ORIGINAL ARTICLE

Correlations between clinical severity, genotype and muscle pathology in limb girdle muscular dystrophy type 2A

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25 May 2007**Background:** Limb girdle muscular dystrophy type 2A (LGMD2A) is characterised by wide variability in clinical features and rate of progression. Patients with two null mutations usually have a rapid course, but in the remaining cases (two missense mutations or compound heterozygote mutations) prognosis is uncertain.**Methods:** We conducted what is to our knowledge the first systematic histopathological, biochemical and molecular investigation of 24 LGMD2A patients, subdivided according to rapid or slow disease progression, to determine if some parameters could correlate with disease progression.**Results:** We found that muscle histopathology score and the extent of regenerating and degenerating fibres could be correlated with the rate of disease course when the biochemical and molecular data do not offer sufficient information. Comparison of clinical and muscle histopathological data between LGMD2A and four other types of LGMD (LGMD2B–E) also gave another important and novel result. We found that LGMD2A has significantly lower levels of dystrophic features (ie degenerating and regenerating fibres) and higher levels of chronic changes (ie lobulated fibres) compared with other LGMDs, particularly LGMD2B. These results might explain the observation that atrophic muscle involvement seems to be a clinical feature peculiar to LGMD2A patients.**Conclusions:** Distinguishing patterns of muscle histopathological changes in LGMD2A might reflect the effects of a disease-specific pathogenetic mechanism and provide clues complementary to genetic data.

Autosomal recessive (type 2) limb girdle muscular dystrophies (LGMDs) are a clinically and genetically heterogeneous group of disorders, including at least 11 different genetic entities,¹ which are characterised by progressive involvement and wasting of the proximal limb girdle muscles. LGMD2A is the most common form of LGMD in European countries, where it affects about 1:100 000 people.^{2–3} It is the most common neuromuscular autosomal recessive disorder after spinal muscular atrophy.

LGMD2A is caused by mutations in the *CAPN3* gene, encoding for a muscle-specific proteolytic enzyme, calpain-3.⁴ Calpain-3 is a multi-domain protein, which shares Ca²⁺-dependent proteolytic activity with ubiquitous calpains;⁵ however, the search for the specific substrates of calpain-3 has been to date unsuccessful. Several interesting lines of research have investigated the pathogenesis of calpainopathy. Calpain-3 could be involved in the regulation of transcription factors controlling survival genes and apoptosis,⁶ and in the degradation and disassembly of cytoskeletal or myofibrillar proteins (sarcomere remodelling).^{7–10}

Since the identification of mutations in the *CAPN3* gene in LGMD2A patients, many clinical and molecular studies on large series of patients with calpainopathy have been reported. These studies have highlighted the heterogeneity in the clinical, molecular and protein characteristics of the disease.^{3 11–18} Molecular diagnosis has revealed the wide clinical spectrum of calpainopathy, which confirms the original clinical descriptions of the LGMDs, which grouped the atrophic pelvifemoral Leyden–Moebius form, the scapulohumeral Erb form and the late-onset Nevin form.^{19–21} The onset of muscle weakness may occur either in the lower or in the upper girdle and at variable ages ranging from early infancy to adult life, and the degree of muscle impairment may range from severe (with early loss of ambulation) to mild myopathies in late adulthood.

The variability of both the clinical phenotype and disease course may be only partly attributable to the genotype.

Although null gene mutations are usually associated with absent calpain-3 protein in muscle, usually resulting in a severe phenotype, the effect of missense mutations (which account for about 70% of mutations in this gene) on both the protein and the phenotype is extremely unpredictable.^{3 12 13} Furthermore, variable degrees of muscle involvement and disease progression have been reported in patients sharing the same mutation, suggesting that additional, as yet unknown genetic or environmental factors may be playing a role in modulating the phenotype.

Biochemical and molecular identification of LGMD2A patients is crucial in order to offer genetic counselling and to properly manage such patients, even though there is currently no treatment for this disorder. Diagnostic analysis of muscle biopsies in LGMD2A has revealed several types and various degrees of myopathic and dystrophic changes, which are non-specific to this disorder and which depend on the extent of muscle involvement at the time at biopsy. However, systematic clinical, histopathological, biochemical and molecular investigations of a large group of patients with calpainopathy need to be conducted to provide detailed histopathology correlates.

