

Clinical and Pathological Characteristics of Four Korean Patients with Limb-Girdle Muscular Dystrophy type 2B

Limb-girdle muscular dystrophy type 2B (LGMD2B), a subtype of autosomal recessive limb-girdle muscular dystrophy (ARLGMD), is characterized by a relatively late onset and slow progressive course. LGMD2B is known to be caused by the loss of the dysferlin protein at sarcolemma in muscle fibers. In this study, the clinical and pathological characteristics of Korean LGMD2B patients were investigated. Seventeen patients with ARLGMD underwent muscle biopsy and the histochemical examination was performed. For the immunocytochemistry, a set of antibodies against dystrophin, α , β , γ , δ -sarcoglycans, dysferlin, caveolin-3, and β -dystroglycan was used. Four patients (24%) showed selective loss of immunoreactivity against dysferlin at the sarcolemma on the muscle specimens. Therefore, they were classified into the LGMD2B category. The age at the onset of disease ranged from 9 yr to 33 yr, and none of the patients was wheelchair bound at the neurological examination. The serum creatine kinase (CK) was high in all the patients (4010-5310 IU/L). The pathologic examination showed mild to moderate dystrophic features. These are the first Korean LGMD2B cases with a dysferlin deficiency confirmed by immunocytochemistry. The clinical, pathological, and immunocytochemical findings of the patients with LGMD2B in this study were in accordance with those of other previous reports.

Key Words : Muscular Dystrophies; Dysferlin; Immunocytochemistry

Seung-Hun Oh^{*,†}, Seong-Woong Kang[†],
Jin-Goo Lee^{*}, Sang-Jun Na^{*},
Tai-Seung Kim^{*}, Young-Chul Choi^{*}

Department of Neurology* and Rehabilitation Medicine[†],
Yongdong Severance Hospital, Seoul; Department of
Medicine, Euijong-bu Collectional Institution[‡],
Euijong-bu; Department of Pathology[§], Severance
Hospital, Brain Korea 21 Project for Medicine, Yonsei
University College of Medicine, Seoul, Korea

Received : 18 December 2003

Accepted : 2 March 2004

Address for correspondence

Young-Chul Choi, M.D.
Department of Neurology, Yongdong Severance
Hospital, 146-92 Dogok-dong, Kangnam-gu,
Seoul 135-270, Korea
Tel : +82.2-3497-3323, Fax : +82.2-3462-5904
E-mail : ychoi@yumc.yonsei.ac.kr

INTRODUCTION

Autosomal recessive limb-girdle muscular dystrophy (ARLGMD) is a clinically and genetically heterogeneous disorder affecting mainly the pelvic and shoulder girdle muscles. According to the genetic classification of ARLGMD, it has been divided into ten subtypes, from LGMD type 2A to LGMD type 2J (1). LGMD type 2B (LGMD2B) is caused by mutations in the dysferlin gene (*DYSF*, MIM*603009) located on chromosome 2p13.3, which induces a dysfunction of dysferlin at the protein level (2). Dysferlin is a member of the FER-1 protein family and contains six putative C2 domains, which can bind to phospholipids, inositol polyphosphates, Ca²⁺ and intracellular proteins (3). Dysferlin is expressed predominantly in the skeletal muscle and localizes to the plasma membrane of the muscle fibers. In addition, it has been suggested to be involved in membrane fusion (4, 5). A recent animal study has suggested that dysferlin plays a role in the sarcolemma repair process (6). The *DYSF* has been also shown to cause Miyoshi myopathy (MM), which is a rare form of distal myopathy with the calf muscles weakness. Identical mutations in patients with LGMD2B or Miyoshi myopathy have been noted in several reports, suggesting a role for the modifier gene(s) (7-11). Both LGMD2B and Miyoshi myopa-

thy are referred to as "dysferlinopathy" because of the deficiency of the same protein.

