A Severe Case of Duchenne-Like Muscular Dystrophy Due to a Mutation in the α-Sarcoglycan (Adhalin) Gene

Marina Fanin, Francesco Martinello, David J. Duggan[1], J. Raphael Gorospe[1], Maria Pia Freda, Elena Pegoraro[1], Gianni Sorarù, Maria Luisa Mostacciuolo[2], Carlo P. Trevisan, Eric P. Hoffman[1] and Corrado Angelini

Neuromuscular Center, Department of Neurology and (2) Biology, University of Padova, Italy and (1) Department of Molecular Genetics and Biochemistry, University of Pittsburgh, USA

Abstract

We report the case of an 11-year-old boy affected with a childhood onset muscular dystrophy who was wheelchair-bound at the age of 10 years. The patient recently developed a mild cardiomyopathy consisting of reduced left ventricular ejection fraction. A first degree cousin of the patient (born from consanguineous parents) was also affected, indicating an autosomal recessive pattern of inheritance. To identify the molecular defect responsible for the disease, we tested the muscle biopsy for dystrophin and other components of dystrophin-glycoprotein complex (DGC). Immunohistochemical and Western blot analyses revealed a total defect of adhalin (α-sarcoglycan), associated with a secondary reduction of dystrophin amount; β-dystroglycan (43 kDa dystrophin-associated glycoprotein) and merosin (laminin alpha2) were normally expressed. Biochemical adhalin deficiency may be primary or secondary to mutations in one of the different genes for the sarcoglycan complex. The identification of a novel missense mutation (L158F) in the α-sarcoglycan gene confirmed the diagnosis of autosomal muscular dystrophy (primary adhalinopathy). Cardiac involvement has been noted in only one other case of primary adhalinopathy, and subclinical involvement has been proposed in all cases given the sarcoglycan complex is present in both skeletal and cardiac muscle [6]. Patients with adhalinopathy require careful monitoring so as to avoid unforeseen cardiac conditions.

Key words: autosomal muscular dystrophy, SCARMD, adhalin, sarcoglycan, missense mutation.

BAM 6 (2): 95-100, 1996

The dystrophin-glycoprotein complex (DGC) of the skeletal muscle membrane [8] consists of dystrophin and dystrophin-associated complexes, i.e. dystroglycan and sarcoglycan complexes [25]. The dystroglycan complex is composed of α- and β-dystroglycan (156 kDa and 43 kDa/A3a, respectively) derived from a single precursor protein encoded by a gene mapped on chromosome 3. The sarcoglycan complex is composed of 3 subunits: α- (50 kDa/A2, adhalin), β- (43 kDa/A3b) and γ-sarcoglycan (35 kDa/A4), encoded by genes located on chromosomes 17, 4, 13, respectively [17]. In contrast to dystroglycan, which is expressed in wide variety of tissues, sarcoglycan is expressed only in striated muscles [24]. In the skeletal muscle membrane of patients with severe childhood autosomal recessive muscular dystrophy (SCARMD), adhalin and the other sarcoglycan components are either absent or severely deficient [10, 11, 13, 15]. This observation suggests that the development of the disease, whatever the primary cause of SCARMD, is the destabilization of the DGC due to the lack of sarcoglycan complex [14, 16]. Since adhalin cDNA have been sequenced [21], a number of different gene mutations have been described in primary adhalinopathy [6, 12, 19, 21]. Adhalin deficient patients show a spectrum of clinical severity ranging from severe DMD-like dystrophy to a phenotype similar to later onset limb-girdle muscular dystrophy [6, 12, 22, 23, 26]. These patients might be misdiagnosed unless tested for both dystrophin and adhalin.

Here we report the case of a patient with primary adhalin deficiency, showing a severe muscular dystrophy associated with cardiac involvement and evidence of autosomal recessive inheritance.

