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Neural

Limb-girdle muscular dystrophy subtypes

First-reported cohort from northeastern China

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Research Highlights

(1) The relative frequencies of the different forms of limb-girdle muscular dystrophy in China are similar to those reported in European countries. Calpainopathy is the most common form of limb-girdle muscular dystrophy in China, followed by dysferlinopathies. According to most cohorts studied to date, caveolinopathy appears to be a rare autosomal dominant form of limb-girdle muscular dystrophy, compared with other subtypes in China.

(2) Disease activity was associated with limb-girdle muscular dystrophy type 2B, while chronicity was associated with limb-girdle muscular dystrophy type 2A biopsies. Acid phosphatase staining is a powerful tool for detecting macrophage distribution in muscle biopsies. Cellular infiltrates are rare, but do not exclude limb-girdle muscular dystrophy type 2D, as is the case for rimmed vacuoles in dysferlinopathies.

Abstract

The relative frequencies of different subtypes of limb-girdle muscular dystrophies vary widely among different populations. We estimated the percentage of limb-girdle muscular dystrophy subtypes in Chinese people based on 68 patients with limb-girdle muscular dystrophy from the Myology Clinic, Neurology Department, First Hospital of Jilin University, China. A diagnosis of calpainopathy was made in 12 cases (17%), and dysferlin deficiency in 10 cases (15%). Two biopsies revealed α -sarcoglycan deficiency (3%), and two others revealed a lack of caveolin-3 (3%). A diagnosis of unclassified limb-girdle muscular dystrophy was made in the remaining patients (62%). The appearances of calpain 3- and dysferlin-deficient biopsies were similar, though rimmed vacuoles were unique to dysferlinopathy, while inflammatory infiltrates were present in both these limb-girdle muscular dystrophy type 2D biopsies. Macrophages were detected in seven dysferlinopathy biopsies. The results of this study suggest that the distribution of limb-girdle muscular dystrophy subtypes in the Han Chinese population is similar to that reported in the West. The less necrotic, regenerating and inflammatory appearance of limb-girdle muscular dystrophy type 2A, but with more lobulated fibers, supports the idea that calpainopathy is a less active, but more chronic disease than dysferlinopathy. Unusual features indicated an extended limb-girdle muscular dystrophy disease spectrum. The use of acid phosphatase stain should be considered in suspected dysferlinopathies. To the best of our knowledge, this is the first report to define the relative proportions of the various forms of limb-girdle muscular dystrophy in China, based on protein testing.

Key Words

neural regeneration; limb-girdle muscular dystrophy; calpain 3; α -sarcoglycan; dysferlin; caveolin-3; grants-supported paper; neuroregeneration

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INTRODUCTION

Limb-girdle muscular dystrophies are defined by their clinical and genetic heterogeneities, with common features of progressive weakness and wasting of proximal limb-girdle muscles. Limb-girdle muscular dystrophy represents a variety of autosomal recessive and dominant disorders^[1]. The clinical characteristics of limb-girdle muscular dystrophies include proximal (pelvic/scapular) and/or distal muscle involvement with typical sparing of facial muscles, and high serum creatine kinase levels. However, wide discrepancies in terms of age of onset, distribution of weakness, cardiac or respiratory muscle involvement and speed of progression, have been observed both within and between families^[2].

The CAPN3 (15q15.1–q21.1) gene encodes the muscle-specific protease calpain 3^[3], which is an intracellular calcium-sensitive cysteine protease involved in muscle repair^[4]. The exact function of calpain 3 is poorly understood, but it is believed to have both structural and proteolytic-mediated sarcomere-remodeling functions during physical stress^[5]. CAPN3 mutations cause limb-girdle muscular dystrophy type 2A or calpainopathy, which can present with different phenotypes, based on the initial distribution of muscle weaknesses. These include pelvi-femoral limb-girdle muscular dystrophy (Leyden-Möbius), which is the most commonly observed phenotype, scapulohumeral limb-girdle muscular dystrophy (Erb), which is often milder, and asymptomatic hypercreatinemia, which is usually seen in children and young adults^[6]. The diagnosis of limb-girdle muscular dystrophy type 2A is based on typical phenotypic findings including high creatine kinase level, a myopathic pattern on electromyography, myonecrosis and regeneration on muscle biopsy. The diagnosis is eventually confirmed by immunoblot analysis followed by gene testing. However, the high cost of genetic analysis still hinders investigations in many parts of the developing world, including India, Brazil, Mexico and China, and western blot assay remains the mainstay of diagnosis and counseling^[7-10]. Limb-girdle muscular dystrophy type 2B, Miyoshi myopathy, distal anterior tibial myopathy, rigid spine syndrome and recently bent-spine syndrome all represent a single disease entity characterized by dysferlin gene mutation^[11-13]. These conditions have sometimes been classified together as dysferlinopathies, given the lack of differences in disease progression, prognosis, genotyping or magnetic resonance imaging pattern^[14]; however, practicing clinicians still favor the individual definitions. Dysferlin, encoded by the DYSF gene (2p13), is a 230-kDa protein that is widely expressed

in tissues, especially in striated muscles and leukocytes^[15]. Dysferlin is a sarcolemmal protein believed to be vital for calcium-mediated vesicle fusion with the sarcolemma and membrane repair of muscle fibers^[16]. The relatively large size of the gene made researchers shift from gene analysis to protein testing to confirm diagnosis, and patients with a lack or near lack of dysferlin labeling in muscles or on blots are presumed to harbor DYSF gene mutations, as supported by four studies performed in different populations^[17-20]. Adhalin or α -sarcoglycan is encoded by the SGCA gene (17q12–q21.33). The protein is involved in sarcolemmal integrity^[21]. Patients with limb-girdle muscular dystrophy type 2D have adhalin or α -sarcoglycan gene mutations. Limb-girdle muscular dystrophy type 1C is an autosomal dominant disease, and one of multiple phenotypes caused by mutation of the CAV3 gene (3p25) CAV3 encodes caveolin-3, a muscle-specific membrane protein involved in the formation of flask-shaped membrane-repair invaginations in muscle fibers^[22]. The most common limb-girdle muscular dystrophy subtype in Italy, Spain, Turkey, Russia, Brazil and Australia is calpainopathy (limb-girdle muscular dystrophy type 2A)^[23-29], while dystroglycanopathy (limb-girdle muscular dystrophy type 2I) is the commonest form in Norway, Denmark and northern England^[30, 31]. In India, sarcoglycanopathies (limb-girdle muscular dystrophy type 2C-2F) are the most prevalent^[32-33], while dysferlinopathies are the most frequent type in USA and Japan^[34-35].

