
Mutations in LAMA2 and CAPN3 genes associated with genetic and phenotypic heterogeneities within a single consanguineous family involving both congenital and progressive muscular dystrophies

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Synopsis
LGMD (limb-girdle muscular dystrophy) and CMD (congenital muscular dystrophy) are two common forms of neuromuscular disorders which are distinguishable by their age of onset but with probably a similar underlying pathway. In the present study, we report immunohistochemical, Western-blot and genetic analyses in a large consanguineous Tunisian family with two branches, including seven patients sharing similar LGMD2 phenotype in one branch and one CMD patient in the other branch. Linkage analyses were compatible with the LGMD2A locus in one branch and the MDC1A (muscular dystrophy congenital type 1A) locus in the other branch. This result was supported by deficiency in merosin and calpain3 in the CMD patient and LGMD patients respectively. Mutation analysis revealed two distinct mutations: a c.8005delT frameshift deletion in exon 56 of the LAMA2 (laminin-α2) gene (MDC1A) was found in the CMD patient and a new homozygous mutation c.1536+1G>T in the donor splice site of intron 12 of the CAPN3 (calpain3) gene (LGMD2A) was found in the LGMD patients. RT–PCR (reverse transcription–PCR) performed on total RNA from a LGMD2A patient’s muscle biopsy showed complete retention of intron 12 in CAPN3 cDNA, generating a PTC (premature termination codon) that potentially elicits degradation of the nonsense mRNA by NMD (nonsense-mediated mRNA decay). Our results indicate that mRNA analysis is necessary to clarify the primary effect of genomic mutations on splicing efficiency that alters mRNA processing and expression level.

Key words: genetic and phenotypic heterogeneities, intron retention, limb-girdle muscular dystrophy type 2A, muscular dystrophy congenital type 1A, nonsense-mediated mRNA decay

INTRODUCTION
Muscular dystrophies are inherited myogenic disorders characterized by progressive muscle wasting and a weakness of variable distribution and severity. On the basis of distribution of predominant muscle weakness, different forms can be delineated. Two common forms of muscular dystrophies are distinguishable by their age of onset: CMD (congenital muscular dystrophy) [1] and LGMD (limb-girdle muscular dystrophy) [2]. In CMD, onset of symptoms is at birth or within the first few months of life, whereas in LGMD, they can occur in late childhood, adolescence or even adult life. Inheritance in LGMD can be either autosomal dominant (LGMD type 1) or autosomal recessive (LGMD type 2) [1], whereas CMD is always recessively inherited [3]. Several subgroups of CMD have been defined on clinical, immunocytochemical and genetic grounds. Some CMDs show clinical features predominantly derived from the muscle dystrophy called classical CMD [MDC1A (muscular dystrophy congenital type 1A)].

Abbreviations used: CAPN3, calpain3; CMD, congenital muscular dystrophy; FKRP, Fukutin-related protein; LAMA2, laminin-α2; LGMD, limb-girdle muscular dystrophy; MDC1A, muscular dystrophy congenital type 1A; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon; RT–PCR, reverse transcription–PCR.

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Figure 1  Pedigree of the Tunisian family showing the segregation of CAPN3 and LAMA2 haplotypes and mutations (the c.1536+1G>T splicing site and the c.8005delT alteration)

deficiency in the laminin-α2 chain (merosin) occurs in 40–50% of cases and is caused by defects in the laminin-α2 gene (LAMA2) on chromosome 6q22 [4]. Autosomal recessive LGMD2 are a heterogeneous group of disorders, which are characterized by progressive involvement and wasting of proximal limb-girdle muscles, and include at least 14 different genetic entities (LGMD2A–LGMD2N) [5]. The causative genes encode highly diverse classes of proteins with different localizations within or at the surface of the skeletal muscle fibre, including sarcolemmal (dysferlin, sarcoglycans) [6,7], sarcoplasmic [calpain3, TRIM32 (tripartite motif-containing protein 32)] [8,9] and sarcomeric (telethonin, titin) [10,11] proteins and the glycosylation pathway enzymes [FKRP (Fukutin-related protein), POMT1 (protein O-mannosyl-transferase 1), FKTN (Fukutin), POMGnT1 (protein O-linked–mannose β-1,2-N-acetylgalcosaminyltransferase 1), POMT2 and LARGE] [12].