Because of the clinical and genetic heterogeneity of the LGMD, the LGMD subtypes are classified based on genetic or protein analysis. The development of antibodies directed against dysferlin has recently been shown to be useful for making a diagnosis of LGMD2B by an examination of a muscle biopsy with immunostaining or immunoblotting techniques (4, 5, 10). In the current study, immunocytochemical examination for dysferlin was tested on a muscle biopsies from 17 Korean ARLGMD patients, and the clinical and pathological characteristics of four Korean LGMD2B patients, who showed the complete loss of the dysferlin protein on a muscle biopsy, were investigated.

MATERIALS AND METHODS

Patients Selection and Muscle biopsy

From March 2001 to November 2002, 17 unrelated, non-consanguineous LGMD patients were enrolled in this study. The diagnosis of LGMD was established by the clinical history and physical examination, the family history, a normal

nerve conduction study and the myopathic pattern on electromyography (12). All the patients underwent an open biopsy from the quadriceps femoris or biceps brachii muscle under local anesthesia. In all the cases, informed consents were obtained from the patients or their parents. Transverse serial frozen muscle sections (7 μ m thickness) were stained with hematoxylin and eosin (H&E), modified Gomori trichrome, and a battery of histochemical techniques (NADH-TR, ATPase pH 9.4/4.6/4.3) were applied. All of them showed normal immunostaining for three domains of dystrophin (N-terminus, C-terminus and Rod-domain) on the muscle specimens, and the deletion was not found on multiplex PCR for selected exons of dystrophin gene.

Immunocytochemical staining of muscle specimens

The tissues were processed for immunocytochemistry as follows: the 7 μ m serial sections were fixed in acetone at 4°C for 10 min, rinsed in 0.05 mol/L Tris-buffered saline (pH 7.5) for 15 min, and incubated for 30 min with a blocking solution containing 2% bovine serum albumin and 5% normal goat serum as described (13). The sections were then

incubated overnight at 4°C with one of the following antibodies: α -sarcoglycan (NCL- α -SARC, Novocastra), β -sarcoglycan (NCL- β -SARC, Novocastra), γ -sarcoglycan (NCL- γ -SARC, Novocastra), δ -sarcoglycan (NCL- δ -SARC, Novocastra), dysferlin (NCL-DYSE, Novocastra), β -dystroglycan (NCL- β -DG, Novocastra, Newcastle Upon Tyne, U.K.), and caveolin-3 (Transduction laboratory, Lexington, KY, U.S.A.). These affinity-purified antibodies were well characterized and did not cross-react with each other. After washing for 30 min in Tris-buffered saline, the sections were examined using secondary goat anti-mouse IgG antibodies conjugated to peroxidase and visualized by a DAB (diaminobenzidine)-peroxidase reaction (Vector Laboratories, CA., U.S.A.). The sections were also incubated with biotinylated goat anti-mouse IgG, which was followed by fluorescent isothiocyanate (FITC) avidin D (Vector Laboratory, CA., U.S.A.) for dysferlin.

RESULTS

Four (Patient No. 1-4) of the 17 ARLGMD patients group (24%) showed a selective loss of dysferlin by both DAB and

Table 1. The Immunocytochemical findings of the 17 ARLGMD patients

Patient No.	Sex/Age (yr)	DPH (DAB)	α -SG (DAB)	β -SG (DAB)	γ -SG (DAB)	δ -SG (DAB)	Dysf (DAB)	Dysf (FITC)	Cav3 (DAB)	β -DG (DAB)
1	F/35	+	+	+	+	+	-	-	+	+
2	F/41	+	+	+	+	+	-	-	+	+
3	F/43	+	+	+	+	+	-	-	+	+
4	M/15	+	+	+	+	+	-	-	+	+
5	F/21	+	+	+	+	+/-	+	+	+	+
6	M/40	+	+	+	+	+	+	+	+	+
7	M/13	+	+	+	+	+/-	+	+	+	+
8	F/25	+	+	+	+	-	+/-	+	+	+
9	M/5	+	+	+/-	+	-	+	+	+	+
10	M/7	+	+	+/-	+	-	+	+	+	+
11	M/17	+	+	+	+	+	+	+/-	+	+
12	M/10	+	+	+	+	+	+	+	+	+
13	M/5	+	+	+	+	+	+	+	+	+
14	F/10	+	+	+	+	+	+	+/-	+	+
15	F/9	+	+	-	+/-	-	+	+/-	+	+
16	M/9	+	+	+	+	+/-	+	+	+	+
17	M/23	+	+	+	+	+	+/-	+	+	+

DAB, diaminobenzidine; FITC, fluorescent isothiocyanate; DPH, dystrophin; α -SG, α -sarcoglycan; β -SG, β -sarcoglycan; γ -SG, γ -sarcoglycan; δ -SG, δ -sarcoglycan; Dysf, dysferlin; Cav3, caveolin-3; β -DG, β -dystroglycan; +/-, decreased or weakly positive.