Confirmation of diagnosis of some limb-girdle muscular dystrophy subtypes may lead to changes in genetic counseling strategies, e.g., in some sarcoglycanopathies previously diagnosed as Becker muscular dystrophy (an X-linked disease). Furthermore, different limb-girdle muscular dystrophy subtypes may require different therapeutic approaches e.g., myostatin blockade is evolving as therapeutic rationale in cases of limb-girdle muscular dystrophy type 1C, whereas targeting inflammatory cascades is the main approach in dysferlinopathies. In addition, identification of patients with sarcoglycanopathy makes gene therapy a possibility in this group of patients.

Though much is known about the clinical features that distinguish calpainopathy from dysferlinopathy^[6], little is known about their respective pathological findings. Many reports have described the clinical features and pathological findings of each disease separately, and have correlated their genotypes and phenotypes to provide a well-characterized outline for each subtype^[36]. Nevertheless, the comparable pathological consequences of most dystrophic diseases mean that few morphological results have been reported, except for unique findings.

However, the wide array of disease activities ranging from slowly progressive to malignant limb-girdle muscular dystrophy may possibly be reflected by morphological features. A rigorous comparison of the morphological features of biopsy specimens between limb-girdle muscular dystrophy type 2A and limb-girdle muscular dystrophy type 2B was therefore warranted. In China, the problem is complicated by the fact that, to the best of our knowledge, only two cohorts of patients with limb-girdle muscular dystrophy have been published, each of which considered only one form of limb-girdle muscular dystrophy^[10, 37]. To the best of our knowledge, no cohort study rating the relative frequencies of different limb-girdle muscular dystrophy subtypes has been conducted in China. The current study therefore summarized the main clinical features and pathological characteristics of the different forms of limb-girdle muscular dystrophy, and compared the morphological findings between the two major limb-girdle muscular dystrophy subgroups.

examinations and muscle biopsies, which were subjected to histochemical, immunohistochemical and western blot analyses to reach a final diagnosis. On this basis, the cases were divided into five groups: calpainopathy ($n = 12$), dysferlinopathies ($n = 10$), α -sarcoglycanopathy ($n = 2$), caveolinopathy ($n = 2$) and unclassified ($n = 42$). Limb-girdle muscular dystrophies were defined according to a complete lack of protein bands on blots and/or total absence of protein staining in muscles (Figure 1). Calpain 3 was deficient in 12 cases (17%) and reduced in eight cases (excluded), dysferlin was lacking in ten patients (15%) and reduced in four patients (excluded), two patients showed a significant lack of adhalin (3%) and three expressed only residual amounts (omitted), and two (3%) biopsies revealed a lack of caveolin-3 (Figure 1). Expression levels of these proteins were not obviously affected in the remaining patients. The most common subtype was therefore limb-girdle muscular dystrophy type 2A, followed by dysferlinopathies (limb-girdle muscular dystrophy type 2B, Miyoshi myopathy).

RESULTS

Quantitative analysis of subjects

Sixty-eight patients were retrospectively enrolled into our cohort. All patients underwent clinical and physiological

Main clinical features of limb-girdle muscular dystrophy subtypes

Table 1 presents the main clinical features of the biochemically confirmed limb-girdle muscular dystrophy subtypes.

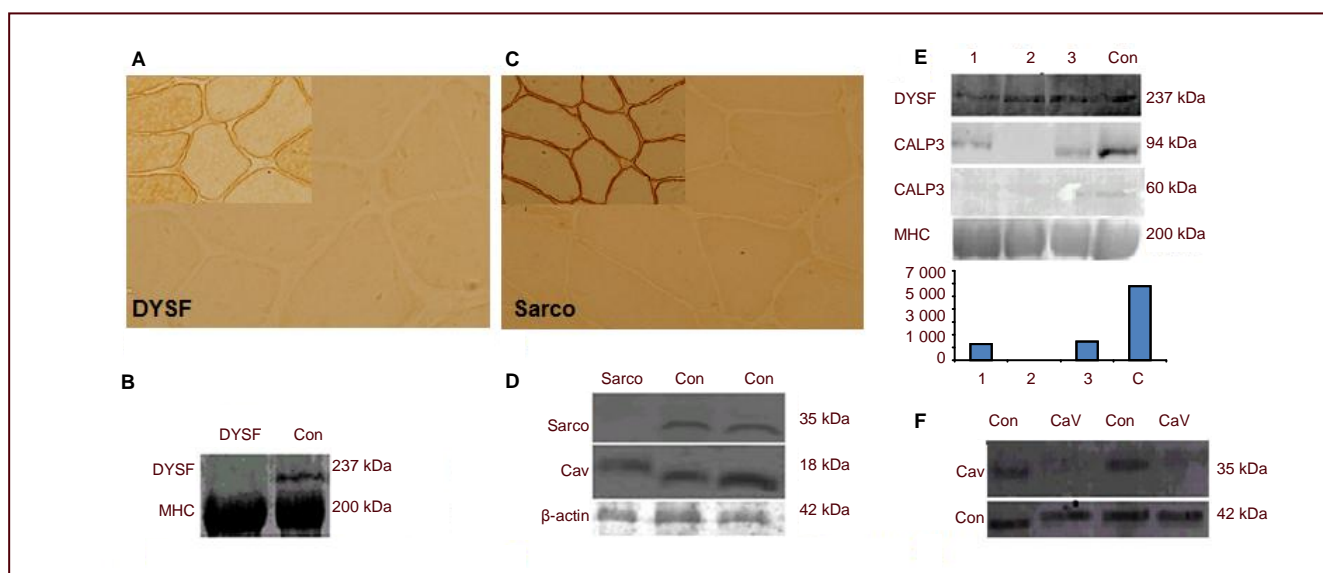


Figure 1 Biochemical assays in patients with limb-girdle muscular dystrophies.

