

Telethonin protein expression in neuromuscular disorders

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Abstract

Telethonin is a 19-kDa sarcomeric protein, localized to the Z-disc of skeletal and cardiac muscles. Mutations in the telethonin gene cause limb–girdle muscular dystrophy type 2G (LGMD2G). We investigated the sarcomeric integrity of muscle fibers in LGMD2G patients, through double immunofluorescence analysis for telethonin with three sarcomeric proteins: titin, α -actinin-2, and myotilin and observed the typical cross striation pattern, suggesting that the Z-line of the sarcomere is apparently preserved, despite the absence of telethonin. Ultrastructural analysis confirmed the integrity of the sarcomeric architecture. The possible interaction of telethonin with other proteins responsible for several forms of neuromuscular disorders was also analyzed. Telethonin was clearly present in the rods in nemaline myopathy (NM) muscle fibers, confirming its localization to the Z-line of the sarcomere. Muscle from patients with absent telethonin showed normal expression for the proteins dystrophin, sarcoglycans, dysferlin, and calpain-3. Additionally, telethonin showed normal localization in muscle biopsies from patients with LGMD2A, LGMD2B, sarcoglycanopathies, and Duchenne muscular dystrophy (DMD). Therefore, the primary deficiency of calpain-3, dysferlin, sarcoglycans, and dystrophin do not seem to alter telethonin expression.

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1. Introduction

The limb–girdle muscular dystrophies (LGMDs) include a heterogeneous group of progressive disorders mainly affecting the pelvic and shoulder girdle musculature. The inheritance may be autosomal dominant (LGMD1) or recessive (LGMD2). Clinically, it ranges from severe forms with onset in the first decade and rapid progression, to milder forms with later onset and slower progression [1,2].

Six autosomal dominant LGMD forms, designated LGMD1A to LGMD1F, were already mapped respectively at 5q22–q34 (myotilin) [3,4], 1q11–21 (lamin A/C [5]), 3p25 (caveolin-3 [6,7], 6q23, 5q31, and 7q (neuromuscular disorders—gene location, September 2001). At present, nine AR forms have been mapped and, with the exception of LGMD2H at 9q31–33 [8] and LGMD2I at 19q13.3 [9], all the others have had their protein products identified. Four of them, mapped at 17q21, 4q12, 13q12, and 5q33, encode respectively for α -sarcoglycan (α -SG), β -SG, γ -SG, and δ -SG, which are glycoproteins of the sarcoglycan sub-complex of the dystrophin–glycoprotein complex (DGC), which plays an important role in muscle fiber integrity [10,11]. Mutations in these genes cause, respectively, LGMD2C, 2D,

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2E, and 2F and constitute a distinct subgroup of LGMDs, the sarcoglycanopathies [12–23]. The three other identified forms are LGMD2B at 2p31, coding for dysferlin [24–26]; LGMD2A at 15q15.1, coding for calpain-3 [27–29] and LGMD2G at 17q11–12, coding for telethonin [22,23]. In addition to the mechanical and structural function of the DGC, it has been recently shown that this complex might play a role in cellular communication [30] as well as interact with the sarcomeric network, through the binding of dys-trophin to F-actin [see revision in [31]].

Telethonin is a sarcomeric protein of 19 kDa possibly localized to the Z-disc of adult striated skeletal and cardiac muscles, where it interacts with the protein titin [32]. The sarcomere is the unit of skeletal and cardiac muscle contraction. In the past few years, there have been major advances in the elucidation of the role of skeletal and cardiac muscle proteins, and mutations in several sarcomeric proteins such as actin [33], tropomyosin 3 and 2 [34,35], Troponin T1 [36], and myotilin [4] were shown to cause human muscle diseases.

Recently, we have shown that mutations in the telethonin gene cause LGMD2G, a relatively mild form of autosomal recessive LGMD, originally mapped in three Brazilian families. This was confirmed through immunohistochemistry and Western blot analysis using an anti-telethonin antibody in muscle of affected patients that presented no protein expression [22,23].