Case report

Since the patient has an unknown father, no data on the paternal line are available. A first degree maternal cousin (born from first degree cousins) who also suffered from progressive muscular dystrophy, became wheelchair-bound in childhood and died at 10 years of age. The onset of symptoms in our patient was at 18 months, when he started walking on tiptoes. He than developed a rapid progressive weakness of proximal muscles. At the age of 5 years he was unable to run and had difficulty in climbing stairs. Serum creatine kinase (CK) was 11,000 U/l (n.v. 0-195). On neurological examination he had waddling gait, calf hypertrophy, positive Gower's sign, diffuse muscle hypotonia and hypotrophy. Despite administration of steroid drug treatment and physiotherapy, the patient lost the abilities to raise from the floor, to climb stairs and to walk independently at 9, 9.5 and 10 years, respectively. Since the age of 10 years he was unable to stand unassisted and to lift arms over the head. The boy is currently 11 year-old, with pronounced proximal muscle weakness, marked joint contractures, he requires help performing daily light activity, including eating and rolling in bed. Electrocardiography revealed incomplete right bundle branch block. Echocardiography showed a mild reduction of left ventricular ejection fraction (55%). Spirometry showed mild restrictive respiratory insufficiency. He has no evidence of mental retardation.

Material and Methods

Immunostaining of muscle biopsy cryosections

Muscle biopsy was obtained from quadriceps muscle after written informed consent from patient's mother. Muscle specimens were frozen in isopentane cooled in liquid nitrogen and stored at -70°C until processed. Unfixed serial cryostat sections 6 µm thick were placed on gelatin-coated slides and incubated overnight at room temperature with the following monoclonal antibodies: dystrophin C-terminal (Dys-2, Novocastra, 1:200), 43 kDa DAG (β-dystroglycan, 1:100), adhalin ($IVD3_1$, 1:20), merosin (laminin α2 chain, Chemicon, 1:2000), β-spectrin (Spec-2, Novocastra, 1:50). Antibodies were diluted in phosphate buffered saline (PBS). Indirect immunofluorescence visualization was obtained by avidin-biotin system: a secondary anti-mouse biotinylated antibody (Amersham, 1:250) was incubated for 30' and was followed by streptavidin-Texas Red (Amersham, 1:100) for 15'. Sections were examined with a Zeiss Axioskop fluorescence photomicroscope.

Dystrophin and adhalin immunoblot analysis on muscle biopsy

Muscle biopsy specimen was serial cryostat cut: about 20 sections were used for the measurement of total non-collagen protein amount by Lowry method and additional 20 sections were dissolved in 100 µl of loading buffer (10% SDS, 50 mM DTT, 10 mM EDTA, 0.1 M TRIS, 0.001% bromophenol blue, at pH = 8). Equal amounts of muscle proteins from patient's and control's muscles were loaded in adjacent lanes of 3.5-15% gradient-discontinuous SDS-PAGE gels, to determine both protein relative abundance and molecular weight. Electrophoresis was performed at 120 V overnight. Proteins were blotted for 5 hours at 1980 mA on nitrocellulose sheet. Filters were air dried, saturated with 5% BSA in TTBS and incubated overnight with a mixture of monoclonal antibodies against dystrophin C-terminus (Novocastra, 1:1000 in TTBS) and adhalin (Novocastra, 1:300). The reaction was developed using a secondary anti-mouse biotinylated antibody (Amersham, 1:1000) for 1 hour, streptavidin-biotinylated horseradish peroxidase complex (Amersham, 1:1000) for 1 hour and exposed on Kodak X-OMAT films by enhanced chemioluminescence method (ECL-Amersham). Dystrophin and adhalin relative abundance, expressed as percentage of normal control, were determined by densitometric analysis of protein bands on the same film and by comparison with myosin heavy chain relative amount in the post-transfer Coomassie blue stained gels.

Identification of adhalin gene mutation

Messenger RNA was isolated from muscle biopsy specimen and its integrity and concentration was estimated by agarose gel electrophoresis. RNA was reverse transcribed into cDNA using oligo dT primer and 5 overlapping regions of the adhalin coding sequence amplified by RT-PCR [6]. Each region of the adhalin coding sequence was tested on three different single-strand conformation polymorphism (SSCP) conditions. Aberrant conformers were excised from dried gels and directly sequenced by fluorescence dydeoxy-cycle sequencing. Sequence data were downloaded onto the Pittsburgh Supercomputer Center for analysis. Comparison to the normal adhalin sequence, protein translation and conservation of aminoacid substitutions of patient sequence data was performed using the University of Wisconsin Genetics computer Group software [5].

Results

Muscle biopsy histopathology showed changes consistent with severe muscular dystrophy: increased fiber size variation, central nuclei and fibrosis, hypertrophic, degenerating and regenerating fibers.

