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Muscle pathology in 31 patients with calpain 3 gene mutations

Zmiany histopatologiczne w biopsji mięśnia u 31 chorych z mutacjami w genie kodującym kalpainę 3

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Abstract

Background and purpose: At present, more than 20 different forms of limb-girdle muscular dystrophies (LGMDs) are known (at least 7 autosomal dominant and 14 autosomal recessive). Although these different forms show some typical phenotypic characteristics, the existing clinical overlap makes their differential diagnosis difficult. Limb-girdle muscular dystrophy type 2 (LGMD2A) is the most prevalent LGMD in many European as well as Brazilian communities and is caused by mutations in the gene *CAPN3*. Laboratory testing, such as calpain immunohistochemistry and Western-blot analysis, is not totally reliable, since up to 20% of molecularly confirmed LGMD2A show normal content of calpain 3 and a third of LGMD2A biopsies have normal calpain 3 proteolytic activity in the muscle. Thus, genetic testing is considered as the only reliable diagnostic criterion in LGMD2A.
Material and methods: In an attempt to find a correlation between genotype and muscle pathology in limb-girdle muscular dystrophy 2A we performed histopathological investigation of a group of 31 patients subdivided according to the type of pathologic *CAPN3* gene mutation.
Results: In all biopsies typical features of muscular dystrophy such as fiber necrosis and regeneration, variation in fiber size and fibrosis were noted. Lobulated fibers were often

Streszczenie

Wstęp i cel pracy: Dotychczas opisano ponad 20 różnych form dystrofii obręczowo-kończynowej (*limb girdle muscular dystrophy* – LGMD) (co najmniej 7 rodzajów o dziedziczeniu autosomalnym dominującym oraz 14 o dziedziczeniu autosomalnym recesywnym). Pomimo że część z tych chorób można różnicować na podstawie obrazu klinicznego, diagnostykę utrudnia często podobieństwo objawów. Dystrofia obręczowo-kończynowa typu 2A (*limb-girdle muscular dystrophy type 2* – LGMD2A), najczęstsza dystrofia mięśniowa w wielu społecznościach (np. w Europie i Brazylii), spowodowana jest przez mutacje w genie kalpainy 3 (*CAPN3*). Badanie immunohistochemiczne kalpainy czy też metodą Western blot nie są wystarczające do ustalenia właściwego rozpoznania (w odpowiednio 1/3 i 20% potwierdzonych genetycznie LGMD2A badania te wypadają prawidłowo). Podstawę rozpoznania tej miopatii stanowi badanie genetyczne.
Materiał i metody: W pracy przedstawiono wyniki badania zależności między genotypem a analizą histopatologiczną biopsji mięśnia u 31 chorych na LGMD2A. Chorzy podzieleni zostali na grupy według wyników badania genetycznego genu *CAPN3* odpowiedzialnego za tę chorobę.
Wyniki: We wszystkich badanych biopsjach stwierdzano typowe zmiany dystroficzne, takie jak obecność włókien martwiczych

encountered in the muscle biopsies of LGMD2A patients. Such fibers were more frequent in patients with 550delA mutation.

Conclusions: These findings may be helpful in establishing diagnostic strategies in LGMD.

Key words: limb girdle muscular dystrophy, *CAPN3*, calpain, muscle biopsy.

i regenerujących, zróżnicowaną wielkość włókien oraz włóknienie. Włókna o nierównomiernym rozkładzie barwień na enzymy oddechowe (*lobulated fibers*) były często obserwowane w biopsjach chorych z LGMD2A. Tego typu włókna szczególnie często występowały u chorych z mutacją 550delA.

Wnioski: Wyniki pracy wnoszą nowe informacje ułatwiające diagnostykę LGMD.

Słowa kluczowe: dystrofia obręczowo-kończynowa, *CAPN3*, kalpaina, biopsja mięśni.