The protein α -actinin is localized in the Z-disk of the sarcomere [37], while the recently identified myotilin is a 57-kDa cytoskeletal protein, with two Ig-like domains homologous to titin, which co-localizes with α -actinin in the Z-line of the sarcomere [3].

Here, we are reporting the results from

- (a) The analysis of the sarcomeric structure of muscle fibers in patients with LGMD2G, through double co-localization immunofluorescence for telethonin with the Z-band proteins α -actinin-2 and myotilin, as well as through electron microscopy (EM) analysis.
- (b) The possible interaction of telethonin with other proteins associated with several forms of AR-LGMD (2A, 2B, SGpathies), Duchenne muscular dystrophy (DMD), nemaline myopathy (NM), and spinal muscular atrophy (SMA).

2. Patients and methods

2.1. Patients

A total of 42 myopathic patients with known mutations were included in this investigation: 6 LGMD2G (4 families), 9 LGMD2A, 14 LGMD2B, 2 LGMD2C, 3 LGMD2D, 2 LGMD2E, 1 LGMD2F, 3 DMD, and 2 NM. One of the LGMD2G patients was identified through telethonin analysis in a sample of 48 patients: 35 still unclassified LGMD patients and 13 with clinical diagnosis of Kugelberg–Welander SMA. Telethonin was also studied in 2 muscle samples from patients with known mutations in the SMA gene, for analysis of muscle under denervation.

The diagnosis of the patients was established through clinical examination and course of the disease, family history, serum creatine-kinase levels [3], DNA analysis (linkage or screening for mutations), and/or protein studies on muscle biopsies (dystrophin, the four sarcoglycans, calpain-3, dysferlin, and telethonin).

2.2. Muscle biopsies

Muscle samples were obtained from bicep biopsies (for diagnostic purposes or after informed consent) frozen in liquid nitrogen immediately after removal and stored at -70°C until use. Routine histological and histochemical procedures were done, with staining for HE, modified Gomori trichrome, NADH, ATPase 9.4, 4.3, and acid and alkaline phosphatase [38]. One female patient (Table 1, #2) underwent two muscle biopsies within a 10 year interval.

The fiber typing was determined by counting 300–500 fibers from each patient in ATPase 9.4 and 4.3 reactions, and by calculating the percentage of type I and type II fibers.

2.3. Protein analyses

Mouse anti-telethonin antibody was raised against a recombinant fragment of telethonin consisting of 128 C-terminus amino acids [39]. Additional antibodies used were: mouse anti-titin (Novacastra and Sigma), rabbit α -actinin-2 [37], rabbit myotilin antibody [4], rabbit polyclonal N-terminal 303-8 for dystrophin (kindly provided by J. Cham-

Table 1
Clinical and molecular data of the LGMD2G patients at time of biopsy

Patients (biopsy number)	Family	Age	CK (fold \uparrow)	Clinical stage (maximal motor ability)	Pattern of weakness (proximal/distal)	Mutation (telethonin gene)
1 (206)	1	male 17	13	walks, climbs stairs assisted	proximal	157C>T/157C>T
2A (207) (biopsy 1)	1	female 18	6	asymptomatic	–	157C>T/157C>T
2B (912) (biopsy 2)		female 28		walks, climbs stairs unassisted	proximal	
3 (913)	1	female 22		walks, climbs stairs unassisted	distal	157C>T/157C>T
4 (201)	2	female 30	15	walks, climbs stairs unassisted	proximal	157C>T/157C>T
5 (208)	3	male 21	3	walks, climbs stairs assisted	distal	157C>T/D637GGGG
6 (927)	4	male 27	20	walks, can not climb stairs	distal and proximal	157C>T/157C>T

Table 2
Histological and histochemical data in the 7 studied biopsies, from 6 LGMD2G patients