During the past decade, exciting progress has been made in the field of CMD and LGMD, emphasizing differences as well as commonalities between them. It is now clear, both clinically and genetically, that CMD and LGMD can overlap, suggesting that the underlying pathology in these diseases may follow a similar pathway [13]. It has now been recognized that mutations in the same protein can give rise to very different phenotypes. In fact, FKRP mutations can manifest with variable severity ranging from a severe CMD (including type 1C, CMD with cerebellar cysts and Walker–Warburg syndrome) to a milder adult-onset LGMD (type 2I) [3,13]. Furthermore, previous studies described patients with LGMD phenotype linked to the LAMA2 locus [14].

In the present study, we report immunohistochemical and genetic analyses of patients belonging to the same large consanguineous Tunisian family with both LGMD2 and CMD phenotypes.

PATIENTS AND METHODS

Patients

In Figure 1, we describe eight patients belonging to a large extended consanguineous family originally from the south-east of Tunisia, where consanguineous marriage is the norm.

A total of 20 family members were examined and seven patients showed a clinical phenotype associated with the diagnosis
of LGMD and different from the index patient who presented with a CMD phenotype (Figure 1). Informed consent was obtained from each of the subjects, and the study was approved by the ethics committee of l’hôpital universitaire Hédi Chaker de Sfax.

**Index patient**

The index patient (V:3), a 5-year-old son, third child of healthy parents who are first cousins, was born at term following spontaneous vaginal delivery. Since his birth he developed a severe muscle hypotonia and a generalized muscle weakness with joint contractures of variable severity. On his first hospitalization, at the age of 2, neurological examination showed generalized hypotonia and muscle weakness with a light calf hypertrophy. He had delayed motor milestones, but he was able to stand up. The deep tendon reflex was abolished, and head control was perfect. He had normal head circumference and no cognitive impairment. Brain MRI (magnetic resonance imaging), performed at 2 years of age, showed abnormal signals of the periventricular white matter without cerebral cysts.

**Family study**

Neurological examination showed, in all LGMD cases, that the first complaints were difficulties in running, climbing stairs and frequent falls. At all stages of the disease, muscle weakness predominated in limb-girdle and trunk muscles and was almost symmetrical, with very selective involvement of certain groups of muscles. Serum creatine kinase is markedly increased especially for the patient V:6 in the early phases of the disease. They displayed similar mild limb-girdle muscular dystrophy phenotypic features, but heterogeneity was expressed by variability in the age of onset, in the age of wheelchair-bound state and in the course of the disease. The cases IV:15 and V:6, whose ages of onset are earlier, reported a pronounced atrophy of quadriceps muscle and biceps and triceps muscles respectively. Facial and ocular muscles were not affected. All the LGMD patients were clinically evaluated according to the Vignos scale [15]. The main clinical features of the eight patients are summarized in Table 1.

**Methods**

**Immunohistochemical analysis**

Muscle biopsies were taken from deltoid muscles after the patients’ informed consent was obtained, frozen in liquid nitrogen immediately after removal and stored at –80°C until use. Serial 4 μm cryostat sections of available muscle biopsies from normal control tissue, one CMD patient (V:3) and three LGMD patients (IV:12, V:5 and V:6) were prepared and mounted on to superFrost® Plus slides. Immunohistochemical expression of dystrophin, γ-sarcoglycan, α-sarcoglycan, α-dystroglycan and laminin-α2 were evaluated using the following antibodies: DYS1 and DYS2 (Novocastra, Newcastle, U.K.; diluted both: 1:30) recognizing the mid-rod domain and the C-terminus of dystrophin respectively, anti-γ-sarcoglycan (AbC10-G005; diluted: 1:100), anti-α-sarcoglycan (AbC10-A015; diluted: 1:200), anti-α-dystroglycan (VIA4-1; Upstate Biotechnology; diluted: 1:100), anti-human merosin 80 kDa fragment toward the N-terminus of dystrophin respectively, anti-γ-sarcoglycan (AbC10-G005; diluted: 1:100), anti-α-sarcoglycan (AbC10-A015; diluted: 1:200), anti-α-dystroglycan (VIA4-1; Upstate Biotechnology; diluted: 1:100), anti-human merosin 80 kDa fragment toward the C-terminus (AbCys; diluted: 1:300) and antihuman merosin 300 kDa fragment toward the N-terminus (NLCmerosin; Novocastra; diluted: 1:200) respectively. All dilutions and washings were made in PBS. All primary antibodies were applied for 1 h and developed using an appropriate secondary antibody (FITC-conjugated rabbit anti-mouse immunoglobulins; Dako). The sections were examined under a Zeiss Axioplan fluorescence microscope.

