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Myopathy with lobulated muscle fibers: evidence for heterogeneous etiology and clinical presentation ☆

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Abstract

The clinico-pathological features of 17 patients displaying a myopathy with lobulated (trabeculated) fibers are reported. All these patients had a limb girdle phenotype and at least 20% of lobulated fibers in their muscle biopsies. There were ten females and seven males. The onset of symptoms ranged from 2 to 55 years (mean 24). The average age at the time of muscle biopsy was 39 (range 3–63). Interestingly, in six patients, high prevalence of lobulated fibers was observed at the second biopsy only, performed on average 11 years after the first or in another muscle. Six patients had a suggestively positive family history. Facial weakness was noted in two patients (genetic study confirmed FSH dystrophy). The course and the severity of weakness varied from one patient to another. Immunohistochemistry and Western blot analyses revealed one Duchenne carrier, one α-sarcoglycanopathy, no dysferlinopathy and four calpain deficiencies (including one patient with FSH dystrophy), but SSCP revealed mutation in the calpain gene in only one of the patients. These results show that (1) myopathies with lobulated fibers are clinically and genetically heterogeneous, (2) lack of calpain expression by Western blot analysis is not always associated with null mutation, (3) a molecular diagnosis is made in less than 40% of myopathy with lobulated fibers, (4) when observed, lobulated fibers are most prominent in proximal muscles and require time to appear.

Keywords: Limb-girdle dystrophy; Lobulated (trabeculated) fibers; Calpain; Dysferlin; Dystrophin

1. Introduction

Lobulated (or trabeculated) muscle fibers were characterized by a peculiar pattern of oxidative enzyme reaction on histochemical preparations of muscle biopsies reflecting an abnormal spatial distribution of the intermyofibrillar mitochondria network. These fibers represented a non-specific muscle change and have been reported in various neuromuscular disorders [1]. In the facio-scapulo-humeral and limb girdle forms of muscular dystrophy, however, lobulated fibers were often conspicuous [2].

In a recent report, Weller et al. [3] named 'myopathy with trabeculated (lobulated) muscle fibers', a clinicopathological entity characterized by the high prevalence (20–90%) of lobulated fibers, as the dominant pathology in patients displaying limb girdle clinical phenotype.

This report prompted us to search for this entity in a series of 3392 muscle biopsies performed between 1992 and 1999. Seventeen patients fulfilled the criteria previously described [3]. Although these patients had a limb girdle muscular myopathy, clinical presentation was heterogeneous. In addition, searches for abnormal expression of the dystrophin complex proteins, dysferlin and calpain also revealed genetic heterogeneity.

2. Material and methods

2.1. Muscle biopsies

This study was carried out on 17 patients. All presented with a limb girdle phenotype and at least 20% of lobulated fibers in their muscle biopsies. Muscle biopsies were performed under local anaesthesia in quadriceps (patients

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1, 3, 4, 7, 8, 11, 14, 15, 17), deltoid (patients 1, 5, 9, 10, 13, 16), biceps (2, 4, 6, 7, 12), peroneus longus (patient 2) and gastrocnemius (patient 9) muscles. Muscle biopsies were performed twice on some patients (1, 2, 4, 7, 9, 14–16). Muscle specimens were flash frozen in isopentane cooled by liquid nitrogen and stored at −80°C until use.

Histoenzymology was carried out on cryostat sections as previously described [4]. The NADH-tetrazolium reductase stain was used to count the lobulated fibers. Four hundred to seven hundred muscle fibers were counted in each case and the number of lobulated fibers was given in percentage. Only typical lobulated fibers which were small type I fibers on serial sections with ATPase stainings were counted.

The large fibers demonstrating little accumulation of NADH-TR end product were not considered as lobulated and not quantified.

2.2. Antibodies

Anti-dystrophin [(Dys1 (clone Dy4/6D3, Dys2 (clone Dy8/6C5) Dys3 (clone Dy10/12B2)], anti-sarcoglycans [α (clone Ad1/20A6), β (clone β Sarc1/5B1), δ (clone δSarc/12C1), γ (clone 35DAG/21B5)], anti-dystroglycan [β (clone 43DAG1/8D5)], anti-β spectrin (clone NCL spect1), anti-calpain [CALP-2C4 (clone Calp3d/2C4), CALP-12A2 (clone Calp3c/12A2)] anti-dysferlin (clone Ham 1/7β6) were purchased from Novocastra.

Anti-merosin (clone 5H2) was purchased from Chemicon.