Patients	Histology							ATPase	
	Size variation	Connective tissue	Central nuclei	Splitting	Hyaline fibres	Macrophagic fibres	Rimmed vacuoles	Type I/II (%)	Atrophy
1	+++	+	+++	+++	0	2/500	1/500	55/45	type I
2A	++	0	+	+	0	2/200	0	52/48	type I
2B	++	+	+	+	0	2/200	0	62/38	type I
3	++	0	+	0	0	1/500	3/500	80/20	type I
4	+++	+	+++	+++	0	0	8/500	66/34	type I
5	+++	+	+++	+++	1/250	5/250	10/80	44/56	type I
6	++	0	++	++	1/300	3/500	0	12/88	type II

berlain) and C-terminal monoclonal Dy8/6C5 for dystrophin (kindly provided by L.V.B. Anderson), monoclonal antibodies for sarcoglycans (described in Ref. [40]), calpain-3 [41], and dysferlin [42].

Immunohistochemical staining of frozen sections was performed using double labeling reactions for α -actinin-2 and telethonin, titin or myotilin and analyzed by fluorescence or confocal microscopy (IBUSP), as described previously [43]. Secondary antibodies were FITC-labeled anti-rabbit and CY3-conjugated anti mouse (Sigma).

Western blot analysis was performed using 6% or 13% SDS-PAGE gels, and then proteins were transferred at 150 V for 1 h [44]. The blots were reacted with antibodies to telethonin or a mix of dystrophin, dysferlin, and calpain-3. The incubations with primary antibodies were done overnight, and the detection was done using alkaline phosphatase-conjugated second antibody.

3. Results

3.1. LGMD2G patients

The results of the clinical and histological analysis done in the six LGMD2G patients are described in Table 1. With

exception of patient 5, who is a compound heterozygote (157C>T/ Δ 637GG), the others have the same homozygous 157C>T missense mutation, which creates a premature stop codon (Q53X) in the telethonin gene.

3.1.1. Histological analysis

Histological analysis showed a dystrophic pattern of muscle degeneration in all six patients, including fiber size variation, slight degree of connective tissue infiltration, presence of high number of internally localized nuclei, splitting, and a variable degree of rimmed vacuoles (Table 2 and Fig. 1). Only one patient (927) showed the presence of spread ghost fibers (see HE in Fig. 4).

Histochemical analysis revealed the presence of a mosaic pattern of type I/II fibers, with a type I predominance in two patients (#3 and 4) and type II predominance in patient 6. Small groups of fibers from the same type were observed in three patients (#1, 2, 5), a type I fiber atrophy in five patients (#1–5), while patient 6 showed type II atrophy (Table 2).

Patient #2, underwent a second muscle biopsy after 10 years, and the same anatomopathological alterations, with a very similar degree of muscle degeneration, were seen in both samples. The only observed difference was in type I/II fiber proportions, with an increase in type I predominance.

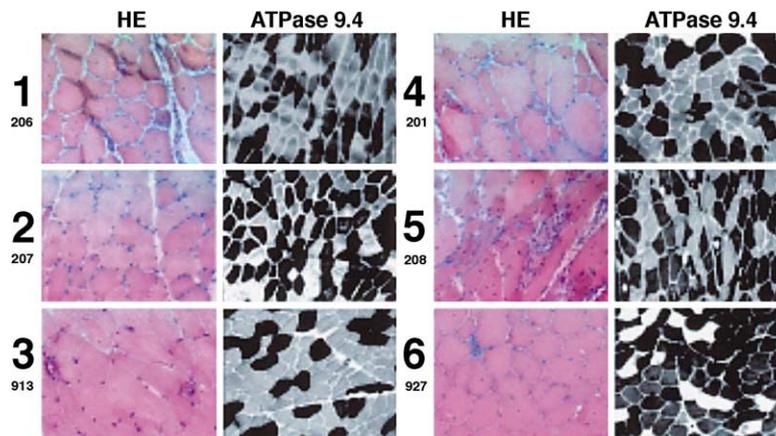


Fig. 1. Histological analysis (Hematoxylin & Eosin staining) and histochemical analysis (ATPase 9.4) in the 6 LGMD2G patients. Magnification: HE \times 200, ATPase \times 100.