Table 2. The clinical and laboratory characteristics of the four LGMD2B patients

Patient No.	Sex/Age (yr)	Age at onset (yr)	Distribution of muscle weakness						Cardiac/pulmonary insufficiency	Bony abnormality	Calf muscle hypertrophy	Serum CK level (IU/L)	
			Face	Neck	U/Ex		L/Ex						
					prox	dist	prox	dist					
1	F/35	27	-	-	4	4+	4	4+	-	-	+	(symmetric)	5310
2	F/41	26	-	-	4	4+	4-	4	-	-	-	-	4010
3	F/43	33	-	-	4+	5	4	4+	-	-	-	-	4460
4	M/15	9	-	-	4-	4+	4-	4	-	-	+	(symmetric)	4689

U/Ex, upper extremity; L/Ex, lower extremity; prox, proximal; dist, distal; CK, creatine kinase.

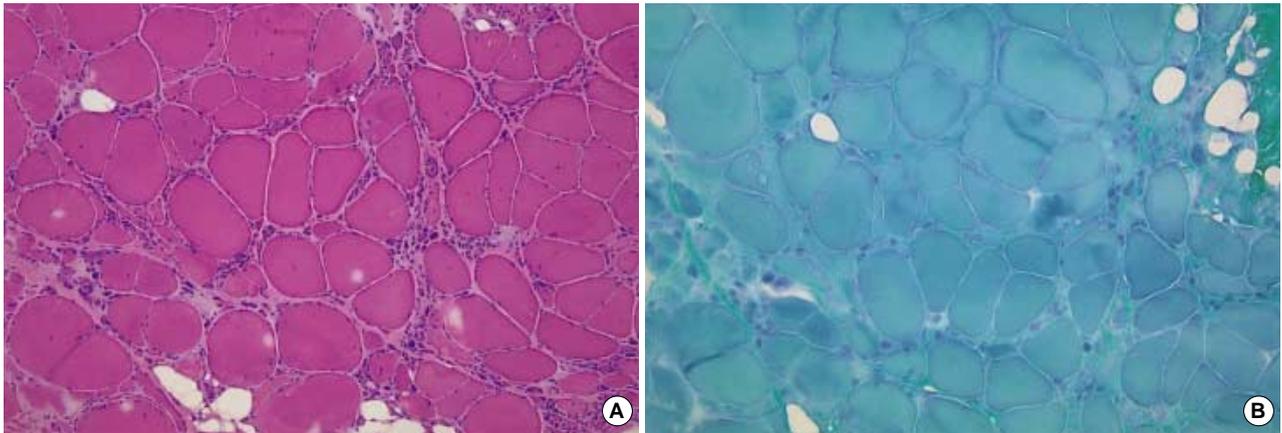


Fig. 1. Muscle specimens of the quadriceps femoris muscle (from patient No. 1) stained by hematoxylin-eosin (A) and modified Gomori Trichrome (B). There were great variation in the size of fibers, fiber splitting with internal nuclei, and clumps of small fibers ($\times 200$).

