al. 2015)
c.C1385T (p.L462R)	Exon 11	NA	NA		Total deficiency	Normal expression	Normal expression	(Fanin et al. 2009)
c.G1469A (p.R490Q)	Exon 11	Early	Mild		Normal expression	Normal expression	Normal expression	(Dinçer et al. 1997; Fanin et al. 2006)
c.C1611A (p.Y537X)	Exon 13	Early	Mild		Deficient	NA	Normal expression	(Dinçer et al. 1997)
c.C1621T (p.R541W)	Exon 13	14 years	Mild		Total deficiency	Normal expression	Normal expression	(Fanin et al. 2004; Piluso et al. 2005)
c.G1699T (p.G567W)	Exon 13	NA	NA		Deficient	NA	Normal expression	(Brenguier et al. 1997)
c.C1714T (p.R572W)	Exon 13	12 years	Severe		Partial deficiency	Normal expression	Normal expression	(Richard et al. 1999; Fanin et al. 2004)
c.G1715A (p.R572Q)	Exon 13	NA	NA		Deficient	NA	Normal expression	(Richard et al. 1999)

Immunohistological and immunoblotting studies are helpful to investigate patients with muscular dystrophy. If the results are confusing with multiple-protein deficiency, a genetic study becomes necessary to identify the exact type of LGMD.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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