2.3. Immunohistochemistry

Automate (VENTANA) immunoperoxidase detection of the antigens described above (except calpain) was performed on serial 4 μm thick cryostat sections in all patients and in four normal muscles. Controls included omission of primary antibodies and irrelevant IgG.

2.4. Western blot analyses

Tissue samples were prepared from all patients' muscles according to the procedure previously described [5]. Normal muscles were also used as controls.

Polyacrylamide gel preparation, migration and blotting were done according to the procedure previously described [6].

In each case, one nitrocellulose sheet was probed with a cocktail of antibodies directed against dystrophin (anti-Dys1 diluted 1/150[e]) and calpain (12A2 diluted 1/100) and the other one was probed with antibodies directed against dystrophin (anti-Dys2 1/25) and sarcoglycans (anti-α 1/75 and γ 1/50). A third nitrocellulose sheet was incubated with anti-dysferlin only (1/500). Incubation was performed for 12 h at 4°C under shaking. Afterwards, the rinsed blots were incubated with goat anti-mouse peroxidase conjugated antibody at a dilution of 1/5000[e] for 1 h at room temperature. The enhanced chemiluminescence system was used for detection of the bound peroxidase conjugated anti-mouse antibody. In the case of certain patients (2, 4, 6 and 16) presenting abnormal calpain expression with anti-12A2 antibody, Western blot analysis was performed with anti-2C4 antibody.

2.5. Molecular genetic studies

2.5.1. FSH gene

Searches for abnormal FSH gene expression were performed on patients 2, 7, 8 and 10. Briefly, 10 μg genomic DNA was doubly digested with *Eco*RI and *Bin*I. Restricted DNA fragments were separated on 0.5% agarose gels, then a Southern blotting procedure was carried out. They were hybridized with ^{32}P-labelled probe p13E.11 and exposed to X-ray films for 2–7 days [7].

2.5.2. Calpain gene

Searches for abnormal calpain gene expression were performed on patients 2, 4, 7 and 16 by SSCP analysis. Five microliters of the PCR products were mixed with 10 μl of loading buffer (95% formamide and 0.1% bromophenol blue), denatured at 95°C for 5 min and cooled on ice. The samples were separated on 0.4-mm-thick polyacrylamide (5, 6 or 7.5% depending on DNA fragment size) 5% glycerol in 0.5 × Tris–borate EDTA. Gels were run at room temperature at 6, 7 or 8 W, depending on exon size, for 16 h. The samples were transferred to Hybond N + membranes (Amersham-Pharmacia) and hybridized with the primers used in the PCR, according to the ECL protocol (Amersham-Pharmacia), as described [8]. Identification of the variant sequences was performed by DNA sequencing. For this purpose, 100 ng of DNA were amplified by PCR. Each exon was amplified by specific primers, chosen in introns. PCR products were directly sequenced, with the same primers by dye-dideoxy sequencing, after purification (Qiaquick kit, Qiagen).

2.5.3. Dystrophin gene

Search for a deletion in the dystrophin gene was carried out in patient 14 by multiplex PCR analysis as previously described [5].

3. Results

3.1. Selection of the patients

Among the 3392 muscle biopsies performed between 1992 and 1999 and registered on computer databases, 41 patients were selected on the basis of the association of the two items 'limb girdle dystrophy and lobulated fibers'. Frozen specimens were still available for 24 out of 41 cases.

Histopathological features of these 24 cases were reviewed by three of the investigators (D.F.B., M.E.D. and J.F.P.) and only 17 cases fulfilled the pathological criteria of myopathy with lobulated fibers [3]. These criteria

included: (1) the accurate diagnosis of lobulated fibers; and (2) the threshold of 20% of lobulated fibers in muscle biopsy. Lobulated fibers were defined as 'fibers showing an irregular pattern of oxidative enzyme reaction on cross section, in which the normal lattice pattern of the reaction was replaced by an irregular lobulated and coarse granular appearance'. This definition excluded other abnormalities of the intermyofibrillar network such as 'pseudo' core or multicore appearance for example. The threshold of 20% of lobulated fibers excluded patients with other distinctive myopathological changes or other evidence of a specific muscle disease [3].

3.2. Clinical features

Clinical features and laboratory data of the 17 patients are given in Table 1.

Of the 17 patients, ten were females and seven were males. Age of onset ranged from 2 to 55 years (mean 24.4). Six patients had a suggestive positive family history. Consanguineous marriage featured in the families of patients 2 and 12. The mode of inheritance was autosomal dominant (patient 10), autosomal recessive (patient 3, 12), X-linked (patient 14) or not defined (patient 2). Patient 14 was the mother of a child who had died from Duchenne muscular dystrophy. Cramps were observed in one patient (patient 10), cardiac and respiratory involvement in another one (patient 5). Facial involvement was observed in two patients (2 and 10) and joint contracture in five (patients 4–6, 15, 16).