Immunostaining for dystrophin carboxy-terminal showed dystrophin-negative fibers and other fibers with reduced staining intensity (Fig. 1). Dystrophin Western blot (with the same antibody) showed normal amount and molecular weight (Fig. 2). Adhalin immunohistochemistry and Western blot analysis showed a total protein deficiency (Fig. 1, 2). Two additional antibodies, β-dystroglycan (43 kDa DAG) and merosin, were used to determine if additional dystrophin-associated protein deficiencies could be identified in this patient. The staining with both antibodies was normal. β-spectrin labeling (used as marker of mem-

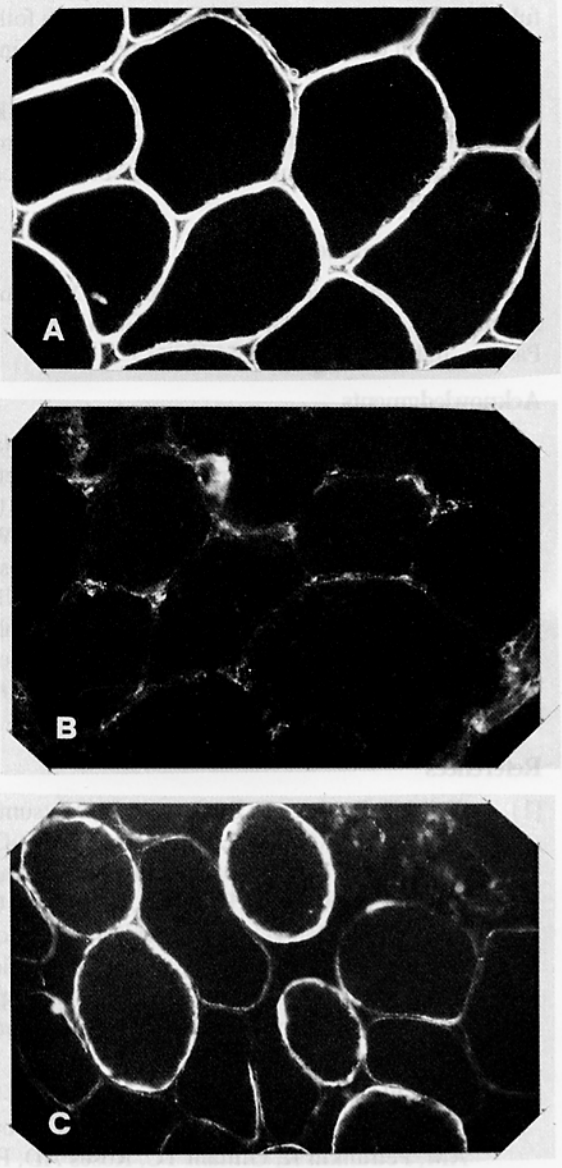

Figure 1. Immunofluorescent staining for adhalin in control muscle biopsy (A) showing a bright and continuous labeling on plasma membrane and in patient's muscle biopsy (B) showing a complete absence of reaction on plasma membrane (magnification x 400). Immunofluorescent staining for dystrophin in patient's muscle biopsy (C) showing several muscle fibers with marked reduction of intensity of staining (magnification x 400).

brane integrity) was normal except for some negative fibers undergoing degeneration.

Muscle RNA was isolated from the biopsy of the patient and a control. RT, PCR and SSCP analysis of the patient's

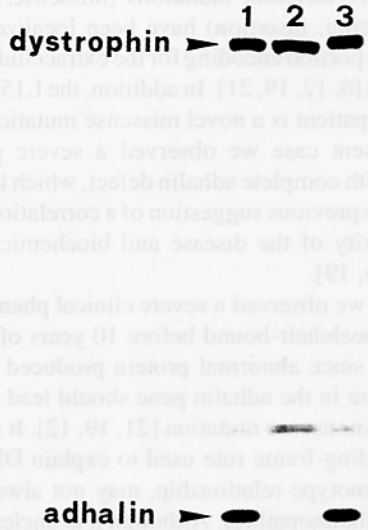

Figure 2. Double labelled dystrophin and adhalin immunoblot in patient's (lane 2) and control muscle (lanes 1, 3). The patient's samples shows normal amount and molecular weight dystrophin (400 kDa) whereas adhalin was completely absent (50 kDa).

RNA showed aberrant SSCP conformers of an extracellular domain region. The aberrant SSCP conformer was directly sequenced and a single base change corresponding to a C → T transition at nucleotide 472 in exon 5 was identified resulting in a leucine to phenylalanine aminoacid substitution (L158F).