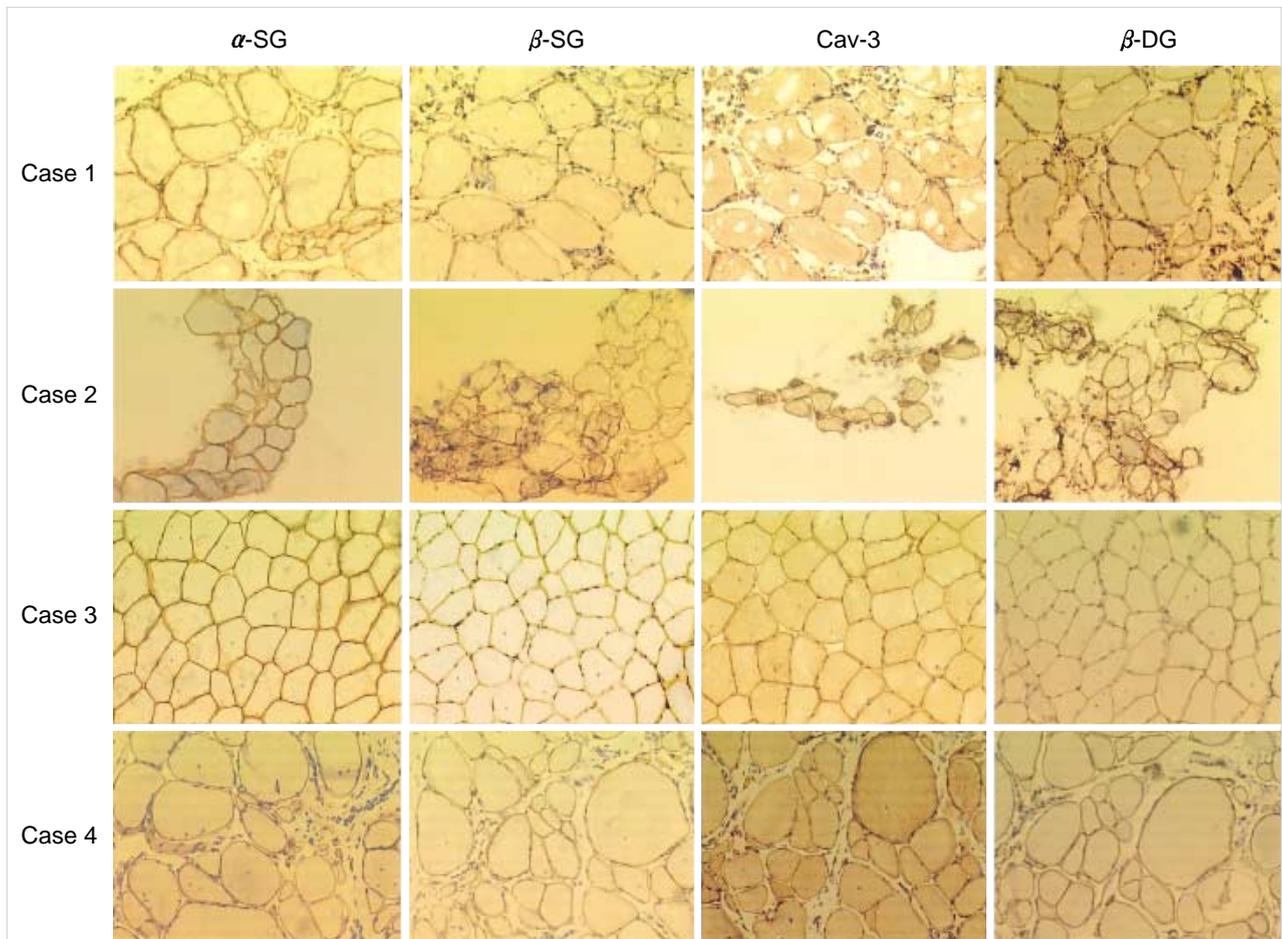


Fig. 2. Immunostaining against α -sarcoglycan (α -SG), β -sarcoglycan (β -SG), caveolin-3 (Cav-3), and β -dystroglycan (β -DG) on the muscle specimen in the four LGMD2B patients by the diaminobenzidine (DAB)-peroxidase method. All four LGMD2B patients showed a normal immunoreactivity against α -sarcoglycan, β -sarcoglycan, caveolin-3, and β -dystroglycan ($\times 200$).