All patients had limb muscle weakness predominantly in the proximal distribution. One patient (case 8) had only lower limb weakness. Distal involvement was also observed in 11 patients. Mild neck muscle flexor weakness was present in one case. The severity of the weakness was scored according to Walton [9] and ranged from 1 to 8.

Laboratory data showed increased CK level in all patients except two (cases 2 and 5). Mean CK value was 594.6 ($Nl < 130$). Electromyography performed in 16 cases showed a myopathic pattern in all cases (low amplitude and polyphasic motor unit potential with hyper-recruitment). It was associated in three patients with spontaneous activity (patients 7 and 11) and in one with neurogenic pattern (patient 8).

3.3. Histological and immunohistochemical data (Table 2, Figs. 1–3)

Muscle biopsy was performed in all patients at the mean age of 39.2 years (range 3–63). In eight patients a second biopsy was performed on average 11 years after the first one (range 1–18). In these biopsies, the number of lobulated fibers was higher than in the first biopsy. Moreover, in three patients (cases 4, 7 and 9), LFs were observed at the second biopsy only (Fig. 1).

In these patients the second biopsy was done on a proximal muscle (quadriceps or deltoid) whereas the first was performed on biceps or gastrocnemius. The prevalence of lobulated fibers was very high (60%) in four patients, high (40–60%) in eight patients and medium (less than 40%) in five. In most muscle biopsies, type I muscle fibers were selectively atrophic and predominant.

Table 1
Clinical pictures and laboratory data of 17 patients with TF myopathy[a]

Patients	Sex	Age of onset	Family history	Cramps	Weakness			Facial involvement	Severity of weakness (Walton score)	CK	EMG
					Proximal	Distal	Neck flexors				
1	F	30	−	−	+	−	−	−	6 (59)	240	Myopathic
2	M	38	+	−	+	+	−	+	6 (59)	N	Myopathic
3	F	30	+	−	+	+	−	−	6 (56)	403	Myopathic
4**	F	2	−	−	+	+	−	−	7 (39)	406	Myopathic
5*/**	F	5	−	−	+	+	+	−	7 (25)	N	Myopathic + SA
6**	F	8	−	−	+	+	−	−	4 (25)	1418	Myopathic
7	M	55	+	−	+	+	−	−	7 (60)	158	Myopathic + SA
8	F	30?	−	−	+	+	−	−	5 (49)	1316	Myopathic Neurogenic
9	M	18	−	−	+	+	−	−	3 (40)	644	?
10	M	17	+	+	+	+	−	+	4 (56)	220	Myopathic
11	F	55	−	−	+	+	−	−	6 (63)	144	Myopathic + SA
12	M	44	+	−	+	−	−	−	5 (60)	1094	Myopathic
13	F	14	−	−	+	−	−	−	7 (42)	563	Myopathic
14	F	30	+	−	+	+	−	−	2 (50)	567	Myopathic
15**	M	3	−	−	+	−	−	−	8 (13)	389	Myopathic
16**	F	12	−	−	+	−	−	−	7 (26)	300	Myopathic
17	M	25	−	−	+	−	−	−	1 (62)	1058	Myopathic

[a] *Cardiac and respiratory involvement, **joint contractures, CK value <130, N, normal, SA, spontaneous activity. The severity of weakness was evaluated at the last neurological examination, age is given in parenthesis.

Table 2
Pathological, immunohistochemical and Western blot analysis of 17 patients with TF myopathy

	Biopsy 1			Biopsy 2				
Patients	Age*	Muscle	%TF	Age	Muscle	%TF	Immunohistochemistry	Western blot
1	49	Quadriceps	25	59	Deltoid	25	N	N
2[a]	38	Peroneus	60	56	Biceps	40	N	Calpain deficiency
3	56	Quadriceps	40	–	–	–	N	N
4	29	Biceps	0	35	Quadriceps	60	N	Calpain deficiency
5	25	Deltoid	50	–	–	–	N	N
6	19	Biceps	50	–	–	–	N	Calpain deficiency
7	59	Biceps	0	60	Quadriceps	40	N	N
8	49	Quadriceps	40	–	–	–	N	N
9	24	Gastrocnemius	0	39	Deltoid	40	N	N
10[a]	54	Deltoid	60	–	–	–	N	N
11	59	Quadriceps	20	–	–	–	N	N
12	49	Biceps	50	–	–	–	N	N
13	45	Deltoid	30	–	–	–	α Sarcoglycan	Sarcoglycanopathy
14	33	L–Quadriceps	10	47	R–Quadriceps	60%	Mosaicism(Dystrophin)	N
15	3	R–Quadriceps	10	19	L–Quadriceps	25%	N	N
16[a]	13	L–Deltoid	35	22	R–Deltoid	40%	N	Calpain deficiency
17	63	Quadriceps	20	–	–	–	N	N

[a] These cases have a proven molecular diagnosis (patients 2 and 10 FSH, patient 16 calpainopathy). *At biopsy; N, normal; ND, not done.