**Western-blot analysis**

Expression of calpain3 and laminin-α2 was also measured by Western-blot analysis on muscle biopsy samples for the three LGMD patients (IV:12, V:5 and V:6) as well as one LGMD2C patient and a normal tissue used as a control. For the index patient (V:3), no muscle sample was available for protein analysis. The 94 kDa calpain3 band was analysed using two monoclonal antibodies against epitopes in the muscle-specific calpain protein (NCL-Calp 3d/2C4 and NCL-Calp3c/12A2; Novocastra). The anti-80 kDa monoclonal antibody directed against the C-terminal domain of the laminin-α2 chain was also used to evaluate laminin-α2 expression. Conventional immunoblot
Table 2 Primer pairs of \textit{CAPN3} gene used for detection of mutation by DNA sequencing

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer (5′–3′) Upper</th>
<th>Primer (5′–3′) Lower</th>
<th>PCR product size (bp) (genomic DNA)</th>
<th>PCR temperature (°C)</th>
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<td>CAPN3-Ex1</td>
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<tr>
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<tr>
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danalysis of calpain3 and laminin-α2 was conducted as described previously [16].

\textbf{Linkage analysis}

After informed consent, blood samples were obtained from patients and the available family members. Genomic DNA was extracted from whole blood using standard procedures [17]. Linkage analysis was carried out with the highly informative (CA)\textsubscript{n} polymorphic microsatellite markers following microsatellite primers spanning the LGMD2A locus (D15S514, \textit{CAPN3}, D15S780, D15S781), the LGMD2B locus (D2S2113, \textit{DYSF}, D2S2112), the LGMD2C locus (D13S232, \textit{SGCG}, D13S1243, D13S1285), the LGMD2D locus (D17S806, D17S1319), the LGMD2E locus (D4S1536, \textit{SGCB}, D4S1577, D4S2996), the LGMD2F locus (D5S640, D5S673, \textit{SGCD}, D5S412), the LGMD2I locus (D19S219, FKRP52, \textit{FKRP}, D19S606) and the MDC1A locus (D6S407, D6S1620, \textit{LAMA2}, D6S1705) (http://genome.ucsc.edu/cgibin/hgGateway). The microsatellite markers were typed in all available family members using radioactive genotyping analysis.

\textbf{Mutation analysis}

The 24 exons and flanking intron region of the calpain3 gene were tested for mutation first in one of the seven LGMD patients by sequence analysis. PCR amplification of all 24 \textit{CAPN3} (calpain3) exons was performed using the primer sets as described in Table 2. Mutation analysis for the CMD case (V:3) was performed by PCR amplification of each of the 65 encoding exons of the \textit{LAMA2} gene and the intron–exon junctions as previously published [18]. Each PCR product was purified by enzyme reaction (exonuclease I; 20 units/μl; Fermentas), and directly sequenced using a Big-Dye di-deoxy-terminator cycle sequencing kit and an ABI-PRISM 3100 automated sequencer (Applied Biosystems). The BLAST homology searches were performed using the programs available at the NCBI (National Center for Biotechnology Information) website and compared the human \textit{CAPN3} and \textit{LAMA2} gene sequences with the wild-type sequences.