FITC methods on the muscle specimen (Table 1). Therefore, they were classified as having LGMD type 2B (LGMD2B). Although three patients (patient No. 8-10) showed isolated

deficiency of δ -sarcoglycan, it was regarded as a technical error since the δ -sarcoglycanopathy had always showed combined deficiency of other sarcoglycans (α , β , and γ) at exam-

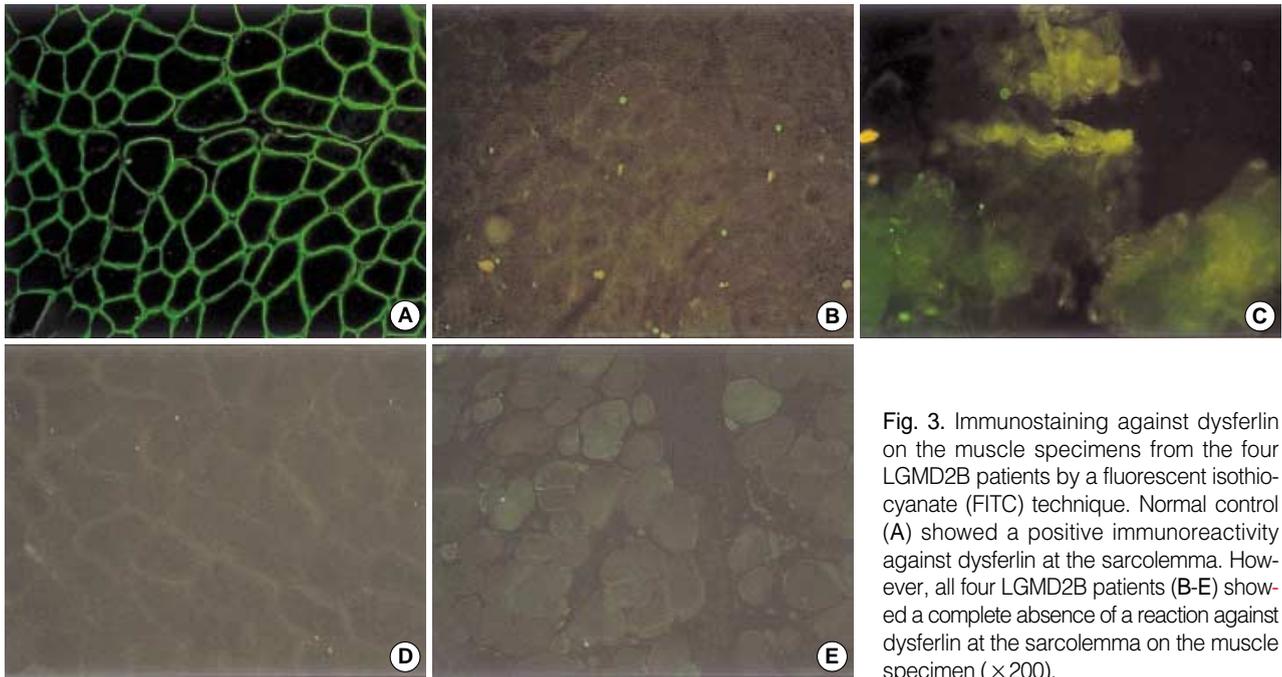


Fig. 3. Immunostaining against dysferlin on the muscle specimens from the four LGMD2B patients by a fluorescent isothiocyanate (FITC) technique. Normal control (A) showed a positive immunoreactivity against dysferlin at the sarcolemma. However, all four LGMD2B patients (B-E) showed a complete absence of a reaction against dysferlin at the sarcolemma on the muscle specimen ($\times 200$).

ination by immunocytochemistry (14). One patient (patient No. 15) showed loss of β and δ -sarcoglycan with partial deficiency of γ -sarcoglycan. This patient might be tentatively classified as β or δ -sarcoglycanopathy although molecular genetic diagnosis was not performed.

Clinical characteristics and course of disease in four patients with LGMD2B

The age of patients at the onset of the disease varied from 9 yr to 33 yr, and the disease duration ranged from 6 yr to 15 yr (Table 2). All four patients showed symmetrical proximal weakness. Although all the patients showed a functional disability in walking or running, none was wheelchair-bound at the examination. While symmetrical hypertrophy of the calf muscle was noted in two patients (patient No. 1 & 4), muscle atrophy was not found in any of the four patients. None of the four patients had a cardiac or respiratory insufficiency nor did they have any bony abnormalities. The serum creatine kinase (CK) level was elevated to approximately 20 times of the normal level in all the patients. (4010-5310 IU/L). One patient (patient No. 1) had a positive family history. One of her elder brother (41 yr old) had similar symptoms, which presented as a slowly progressive proximal weakness over 15 yr.