(A) DYSF deficiency in muscle section using immunohistochemical staining ($\times 400$). (B) Western blot assay for DYSF expression. MHC served as internal reference. (C) Lack of Sarco on immunohistochemical staining ($\times 400$). (D) Western blot assay for Sarco expression. Normal Cav expression was found. β -actin served as internal reference. (E) Western blot assay for CALP3 expression. Lanes (1) and (3) showed partial protein deficits (excluded), and lane (2) with complete absence of CALP3. Normal DYSF expression was found. MHC served as an internal reference. CALP3 expression expressed as the absorbance ratio of CALP3 to β -actin. (F) Western blot assay for Cav expression. DYSF: Dysferlin; MHC: heavy chain myosin; Sarco: α -sarcoglycan; Cav: caveolin-3; CALP3: calpain 3; Con: control.

All but one patient with Miyoshi myopathy (1/26) presented with initial proximal muscle involvement of either Leyden-Möbius (pelvic) type, which was the most common (50%), generalized weakness of all four limbs (about 27%), or Erb (scapular) phenotype, which was relatively rare (about 19%). Most patients initially complained about one group of proximal muscles, but all four limbs became weak after several years. The mean onset age was generally late in all groups, compared with those in cohorts from other countries, with an evident male-to-female predominance (20:6). However, it is possible that our inclusion of only definite cases might have introduced some bias. The onset age ranged from 15–71 years.

Regarding calpainopathy, 11 out of 12 patients were male. The mean onset age was around 32 years, with a wide range from adolescence to old age, and the mean illness duration was about 7 years. Seven patients had early onset (≤ 30 years), and five were affected late (> 30 years). The mean creatine kinase level was about eight times the upper normal limit (200 IU). The pelvic muscles were the first affected in four patients (Leyden-Möbius), while four had scapular-muscle onset (Erb limb-girdle muscular dystrophy), and the other four presented with generalized weakness. Mobility status was relatively well-preserved, except in one patient who was wheelchair bound. Difficulty in climbing stairs, getting up from the squat position, and

exertion when combing hair were reported by most patients. Six patients had winging of scapulae, and three had calf hypertrophy (Figure 2). The ratio of sporadic to familial cases was 9:3. Table 2 shows the clinical features of the twelve calpain 3-deficient patients.

Dysferlinopathies were the second most common subtype after limb-girdle muscular dystrophy type 2A, accounting for ten out of the 68 limb-girdle muscular dystrophy patients (about 15%). We noted several different phenotypes: five patients had pelvic-muscle weakness (Leyden-Möbius limb-girdle muscular dystrophy); three had generalized weakness, one had shoulder-muscle weakness (Erb limb-girdle muscular dystrophy), and one had Miyoshi distal phenotype. The male-to-female ratio was 7:3. The mean age was around 29 years, with an extremely wide range (15–71 years), but if patient M677 was excluded (71 years old), the mean age of the remaining nine was about 24 years. Seven had early onset (≤ 30 years) and three described late onset (> 30 years). The mean illness duration was about 6 years from the first complaint, and longer duration of illness was associated with milder disease. Creatine kinase levels were highest among all the forms of limb-girdle muscular dystrophy, and were 30-fold the upper normal limit. Only one patient was crippled and another moved with the support of a stick. Seven cases were sporadic, two reported other affected sibs, and the remaining case had consanguineous parents (Table 3).

Table 1 Clinical features of different forms of limb-girdle muscular dystrophy (LGMD)

	<i>n</i>	Sex (male/female, <i>n</i>)	Onset [mean±SD (range), year]	Duration (mean±SD, year)	Creatine kinase (mean±SD, IU)	Scapular winging (<i>n</i>)	Calf hypertrophy (<i>n</i>)	Family history (<i>n</i>)
LGMD2A	12	11/1	32.6±13.3(16–53)	7.2±8.8	1 493.9±1 649.8	6	3	3
LGMD2B/MM	10	7/3	29.3±16.4(15–71)	5.8±6.4	6 059.1±5 540.9	0	1	2
LGMD2D	2	1/1	43.8±10.9(36–51)	1.8±1.8	3 698.5±4 008.2	0	1	0
LGMD1C	2	1/1	25.5±0.7(25–26)	7.0±4.2	1 906.1±21 51.2	1	1	0

Table 2 Clinical features of limb-girdle muscular dystrophy (LGMD) type 2A

Patient No.	Sex	Age at presentation (year)	Age at onset (year)	Creatine kinase (IU)	Phenotype	Ambulation	Family history
M479	Male	58	30	592	Leyden-Möbius	Mobile	Positive
M486	Female	24	18	1 569	Leyden-Möbius	Mobile	Negative
M510	Male	51	50	2 783.3	Weakness	Mobile	Negative
M535	Male	23	22	2 300.7	Leyden-Möbius	Mobile	Negative
M548	Male	32	30	105	Erb LGMD	Crippled	Negative
M573	Male	18	16	5 647	Leyden-Möbius	Mobile	Negative
M806	Male	47	27	505	Erb LGMD	Mobile	Negative
M808	Male	44	34	1 806	Weakness	Mobile	Positive
M816	Male	52	52	Not done	Erb LGMD	Mobile	Negative
M817	Male	27	20	442	Weakness	Mobile	Negative
M825	Male	54	53	154	Weakness	Mobile	Negative
M833	Male	49	39	529	Erb LGMD	Mobile	Positive