The c.1536+1G>T mutation identified in the exon 12 fragment of the \textit{CAPN3} gene was investigated by direct sequencing in 100 Tunisian healthy control individuals. The c.8005delT mutation, occurring in the \textit{LAMA2} gene, induces the creation of an \textit{EcoRI} restriction site. The \textit{EcoRI} restriction pattern of the exon 56 fragment (255 bp) was used to confirm the presence of the mutation in patients’ relatives and to screen 100 unrelated healthy Tunisian individuals. Digestion of PCR products was performed according to the manufacturer’s instructions (Jena Bioscience), followed by separation on 2% Nusieve gels.
RNA isolation and RT–PCR (reverse transcription–PCR)

Total RNA was extracted from frozen muscle samples with the SV Total RNA Isolation System (Promega). RT–PCR, covering the coding sequence of exons 11–14, was performed for one LGMD2A patient using the following primers: 5′-CTGGGTACGGGGTTGCTCT-3′ (forward primer 44 pb downstream of exon 11) and 5′-CGATCCACGAGATGGTATT-3′ (reverse primer 30 pb upstream of exon 15), with the PrimeScriptTMRT-PCR kit (Takara Bio) according to the manufacturer’s recommendations. The PCR was performed for 30 rounds, each consisting of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. The expected PCR products of 465 bp were separated and visualized under UV light by electrophoresis on 2% agarose gels stained with ethidium bromide.

Bioinformatic analysis

The prediction of the potential pathogenetic effect of the frameshift mutation c.8005delT at the protein level was done by in silico (in or by means of a computer simulation) analysis using specific software programs (http://www.expasy.ch/tools/dna.html and http://www.expasy.ch/prosite/swiss-Prot/LAMA2). The multiple alignments of the laminin-α2 peptide sequences were performed using the ClustalW program. Sequences from the species were obtained from the NCBI database.

RESULTS

Immunohistochemical and Western-blot analyses

Muscle biopsy revealed histological changes characteristic of muscular dystrophy in all patients, although the muscle specimen from the CMD patient was more severely affected and was characterized by connective tissue proliferation (Figure 2). Laminin-α2 was partially expressed with both antibodies and positivity on muscle fibre surfaces was patchy, with varied intensity from fibre to fibre and fascicle to fascicle (Figure 2). α-Dystroglycan, α-sarcoglycan, γ-sarcoglycan and dystrophin immunostaining were slightly reduced on muscle fibre surfaces of patient V:3 (Figure 2).

On the basis of clinical and immunohistochemical data, we suggest that the patient V:3 with hypotonia, white matter changes, normal intelligence and laminin-2 deficiency could be affected by the classical CMD form (MDC1A), which is due to LAMA2 gene defects. Immunofluorescence analysis of skeletal muscle sections taken from patients IV:12 and V:5 diagnosed with LGMD was performed using monoclonal antibodies recognizing laminin-α2, α-dystroglycan, α- sarcoglycan, γ-sarcoglycan and dystrophin. A near normal level of expression of these proteins was detected in both patients when compared to normal control tissue (Figure 2). However, a slight reduction in the staining of all muscle sections studies in this series was seen in patient V:6, except for a monoclonal antibody raised against the N-terminal region of the laminin-α2 in which case it showed a near normal staining in immunohistochemical studies (Figure 2). Western blots performed for these three patients using calpain3 antibodies revealed a total deficiency in calpain3 (absence of the 94, 60 and 30 kDa bands in the blot; Figures 3a and 3b), whereas normal protein levels for calpain3 were observed in normal and LGMD2C muscle biopsies used as a control. Western-blot analysis using an antibody that recognizes the 80 kDa merosin fragment revealed a normal amount in all samples of biopsies (Figure 3c). In summary, all these immunohistochemical and Western-blot findings were compatible with a calpainopathy in the LGMD cases.