Introduction

Limb girdle muscular dystrophies (LGMDs) represent a heterogeneous group of disorders with variable clinical and genetic features. Limb girdle muscular dystrophies are characterized clinically by progressive muscle weakness and atrophy mainly of the pelvic and shoulder girdle muscles but without affecting facial muscles. The clinical course may be variable, ranging from severe forms with early onset and rapid progression to milder forms with later onset and minor physical disability [1,2]. However, in all cases serum creatine kinase is elevated and a dystrophic pattern with necrosis and regeneration on the muscle biopsy is observed. At present, more than 20 different forms of LGMDs are known (at least 7 autosomal dominant and 14 autosomal recessive) [3]. Although these different forms show some typical phenotypic characteristics, the existing clinical overlap makes their differential diagnosis difficult [3,4]. Laboratory testing, such as calpain immunohistochemistry [5] and Western-blot analysis, is not totally reliable, since up to 20% of molecularly confirmed limb-girdle muscular dystrophy type 2 (LGMD2A) show normal content of calpain 3 [6-9] and a third of LGMD2A biopsies have normal calpain 3 proteolytic activity in the muscle [10]. Thus, genetic testing is considered as the only reliable diagnostic criterion in LGMDs [11-14].

A classic LGMD2 phenotype was described by Fardeau in 1996 [15] in a small community living in the Reunion Island. Since then, LGMD2A has been identified in many countries [3,13,21-24]. Subsequently, wide intra- and interfamilial variability has been reported [8,14,25,26]. Limb-girdle muscular dystrophy type 2 is the most prevalent LGMD in many European as well as Brazilian communities [20,21,23,27,28].

Mutations in the calpain (*CAPN3*) gene have been proven to be responsible for LGMD2A (MIM# 53600) [29,30]. Until now, more than 160 pathogenic muta-

tions have been identified. However, lack of defined mutational hotspots makes molecular analysis laborious and expensive [14].

Calpain 3 is a skeletal, muscle specific, Ca^{2+}-activated neutral protease [30]. The physiological role of calpain 3 in muscle cell is still uncertain [14]. Spencer *et al.*,[25] and Keira *et al.* [31] described calpain 3 binding to the N2 and M-line regions of titin. This binding is thought to stabilize calpain 3, preventing its autolysis. It has been shown that calpain 3 is involved in the disassembly of myofibrils during early stages of turnover [32] and plays a role in the postfusion defect in muscle maturation and differentiation [33,34]. As for the LGMD2 pathological mechanism, it is now well accepted that loss of function mutation leads to abnormal sarcomere formation and impairment of muscle protein capacity [35].

Numerous myopathological changes typical for muscular dystrophy have been described in LGMD2A patients [19,36,37]. Basic histological and histochemical features of the muscle biopsy are relatively non-specific. However, the presence of abnormal lobulation of type 1 fibers in the end stage of the disease was found by Fardeau *et al.* [15] already in the Reunion Island patients. Subsequently, the high proportion of lobulated fibers in muscle biopsy was considered as a hallmark of pathological lesions of calpainopathy [38,39].

Fanin *et al.* [40] in their description of 24 LGMD2A patients found significantly lower levels of dystrophic features (i.e. degenerating and regenerating fibers) and higher levels of chronic changes (i.e. lobulated fibers) as compared with other types of LGMD2A. Lobulated fibers, so called because of their peculiar pattern of oxidative enzyme reaction on histochemical preparations, represent a non-specific muscle pathology, observed in a variety of neuromuscular disorders [41]. They have been described in both genetic (facioscapulohumeral muscular dystrophy, Duchenne carrier, alpha-sarcoglycanopathy, myotonic dystrophy type 2) [10,42,43] and acquired myopathies (dermatomyositis, polymyositis and

Aleksandra A. Nadaj-Pakleza, Małgorzata Dorobek, Klaudia Nestorowicz, Barbara Ryniewicz, Elżbieta Szmidt-Sałkowska, Anna M. Kamińska

adult nemaline myopathy) [44-47]. Interestingly, they were also reported in neurogenic muscle disease [48] and dystonia [49].

The exact pathomechanism leading to the formation of lobulated fibers remains to be elucidated. In LGMD2A, the loss of calpain activity on myofibrils leads to incomplete muscle maturation and this hypothesis is currently taken into consideration [25,38]. The question of selective involvement of type 1 muscle fibers still remains unanswered.