Table 3 Clinical features of dysferlinopathy patients

Patient No.	Sex	Age at presentation (year)	Age at onset (year)	Phenotype	Creatine kinase (IU)	Ambulation	Family history
M542	Female	42	32	Leyden-Möbius	2 562.3	With support	Negative
M544	Male	22	17.5	Leyden-Möbius	3 147	Ambulant	Negative
M620	Female	30	28	Leyden-Möbius	8 062	Ambulant	Negative
M677	Male	71	70.5	Weakness	16 000	Ambulant	Negative
M708	Female	46	26	Weakness	817	Ambulant	Positive
M715	Male	22	18.5	Miyoshi myopathy	8 252	Ambulant	Consanguinity
M736	Male	39	29	Leyden-Möbius	2 886	Crippled	Negative
M766	Male	40	39	Weakness	3 930	Ambulant	Negative
M832	Male	18	17	Leyden-Möbius	14 606	Ambulant	Negative
M849	Male	15	15	Erb LGMD	329	Ambulant	Positive

LGMD: Limb-girdle muscular dystrophy.

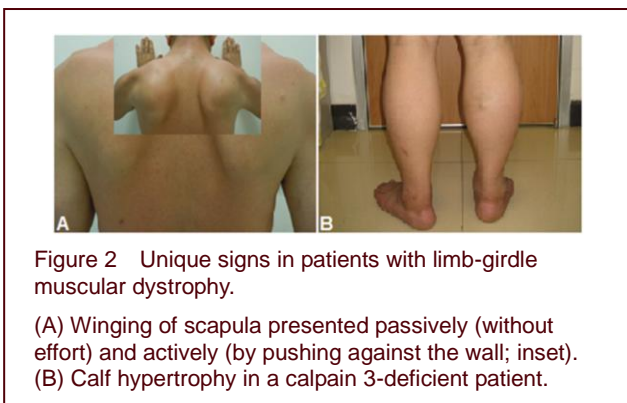


Figure 2 Unique signs in patients with limb-girdle muscular dystrophy. (A) Winging of scapula presented passively (without effort) and actively (by pushing against the wall; inset). (B) Calf hypertrophy in a calpain 3-deficient patient.

Regarding limb-girdle muscular dystrophy type 2D, only two patients showed absence of sarcoglycan- α on blots and in muscles. Mild facial weakness was discerned in the female patient, and the mean creatine kinase level was around 18-fold the upper normal limit.

Two patients showed an absence of caveolin-3 on blots. Both these patients presented with pelvic-muscle weakness, but calf hypertrophy and winging of scapulae were only seen in the female patient. The mean creatine kinase level was around 10 times the upper normal limit.

Pathological features of limb-girdle muscular dystrophy subtypes

Deviations from normal in terms of muscle biopsy results were defined as dystrophy (necrosis, regeneration, opaque fibers and fibro-fatty replacement), myopathy (splitting fibers, internal nuclei and fiber-size variation), neuropathy (pyknotic nuclear clumps, angulated fibers and fiber-type grouping) inflammation (perimysial, endomysial, perivascular and in necrotic fibers), chronicity and disease progression (cytochrome c oxidase-negative fibers, lobulated fibers), activity (more degeneration, regeneration, infiltrating cells), and miscellaneous (rimmed vacuoles, ragged red, ring and moth-eaten fibers) (Figure 3).

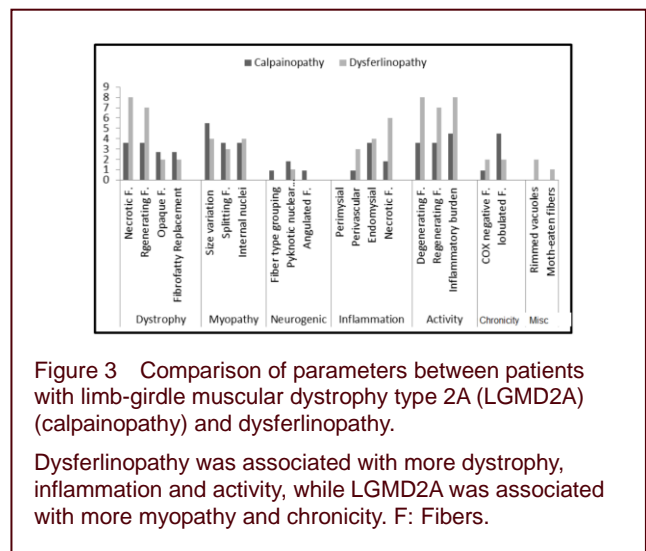


Figure 3 Comparison of parameters between patients with limb-girdle muscular dystrophy type 2A (LGMD2A) (calpainopathy) and dysferlinopathy.

Dysferlinopathy was associated with more dystrophy, inflammation and activity, while LGMD2A was associated with more myopathy and chronicity. F: Fibers.

In limb-girdle muscular dystrophy type 2A biopsies, mild-to-moderate dystrophic changes were noted in all but two biopsies (M479, M833); where muscle fibers were mostly replaced by fibro-fatty tissues. Two others showed clear neurogenic patterns (M548, M817), and the morphological changes were very mild in a further two biopsies (M573, M825). The remaining six patients showed mild-to-moderate myopathic structures. Lobulated fibers were detected in five muscle biopsy sections, and cellular infiltrates were seen in four.

Concerning dysferlinopathies, inflammation and dystrophy were the main findings. We detected cellular infiltrates in eight biopsies, mainly in necrotic fibers (Figure 4). The most common inflammatory infiltrates were macrophages. We used the acid-phosphatase reaction to distinguish between macrophages and other cell types and to determine their numbers and distribution. Seven biopsies displayed macrophages that were frequently encountered in necrotic fibers and the perivascular space, but seldom in the endomysium (Figure 5). Regarding dystrophy, eight patients showed necrotic fibers,

regenerating fibers were identified in seven, while fibro-fatty replacements and hyper-contracted fibers were each visible in two biopsies.