METHODS

We carried out a systematic clinical, histopathological, immunohistochemical, biochemical and molecular investigation of a large group of patients with calpainopathy, in order to examine the hypothesis that LGMD2A has lower levels of dystrophic muscle changes (eg. regeneration and degeneration) compared with other forms of recessive LGMD. Furthermore, we tested whether the combined use of an accurate muscle histopathological study and biochemical–molecular analysis could offer data of prognostic value.

Abbreviation: LGMD, limb girdle muscular dystrophy

Selection criteria of patients

We selected 24 patients from a total of 82 with a molecular diagnosis of LGMD2A attending the Neuromuscular Center of Padova, based on the availability of sufficient muscle tissue to conduct the planned analysis. To compare histopathological muscle data of LGMD2A with other forms of LGMD, we enrolled a further 44 patients (26 LGMD2B, 6 LGMD2D, 7 LGMD2E, 5 LGMD2C), some of whom had previously been studied using the same histopathological and clinical methods.^{22, 23} These cases were selected to match the LGMD2A patients as closely as possible for age at biopsy, age at onset and disease duration.

Clinical data

Age at onset ranged from 3 to 47 years (mean 16.5); 11 cases had early onset (<12 years) and 13 had adult or late onset (table 1). In most patients (20/24), the weakness initially involved the lower girdle muscles; in four it involved the upper girdle.

Calculation of disease duration was not possible in one patient who had a biopsy taken 6 years before the onset of the disease (the patient had only increased creatine kinase). In the remaining 23 patients, the disease duration varied widely (from 1 to 46 years, mean 13.2). In seven patients the biopsy was taken within 2 years of the onset, but in six cases it was obtained after >20 years from onset.

Calculation of the rate of disease course was not possible in two patients who had an insufficiently long clinical follow-up (<6 years). In the remaining 22 patients, the rate of progression was rapid in 12, and slow in 10.

Neuromuscular clinical evaluation

Retrospective analysis of the clinical records collected at onset, at the time of biopsy and during the subsequent outpatient examinations was used to assess:

- age at onset of symptoms: early (<12 years), typical (12–30 years), late (>30 years);

- clinical phenotype: LGMD (early lower girdle involvement), Erb (early upper girdle involvement);
- disease duration: time elapsed between the onset of the disease and muscle biopsy;
- functional clinical grade using the modified 10-graded scale of Walton and Gardner-Medwin;²¹
- disease progression (or course), calculated from data obtained in ≥ 2 different clinical examinations and graded as rapid (worsening ≥ 3 grades of the functional scale in 6 years), or slow (worsening <3 grades in 6 years).

Calpain-3 protein analysis and CAPN3 gene mutations

Specimens of muscle biopsies were analysed by multiple western blots using a mixture of monoclonal antibodies against calpain-3 (Calp12A2, diluted 1:800), α -sarcoglycan (diluted 1:300), dystrophin (Dys-2, diluted 1:1000), and dysferlin (Hamlet, diluted 1:1000) (all purchased from Novocastra, Newcastle Upon-Tyne, UK).²⁴ The protein quantity of each sample was normalised to the amount of tissue loaded, as determined by the skeletal myosin bands in the post-transfer Coomassie blue-stained gels. The protein level in patients was determined by densitometry (ImageJ software V.1.34) and expressed as percentage of control. Muscle biopsies from patients showing normal calpain-3 levels by conventional immunoblotting underwent a further biochemical assay to test calpain-3 autocatalytic activity as described previously.²⁴

Screening of mutations in the *CAPN3* gene was conducted using single strand conformational polymorphism analysis and denaturing high-performance liquid chromatography, as previously described.^{3, 13}

Morphological and immunohistochemical study of muscle biopsy

Open muscle biopsies (mostly of quadriceps femoris, seldom of biceps/triceps brachii), were taken as part of the diagnostic procedure, after written consent from the patients or their parents.