To provide detailed histopathological characteristics in a large group of patients with calpainopathy, we evaluated muscle biopsies of 31 patients with genetically proven LGMD2A.

Material and methods

Patients

Out of our group of 68 patients with genetically proven LGMD2A, we have studied 31 cases (15 females and 16 males) who had a muscle biopsy taken for diagnostic purposes during the period from 1992 to 2002.

Table 1. Clinical and genetic description of subjects. Group 1: Patients with homozygous del550A mutation

Patient number	Sex	Age at onset	Age at biopsy
1	F	12	12
2	M	14	23
3	F	12	14
4	F	13	15
5	M	9	17
6	M	6	13
7	F	18	25
8	M	12	14
9	F	11	13
10	F	12	16
11	F	10	27
12	F	5	18
13	M	14	15
14	M	14	14
15	F	2	23
16	M	11	12

F – female, M – male

There was no family history of myopathy in all diagnosed patients.

Molecular genetic analysis: DNA testing for calpain 3 gene mutations

Mutation analysis in the *CAPN3* gene was done by single strand conformation polymorphism (SSCP) and heteroduplex (HE) analysis according to previously described procedures [30,50]. Polymerase chain reaction products revealing an abnormal SSCP/HE migration pattern were directly sequenced on an ABI PRISM 377 automated fluorescent DNA sequencer (Applied Biosystems) in the DNA Sequencing and Oligonucleotide Synthesis Laboratory, IBB PAN, Warsaw, Poland.

Muscle biopsy specimens

A muscle biopsy was performed on the vastus femoris muscle in all patients according to a previously described method [51]. Detailed data on light microscopic evaluations were performed on transverse sections conventionally stained with hematoxylin-eosin (H&E), Gomori trichrome, NADH dehydrogenase, succinic dehydrogenase (SDH), lactic dehydrogenase (LDH) and ATPase after preincubation at pH 9.4 and 4.35.

For the purpose of this study, all samples were reevaluated by two authors in non-blinded analysis (A.N.-P. and A.K.). Pathological changes such as necrosis and regeneration, fibrosis, hyaline fibers, ring fibers and muscle fiber type composition were assessed semi-quantitatively in every case. Changes were graded on a 4-point scale: none (–), mild (+), moderate (++), or advanced (+++). Necrosis and other structural changes were assessed in selected areas in H&E staining, whereas fiber type composition and lobulated fibers were assessed in serial sections in the same area in SDH.

Lobulated fibers were defined as fibers with a characteristic peculiar pattern of oxidative enzyme reaction on histochemical preparations.

As for fiber type composition [52], the following approximate proportions were accepted: type 1 – 30-40% and type 2 – 60-70%.

Results

Clinical and genetic data of 31 patients are summarized in Tables 1, 2 and 3.

Table 2. Clinical and genetic description of subjects. Group 2: Patients with different homozygous mutations other than del550A

Patient number	Sex	Age at onset	Age at biopsy	Mutation
1	M	2	11	hz 418dupC,fs
2	M	8	14	hz 418dupC,fs
3	M	20	30	hz 598-612del15ntd Phe200_Leu204del
4	F	11	12	hz 598-612del15ntd Phe200_Leu204del
5	M	13	18	hz 598-612del15ntd Phe200_Leu204del

M – male, F – female

Table 3. Clinical and genetic description of subjects. Group 3: Patients with different heterozygous mutations

Patient number	Sex	Age at onset	Age at biopsy	Mutation
1	F	10	12	htz del550A, 2242C>T Arg 748stop
2	M	7	9	02 htz del550A, htz del598-612, The 200-204 FWSAL
3	F	10	15	htz del550A, htz509A>G Tyr170Cys(new)
4	M	13	15	htz del550A, 2242C>T Arg 748stop
5	F	12	15	htz del550A, htz649G>A, Glu 217Lys
6	M	12	15	htz del550A, 1722delC fs(new)
7	F	15	19	htz del550A, htz del598-612, The 200-204
8	M	6	6	htz del550A, htz1356insT,fs
9	M	14	20	htz 1063C>T Arg355Trp, htz 1194-9A>G fs
10	M	10	10	htz del550A, 418dupC fs