Pathological and immunocytochemical findings of muscle biopsy in four patients with LGMD2B

All the patients showed the following non-specific myopathic changes in the muscle: increased internal nuclei, a few

or many atrophic fibers, degenerating fibers with regenerating fibers, and an increased fiber size variation (Fig. 1). Type 1 fiber predominance ($>60\%$) was noted in one patient (patient No. 1). There was no endomysial or perivascular inflammatory cell infiltrations in all the patients.

In all the patients with LGMD2B, immunocytochemical reaction against all the subtypes of sarcoglycans, β -dystroglycan, and caveolin-3 was normal (Fig. 2, Table 1), while the reaction against dysferlin showed complete loss of reactivity at the sarcolemma of the muscle fibers (Fig. 3).

DISCUSSION

The clinical features of our patients with LGMD2B was similar to those of previous reports (12, 15-17). Symptoms of the disease generally are manifested during the early adulthood (average onset is noted between the age of 17-30 yr), and the disease generally has a benign course, and confinement to a wheelchair may occur in approximately 10% of patients (15, 18). Despite of the slow clinical evolution, extremely high CK level (10-20 times of normal value) was characteristic of LGMD2B, being indicative of protein's role in normal muscle homeostasis ("leaky" membrane) rather than being essential for structural stabilization of the skeletal muscle (6, 19). A few LGMD2B patients showed symmetrical or asymmetrical calf muscle hypertrophy, as was observed in two of the patients in this study. Up to date, only several estimates of the frequency of dysferlinopathy exist. The proportion of LGMD2B is thought to be relatively high in Brazilian and Japanese population (estimated to be 19-25% of all

ARLGMD cases) (15, 20) and there is founder mutation of *DYSF* appears to be present in the Libyan Jews (17). However, another recent study reported a relatively lower frequency of the dysferlin deficiency in Caucasian patients with the LGMD phenotype (approx. 1%), indicating a frequency variation according to the ethnicity (21). Although it is not enough to estimate the frequency of LGMD2B in the Korean ARLGMD patients due to a small number of subjects, these results suggest that the frequency of LGMD2B is relatively high among ARLGMD in Korea. A detailed population-based study will be needed to determine the exact frequency of the LGMD2B in Korea.

In all the cases with dysferlin deficiency, the immunocytochemical study showed normal reactivity against dystrophin, α -, β -, γ -, δ -sarcoglycans, β -dystroglycan on muscle specimen, which suggests that there are no interactions between dysferlin and the dystrophin-glycoprotein complex (DGC). This finding is in accordance with previous studies (22, 23). The *DYSF* is large, comprising 55 exons that span the genomic region of >150 kb (24). Although direct gene analysis provides the most reliable diagnosis, it is costly, time-consuming, and labor intensive because of the large size of the *DYSF*. Moreover, defects in the dysferlin gene involve mostly single nucleotide changes with no common mutations, gross rearrangements, or mutational hotspots that could aid detection (2). For this reason, it would be better to initiate screening for the dysferlin deficiency using antibodies against this protein in muscle biopsies. Complete loss of dysferlin without deficiency of other proteins appears to be specific for primary dysferlinopathy on immunocytochemistry (24). However, it should be noted that some cases with sarcoglycanopathy, caveolinopathy can show partial deficiency of dysferlin (secondary deficiency) (25, 26). Therefore, it is recommendable to perform immunocytochemical analysis using antibodies against other sarcolemmal proteins in addition to the antibodies against dysferlin. When partial deficiency of dysferlin is present on immunocytochemistry, direct sequencing of *DYSF* will be needed.

In conclusion, the clinical, pathological, and immunocytochemical findings of patients with LGMD2B in this study were in accordance with those of previous reports.