Table 1 Clinical, molecular, and pathological data

Patient no	Clinical phenotype	Age at onset (years)	Age at biopsy (years)	Disease duration (years)	Disease course	Calpain (%)	CAPN3 gene mutations	Regenerating fibres (%)	Degenerating fibres (%)	Lobulated fibres (%)	Severity score
1	Eo LGMD	3	31	28	Rapid	100	G496R/C328X	2.7	0	15.8	Moderate
2	Eo LGMD	4	50	46	Slow	0	P82L/G233R	0.3	0	3.0	Moderate
3	Eo LGMD	7	8	1	Rapid	0	550delA/R748X	3.5	0.7	0	Severe
4	Eo LGMD	8	13	5	Rapid	0	100delG/Y763C	2.1	0.6	0	Severe
5	Eo LGMD	8	19	11	Rapid	0	550delA/?	1.8	0.1	2.3	Severe
6	Eo LGMD	10	11	1	Rapid	0	550delA/801+1G→A	2.6	0.4	0.2	Severe
7	Eo LGMD	10	12	2	Rapid	0	550delA	8.1	0	0.2	Mild
8	Eo LGMD	10	21	11	Rapid	0	2230-2231insAACA	2.7	0	21.6	Advanced
9	Eo LGMD	11	22	11	Slow	0	I178T	7.6	0.7	0	Severe
10	Eo LGMD	12	20	8	Rapid	0	550delA/I777T	12.0	0.8	0	Moderate
11	Eo LGMD	12	49	37	Rapid	0	R440Q/R448H	1.5	0	1.2	Advanced
12	LGMD	15	23	8	Rapid	5	N449H	5.5	0.4	0	Moderate
13	LGMD	15	17	2	Rapid	10	V354G	10.9	4.6	0	Moderate
14	LGMD	16	56	40	Slow	10	R572W/Y537X	2	0.8	14.4	Moderate
15	LGMD	16	10	—	Slow	100	R490Q	0.3	0	0	Mild
16	LGMD	18	21	3	Rapid	0	R748X/?	5.8	0.1	6.1	Severe
17	LGMD	19	38	19	Slow	0	R748Q	0.7	0	6.7	Advanced
18	Erb	19	43	24	Slow	100	R490Q	0.3	0	0.1	Mild
19	Erb	21	31	10	Rapid	0	V431M/R448C	1.6	0.2	0	Moderate
20	LGMD	24	46	22	Slow	0	R448H/1992+1G→T	1.6	0.3	9.6	Severe
21	Erb	35	36	1	—	100	259-260insT/T184M	0.2	0	0	Mild
22	Erb	28	30	2	Rapid	100	R490Q	8.3	0.6	5.8	Severe
23	LGMD	28	38	10	Slow	100	T192I/Y537X	0.1	0	0	Mild
24	Lo LGMD	47	49	2	—	100	R490Q	0.2	0.1	0	Mild

?, Mutant allele not identified; —, impossible to determine; Eo, early onset; Lo, late onset. Homozygous mutations are in bold type.

A set of seven serial sections was obtained for each biopsy. The first two sections (10 µm thick) were routinely stained with H&E and NADH-tetrazolium reductase.²⁵ The total number of fibres per section was counted using overlapping microscope images of H&E-stained sections (Zeiss Axioskop epifluorescence photomicroscope, equipped with CoolSnap Photometrics digital camera and Roper Scientific imaging software). The same sections were used to analyse and measure the following parameters: fibre size diameter, connective tissue proliferation (fibrosis), degenerating fibres (including necrotic, phagocytosed, hyaline and opaque fibres, and expressed as a percentage of total fibres), lobulated fibres (evaluated with NADH-tetrazolium reductase stain and expressed as a percentage of total fibres).