No other pathological changes were observed except centrally located nuclei and occasional necrotic fibers in some muscle biopsies. In addition, the second biopsy of patient 7 showed numerous rimmed vacuoles.

Immunohistochemical analysis was normal in all patients except two. One patient (case 14) had a mosaic pattern with all anti-dystrophin antibodies whereas β-spectrin and β-dystroglycan expression were normal in fibers lacking dystrophin. According to her family history, this patient was classified as a Duchenne carrier (Fig. 2).

The other one (case 13) had abnormal sarcoglycans expression with a strong decrease of αsarcoglycan, a moderate diminution of γ and δ sarcoglycan, whereas the β sarcoglycan was normal (Fig. 3).

3.4. Western blot analysis

On Western blot analysis, dystrophin and dysferlin expression were normal in all patients, α and γ sarcoglycans were strongly reduced in patient 13 (Fig. 4). Calpain expression was abnormal in four patients, (patients 2, 4, 6 and 16) (Fig. 5). In these patients Western blot was carried out at least twice. In patient two, the 94 and 30 kDa bands were absent while the 60 kDa band was strongly reduced. In patients 4, 6 and 16 the three bands were lacking (Fig. 5A,B).

3.5. Molecular genetic studies

The FSH gene was normal in patients 7 and 8 whereas it demonstrated a p13E-11 *Eco*RI-*Bln*I abnormal fragment in patients 2 and 10. Patient 2 exhibited a particular pattern with two abnormal fragments of 12 and 25 kb. This patient likely has a recessive form of FSH. Patient 10 exhibited an abnormal fragment of 12 kb plus a normal fragment longer than 50 kb.

Mutations in the Calpain 3 gene were not found in patients 2, 4 or 7 whereas two mutations were observed in patient 16 in exons 8 and 13. The heterozygous mutation W360X was observed in exon 8 and the mutation (heterozygous) E553K was seen in exon 13.

The first mutation involved a change of an amino acid which is strictly conserved among the mammalian and non-mammalian calpains and created a stop codon. The E553K mutation also involved a highly conserved residue and has been detected in another French LGMD family (Sàenz and Cobo, unpublished data).

Genetic analysis of the dystrophin gene disclosed no deletion in patient 14. Search for mutation was not done. A Becker muscular dystrophy was excluded in male patients because of lack of characteristic clinical history, and normal immunohistochemical and biochemical detection of dystrophin. In addition, although genetic study of Steinert myotonic dystrophy was not done, search for electromyographically myotonic discharges was negative in all patients.

4. Discussion

The incidence of myopathy with lobulated fibers was difficult to assess. Weller et al. [3] showed that it corresponded to 7% of the muscle biopsies identified as non-inflammatory myopathy but 1% of the total number of muscle biopsies performed.

The selection of the patients in our study was on a quite different basis but the range is almost the same: about 1% of the muscle biopsies examined in Marseille. Lobulated (trabeculated) fiber myopathy was defined by Weller et al.