Linkage and sequencing analyses

Results of linkage analysis revealed an exclusion of the LGMD loci LGMD2C, LGMD2D, LGMD2E, LGMD2F, LGMD2I and LGMD2B in this family including two branches (results not shown), and all LGMD patients (IV:12, IV:13, IV:14, IV:15, V:4, V:5 and V:6) were found to be homozygous for markers surrounding the CAPN3 locus (D15S514, D15S780 and D15S781) on chromosome 15q15, suggesting homozygous mutation in CAPN3 gene (Figure 1). However, the haplotype analysis was compatible with linkage to the LAMA2 locus on 6q22 for only the branch family of the CMD patient V:3 (Figure 1). For the second branch of this family, genetic analysis excluded linkage to the LAMA2 locus. To confirm the genetic linkage results, we performed a mutation screening of all CAPN3 and LAMA2 gene exons. Mutation screening of the CAPN3 coding region and intron–exon boundaries revealed for all seven LGMD patients the same novel homozygous splicing site c.1536+1G>T, resulting in a substitution positioned at the first nucleotide of intron 12, changing a guanine into a thymine residue (Figure 4a). Using direct sequencing, the mutation was found to segregate with the disease in an autosomal recessive fashion in the parents of patients IV:12, IV:13, IV:14, IV:15, V:4, V:5 and V:6. This variant was absent by direct sequencing in 100 control chromosomes.

After sequencing all the 65 coding exons of LAMA2 gene and their intron–exon junctions, we identified a homozygous 1 bp deletion, c.8005delT, in the CMD patient V:3 (Figure 4b). We took advantage of the creation of an EcoRI restriction site induced by this mutation to verify the segregation and to exclude in controls (Figure 4c). Normal control DNA remained uncut at 255 bp, whereas the product containing the mutation in homozygous state (V:3) is cut into two fragments of 155 and 100 bp. Only one of the two alleles was cut for parents (IV:3 and IV:4) and the two siblings (V:1 and V:2) who are heterozygous carriers of the c.8005delT mutation (Figure 4c). This variant was not found in 100 Tunisian healthy control individuals.

mRNA analysis

In order to establish the effect of the splicing site mutation c.1536+1G>T on RNA splicing of the CAPN3 gene, we analysed CAPN3 mRNA obtained from muscle biopsies. Using primers within exons 10 and 14, RT–PCR revealed an amplified fragment of 465 bp in the control, which corresponds to
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Figure 2 Immunofluorescence labelling of muscle biopsies

Immunofluorescence labelling of C-terminal laminin-α2 chain (80 kDa), N-terminal laminin-α2 chain (300 kDa), α-dystroglycan, α- and γ-sarcoglycans (α-SG and γ-SG) and dystrophin (DYS1 and DYS2) in muscle biopsies from normal control, three LGMD (IV:12, V:5 and V:6) patients and one DMC (V:3) patient.

Bioinformatic analysis: prediction of the frameshift mutation c.8005delT effect in LAMA2 gene

In order to predict the effect of c.8005delT mutation on laminin-α2 protein structure and function, we investigated in silico the effect of the deletion, since the CMD patient’s muscle biopsy was not available to perform mRNA assay. We noted first that the deletion of T led to a frameshift from the residue 2669 and the addition of 58 amino acids that are different from the wild-type sequence. By using multiple alignments, a particular stretch of these 58 amino acids was found to be conserved among the sequences aligned (Figure 6). The frameshift caused the expected splicing product. In contrast, RT–PCR experiments displayed a longer band of 1123 bp in the LGMD2A patient (Figure 5a) caused probably by the retention of the 12th intron in the CAPN3 transcript. In order to verify this hypothesis, the RT–PCR products from both the control (Figure 5b) and the patient were sequenced. As expected, sequence analysis revealed retention of the entire intron 12 in LGMD2A patients (Figure 5c), thus confirming that the (G>T) mutation at the highly conserved GU splice donor site completely abolishes intron 12 5′-splicing site selection, leading to the 5′-splicing mutant variant of calpain3.
Genetic and phenotypic variabilities in a single family of muscular dystrophies

Figure 3 Immunoblot analysis of calpain 3 and laminin-α2 proteins
(a–c) Detection of calpain3 and laminin-α2 by Western blot using the monoclonal antibodies NCL-CALP-12A2 (a), NCL-CALP-2C4 (b) and anti-80 kDa fragment (c); lane 1: the control; lane 2: patient V:6; lane 3: patient V:5; lane 4: patient IV:12; and lane 5: LGMD2C patient. (d) Myosin stain acts as a control of the specimen preservation.