The additional five serial sections (6 µm thick) were placed on gelatinised slides and processed for immunohistochemical analyses, using a panel of different monoclonal antibodies to further investigate muscle regeneration: fetal myosin (1:100 dilution; MHCn, Novocastra, Burlingame, California, USA), neural cell adhesion molecule (1:100 dilution; CD56, Novocastra), desmin (1:100 dilution; Monosan, Uden, The Netherlands), vimentin (1:100 dilution; Monosan), laminin α -5 chain (1:2000 dilution; Chemicon, Temecula, USA). We used the term “regenerating fibres” to refer to fetal myosin-positive fibres. Muscle fibres showing a faint MHCn reaction (intermediate stain) and nuclear clumps (with positive MHCn staining) were excluded from the calculation, so that only active regenerating fibres were included.

Biopsies were graded on a 4-point histopathological scale based on the dystrophic changes and all morphological parameters analysed: (1) mild (slight increase in fibre size variability, absent or mild regeneration and degeneration, absent or mild fibrosis); (2) moderate (moderate increase in variability of fibre size, variable degeneration and regeneration, moderate or severe fibrosis); (3) severe (marked increase in variability of fibre size, variable degeneration and regeneration, moderate or severe fibrosis); and (4) advanced (huge increase in variability of fibre size, variable regeneration and degeneration, severe fibrosis).

Two independent observers matched their results when attributing non-numerical graded scores (eg mild, moderate, severe, advanced score or degree of fibrosis), in order to reduce operator-dependent bias.

Statistical analysis

Values are expressed as mean (SD). The Student *t* test was used to compare the mean values of each numerical parameter across different groups of LGMD patients. Linear regression analysis was used to correlate numerical parameters in the same group of patients. Spearman's rank correlation test was used to correlate non-parametric variables (eg. pathology score) in the same group of patients. Significance was set at $p < 0.05$.

RESULTS

Biochemical and molecular data, and genotype–protein–phenotype correlations

The search for mutations in the *CAPN3* gene allowed us to identify both mutant alleles in 22 patients (table 1). In the remaining two patients only one mutant allele was found; the LGMD phenotype and the absence or severe deficiency of calpain-3 protein in these patients exclude the possibility that they are true heterozygotes, and suggest that the second mutant allele escaped identification because of the insufficient sensitivity of the methods used (detection rate 96%). Of the 27 different mutations found, 17 were missense and 10 null (nonsense or frame-shifting deletions or insertions). One mutation (C328X) was novel.

All four patients who were homozygous or compound heterozygous for two null mutant alleles had absent calpain-3 protein, early-onset LGMD and rapid progression (table 1). In contrast, patients who had homozygous missense mutations or were compound heterozygous for two missense alleles or for one missense and one null mutant allele had extremely variable levels of protein and rates of disease progression.

Of 5 patients with normal levels of calpain-3 protein, 3 (60%) had slow disease progression, and 11 of 15 (73%) patients with absent or $<5\%$ protein quantity had rapid progression. Of 11 patients with early onset of weakness, 10 (91%) had absent protein.

The four patients with the same homozygous missense mutation (R490Q) had differences in age of onset (from 16 to 47 years), distribution of muscle involvement (Erb or LGMD), disease progression, fibre regeneration and degeneration, and histopathology severity score.

Muscle histopathology and immunohistochemistry, and clinicopathological correlations

All muscle biopsies analysed showed an increase in fibre size variability (table 1) compared with normal ranges. In 10 patients, there were both atrophic and hypertrophic fibres, but in most cases, the abnormal size variability was due only to atrophic fibres.

Degeneration involved an average of 0.4% of total fibres. Of the nine patients with $>0.4\%$ of degenerating fibres, seven (78%) had rapid disease progression (table 1), suggesting that the extent of muscle degeneration could be an index of disease progression, even in early stages of the disease. We observed a direct correlation between degenerating and regenerating fibres ($p < 0.01$).

On average, 3.4% of regenerating fibres had a positive fetal myosin reaction. There was an inverse correlation between regenerating fibres and age at biopsy ($p = 0.02$), whereas regeneration seemed not to be correlated with disease duration, suggesting that the decreased regenerative capacity of muscle with age is not related to disease course. Of the 11 patients with a degree of regeneration involving $>2.5\%$ of total fibres, 10 (91%) had a rapid disease course, suggesting that the extent of muscle regeneration could be an index of disease progression, even in early stages of the disease.