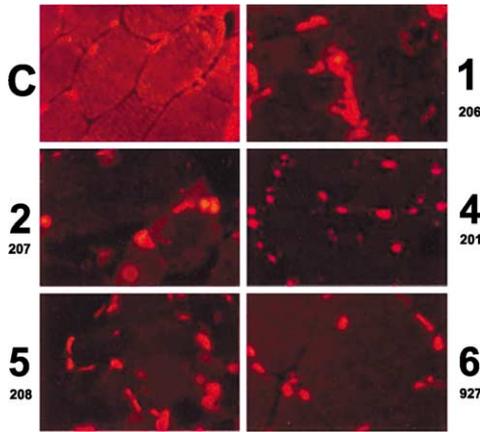


Fig. 2. Immunofluorescence analysis using anti-telethonin antibodies in LGMD2G patients, as compared to a normal control. Magnification: $\times 400$.

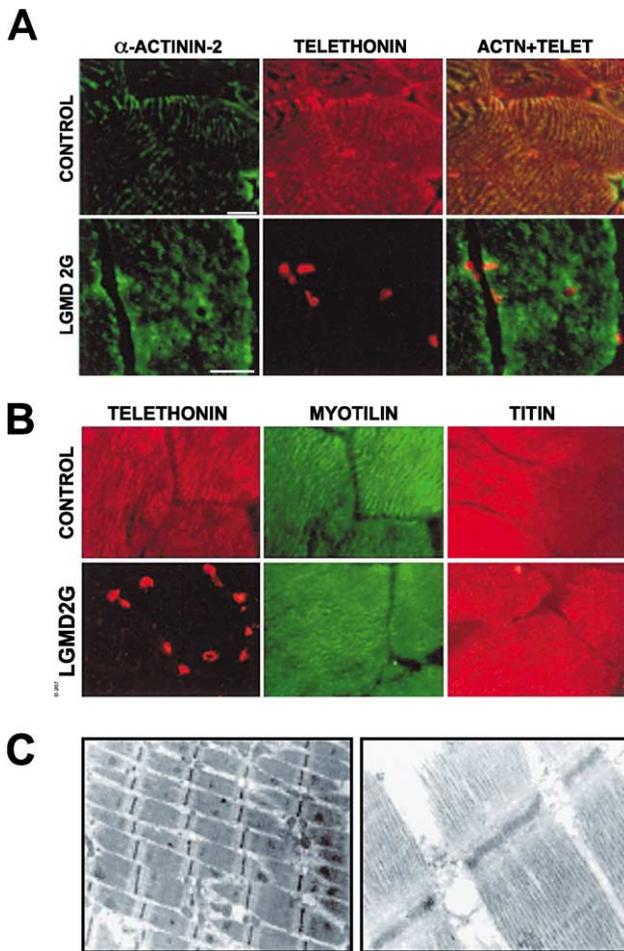


Fig. 3. (A) Double immunofluorescence for α -actinin-2 and telethonin in a normal control and in one LGMD2G patient (#2); (B) double IF for myotilin and telethonin, and the homogeneous reaction for titin (sigma antibody); (C) EM of the same sample of muscle, showing the maintenance of the sarcomeric structure ($\times 40,000$).

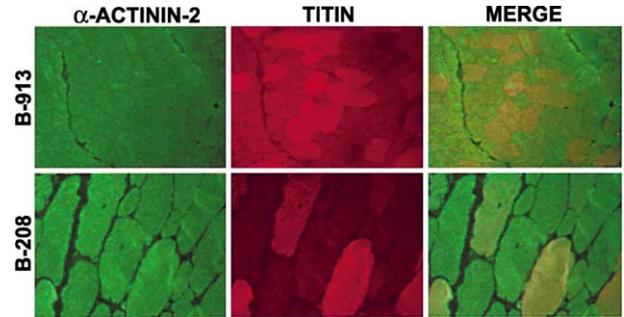


Fig. 4. Double immunofluorescence of α -actinin-2 and titin (Novacastra antibody) in two LGMD2G patients, showing the positive titin pattern with a mosaic of type II fibres with higher labeling for titin (magnification: $\times 200$).