ACKNOWLEDGMENTS

This work was supported by the Brain Korea 21 Project for Medical Science, Yonsei University.

REFERENCES

- Zatz M, de Paula F, Starling A, Vainzof M. *The 10 autosomal recessive limb-girdle muscular dystrophies*. *Neuromuscul Disord* 2003; 13: 532-44.
- Liu J, Aoki M, Illa I, Wu C, Fardeau M, Angelini C, Serrano C, Urtizberea JA, Hentati F, Hamida MB, Bohlega S, Culper EJ, Amato AA, Bossie K, Oeltjen J, Bejaoui K, McKenna-Yasek D, Hosler BA, Schurr E, Arahata K, de Jong PJ, Brown RH Jr. *Dysferlin, a novel skeletal muscle genes, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy*. *Nat Genet* 1998; 20: 31-6.
- Britton S, Freeman T, Vafiadaki E, Keers S, Harrison R, Bushby K, Bashir R. *The third human FER-1-like protein is highly similar to dysferlin*. *Genomics* 2000; 68: 313-21.
- Matsuda C, Aoki M, Hayashi YK, Ho MF, Arahata K, Brown RH Jr. *Dysferlin is a surface membrane-associated protein that is absent in Miyoshi myopathy*. *Neurology* 1999; 53: 1119-22.
- Anderson LV, Davison K, Moss JA, Young C, Cullen MJ, Walsh J, Johnson MA, Bashir R, Britton S, Keers S, Argov Z, Mahjneh I, Fougerousse F, Beckmann JS, Bushby KM. *Dysferlin is a plasma membrane protein and is expressed early in human development*. *Hum Mol Genet* 1999; 8: 855-61.
- Bansal D, Miyake K, Vogel SS, Groh S, Chen CC, Williamson R, McNeil PL, Campbell KP. *Defective membrane repair in dysferlin-deficient muscular dystrophy*. *Nature* 2003; 423: 168-72.
- Weiler T, Greenberg CR, Nylen E, Halliday W, Morgan K, Eggertson D, Wrogemann K. *Limb-girdle muscular dystrophy and Miyoshi myopathy in an aboriginal Canadian kindred map to LGMD2B and segregate with the same haplotype*. *Am J Hum Genet* 1996; 59: 872-8.
- Illarioshkin SN, Ivanova-Smolenskaya IA, Tanaka H, Vereshchagin NV, Markova ED, Poleshchuk VV, Lozhnikova SM, Sukhorukov VS, Limborska SA, Slominsky PA, Bulayeva KB, Tsuji S. *Clinical and molecular analysis of a large family with three distinct phenotypes of progressive muscular dystrophy*. *Brain* 1996; 119: 1895-909.
- Illarioshkin SN, Ivanova-Smolenskaya IA, Greenberg CR, Nylen E, Sukhorukov VS, Poleshchuk VV, Markova ED, Wrogemann K. *Identical dysferlin mutation in limb-girdle muscular dystrophy type 2B and distal myopathy*. *Neurology* 2000; 55: 1931-3.
- Weiler T, Bashir R, Anderson LV, Davison K, Moss JA, Britton S, Nylen E, Keers S, Vafiadaki E, Greenberg CR, Bushby CR, Wrogemann K. *Identical mutation in patients with limb girdle muscular dystrophy type 2B or Miyoshi myopathy suggests a role for modifier gene(s)*. *Hum Mol Genet* 1999; 8: 871-7.
- Linssen WH, Notermans NC, Van der Graaf Y, Wokke JH, Van Doorn PA, Howeler CJ, Busch HF, De Jager AE, De Visser M. *Miyoshi-type distal muscular dystrophy. Clinical spectrum in 24 Dutch patients*. *Brain* 1997; 120: 1989-96.
- Bushby KM. *Making sense of the limb-girdle muscular dystrophies*. *Brain* 1999; 122: 1403-20.
- Choi YC, Park GT, Kim TS, Sunwoo IN, Steinert PM, Kim SY. *Sporadic inclusion body myositis correlates with increased expression and cross-linking by transglutaminases 1 and 2*. *J Biol Chem* 2000; 275: 8703-10.
- Bushby KM. *The limb-girdle muscular dystrophies-multiple genes, multiple mechanisms*. *Hum Mol Genet* 1999; 8: 1875-82.
- Passos-Bueno MR, Vainzof M, Moreira ES, Zatz M. *Seven autosomal recessive limb-girdle muscular dystrophies in the Brazilian population: from LGMD2A to LGMD2G*. *Am J Med Genet* 1999; 82: 392-8.