There was an apparent, although not significant ($p = 0.07$), direct correlation between lobulated fibres and disease duration. Furthermore, patients with a large number of lobulated fibres ($>10\%$ of total) had the worst clinical functional scores (grades 7 and 8) at the time of muscle biopsy (data not shown), suggesting that the occurrence of lobulated fibres is a marker of chronic disease but does not have a prognostic value.

We observed that 3 of the 4 (75%) patients with mild histopathology severity score had a slow disease progression (in 2 additional cases the course was not available), whereas 8 of the 11 (73%) patients with severe or advanced pathology scores had rapid disease progression (table 1), suggesting that the muscle pathology score could predict the rate of progression even in early stages of the disease. The histopathology severity score seemed not to be correlated with disease duration ($r_s = 0.2$, $p > 0.05$).

Comparison between LGMD2A and other types of LGMD

Because of the large number of LGMD2A and LGMD2B cases involved in this study, the comparison between these two forms of LGMD was expected to offer the most reliable results (table 2). Furthermore, sarcoglycanopathy patients have earlier age at onset and age at biopsy than LGMD2A and 2B.

The extent of regeneration and degeneration was significantly lower in LGMD2A than in the other LGMD (table 2,

Table 2 Comparison between different forms of LGMD

	LGMD2A	LGMD2B	LGMD2C-E
Patients (n)	24	26	18
Age range at biopsy (years)	8–56	17–58	1–43
Age at biopsy (years)	28.9 (14.6)†	29.2 (10.4)	17.7 (13.5)
Age at onset (years)	16.5 (10.2)†*	21.7 (8.1) (n=25)	7.5 (8.4) (n=16)
Disease duration (years)	13.2 (13.5) (n=23)	8.5 (8.2) (n=21)	10.8 (11.3)
Regenerating fibres (%)	3.4 (3.5)*	11.8 (13.1)	4.9 (4.7)
Degenerating fibres (%)	0.4 (0.9)†*	0.9 (0.9)	1.4 (1.5)
Lobulated fibres (%)	3.6 (6.0)	1.5 (4.0)	0.9 (2.5)

Values are expressed as mean (SD).

Values in second set of parentheses are the number of cases used in the calculation.

Statistically significant differences: *LGMD2A vs LGMD2B, †LGMD2A vs LGMD2C-E.

figure 1). In particular, regenerating and degenerating fibres were fourfold and twofold lower in calpainopathy than in LGMD2B, respectively ($p < 0.05$).

Lobulated fibres were 2–4-fold higher in LGMD2A than in the other LGMD types (table 2, figure 1), although differences were not significant because of the huge standard deviation. Disease duration, which might influence the frequency of lobulated fibres, was not significantly different across the three groups of LGMD.

DISCUSSION

There is wide clinical variability in the age at onset, distribution of muscle weakness, and rate of progression in LGMD2A.^{11–18} Furthermore, variability in intrafamilial phenotype is not unusual. In most cases, the onset of weakness in childhood is associated with severe muscular dystrophy and early loss of ambulation. However, a rapid worsening of the disease may also occur in patients with adult or late onset, and, conversely, a slow course may occur in patients with early onset. One of the aims of this study was to differentiate between patients with rapid and slow rates of disease progression, independent of age at onset. Because no standardised rules are available for this purpose, we adopted new criteria that resulted in the clear identification of two groups of patients. The disease course was derived by retrospective analysis of the functional motor disabilities over time, but the availability of indices that help to predict the rate of progression in the early stages of the disease could be a valuable tool for the clinical investigation.

Early biochemical and molecular diagnosis is essential for prognosis in LGMD2A. We have shown that identification of gene mutations could enable prediction of the rate of

progression only when the two mutant alleles were null mutations (nonsense or frame-shifting deletions/insertions) that result in the premature truncation of protein synthesis and absence of protein;^{11–13} such patients have an almost homogeneous clinical phenotype, which is invariably characterised by early onset and rapid course. Conversely, no definite correlations can be drawn in cases with two missense mutations or one missense and one null mutation; the age at onset of these patients varies greatly, as do disease progression and protein levels. This unpredictable effect of missense mutations on both the protein and the phenotype is mainly due to the fact that they are located in domains of different functional importance. However, even patients sharing the same homozygous missense mutation may have different degrees of muscle involvement and rates of disease progression, suggesting that unknown genetic and environmental factors might modulate the phenotype.