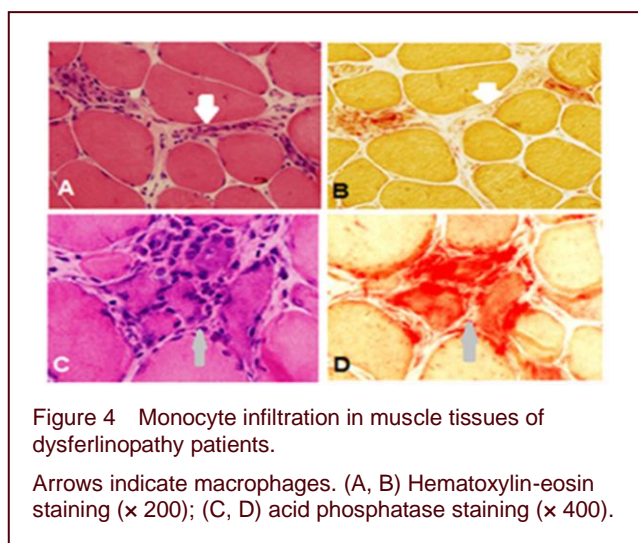


Figure 4 Monocyte infiltration in muscle tissues of dysferlinopathy patients. Arrows indicate macrophages. (A, B) Hematoxylin-eosin staining ($\times 200$); (C, D) acid phosphatase staining ($\times 400$).

Rimmed vacuoles were present in two biopsies (M677, M736), and these patients presented with unexpected generalized and proximal muscle weaknesses, respectively. We detected cytochrome c oxidase-negative fibers in two biopsies (M542, M715). Lobulated fibers were scattered together with cytochrome c oxidase-negative ones in one biopsy (M542), and independently in another (M708). The cases associated with these biopsies were characterized by longer illness durations. Type-I fibers were predominant in two biopsies (M544, M715), and the major histocompatibility complex-I was up-regulated in three biopsies (M677, M736 and M766) (Figure 5).

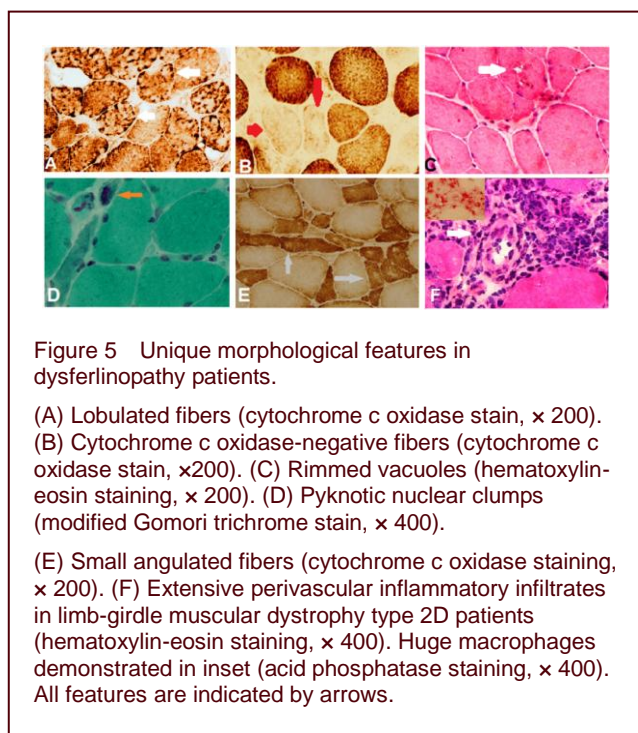


Figure 5 Unique morphological features in dysferlinopathy patients. (A) Lobulated fibers (cytochrome c oxidase stain, $\times 200$). (B) Cytochrome c oxidase-negative fibers (cytochrome c oxidase stain, $\times 200$). (C) Rimmed vacuoles (hematoxylin-eosin staining, $\times 200$). (D) Pyknotic nuclear clumps (modified Gomori trichrome stain, $\times 400$). (E) Small angulated fibers (cytochrome c oxidase staining, $\times 200$). (F) Extensive perivascular inflammatory infiltrates in limb-girdle muscular dystrophy type 2D patients (hematoxylin-eosin staining, $\times 400$). Huge macrophages demonstrated in inset (acid phosphatase staining, $\times 400$). All features are indicated by arrows.

Cellular infiltrates were the main characteristic feature of both limb-girdle muscular dystrophy type 2D biopsies. Most cells were mononuclear cells, with few eosinophils, and mild-to-moderate myopathic structures were also characteristic.

Mild myopathic features with abundant lobulated fibers were apparent in both limb-girdle muscular dystrophy type 1C biopsies.

There was no significant difference between calpainopathy and dysferlinopathy in terms of the different pathological parameters (chi-square test, $P = 0.3$). However, dysferlinopathy biopsies showed nearly twice as many dystrophic, inflammatory structures and a more active progressive course, while calpain 3-deficient patients displayed more myopathic and chronic features (represented by more size variation, splitting fibers and lobulated fibers) (Table 4, Figure 3).

Laboratory results in limb-girdle muscular dystrophy subtypes

Creatine kinase is a pointer of disease activity, and creatine kinase levels were significantly higher in dysferlinopathy patients compared with calpainopathy patients (independent samples t -test: $P = 0.03$). Electromyography showed myopathic damage in all patients, though neurogenic changes were noted in some muscle biopsies. One dysferlinopathy patient showed speckled ST-segment depression and left ventricular hypertrophy on electrocardiography. Serum uric acid levels measured in patients with dysferlinopathy were within normal limits (110–420 $\mu\text{mol/L}$, data not shown).