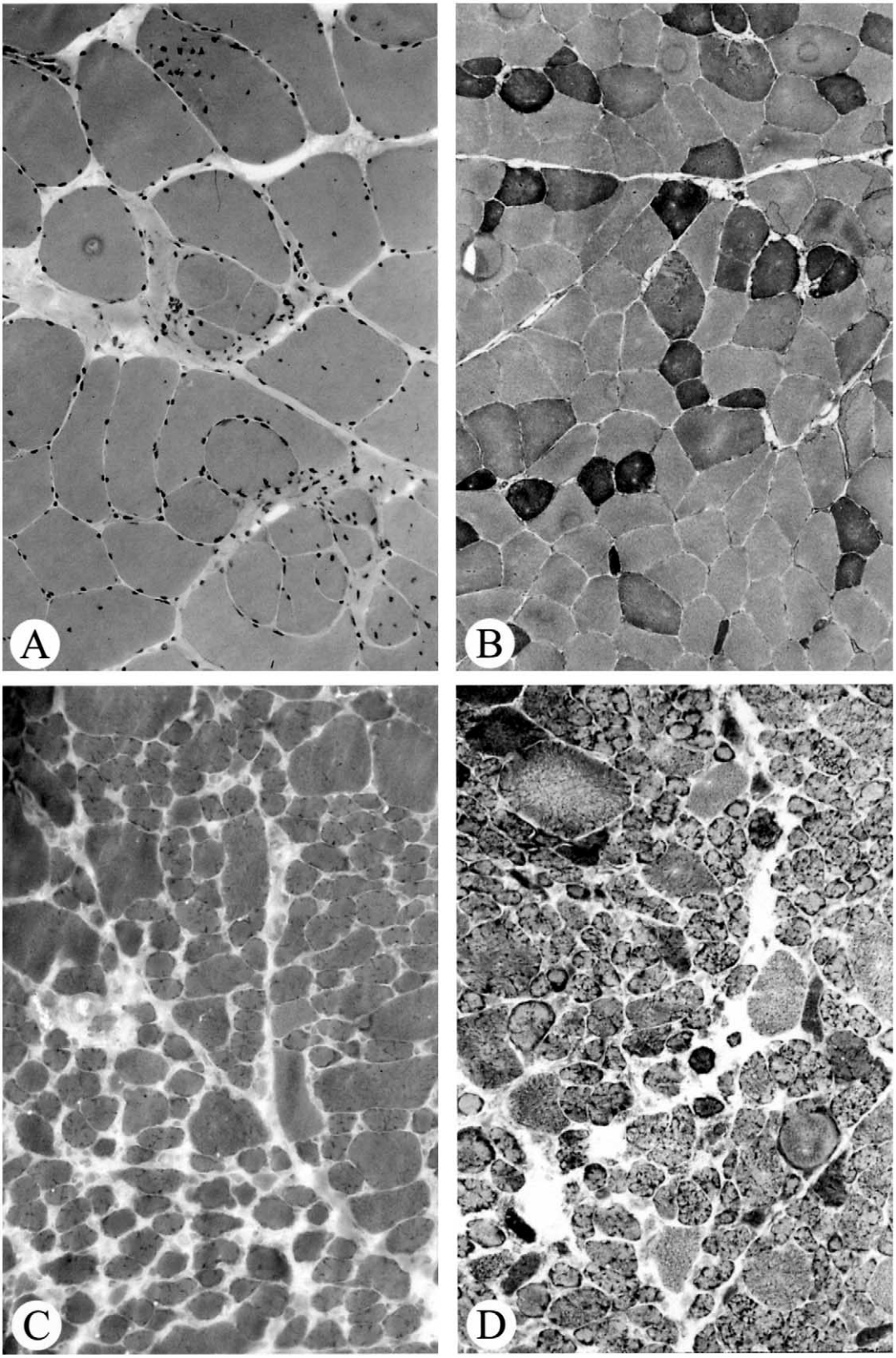

Fig. 1. Patient 4: calpainopathy. First biopsy (biceps at 29 years) showing fibers of various sizes, splitting, centrally located nuclei, fibrosis (A, HE) but lack of LFs (B, NADH-TR). Second biopsy (quadriceps at 35 years) showing small type 1 fibers (C Gomori trichrome) and 60% of LFs (D, NADH-TR).