F – female, M – male

All patients were sporadic and had limb-girdle clinical phenotype. Patients were divided according to the mutation. In 16 patients, the homozygous occurrence of 550delA mutation was detected (group 1). In 5 patients different homozygous mutations other than 550delA were demonstrated (group 2) and the remaining 10 patients were compound heterozygotes for previously described mutations in the *CAPN3* gene (group 3).
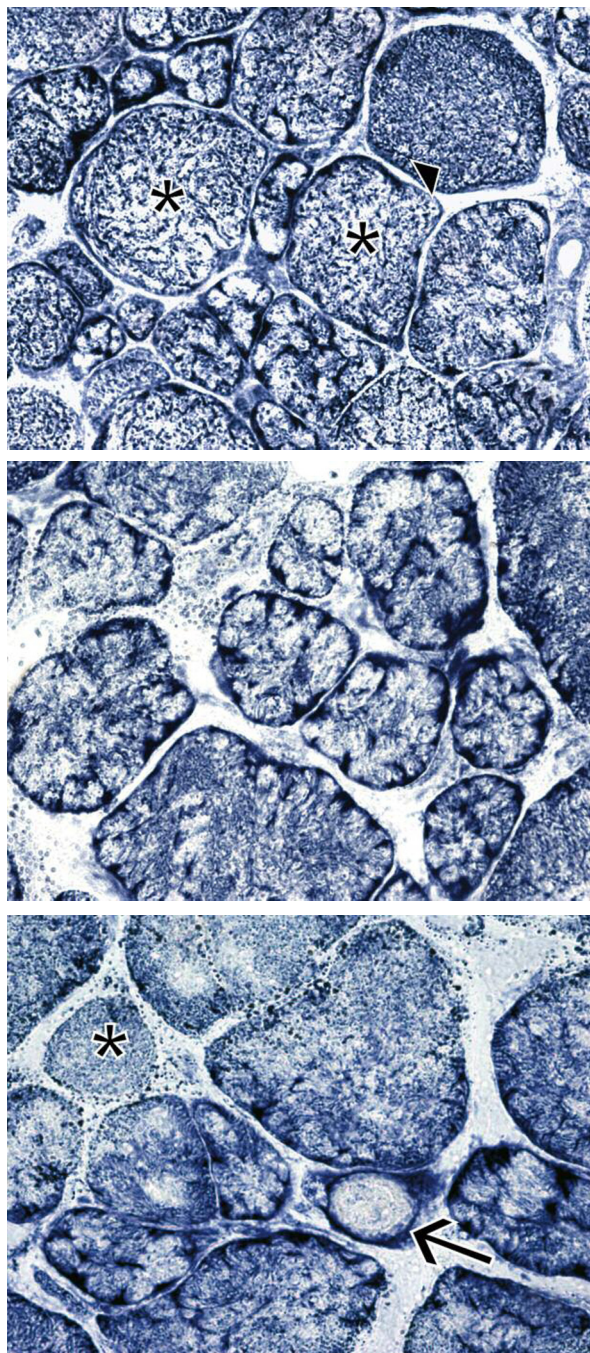
Group 1 (Table 1) consisted of 16 patients (9 males and 7 females). The mean age at onset of muscle symptoms was 10.9 years (range: 2-18 years). Group 2 (Table 2) consisted of 5 patients (1 female and 4 males). The mean age at onset was 12.5 years (range: 6-15 years). Group 3 (Table 3) consisted of 10 patients (4 females and 6 males). The mean age of onset was 15 years (range: 2-20 years).

All examined patients had myopathic changes in muscle biopsy. A constant feature, observed in all examined biopsies, was increased variability in fiber size and fibrosis. The other common feature typical for muscular dystrophy was fiber necrosis (27/31) and regeneration (30/31). Only one biopsy showed neither necrosis nor regeneration. The occurrence of hyaline fibers was frequent (21/31), whereas ring fibers were rarely encountered (5/31).