One important conclusion drawn from our study is that although rapid disease course was predicted in patients presenting two null mutations, muscle histopathology in the remaining cases (21/25 in our series) could correlate with disease progression, although this was sometimes unpredictable. In particular, muscle histopathology score and extent of fibre regeneration and degeneration seem to be helpful prognostic tools when molecular and biochemical data are insufficient for prognosis.

Our results have shown that muscle biopsy is an important procedure for diagnosis of LGMD2A; protein analysis and muscle pathology gives more information than molecular diagnosis by genomic DNA analysis alone.

A novel and significant conclusion drawn from our study has come out of the comparison of the data between LGMD2A and

Figure 1 Histograms showing the extent of muscle fibre regeneration, degeneration and lobulation (left to right, respectively) in the five different types of LGMD examined. Note that in comparison with the other forms of LGMD, LGMD2A has a lower degree of active dystrophic changes (regenerating and degenerating fibres), and a higher degree of chronic features (lobulated fibres).

different types of recessive LGMD: compared with other LGMD, LGMD2A has significantly lower levels of active muscle changes (ie regeneration and degeneration) and higher levels of chronic features, such as lobulated fibres. These results might explain the clinical observation that atrophic muscle involvement seems to be a clinical feature peculiar to patients with calpainopathy.

Distinguishing patterns of muscle histopathological changes in LGMD2A might reflect the effects of a disease-specific pathogenetic mechanism. According to observations in recent studies, calpain-3 deficiency affects sarcomere organisation and its maintenance in adult muscle fibres.^{8 9 28 29} Skeletal muscles must constantly adapt to respond to various physiological conditions (metabolic, mechanical, hormonal) by a complex process called sarcomere remodelling, which involves protein synthesis and degradation by the intervention of the proteolytic system. In an initial phase of sarcomere remodelling, ubiquitous calpains and the ubiquitin-proteasome system disassemble and degrade myofibrils, whereas in the recovery phase, when an increase in calpain-3 expression has been found, muscles defective in calpain-3 fail to regain their full weight.³⁰ This complex mechanism would explain the following muscle pathology features observed in LGMD2A:

1. The increased myonuclear apoptosis found in association with subsarcolemmal localisation and accumulation of NF- κ B, which results in perturbation of the I κ B α /NF- κ B pathway due to calpain-3 defects.⁶ However, these findings probably cannot fully explain the pathological mechanism of LGMD2A, especially the formation of lobulated fibres, which are a pathological hallmark in the late stage of the disease.
2. The frequent occurrence of lobulated fibres observed in calpainopathy.^{16 26 27} As the reduced levels of calpain-3 inversely correlate with the levels of muscle-degrading proteases (cathepsins, ubiquitous calpains), dysregulation of these proteases would induce an alteration of the cytoarchitecture of muscle fibres, and abnormal subsarcolemmal distribution of mitochondria and focal Z-line streaming.⁹ Expression profiling studies have shown that increased expression of actin filament-binding proteins may contribute to the changes of the intramyofibre structure observed in lobulated fibres.³¹
3. Muscle atrophy, possibly resulting from both hypotrophy (decrease in fibre size) and hypoplasia (reduction in the number of fibres) by an increase in protein degradation, decrease in protein synthesis, loss of fibres by apoptosis or necrosis, and inability of satellite cells to counteract the decrease in fibre size and number.³²

The different histopathological features we observed in the different forms LGMD appear to result from different pathogenetic mechanisms. The severe damage to muscle fibres caused by the defect in integral plasmalemmal proteins would explain the high levels of active muscle changes in dysferlinopathy and sarcoglycanopathy, and conversely, the abnormal sarcomere remodelling caused by the enzyme defect of calpain-3 might account for the low levels of active muscle pathology changes and the increase in chronic features of LGMD2A.

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