3.1.2. Immunohistochemical analysis

In normal controls, telethonin immunofluorescence analysis showed a strong sarcomeric labeling pattern, with the majority of myonuclei also positively labeled (Fig. 2). Both type I and type II fibers were equally labeled.

Muscle from all 6 LGMD2G patients showed a total absence of telethonin in the sarcomeric network, but some labeling in the nucleus (Fig. 2). Double immunofluorescence analysis for α -actinin 2 and telethonin showed a cross-striation pattern in muscle fibers with the first antibody but no labeling with the second one (Fig. 3A). Myotilin IF analysis also showed a sarcomeric pattern in their muscle fibers (Fig. 3B).

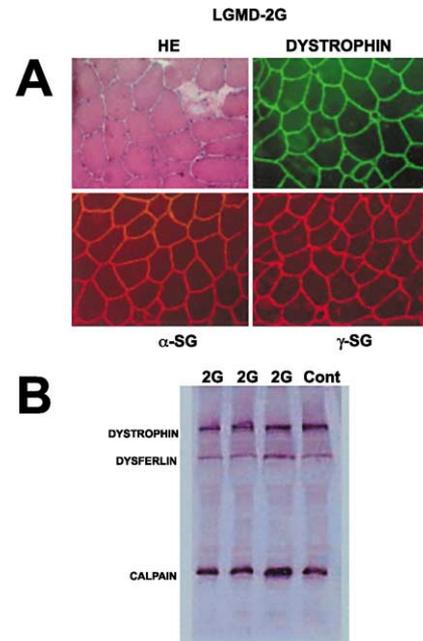


Fig. 5. Immunohistochemical analysis for dystrophin and two sarcoglycans, as well as multiplex western blot for dystrophin, dysferlin and calpain-3 in the LGMD2G patients, showing normal distribution and quantity of the proteins.

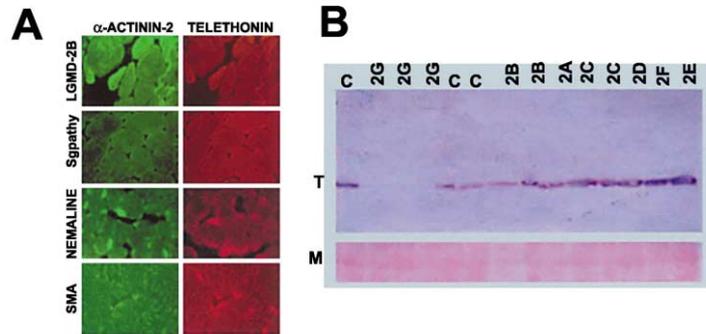


Fig. 6. (A) Double Telethonin and α -actinin-2 analysis in patients with other forms of MD (LGMD2B and sarcoglycanopathy 2D), in a patient with nemaline myopathy, showing the high concentration of telethonin in the rods, and the normal distribution of telethonin in a SMA patient. (B) Western blot analysis in some other forms of LGMD, as compared to control (C) and LGMD2G patients. T=Telethonin; M=myosin content in the ponceau pre-stained blot.

The analysis of titin through two antibodies showed a positive IF pattern with both in LGMD2G patients (Fig. 3), with a mosaic pattern of more positively labeled type II fibers through the antibody from Novacastra (Fig. 4).

Analysis for sarcolemmal proteins showed normal expression of dystrophin, sarcoglycans, dysferlin, and calpain-3 proteins in the LGMD2G patients (Fig. 5).

3.1.3. Ultrastructural analysis

Ultrastructural analysis confirmed the maintenance of the sarcomeric architecture integrity in the LGMD2G patients (Fig. 3C).

3.2. Other forms of neuromuscular disorders

The analysis of telethonin on muscle biopsies from patients with LGMD2A, LGMD2B, SGpathies, and DMD showed a positive sarcomeric pattern in all and a band of the expected MW by Western blot analysis (Fig. 6B). In addition, in muscle fibers from patients with NM, telethonin was clearly present in the rods while in SMA patients, also a normal telethonin IF pattern was observed (Fig. 6A).