16. Ueyama H, Kumamoto T, Horinouchi H, Fujimoto S, Aono H, Tsuda T. *Clinical heterogeneity in dysferlinopathy. Intern Med* 2002; 41: 532-6.
17. Argov Z, Sadeh M, Mazor K, Soffer D, Kahana E, Eisenberg I, Mit-rani-Rosenbaum S, Richard I, Beckmann J, Keers S, Bashir R, Bush-by K, Rosenmann H. *Muscular dystrophy due to dysferlin deficien-cy in Libyan Jews. Clinical and genetic features. Brain* 2000; 123: 1229-37.
18. Zatz M, Vainzof M, Passos-Bueno MR. *Limb-girdle muscular dys-trophy: one gene with different phenotypes, one phenotype with dif-ferent genes. Curr Opin Neurol* 2000; 13: 511-7.
19. Lennon NJ, Kho A, Bacskai BJ, Perlmutter SL, Hyman BT, Brown RH Jr. *Dysferlin interacts with annexins A1 and A2 and mediates sarcolemmal wound-healing. J Biol Chem* 2003; 278: 50466-73.
20. Tagawa K, Ogawa M, Kawabe K, Yamanaka G, Matsumura T, Goto K, Nonaka I, Nishino I, Hayashi YK. *Protein and gene analyses of dysferlinopathy in a large group of Japanese muscular dystrophy patients. J Neurol Sci* 2003; 211: 23-8.
21. Fanin M, Pegoraro E, Matsuda-Asada C, Brown RH Jr, Angelini C. *Calpain-3 and dysferlin protein screening in patients with limb-gir-dle dystrophy and myopathy. Neurology* 2001; 56: 660-5.
22. Vainzof M, Anderson LV, McNally EM, Davis DB, Faulkner G, Valle G, Moreira ES, Pavanello RC, Passos-Bueno MR, Zatz M. *Dysferlin protein analysis in limb-girdle muscular dystrophies. J Mol Neurosci* 2001; 17: 71-80.
23. Anderson LV, Harrison RM, Pogue R, Vafiadaki E, Pollitt C, Davi-son K, Moss JA, Keers S, Pyle A, Shaw PJ, Mahjneh I, Argov Z, Greenberg CR, Wrogemann K, Bertorini T, Goebe HH, Beckmann JS, Bashir R, Bushby KM. *Secondary reduction in calpain 3 expres-sion in patients with limb girdle muscular dystrophy type 2B and Miyoshi myopathy (primary dysferlinopathies). Neuromuscul Dis-ord* 2000; 10: 553-9.
24. Aoki M, Liu J, Richard I, Bashir R, Britton S, Keers SM, Oeltjen J, Brown HE, Marchand S, Bourg N, Beley C, McKenna-Yasek D, Arahata K, Bohlega S, Cupler E, Illa I, Majneh I, Barohn RJ, Urtiz-berea JA, Fardeau M, Amato A, Angelini C, Bushby K, Beckmann JS, Brown RH Jr. *Genomic organization of the dysferlin gene and novel mutations in Miyoshi myopathy. Neurology* 2001; 57: 271-8.
25. Piccolo F, Moore SA, Ford GC, Campbell KP. *Intracellular accu-mulation and reduced sarcolemmal expression of dysferlin in limb-girdle muscular dystrophies. Ann Neurol* 2000; 48: 902-12.
26. Matsuda C, Hayashi YK, Ogawa M, Aoki M, Murayama K, Nishi-no I, Nonaka I, Arahata K, Brown RH Jr. *The sarcolemmal proteins dysferlin and caveolin-3 interact in skeletal muscle. Hum Mol Genet* 2001; 10: 1761-6.