Intensity of all analyzed histopathological changes varied from 'none' to 'advanced' and seemed not to be correlated with either age of the patient or the duration of the disease. It was noted, however, that in group 1 fibrosis tended to increase as the disease progressed. No such relationship was observed in other groups. Occurrence of lobulated fibers was found in more than half of the biopsies (17/31). Such fibers behaved mostly as type 1 fibers.

No significant differences were observed between groups with different mutations except for the high percentage of lobulated fibers in group 1 (patients homozygous for del550A mutation). In this group, lobulated fibers were noted in almost 2/3 of cases (10/16). No correlation between the duration of the disease and the

Aleksandra A. Nadaj-Pakleza, Małgorzata Dorobek, Klaudia Nestorowicz, Barbara Ryniewicz, Elżbieta Szmidt-Sałkowska, Anna M. Kamińska

Fig. 1. Multiple lobulated fibers with irregular aggregations of oxidative activity within the cytoplasm. Note also the presence of the ring fiber (arrow) as well as the presence of normal type I (arrowheads) and type 2 (asterisk) fibers. Succinic dehydrogenase × 20

number of lobulated fibers was noted. For instance, in the group of 4 patients with longstanding disease (< 10 years) only 1 had lobulated fibers. In group 2, only 1 of 5 biopsies showed the occurrence of lobulated fibers. In group 3, lobulated fibers were noted in half

of the patients. Type 1 fiber predominance was observed in one third of biopsies (10/31) while type 2 fiber predominance was very seldom seen (3/31).

Lobulated muscle fibers were characterized by an irregular pattern of oxidative enzyme reaction in light microscopy (Fig. 1). Most of them were atrophied and behaved as type 1 fibers.

Discussion

In our analysis, we confirmed the presence of typical dystrophic pathological changes in the muscle biopsies with genetically proven LGMD2A. As in other muscular dystrophies, LGMD2A muscles show active necrotic and regenerating processes resulting, as the disease progresses, in increased fiber size variation and fibrosis. The intensity of the dystrophic process did not vary across the three groups with different mutations and seemed not to correlate with the age of the onset and disease duration, except for more pronounced fibrosis in patients with longstanding disease.

Histopathological investigations of muscle biopsies in LGMD2A patients are scarce [19,26,36,53]. Three of these studies [19,36,53] dealt with systematic reviews, whereas Vainzof *et al*. [24] described preclinical histopathological changes in muscle biopsies from members of a large consanguineous family. Chae *et al*. [36] in all patients with *CAPN3* mutations found the typical feature of muscular dystrophy with less active necrosis and prominent fibrosis in the advanced stages of the disease. Similar observations were presented by Hermanova *et al*. [19]. In a group of 12 LGMD2A patients, a less active necrotic process was observed in patients with longstanding disease. The extent of fibrosis, however, correlated with the disease duration.

According to Fanin *et al*. [53], in their series of 24 LGMD2A patients the level of dystrophic features (i.e. degenerating and regenerating fibers) was significantly lower than in other types of LGMD2. The authors also found a direct correlation between the extent of muscle fiber degeneration and regeneration and disease progression. In their group, the histopathology severity score seemed not to be correlated with disease duration.

Yet, a correlation between the extent of fibrosis and disease duration was found in our patients only in subgroup 1 of LGMD2A homozygous for del550A mutation. No such correlations were found in the other subgroups.

There are only a few studies that have analyzed the presence of lobulated fibers in genetically confirmed

Table 4. Occurrence of muscle histopathological changes in patients with homozygous del550A mutation (Group 1)

No	Fiber type pre-dominance	Necrosis	Regeneration	Increase in fiber variability	Hyaline fibers	Fibrosis	Ring fibers	Lobulated fibers
1	Type 1	+	+	+	+++	++	−	++
2	Type 1	+++	+++	+++	++	+	−	−
3	Type 1	+	+++	++	+	+	−	−
4	Type 1	++	+++	++	++	+	−	+
5	Type 1	+++	+++	++	++	++	+	−
6	Type 1	+	++	++	−	+	−	+++
7	-	+	++	++	++	+++	++	++
8	-	+++	+++	+++	+	+++	−	+
9	-	+	+++	+	+	++	−	−
10	-	+	+	++	−	++	−	+++
11	-	−	++	+	+++	+++	−	−
12	Type 2	+	+	+	+++	+	+	+++
13	Type 2	+	++	+	+	+	−	−
14	-	+	++	+	++	+	−	++
15	-	−	++	++	+	+	−	+
16	-	++	++	+	−	+	−	+