The occurrence of a PTC (premature termination codon) at position 2727 in the proximal part of the LG3. The truncated protein, if translated, would contain only 2727 residues and leads to the loss of the C-terminal 395 amino acids of merosin including LG4 and LG5 domains of the G globular domain of laminin-α2 representing the functional binding region of merosin to the α-dystroglycan.

DISCUSSION

In the present study, we performed immunohistochemical and genetic analyses of one CMD and seven LGMD2 patients belonging to the same large consanguineous family. So far, LGMD2C is the most common genetic form encountered in Tunisia and most of the LGMD2 Tunisian patients tested so far were found to be homozygous for c.del521T mutation in the SGCG gene. For the first time, we have described here evidence for the locus of LGMD2A that had not been previously reported in Tunisian LGMD families. Although the seven LGMD cases share the same novel mutation c.1536+1G>T in the CAPN3 gene, an intrafamilial clinical variability was noted. The splicing site mutation c.1536+1G>T led to a complete retention of intron 12 in CAPN3 cDNA associated with a total deficiency of calpain3 protein.

Immunohistochemical and Western-blot analyses showed muscle protein expression concordant with calpainopathy. Indeed, immunohistochemical study showed a near normal sarcolemmal labelling with all antibodies tested. Under normal conditions, three calpain3 bands of 94, 60 and 30 kDa are detected in Western blots [19], whereas Western-blot analysis of muscle calpain3 in LGMD2A patients can show a total, partial or (more rarely) no apparent deficiency, with no direct correlation between the amount of the protein and the severity of the phenotype [20]. Concerning our patients, Western-blot analyses were performed using either NCL-CALP-12A2 antibody or the NCL-CALP-2C4 antibody and the result revealed the absence of calpain3. Very low levels or no expression of calpain3 were seen in European and Brazilian patients with the clinical course varying from mild to severe [21].
DNA linkage analysis with both intragenic microsatellite markers and those adjacent to genes implied the involvement of two different disease loci, namely LGMD2A in one family branch (patients IV:12, IV:13, IV:14, IV:15, V:4, V:5 and V:6) and CMD1A in the other one (patient V:3). Mutation analysis of the CAPN3 gene confirmed the presence of a new splice mutation in the donor splice site of intron 12, c.1536+1G>T, in the seven LGMD cases in a homozygous state, and in a heterozygous state in the CMD case (V:3). More than 448 independent pathogenic mutations have been reported up to now in CAPN3 gene. Among these mutations, a few are splice site mutations that have been reported to occur in domain III and a few have been analysed in patients by performing mRNA analysis (see http://www.dmd.nl/CAPN3) [22]. RT–PCR and sequencing analysis of CAPN3 mRNA isolated from a patient’s muscle biopsy showed an abnormal CAPN3 spliced transcript as a result of the retention of the entire intron 12 and containing an in-frame premature translation stop codon at nucleotide c.1536+3. Intron retention is certainly the least studied of all types of alternative splicing, and only a partial intron retention has been reported in the LGMD2A cases described in the CAPN3 gene [23]. However, intron retention has been reported in many human disorders such as in Ullrich congenital muscular dystrophy [24], breast cancer [25] and amyotrophic lateral sclerosis [26].

With regard to the complete absence of calpain3 on Western-blot analysis, two hypotheses may be evoked: either the
degradation of the nonsense mRNA, or possibly the translation of a truncated calpain3 protein lacking a part of domain III and domain IV. The most probable hypothesis is that the resulting nonsense mRNA is probably destroyed by the quality control surveillance mechanism NMD (nonsense-mediated mRNA decay) according to the PTC position rule (>50–55 nt upstream of the last exon–exon junction), which is detected in our patient at position c.1536+3. Often, retention of internal introns restricts the export of these mRNAs and makes them the targets for degradation by the NMD pathway, particularly if they contain PTC [27,28].