DISCUSSION

We determined the relative incidences of the different subtypes of limb-girdle muscular dystrophy in a cohort of northeastern Chinese patients. This study enrolled a relatively small number of patients ($n = 68$) over a little more than a 4-year period, from a renowned tertiary center for neuromuscular diseases that drew patients from most of northeastern China.

The relatively small sample size may reflect the low incidence of this disease in China, and suggests that pediatric and adult neuromuscular specialists need to collaborate to increase sample sizes and avoid sampling bias. Unlike other genetic isolates^[25, 38], all patients were of Chinese Han origin, and morphological and biochemical heterogeneities were apparent among the subjects.

Table 4 Morphological features of different forms of limb-girdle muscular dystrophy (LGMD)

	Light microscopy						
	Dystrophy	Myopathy	Inflammation	Activity	Neurogenic	Chronicity	Unique
LGMD1C	0/2	2/2	0/2	0/2	0/2	2/2	0/2
LGMD2A	4/12	6/12	4/12	4/12	2/12	5/12	0/12
LGMD2B/MM	8/10	4/10	8/10	8/10	1/10	2/10	RV = 2/10
LGMD2D	2/2	1/2	2/2	2/2	0/2	0/2	0/2

Number of cases/total number of patients. RV: Rimmed vacuoles.

Immunohistochemistry and immunoblotting

Patients with residual expression of the study proteins on immunohistochemistry and/or immunoblotting were excluded. These techniques were used to confirm the different limb-girdle muscular dystrophy subtypes for the following reasons: complete lack of calpain 3 on western blot was highly predictive (84%) of calpainopathy^[39-40], and the combination of clinical criteria with biochemical analyses increased the probability of a correct diagnosis to 90–100%^[36, 41]. Furthermore, an absence or marked reduction of dysferlin or caveolin-3 is strongly linked to a primary gene defect^[17, 42]. Although several studies have tried to predict the genotyping of sarcoglycanopathy by the immunolabeling pattern of corresponding sarcoglycans^[43-45], Bioto *et al*^[43, 46] recognized the approach to be futile, except in the case of α -sarcoglycan.

Statistics

Limb-girdle muscular dystrophy type 2A was the most common limb-girdle muscular dystrophy subtype in our cohort, with a relative incidence of 17%. This finding was in agreement with data reported for cohorts from other ethnicities, which indicated variable frequencies ranging from 12% in USA, to 50% in Turkey, and 80% in the Basque Country and Russia (founder effect)^[29]. The second most common form of limb-girdle muscular dystrophy in our series was dysferlinopathy, with a relative incidence of about 15%. The frequency of dysferlinopathies in other populations ranged from 5% in Australia, to 12% in Turkey and northeastern Italy, 14% in Brazil, 18% in USA and Italy, and 19% in Japan^[23-25, 28-29, 34, 47]. Sarcoglycanopathies were underestimated in the present study, possibly because of the early onset of this form of the disease, and their consequent under-representation in adult neurology.

Our results are in general agreement with those from Italy, Turkey, Russia, Brazil and Australia in terms of the relative incidences of the various forms of limb-girdle muscular dystrophy. It is therefore accepted that calpainopathy is the most frequent limb-girdle muscular dystrophy subtype worldwide, followed by dysferlinopathy,

whereas limb-girdle muscular dystrophy type 1C represents only 2–3% in most studied cohorts.

Clinical features

The present study reported some clinical variation represented by a wide age range with generally late onset of disease, and male predominance. However, the clinic from which the patients were enrolled only admitted adults with neuromuscular disorders, which could account for these observations. Likewise, the mild symptoms and maintenance of mobility in most patients and possible underestimate of cases based on the robust criteria would support the idea of clinical heterogeneity of the disease^[48]. Moreover, Takahashi and colleagues recently reported similar results in a cohort of patients with late-onset dysferlinopathy from Japan^[67].

Milder phenotypes in women compared with men have been reported previously in several limb-girdle muscular dystrophy phenotypes, including limb-girdle muscular dystrophy type 2G and limb-girdle muscular dystrophy type 2L, implying a possible effect of estrogens^[49]. Based on our current knowledge of this type of dystrophy, the mechanism responsible for the male predominance reported in the current study requires further study. The steroid deflazacort has entered a clinical trial for the treatment of dysferlinopathies. Angelini *et al*^[68] recently demonstrated that sporting activities, usually performed by males, appeared to have some impact on the pathogenesis of dysferlinopathy. The Leyden-Möbius phenotype was the most prevalent amongst patients, which was consistent with a previously published study^[6]. Sporadic cases were predominant, but this fact should not thwart the diagnosis of inherited diseases.

Based on reports from other parts of Asia including Japan, South Korea, Thailand and south India, the Miyoshi distal phenotype was very rare in the present cohort^[50-52], suggesting the existence of an asymptomatic mild Miyoshi form, or the presence of sampling bias.

We also monitored calf hypertrophy in limb-girdle mus-

cular dystrophy type 2A probands, which sign was considered rare in Central China and other countries^[10, 36, 53], apart from Brazil^[54]. In contrast, scapular winging has often been identified in calpainopathies^[41].

Further multicenter studies throughout China are needed to determine if our sample was representative of the Chinese population.

Morphology

Rimmed vacuoles–myopathy represents clinically and genetically disparate diseases. Differential diagnoses encompass inclusion-body myopathy, distal myopathy with rimmed vacuoles (GNE mutation), X-linked Emery-Dreifuss muscular dystrophy, scapulo-peroneal myopathy with cytoplasmic inclusions, and Miyoshi myopathy. To the best of our knowledge, no studies have reported rimmed vacuoles in limb-girdle muscular dystrophy type 2B. Interestingly, however, we encountered two patients with late-onset proximal muscle dystrophies characterized by rimmed vacuoles and dysferlin deficiency.