4. Discussion

Mutations in the telethonin gene are apparently rare. As stated in our previous report, although the Brazilian population is highly miscigenated, an identical haplotype associated with the mutation 157C>T was found in three of the four families, suggesting a founder effect [23].

Telethonin, which is the 12th most abundant nuclear transcript in adult skeletal muscle [39], has been shown to be one of the substrates of titin, the giant 3–4 MDa sarcomeric protein. Titin acts as a molecular ruler for the assembly of the sarcomere by providing spatially defined binding sites for other sarcomeric proteins over the entire length of the half-sarcomere [45]. After its activation through phosphorylation and Ca^{2+} /calmodulin binding, titin was shown to phosphorylate the C-terminal domain

of telethonin [46]. In addition, it was suggested that the N-terminal region of titin could adopt a closed conformation, which would block the telethonin binding site. In vivo, phosphorylation of the multiple serine–proline phosphorylation sites by developmentally controlled kinases could be the modulation factor controlling titin–telethonin interactions [32].

Telethonin also has putative phosphorylation sites in its C-terminal region [39], and the dual phosphorylation of both titin and telethonin might be important in controlling the interaction of both proteins. The mutations identified in our LGMD2G patients lead to the disruption of this functionally important region of telethonin [23], resulting in the absence of the telethonin protein in muscle fibers from all six studied LGMD2G patients. However, titin was clearly retained, as observed through the positive pattern on IF. Only one titin gene has been identified in the human genome. This gene has several isoforms produced by alternative splicing which are responsible for individual diversity of distribution in human muscles [47]. Therefore, the mosaic pattern of titin observed in our patient with a monoclonal titin antibody could reflect the recognition of an epitope in the C-terminal region, in a similar way to the one seen with myosin isoforms and fiber typing, although the possibility that the differential staining could be due to the accessibility of the epitopes rather than truly differentially expressed isoforms cannot be ruled out.

In cardiac myocytes, it has been shown that both the titin Z1–Z2 domains and telethonin (titin-cap) are required for the structural integrity of sarcomeres. A severe myofibril disruption is observed after over expression of either molecule, suggesting that their interaction is critical in titin filament-regulated sarcomeric assembly [32]. In our LGMD2G patients, immunofluorescence analysis for α -actinin-2 and myotilin, two proteins from the Z-disk of the sarcomere, showed a cross-striation pattern, suggesting that at least part of this myofibrillar structure is retained. Ultrastructural analysis confirmed the maintenance of the sarcomeric architecture in our telethonin deficient patients. These observations suggest that muscle degeneration and

weakness in LGMD2G patients with null mutations in the telethonin gene are more likely to occur due to a functional defect than to an alteration in the sarcomeric structure. On the other hand, the presence of rimmed vacuoles in the muscle fibers from these patients might be due to focal regions of sarcomeric degeneration.

Mues et al. [45] demonstrated that telethonin localizes to the nascent Z-disk in cultured myocytes. Here, the analysis of muscle fibers from patients with NM demonstrated that telethonin was clearly present in the rods, which are formed by proteins from the Z-band of the sarcomere [48]. Therefore, the presence of telethonin in the rods as well as its colocalization with α -actinin and myotilin confirm the presence of telethonin in the Z-disk also in the mature muscle.

In our previous report, we have shown an identical haplotype associated with the mutation 157C>T in three of the four LGMD2G families. Families 1 and 2 (Table 2) are inbred and of Negroid descent, while both parents in family 3, in which the mutation is in homozygosity, have Italian ancestry [23]. Therefore, although these three families apparently have distinct origin, our population is highly miscigenated and the finding of a common haplotype in them suggests a founder effect in Brazil.