For the second branch of our family, the CMD patient showing a linkage to the MDC1A locus, sequencing of all 65 coding exons and their intron–exon junctions revealed homozygous frameshift deletion, c.8005delT, in exon 56 of the \textit{LAMA2} gene. Immunohistochemical studies using two antibodies against the C-terminal and N-terminal regions of the laminin-α2 chain showed a partial expression of the laminin-α2 protein assembled at the myofibre junctions. No muscle sample was available for mRNA analysis. The c.8005delT leads to a change of the 58 amino acids sequence (from residues 2670 to 2727) before the creation of a PTC at position 2728, and thus the loss of the C-terminal 395 amino acids of merosin, including the fourth and the fifth LG modules of the G globular domain of laminin-α2 representing the functional binding region of merosin. In contrast to our family, previously published mutations affecting this domain are associated with a severe phenotype and total absence of laminin-α2 chain in muscle fibres [29].

In the present study, we noted heterogeneous phenotypes between our LGMD2A patients sharing the same mutation. They displayed similar mild LGMD phenotypic features but heterogeneity was expressed by variability in the age of onset, in the age of becoming wheelchair-bound and in the course of the disease. A wide intra- and inter-familial clinical variability ranging from severe to milder forms was reported in a multicentre study of 163 European LGMD2A patients [30]. The mean age at onset was 13.7 years (ranging from 2 to 40 years old) and the mean age of loss of walking ability was 17.3 years (range 5–39 years) after onset, with no sex difference in age at onset or progression. Fardeau et al. [31] noted that approx. 50% of the LGMD2A patients lost mobility around the age of 20 and that the earlier the onset, the faster the evolution. This has not been observed in our family, with the cases IV:12, IV:13 and IV:14, who remained ambulant until around the age of 30 years.

This intra-familial variability, in both clinical phenotype and disease course, encountered in our patients sharing the same mutation could be due to differences in environmental factors or to the genetic background (influence of modifier gene) or both [32]. However, the predominance of phenotypic variability between patients IV:12, IV:13, IV:14 or patients V:4, V:5, V:6, usually living in similar environmental conditions, suggests that genetic factors may play an important role in the expression of this clinical variability rather than the environmental background. The different findings in LGMD2A patients could be partially caused by inter-individual variation in NMD efficiency, related to yet unexplained genetic modifying factors [33,34].

In conclusion, we have described, in the present study and for the first time, a Tunisian family with LGM2A and MDC1A forms. LGM2A patients carried a new splicing site mutation c.1536+1G>T in the \textit{CAPN3} gene, which is, to our knowledge, the first reported mutation that leads to complete retention of intron 12 of the \textit{CAPN3} gene and total calpain3 deficiency. We also reported a single base deletion at position 8005 (c.8005delT) in the \textit{LAMA2} gene associated with a severe form of classical congenital muscular dystrophy and partial merosin deficiency in MDC1A patients belonging to the second branch of the same family. Indeed, the genetic heterogeneity observed in this family drew attention to the difficulty of genetic counselling in inbred populations. Our results illustrate the practical importance of RNA analysis for a reliable establishment of mutation status, and provide an interesting insight into the processes of mRNA decay in cells of LGMD2A patients.

**AUTHOR CONTRIBUTION**

Ikhlass Hadj Salem led the literature review, performed experiments, analysed and interpreted the data, and wrote the manuscript. Fatma Kamoun performed the neurological investigation of the patients. Nacim Louhichi reviewed the paper and contributed to the analysis of the CMD patients’ results. Souad Rouiss provided the necessary equipment and contributed advice for the Western blotting analysis. Mariam Mziou took part in the clinical investigations. Nourhene Fendri-Kriaa contributed to the manuscript preparation and gave conceptual advice. Fatma Makni-Ayadi performed the measurement of the creatine kinase levels in all of the patients. Chahnez Triki supervised the clinical investigations. Faiza Fakhfakh supervised the study from the experiments to writing, analysed and discussed the results and commented on the manuscript at all stages.

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Genetic and phenotypic variabilities in a single family of muscular dystrophies


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