The dilemma of dysferlinopathy and inflammation has yet to be resolved. Previous studies demonstrated that macrophages were the most frequent cells involved in dysferlin-deficient muscles^[55]. We used acid phosphatase staining instead of anti-macrophage antibodies to quantify and locate macrophages in DYSF mutant muscles. This represents an easy and cost-effective method with comparable specificity to monoclonal antibodies^[56]. Confalonieri *et al*^[57] labeled perivascular cuffing and infiltrating macrophages of necrotic fibers, and the results of our biopsies revealed a similar pattern of dissemination.

Lobulated fibers have typically been described in calpainopathy. We detected lobulated fibers in five limb-girdle muscular dystrophy type 2A patients, two with dysferlinopathy, and both patients with caveolinopathy. These observations confirmed the view that lobulated fibers were relatively non-specific findings, and were indicative of chronic changes perturbing mitochondrial enzymatic activities.

Many studies have found inflammatory infiltrates in various limb-girdle muscular dystrophy subtypes^[58], but inflammatory cells have not previously been reported in adhalin-deficient cases. We identified extensive infiltrates in limb-girdle muscular dystrophy type 2D biopsies, mainly involving macrophages (stained by acid phosphatase) with few eosinophils. Remarkably, the two unrelated patients shared many characteristics, including late disease onset, short illness duration, sporadic

non-familial etiology, no scapular winging, and finally dystrophic inflammation in muscle biopsies.

There were no significant differences in onset age or illness duration between the two groups, but dystrophic and inflammatory constituents were around two-fold more common in dysferlinopathics, whereas myopathy and chronicity were associated with limb-girdle muscular dystrophy type 2A. This finding can be interpreted in light of recent studies. Inflammatory infiltrates were often present in DYSF mutant muscles^[55, 57], and moreover, Hauerlev's team documented that muscle regeneration was affected in calpain 3-deficient patients^[58]. Furthermore, Gallardo *et al*^[3] stated that myopathic changes were the main characteristic of limb-girdle muscular dystrophy type 2A. Lobulated fibers, which reflected disease chronicity, were more frequent in calpainopathy. A recent study by Angelini and coworkers suggested that the limb-girdle muscular dystrophy type 2B disease process was more active compared with limb-girdle muscular dystrophy type 2A^[6].

Biochemistry

Creatine kinase levels were ranked as follows, in decreasing order: limb-girdle muscular dystrophy type 2B > limb-girdle muscular dystrophy type 2D > limb-girdle muscular dystrophy type 1C > limb-girdle muscular dystrophy type 2A, indicating that dysferlinopathies are the most active disease, followed by sarcoglycanopathies. These data are in agreement with those from other geographic areas^[27].

The relationship between defective membrane healing in dysferlin-deficient muscles and immunological attack has recently been verified. It was hypothesized that molecules released from dysferlinopathic muscles may activate the complement system. Uric acid was one the molecules proposed to play a role in initiating the inflammatory cascade^[59]; however, serum uric acid levels in the current study appeared normal, suggesting that other, as yet unknown molecules may trigger the aforementioned pathway.

Novel findings

To the best of our knowledge, only two cohorts of limb-girdle muscular dystrophy patients have so far been independently analyzed in China; one cohort of 15 calpainopathy cases was studied in Shanghai^[10], while another cohort of eight dysferlinopathy patients was studied in Jinan^[37]. The main limitations of both these cohorts were their focus on the clinical and pathological characteristics of a single form of limb-girdle muscular

dystrophy. Despite they estimated frequencies of each form, these studies provided no information on the relative incidences of each subtype. In contrast, we elucidated the relative incidences of each limb-girdle muscular dystrophy subtype, with the exception of sarcoglycanopathies, which were undervalued in the present as a result of the shortage of antibody types and the childhood onset of the disease, which hindered the analysis of the real burden of the disease.

In addition, our study provided a detailed evaluation of the differences between limb-girdle muscular dystrophy type 2A and limb-girdle muscular dystrophy type 2B from a morphological perspective, including some unique findings, and investigated potential mechanisms that might be responsible for the inflammatory cascade in dysferlinopathy.

Future prospects

The diagnosis of some genetic diseases in China remains difficult. Gene sequencing followed by restriction enzyme assay to avoid amplification artifacts is the gold standard, accompanied by screening of a large number of matched healthy individuals of the same ethnicity to prove the pathogenicity of the selected mutations. For example, pathogenic mutations in *CAV3* in the American population turned out to be normal variants in Brazilians^[60]. However, this approach is not applicable on a wide scale, even in the developed world where single-stranded conformation polymorphism analysis or denaturing high-performance liquid chromatography (80–90% sensitivity) are the most commonly used methods for diagnosing limb-girdle muscular dystrophies. Recent studies have identified protein analysis as a comparably efficient, cost-effective, reliable and highly sensitive strategy for the diagnosis of limb-girdle muscular dystrophy. There is currently no effective therapy for limb-girdle muscular dystrophy, though a broad range of potential therapies is undergoing trials, and may offer hope for such patients. These therapies include gene transfer, exon skipping, stop-codon suppression, myostatin inhibition, and calcium-channel blockers^[61-62].

The current study represents the first in China to clarify the relative incidences of the different limb-girdle muscular dystrophy subtypes. In addition, the results provide the first comparison of a wide range of pathological parameters between limb-girdle muscular dystrophy type 2A and limb-girdle muscular dystrophy type 2B. Overall, the results suggest a relatively high incidence of calpainopathy in northeastern China, followed by dysferlinopathy. The present cohort was characterized by a wide

range and generally older age at disease onset, pre-eminent proximal myopathy and high creatine kinase levels, mostly sporadic cases, male preponderance, and preservation of mobility. Acid phosphatase staining should be implemented in patients with suspected dysferlinopathies. Rimmed vacuoles were rarely encountered morphological features, but did not exclude a diagnosis of limb-girdle muscular dystrophy type 2B. Future etiology-based treatment opportunities should emphasize the importance of the well-defined, clinical, proteomic and genetic continuum of limb-girdle muscular dystrophy subtypes.