Inheritance of the L158F missense mutation was confirmed by screening the genomic DNA of the parents. The patients' mother was found to be a carrier of the missense mutation, the patient's father was not available. Given that the patient's father was unavailable, the patient may be either a homozygote or a compound heterozygote for this mutation with a deletion of the paternal allele.

Genomic DNA from 100 normal chromosomes (from non-muscular dystrophy cases) was used as controls to screen for the nucleotide changes. The base change identified in this patient was not observed in any of the controls. The missense mutation L158F in the adhalin gene has not been previously reported.

Discussion

It has been recently determined that SCARMD showing biochemical adhalin deficiency may be due to mutations occurring in each of the genes for α, β or γ-sarcoglycan components [1, 2, 3, 7, 17, 18], proving the genetic heterogeneity for this disorder. In this light, a new classification of limb-girdle muscular dystrophy (LGMD) has been pro-

posed, and chromosome 17-linked adhalinopathy was indicated as LGMD-2D [4].

The adhalin gene shows a high degree of allelic heterogeneity, since 15 different mutations (missense, nonsense, aberrant splicing, insertion) have been localized throughout the gene portion encoding for the extracellular domain of the protein [6, 12, 19, 21]. In addition, the L158F change found in our patient is a novel missense mutation.

In the present case we observed a severe phenotype associated with complete adhalin defect, which is in agreement with the previous suggestion of a correlation between clinical severity of the disease and biochemical adhalin abundance [6, 19].

In our case we observed a severe clinical phenotype: the child was wheelchair-bound before 10 years of age. This is surprising since abnormal protein produced by a missense mutation in the adhalin gene should lead to a better course than a nonsense mutation [21, 19, 12]. It seems that the same reading-frame rule used to explain DMD/BMD genotype/phenotype relationship, may not always be applicable in adhalinopathies. Although it is unclear the way by which missense mutations affect protein function, adhalin abnormalities causes an impaired assembly with other sarcoglycan components, leading to the disruption of the linkage between the sarcolemma and the extracellular matrix. This hypothesis might explain the concomitant absence of other sarcoglycan components in adhalin-deficient patients [3, 14, 17], and suggests the term "sarcoglycanopathy" as a collective name for these autosomal muscular dystrophies [17].

In the present case the clinical features and the rapid progression rate of the disease, associated with abnormal dystrophin, could have addressed the diagnosis to severe dystrophinopathy unless adhalin would not have been studied. The analysis of family pedigree strongly indicated that the disease was inherited as an autosomal recessive trait. All muscular dystrophy patients with normal dystrophin should be screened for adhalin by immunohistochemical and biochemical methods. Adhalin mutation studies should be pursued for those patients with abnormal adhalin protein [6, 12]. The incidence of adhalinopathy among muscular dystrophy patients ranges from 2.5 to 5% of total [11, 6], but it is likely underestimated because of the presence of biochemically undetected secondary and partial defects and possible relatively mild clinical course in some patients. Infact, according to the prevalence rate of the disease estimated in Japan (1.0 to 2.1 x 10^{-6}) [11], one should expect about 57-120 patients among Italian population.

The patient reported here is the fourth Italian case describe to date with a primary adhalinopathy [6, 19]. Unfortunately, despite the molecular diagnosis of adhalinopathy, prognosis is difficult to address in individual patients due to the wide range of clinical severity and the variable disease progression. Our case appears to have a poor prognosis, because of the early age at which the deambulation was lost and of the sudden development of cardiomyopathy. A cardiac impairment has been briefly reported in only one case in adhalinopathy [9], although it should be expected being that adhalin is also expressed in cardiac muscle [20, 24]. Thus, in adhalin deficient patients a careful cardiac examination should be carried out; a follow-up may be useful to disclose advanced-stage cardiac involvement.

Our report suggests that clinically severe DMD-like dystrophy may be due to adhalin defect and that an appropriate genetic counselling requires accurate biochemical and molecular diagnosis.

Address correspondence to:

Dr. M. Fanin, Neuromuscular Center Department of Neurology, University of Padova, via Giustiniani 5, 35128 Padova, Italy, fax +39 49 8751770.

Acknowledgments

This work was supported by a grant from Italian National Research Council (CNR) "Finalized Project Genetic Engineering". The financial support of Telethon-Italy (grants no. 695 and 688) is gratefully acknowledged. We are grateful to Drs. LVB Anderson (Regional Neuroscience Center, Newcastle-upon-Tyne General Hospital, UK) and KP Campbell (Department of Physiology and Biophysics, University of Iowa City, USA) for their kind gift of antibodies raised against 43 kDa and 50 kDa DAGs, respectively.