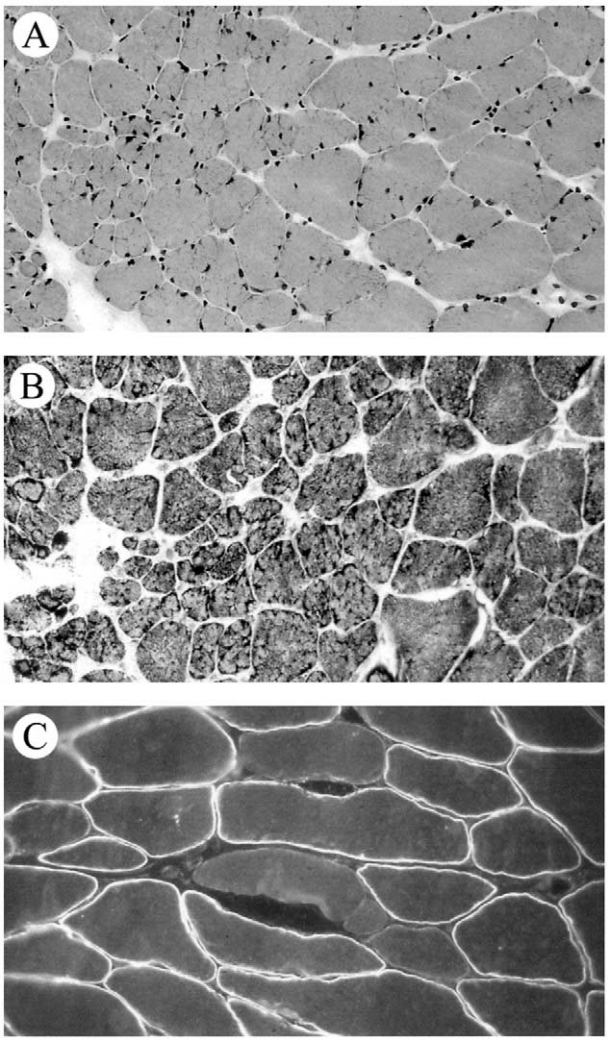

Fig. 2. Patient 14. Duchenne carrier, second biopsy (quadriceps) showing fibers of various sizes (A, HE). LFs are observed in 60% of fibers (B, NADH-TR), mosaic pattern with anti-Dys 2 antibody (C).

[3] as a 'syndrome in which more than 20% lobulated fibers, in the muscle biopsy, constituted the major pathological change while no other pathological alterations were present in the biopsy except for the smallness of lobulated fibers' [3]. On this basis, we could rule out seven patients. These cases showed less than 10% of lobulated fibers and five biopsies also demonstrated numerous myopathological changes such as annulated fibers, high number of centrally located nuclei, sarcoplasmic masses, splitting, necrotic or regenerative fibers and an increase of connective tissue. Three cases were indeed Becker muscular dystrophy, two were proximal myotonic myopathy (PROMM) and two facio-scapulo-humeral (FSH) dystrophy. The 17 remaining cases fulfilled the criteria described previously for lobulated fiber myopathy [3]: the number of lobulated fibers was higher than 20%, and the other pathological changes observed were in most cases type I predominance and type I atrophy. In some biopsies, however, occasional centrally located nuclei, rare necrotic or regenerative fibers and splitting were observed. In the series previously described [3], the clinical phenotype was characterized by an adult onset and a slowly progressive muscle weakness affecting mainly proximal limb musculature, causing little functional deficit, even many years after onset. Therefore the authors [3] suggested that the LFs is a 'distinctive clinico-pathological entity although not necessarily etiologically homogeneous'.

The clinical phenotype of the 17 patients reported here was to some extent heterogeneous. If all patients presented with a limb-girdle phenotype (it was one of the criteria for the selection of the patients) the age of onset varied; five patients had an onset in childhood and three in adolescence. Two had facial involvement and the course of the disease and the severity of the weakness also varied from one patient to another. In addition, molecular and genetic studies also demonstrated genetic heterogeneity. Two patients had FSH dystrophy, one had a sarcoglycanopathy (likely α-sarcoglycanopathy but a search for mutations in the α-sarcoglycanopathy gene was not done), another had a family history of DMD and a mosaic pattern with anti-dystrophin antibodies and was a Duchenne carrier. Western blot analysis of the calpain-3 was abnormal in four patients (patients 2, 4, 6, 16). Patient 2 however, had a FSH dystrophy (confirmed by genetic analysis) and SSCP sequencing of the calpain gene disclosed no abnormality. Therefore in this patient the calpain deficiency was likely to be a secondary phenomenon. In the three remaining patients (4, 6, 16), calpain was undetectable by Western blot analysis. SSCP sequencing of the calpain gene showed mutations in only one patient (patient 16).

It was normal in patients 4 and 6. Interestingly, dysferlin expression was normal in these patients, and it was unlikely that they have a secondary reduction in calpain associated with a primary defect in dysferlin [10]. However, we did not perform genetic analysis of the dysferlin gene in these patients. Because the SSCP technique did not offer 100% sensitivity and did not allow the detection of entire exon deletions, we strongly believe that patients 4 and 6 had a primary calpainopathy. The clinical features of the three patients lacking calpain-3 expression by Western blot analysis were homogeneous and in keeping with a calpainopathy (LGMD2A) [11]. The onset was in childhood [12] and all patients had proximal symmetrical weakness and atrophy with Achilles tendon contractures or more diffuse contractures. Facial muscles and neck muscles were spared. All these patients presented with frequent falls, and difficulty in running or climbing stairs. Confinement to a wheelchair occurred in patients 4 and 16, at 37 and 14 years, respectively after the onset of symptoms, whereas patient 6 was still ambulatory. Finally, dysferlin expression analyzed by both Western blot analysis and immunohistochemistry was normal in all patients. The number of patients was too low to exclude the possibility that dysferlin deficiency might mimic myopathy with lobulated fibers.