SUBJECTS AND METHODS

Design

A clinical retrospective cohort study.

Time and setting

Patients were recruited from the Myology Clinic, Department of Neurology, First Hospital of Jilin University, China, from August 2007 to January 2012. The data were analyzed from February 2012 to October 2012.

Subjects

Inclusion criteria

(1) Patients presented clinically with adolescent- or adult-onset weakness. (2) Proximal, with or without distal, limb muscle weakness. (3) Elevated muscle enzymes. (4) Dystrophic and/or myopathic changes in muscle biopsies. (5) Myopathic changes on electromyography. (6) Patients from northeastern China. (7) Data on history, physical examination, laboratory investigations, electrocardiography, electromyography, ultrasound of muscles, muscle biopsy and images of some candidates available for analysis, after written informed consent from patients with limb-girdle muscle weakness.

Exclusion criteria

(1) Patients with onset before the age of 12 years, with delayed motor milestones, or mentally retarded patients (to exclude congenital muscle dystrophy). (2) Muscle biopsy with glycogen or fat deposit ruled out (to exclude metabolic muscle diseases). (3) Abnormal motor/sensory nerve conduction or positive repeated nerve stimulation, electromyography exempted for suspicion of neurogenic disease, spinal muscle atrophy or myotonic dystrophy. (4) Abnormal dystrophin C, N and Rod antibodies on immunocytochemistry (to exclude dystrophinopathy). (5) Patients with mild facial or ocular muscle weakness or normal pelvic musculature were considered as fas-

cio-scapulohumeral dystrophy (except for one case of biochemically confirmed limb-girdle muscular dystrophy type 2D). (6) Skin rash (to exclude patients with polymyositis). Based on these inclusion and exclusion criteria, a total of 68 patients were recruited and subjected to the following investigations^[10].

The protocols were conducted in accordance with the *Administrative Regulations on Medical Institution*, formulated by the State Council of China^[63].

Methods

Muscle biopsies

At the time of analysis, an open biopsy from the biceps brachii or less commonly from the tibialis anterior muscle was obtained under local anesthesia, after obtaining written informed consent. Samples were frozen and stored in liquid nitrogen.

Histochemical staining

Serial frozen sections were stained with hematoxylin and eosin, modified Gomori trichrome, oil red O, nicotinamide adenine dinucleotide tetrazolium reductase, periodic acidic-Schiff, cytochrome c oxidase and acid phosphatase, as described previously^[64].

Sections were evaluated for the following abnormal features: necrosis, regeneration, opaque fibers, fibro-fatty replacement, splitting fibers, internal nuclei, fiber-size variation, pyknotic nuclear clumps, angulated fibers, fiber-type grouping, necrotic, perimysial, endomysial, perivascular inflammatory cells, cytochrome c oxidase-negative fibers, lobulated fibers, rimmed vacuoles, ragged red, ring and moth-eaten fibers.

Immunohistochemical staining

Further serial sections were placed on gelatinized slides and analyzed immunohistochemically using a panel of purified mouse anti-human monoclonal antibodies to the following proteins: dystrophin (1:50; NCL-DYS1, 2, 3; Novocastra Laboratories, Newcastle, UK), α -sarcoglycan (1:100; Vector Laboratories Inc., Burlingame, CA, USA), dysferlin (1:40; NCL-Hamlet; Novocastra Laboratories) and mouse monoclonal antibodies to major histocompatibility complex class I (1:1 000; ABC, Biolegend, San Deigo, CA, USA). Slides were incubated overnight at 4°C. A secondary peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (1:50; KPL, Gaithersburg, MD, USA) was added for 30 minutes at room temperature. The immunohistochemistry technique has been described previously^[65]. Complete deficiency of a specific protein on the muscle-fiber membrane indicated specific muscular

dystrophy-associated membranopathies.

Western blot assay

Muscle biopsy sections were liquefied in treatment buffer (0.05 mol/L dithiothreitol, 0.1 mol/L ethylenediaminetetraacetic acid, 0.125 mol/L Tris, 4% sodium dodecyl sulfate, 0.005% bromophenol blue), boiled for 5 minutes, and centrifuged at 20 000 $\times g$ for 15 minutes at 4°C. Proteins were resolved by 8–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to Hybond polyvinylidene fluoride membranes (Amersham Bioscience, NJ, USA) for 1 hour at 20°C. Blots were blocked with bovine serum albumin (3–5%) with shaking for 30 minutes at 20°C and then incubated with purified mouse anti-human caveolin-3 monoclonal antibody (1:1 000), (BD Transduction Lab., Franklin Lakes, NJ, USA), purified mouse anti-human calpain-3 monoclonal antibody (1:200; Calp3d/2C4), purified goat anti-dysferlin polyclonal antibody (1:50), purified rabbit anti-sarcoglycan- α polyclonal antibody (1:250). Purified rabbit anti-heavy chain myosin polyclonal antibody (1:250) was used to standardize the amount of protein loaded in each lane. Incubation with primary antibodies was performed overnight at 4°C. Calpain 3, dysferlin, sarcoglycan- α and heavy-chain myosin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Immunoreactive bands were visualized using Cy3-conjugated AffiniPure goat anti-mouse, goat anti-rabbit or donkey anti-goat IgG (1:1 000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 2 hours at 25°C with shaking, and using a Typhoon fluorescence scanner (Amersham Bioscience)^[66]. Image J software (freely available at <http://rsb.info.nih.gov/ij/>) was used to detect the deficiency of the protein of interest compared with control (muscle biopsies with metabolic or neurogenic muscular disorders).

Statistical analysis

Myopathy and specific clinical features were compared using chi-square and independent samples Student's *t*-tests, respectively between the commonest forms of limb-girdle muscular dystrophy (calpainopathy and dysferlinopathy). A *P* value of ≤ 0.05 was considered significant. Data analysis was performed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA).

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