References

[1] Azibi K, Bachner L, Beckmann JS, Matsumura K, Hamouda E, Chaouch M, Chaouch A, Ait-Ouarab R, Vignal A, Weissenbach J, Vinet MC, Leturcq F, Collin H, Tomè FMS, Reghis A, Fardeau M, Campbell KP, Kaplan JC: Severe childhood autosomal recessive muscular dystrophy with the deficiency of the 50 kDa dystrophin-associated glycoprotein maps to chromosome 13q12. *Hum Mol Genet* 1993; 2: 1423-1428.

[2] Ben Othmane K, Ben Hamida M, Pericak-Vance MA, Ben Hamida C, Blel S, Carter SC, Bowcock AM, Petruhkin K, Gilliam TC, Roses AD, Hentati F, Vance JM: Linkage of Tunisian autosomal recessive Duchenne-like muscular dystrophy to the pericentromeric region of chromosome 13q. *Nature Genet* 1992; 2: 315-317.

[3] Bonnemann CG, Modi R, Noguchi S, Mizuno Y, Yoshida M, Gussoni E, McNally EM, Duggan DJ, Angelini C, Hoffman EP, Ozawa E, Kunkel LM: β-sarcoglycan (A3b) mutations cause autosomal recessive muscular dystrophy with loss of the sarcoglycan complex. *Nature Genet* 1995; 11: 266-273.

[4] Bushby KMD, Beckmann JS: The limb girdle muscular dystrophies. Proposal for a new nomenclature. *Neuromusc Disord* 1995; 5: 337-343.

[5] Devereax J, Harbeli P, Smithies O: A comprehensive set of sequence programs for the VAX. *Nucl Acid Res* 1984; 12: 387-395.

[6] Duggan DJ, Fanin M, Pegoraro E, Angelini C, Hoffman EP: α-sarcoglycan (adhalin) deficiency: complete deficiency patients are 5% of childhood-onset dystrophin-normal muscular dystrophy and most partial deficiency patients do not have gene mutations. *J Neurol Sci* 1996 (in press).

[7] El Kerch F, Sefiani A, Azibi K, Boutaleb N, Yahyaoui M, Bentahila A, Vinet MC, Leturcq F, Bachner L, Beckmann J, Campbell KP, Tomè FMS, Fardeau M, Kaplan JC: Linkage analysis of families with severe childhood autosomal recessive muscular dystrophy in Morocco indicates genetic homogeneity of the disease in North Africa. *J Med Genet* 1994; 31: 342-343.

[8] Ervasti JM, Ohlendieck K, Kahl SD, Gaver MG, Campbell KP: Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* 1990; 345: 315-319.

[9] Fadic R, Sunada Y, Waclawick AJ, Buck S, Lewandoski PJ, Campbell KP, Lotz BP: Deficiency of a dystrophin-associated glycoprotein (adhalin) in a patient with muscular dystrophy and cardiomyopathy. *N Engl J Med* 1995; 334: 362-366.

[10] Fardeau M, Matsumura K, Tomè FMS, Collin H, Leturcq F, Kaplan JC, Campbell KP: Deficiency of the 50 kDa dystrophin associated glycoprotein (adhalin) in severe autosomal recessive muscular dystrophies in children native from European countries. *C R Acad Sci* 1993; 316: 799-804.

[11] Hayashi YK, Mizuno Y, Yoshida M, Nonaka I, Ozawa E, Arahata K: The frequency of patients with 50 kDa dystrophin-associated glycoprotein (50 DAG or adhalin) deficiency in a muscular dystrophy patient population in Japan. *Neurology* 1995; 45: 551-554.

[12] Ljunggren A, Duggan D, McNally E, Boyan KB, Gama CH, Kunkel LM, Hoffman EP: Primary adhalin deficiency as a cause of muscular dystrophy in patients with normal dystrophin. *Ann Neurol* 1995; 38: 367-372.

[13] Matsumura K, Tomè F, Collin H, Azibi K, Chaouch M, Kaplan JC, Fardeau M, Campbell KP: Deficiency of the 50k dystrophin-associated glycoprotein in severe childhood autosomal recessive muscular dystrophy. *Nature* 1992; 359: 320-322.

[14] Matsumura K, Campbell KP: Deficiency of dystrophin-associated proteins: a common mechanism leading to muscle cell necrosis in severe childhood muscular dystrophies. *Neuromusc Disord* 1993; 3: 109-118.