Taken together, our results suggest that lobulated fiber

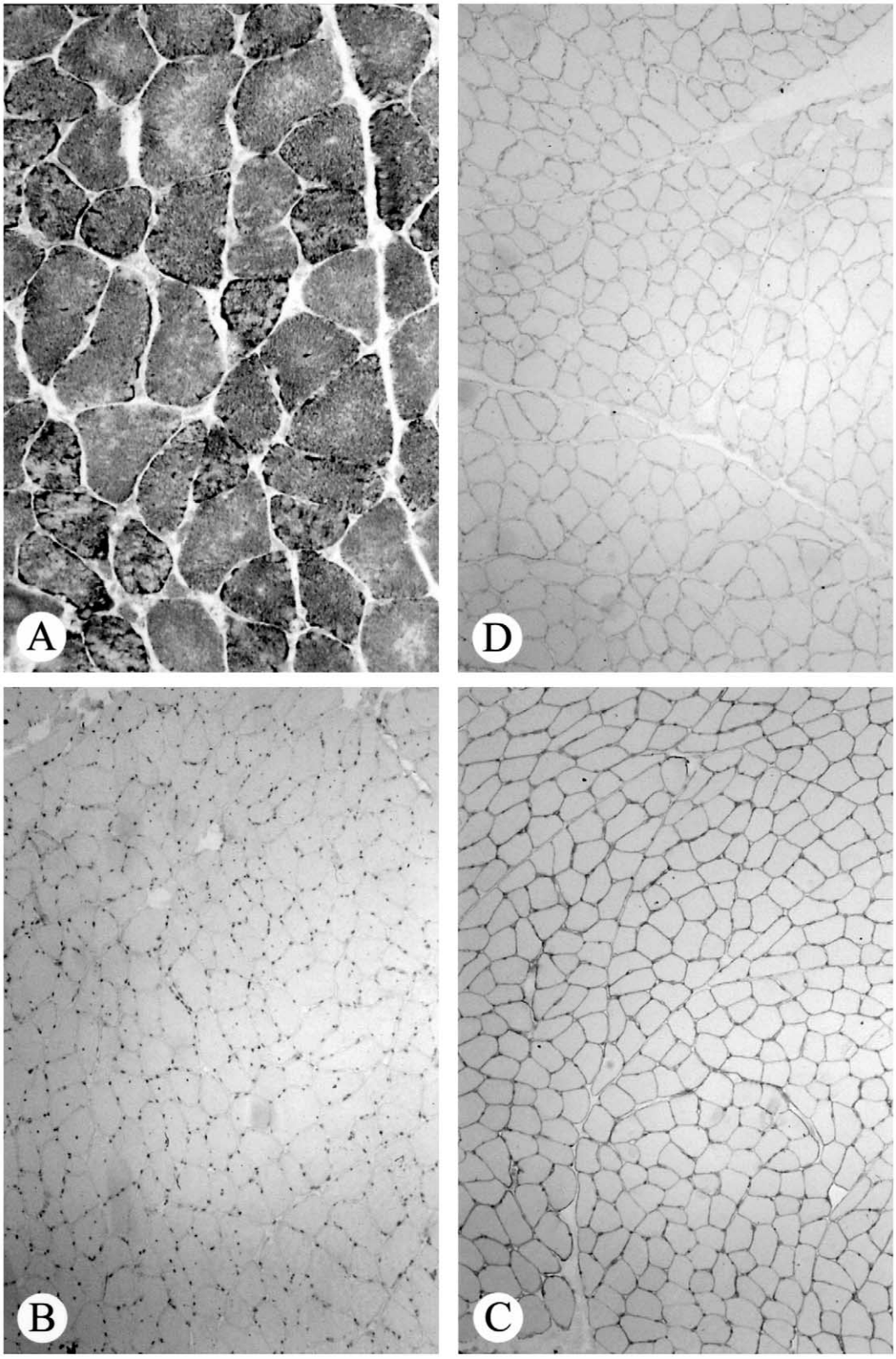

Fig. 3. Patient 13. LFs are observed in 30% of fibers (A, NADH-TR), γ-sarcoglycan is reduced (B), α-sarcoglycan expression is almost absent (C) in comparison with one control (D).