4.1. Histological characterization of LGMD2G patients

In the first histopathological description of patients with LGMD2G, muscle biopsies showed the presence of a high number of rimmed vacuoles [49]. The analysis of four additional LGMD2G patients revealed that although present in the majority of muscle samples, the rimmed vacuoles were not the predominant histopathological marker of this disease. A high variability in the degree of muscle degeneration was observed among the six patients, which did not appear to be correlated to the clinical course.

Interestingly, the analysis of a new muscle biopsy sample of a female patient who was asymptomatic when the first biopsy was taken, but clearly affected 10 years later, showed very few pathological alterations, suggesting that no gross muscle degeneration occurs with time in telethonin-deficient patients.

Muscle fiber composition showed a variability of fiber typing. Five of the patients had a significant type I predominance, while in one patient, a very high type II predominance was observed, thus suggesting no direct correlation with the primary gene defect. As observed in the present patients, small areas of atrophy and fiber type predominance are characteristic of denervation. However, this can also be the result of the splitting of larger fibers, which is very common in the dystrophic process [38]. Therefore, caution should be taken in interpreting these type of results.

The analysis of the amount of the telethonin transcript in a number of mice muscles of different types, including slow-twitch postural (soleus) and fast-twitch (EDL) muscles, showed that there is no statistically different levels of expression between muscle of different fiber type com-

position [50]. In the present study, both type I and type II of human mature fibers expressed the same pattern of telethonin. Therefore, the apparent increase in proportion of type I fibers in five of the six patients (and also common in congenital myopathies [38]) appears not to be related to a preferential expression of telethonin in a specific type of fiber.

On the other hand, in one family, only proximal weakness occurred, while in three families, both distal and proximal involvement were observed. A mixed pattern of proximal and distal pattern of weakness may also be present in other forms of LGMD, such as the proximal LGMD2B and distal Miyoshi Myopathy, both associated with mutations in the dysferlin gene [25,26].

4.2. Denervation

Mason et al. [50] have shown that the mouse telethonin transcript is down-regulated in response to denervation. Short-term denervation (2 days) was sufficient to lead to a 50% fall in the level of telethonin in the muscle of the denervated animals, suggesting that innervation and/or muscular activity was necessary for the maintenance of telethonin expression. Our analysis of telethonin in muscle biopsies from SMA patients (genetically denervated) showed a sarcomeric pattern of labeling which did not differ from normal controls, suggesting no telethonin alteration in these cases.

4.3. Interactions

In our first studies, the observation of normal dystrophin and α -sarcoglycan staining in the muscle of LGMD2G patients [23] would indicate that the product of this gene apparently did not interact with the DGC. Here, we confirm that muscle from patients with LGMD2G showed normal expression of dystrophin, the four sarcoglycans, dysferlin and calpain-3. In addition, the analysis of telethonin on muscle biopsies from patients with LGMD2A, LGMD2B, SGpathies, and DMD showed normal localization, suggesting that the deficiencies of calpain-3, dysferlin, sarcoglycans, and dystrophin do not seem to alter telethonin expression.

4.4. Differential diagnosis

Since our LGMD2G patients showed a broad range of phenotypes, ranging from Kugelberg–Wellander-like to typical LGMD, we screened for telethonin deficiency in muscle biopsies from 48 patients with these phenotypes, in an attempt to identify additional cases of LGMD2G. One additional patient (#5), with a total deficiency of telethonin was detected. Complementary DNA studies detected homozygosity for the 157C>T/157C>T mutation in his telethonin gene.

In a recent report from our Center, we studied a total of 153 LGMD families (118 classified and 35 unclassified) by DNA and protein analysis [51]. Considering that in four of

these families the patients are affected by LGMD2G, the frequency of telethonin deficiency is estimated as approximately 2.5% of all cases of LGMD in our population.

In conclusion, our data show that muscles from patients with LGMD2G have a normal expression for the proteins dystrophin, sarcoglycans, dysferlin, calpain-3, and titin, and the primary deficiencies of that proteins do not seem to alter telethonin expression. The observed maintenance of the sarcomeric architecture suggests that the primary absence of telethonin apparently has no effect on the formation and composition of the sarcomere in the LGMD2G patients.

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