[15] Mizuno Y, Noguchi S, Yamamoto H, Yoshida M, Suzuki A, Hagiwara Y, Hayashi YK, Arahata K, Nonaka I, Hirai S, Ozawa E: Selective defect of sarcoglycan complex in severe childhood autosomal recessive muscular dystrophy muscle. *Biochem Biophys Res Comm* 1994; 203: 979-983.

[16] Mizuno Y, Noguchi S, Yamamoto H, Yoshida M, Nonaka I, Hirai S, Ozawa E: Sarcoglycan complex is selectively lost in dystrophic hamster muscle. *Am J Pathol* 1995; 146: 530-536.

[17] Noguchi S, McNally EM, Ben Othmane K, Hagiwara Y, Mizuno Y, Yoshida M, Yamamoto H, Bonnemann CG, Gussoni E, Denton PH, Kyriakides T, Middleton L, Hentati F, Ben Hamida M, Nonaka I, Vance JM, Kunkel LM, Ozawa E: Mutations in the dystrophin-associated protein γ-sarcoglycan in chromosome 13 muscular dystrophy. *Science* 1995; 270: 819-822.

[18] Passos-Bueno MR, Oliveira JR, Bakker E, Anderson RD, Marie SK, Vainzof M, Roberds S, Campbell KP, Zatz M: Genetic heterogeneity for Duchenne-like muscular dystrophy (DLMD) based on linkage and 50 DAG analysis. *Hum Mol Genet* 1993; 2: 1945-1947.

[19] Piccolo F, Roberds SL, Jeanpierre M, Leturcq F, Azibi K, Beldjord C, Carriè A, Recan D, Chaouch M, Reghis A, El Kerch F, Sefiani A, Voit T, Merlini L, Collin H, Eymard B, Beckmann JS, Romero NB, Tomè FMS, Fardeau M, Campbell KP, Kaplan JC: Primary adhalinopathy: a common cause of autosomal recessive muscular dystrophy of variable severity. *Nature Genet* 1995; 10: 243-245.

[20] Roberds SL, Anderson RD, Ibraghimov-Beskrovnaya O, Campbell KP: Primary structure and muscle specific expression of the 50 kDa dystrophin-associated glycoprotein (adhalin). *J Biol Chem* 1993; 268: 23739-23742.

[21] Roberds SL, Leturcq F, Allamand V, Piccolo F, Jeanpierre M, Anderson RD, Lim LE, Lee JC, Tomè FMS, Romero NB, Fardeau M, Beckmann JS, Kaplan JC, Campbell KP: Missense mutations in the adhalin gene linked to autosomal recessive muscular dystrophy. *Cell* 1994; 78: 625-633.

[22] Romero NB, Tomè FMS, Leturcq F, El Kerch F, Azibi K, Bachner L, Anderson RD, Roberds SL, Campbell KP, Fardeau M, Kaplan JC: Genetic heterogeneity of severe childhood autosomal recessive muscular dystrophy with adhalin (50 kDa dystrophin-associated glycoprotein) deficiency. *C R Acad Sci* 1994; 317: 70-76.

[23] Sewry CA, Sansome A, Matsumura K, Campbell KP, Dubowitz V: Deficiency of the 50 kDa dystrophin-associated glycoprotein and abnormal expression of utrophin in 2 south Asian cousins with variable expression of severe childhood autosomal recessive muscular dystrophy. *Neuromusc Disord* 1994; 4: 121-129.

[24] Yamamoto H, Mizuno Y, Hayashi K, Nonaka I, Yoshida M, Ozawa E: Expression of dystrophin-associated protein 35 DAG (A4) and 50 DAG (A2) is confined to striated muscle. *J Biochem* 1994; 115: 162-167.

[25] Yoshida M, Suzuki A, Yamamoto H, Noguchi S, Mizuno Y, Ozawa E: Dissociation of the complex of dystrophin and its associated proteins into several unique groups by n-octyl β-D-glucoside. *Eur J Biochem* 1994; 222: 1055-1061.

[26] Zatz M, Matsumura K, Vainzof M, Passos-Bueno MR, Pavanello RCM, Marie SK, Campbell KP: Assessment of the 50-kDa dystrophin-associated glycoprotein in Brazilian patients with severe childhood autosomal recessive muscular dystrophy. *J Neurol Sci* 1994; 123: 122-128.