Table 5. Occurrence of muscle histopathological changes in patients with different homozygous mutations other than del550A (Group 2)

No	Fiber type pre-dominance	Necrosis	Regeneration	Increase in fiber variability	Hyaline fibers	Fibrosis	Ring fibers	Lobulated fibers
1	−	−	−	++	+	+	−	−
2	−	++	++	++	+	++	+	++
3	Type 1	−	−	+	−	+	−	−
4	−	+++	+++	++	++	++	−	−
5	−	+	++	+	++	+++	−	−

LGMD2A patients. None of them, however, correlated the histopathological findings with the type of *CAPN3* mutation. Chae *et al.* [36] found a high proportion (67%) of lobulated fibers in his series of 21 patients as well as a positive correlation between the number of lobulated fibers and the stage of the disease.

Hermanova *et al.* [19], in their material of 14 muscle biopsies, revealed lobulated fibers in half of the specimens. They observed that most lobulated fibers were found in muscle biopsies performed after a longstanding disease course and were not found in the early stages of the disease. Fanin's findings [53] also confirmed the occurrence of lobulated fibers in a high percentage of cases (13 from a group of 24 patients). Similarly, they observed a correlation between the number of lobulated fibers and disease duration.

In our patients, lobulated fibers were found in more than half of the biopsies in the group of 31 LGMD2A cases. No obvious association between lobulated fibers and disease duration was observed in the analyzed group.

We performed the first, to our knowledge, histopathological investigation of a group of LGMD2A patients, subdivided according to the type of pathogenic mutation.

Aleksandra A. Nadaj-Pakleza, Małgorzata Dorobek, Klaudia Nestorowicz, Barbara Ryniewicz, Elżbieta Szmidt-Sałkowska, Anna M. Kamińska

Table 6. Occurrence of muscle histopathological changes in patients with different heterozygous mutations (Group 3)

No	Fiber type pre-dominance	Necrosis	Regeneration	Increase in fiber variability	Hyaline fibers	Fibrosis	Ring fibers	Lobulated fibers
1	Type 1	+ +	+	+	+ +	+ +	−	+ + +
2	−	+ +	+	+	+	+	+	+ + +
3	−	−	+	+ +	−	+	−	−
4	−	+ +	+	+	−	+ +	−	−
5	−	+ +	+ +	+ +	+	+	−	−
6	Type 1	+ +	+ +	+ +	−	+	−	+ + +
7	−	+ +	+ + +	+ + +	+	+	−	+
8	Type 1	+ +	+ +	+ +	−	+	−	−
9	Type 2	+	+	+ + +	−	+	−	+ +
10	−	+	+	+	−	+	−	−

The only relation we found was a higher occurrence of lobulated fibers in patients homozygous for del550A mutation.

Genotype-phenotype correlations in LGMD2A have been described so far in only two papers [19,53]. Fanin *et al.* [53], however, concentrated on the relationship between genotype and protein content in muscle. Saenz *et al.* [50] analyzed the clinical status of patients with different mutations and suggested that patients with homozygous mutations tend to have a more severe phenotype and faster clinical course. Neither of these studies took muscle morphology into consideration.

Limb-girdle muscular dystrophy type 2 remains one of the most difficult of all recessive LGMDs to diagnose. The variability of clinical phenotype, incomplete sensitivity and specificity of calpain 3 protein biochemical analysis [30], and the effort required to identify point mutations in a relatively large gene make the diagnostic process a complicated, laborious and expensive task. Yet, an unequivocal diagnosis is necessary, both when offering genetic counseling and when selecting patients for future clinical trials with drug or gene therapy.

Conclusions

In our opinion, distinguishing the patterns of muscle histopathological changes in LGMD2A is a very informative step in clinical diagnosis, helpful in establishing diagnostic strategies in LGMD patients.

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Disclosure

Authors report no conflict of interest.

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