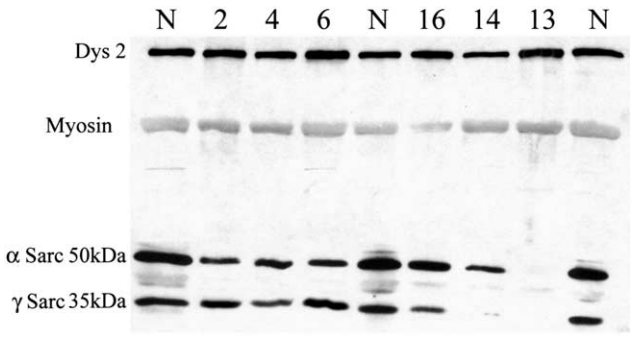

Fig. 4. Western blot analysis Dystrophin (anti-Dys 2) and sarcoglycans (anti-α and γ) in patients and controls (N). Compare with myosin content in muscle biopsies. Severe reduction of sarcoglycans (α and γ) is observed in patient 13.

myopathy is clinically heterogeneous reflecting genetic heterogeneity. However, this study also shows that in spite of the systematic analysis of the expression of the proteins involved in the dystrophin membranous complex, the calpain-3 or dysferlin, the genetic identification of lobulated fiber myopathy remains unknown in 60% of cases. Therefore, lobulated fiber myopathy still remains a distinctive pathological entity occurring in patients with a limb-girdle phenotype.

Lobulated fibers are associated with an abnormal distribution pattern of the intermyofibrillar mitochondria but the mechanism leading to this maldistribution is unknown. Oxidative enzymes such as succino-dehydrogenase and cytochrome C oxidase are normally expressed in lobulated fibers suggesting that they are biochemically normal at least regarding these enzymes. Moreover, electron microscopy disclosed structurally normal but misplaced mitochondria.

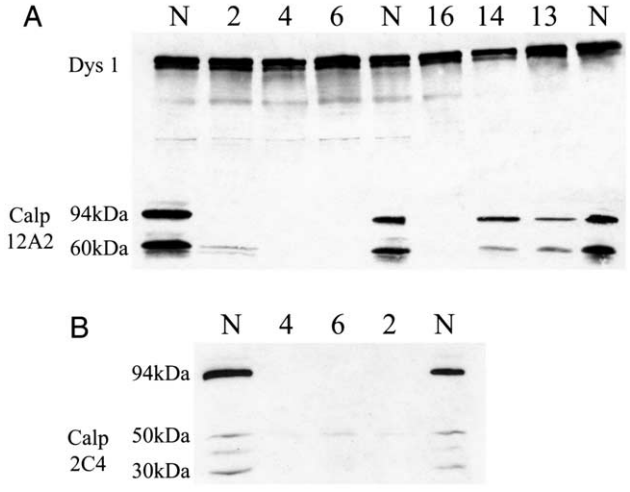

Fig. 5. Western blot analysis. (A) Dystrophin (anti-Dys1) and calpain (anti-12A2) expression in patients and controls (N): lack of calpain expression in patients 4, 6 and 16. Only slight expression of the 60 kDa band of calpain is observed in patient 2. (B) Lack of calpain (anti-2C4) expression in patients 2, 4 and 6 whereas calpain expression in controls (N) is normal.

Expression of various cytoskeletal molecules that might be present in the putative mitochondrial anchoring system has been previously studied. The expression of these molecules including desmin, vimentin, β-actin and β-tubulin, talin and vinculin was normal in lobulated fibers [3,13]. Titin expression was not studied. Calpain-3 interacted with titin via one of these muscle-specific signal-like sequences [14]. Abnormal calpain-3 expression was found in 4 out of the 17 patients. Whether abnormal calpain-3 expression contributed to lobulated fiber formation remains to be clarified but it is worth noting that large amounts of lobulated fibers were not found consistently in calpainopathy.

Moreover we have observed that in some patients lobulated fibers were seen only at the second biopsy (patients 3, 4, 7, 9), or the number of lobulated fibers was higher at the second biopsy than at the first (patients 14–16). These biopsies were performed 1–16 years (mean 10 years) after the first one. The number of lobulated fibers did not correlate with the severity of the disease. Interestingly, only one pediatric case was reported in this series (patient 15) and it has previously been emphasized that lobulated fibers usually occur in adult patients only [2]. Also, the muscle biopsied was usually different in the second biopsy and it is clear from our results that the number of lobulated fibers is higher in proximal muscles (deltoid or quadriceps) than in more distal ones. Taken together, these results suggest that lobulated fibers are muscle dependent and might require time to appear. Moreover, it also suggests that sampling might be a major factor for the detection of lobulated fibers.

4. Note added in proof

Since the acceptance of this paper, we have observed a myopathy with lobulated muscle fibers in a patient presenting lask of dysferlin expression. Genetic analysis is in progress.

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