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IMMUNOHISTOCHEMICAL ALTERATIONS OF DYSTROPHIN IN CONGENITAL MUSCULAR DYSTROPHY

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SUMMARY - The dystrophin distribution in the plasma muscle membrane using immunohystochemistry was studied in 22 children with congenital muscular dystrophy. The dystrophin was detected by immunofluorescence in muscle biopsy through a polyclonal antibody. All the cases had patchy interruptions of the fluorescence in the plasma membrane. A large patchy interruption of the sarcolemma was found in 17 cases, small interruption in 12, and a combination of large and small patchy discontinuity in 7. Small gaps around the fiber like a rosary were found in 15 cases. The frequency of these abnormalities ranged cases from: all fibers in 5 cases, frequent in 8, occasional in 5, and rare in 4. Five cases had total absence of immunofluorescence. These results suggest that the dystrophin expression is abnormal in this group of children and that this type of abnormalities can not be differentiated from early Becker muscular dystrophy nor childhood autosomal recessive muscular dystrophy through immunohystochemistry alone.

KEY WORDS: congenital muscular dystrophy, dystrophin, congenital myopathies.

Alterações imuno-histoquímicas da distrofina na distrofia muscular congênita

RESUMO - Foi estudada a distribuição da distrofina na membrana plasmática das fibras musculares em 22 crianças com distrofia muscular congênita, através de técnicas de imuno-histoquímica. A distrofina foi identificada nas biópsias musculares processadas a fresco, por técnicas de imunofluorescência utilizando anticorpos policlonais. Todos os casos tinham interrupções da imunofluorescência na membrana plasmática. Em 17 elas eram grandes, em 12 eram pequenas e em 7 eram de ambos os tipos. Fibras com interrupções pequenas e constantes, como um rosário, foram vistas em 15 casos. Essas anormalidades estavam presentes em todas as fibras em 5 casos, eram frequentes em 8, ocasionais em 5 e raras em 4. Cinco casos mostraram fibras sem distrofina. Esses dados sugerem que a expressão da distrofina é anormal nesse grupo de crianças. Essas anormalidades podem também ser encontradas em casos precoces de distrofia muscular de Becker e distrofia autossômica recessiva da infância. Portanto, isoladamente a imuno-histoquímica não permite a diferenciação.

PALAVRAS-CHAVE: distrofia muscular congênita, distrofina, miopatias congênitas.

The characterization of dystrophin as a product of the Duchenne muscular dystrophy locus led to the demonstration of their localization in the normal sarcolemma of muscle cells, and cerebral and spinal neurons [5,9,10]. The dystrophin is deficient in most fibers of Duchenne muscular dystrophy. It has an abnormal distribution in some fibers of Becker muscular dystrophy, and also in female carriers for the gene of Duchenne muscular dystrophy [5,6,23]. The abnormal distribution of dystrophin is very important in the diagnosis of these diseases, and further studies are necessary in other muscular dystrophies, such as the congenital muscular dystrophy [1,2].

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In this paper, the pattern of the dystrophin distribution in 22 cases of congenital muscular dystrophies is reported.

MATERIAL AND METHODS

Twenty-two patients with congenital muscular dystrophies are studied, 13 males and 9 females. All the patients had a delay in the motor milestones with severe hypotonia and some had arthrogryposis. The mean age at the time of the biopsy was 3.54 years-old, and at the time the first symptoms were observed, mean age was 0.12 years-old. Mean creatinekinase was 10.46 times the normal value (TNV), lactic dehydrogenase was 0.77 TNV, aldolase was 2.33 TNV, oxalacetic transaminase was 0.27 TNV, and glutamic transaminase was 0.16 TNV. In 10 cases the electromyography was compatible with myopathy and in one of them mixed (neuromyopathic) patterns were found (Table 1).

All patients had a muscle biopsy studied by fresh-frozen section stained for modified Gomori trichrome, hematoxiline-eosin, oil red O, PAS and cresyl violet. Also all the biopsies had histochemical reactions for ATPase pH 9.4, 4.3 and 4.6, NADH-tetrazolium reductase, myophosphorilase, non-specific esterase, succinic dehydrogenase, acid and alkaline phosphatase [22] (Table 1). The histological diagnosis of this group was active myopathy in 2, chronic myopathy in 6, chronic myopathy with activity in 11, inflammatory myopathy in 1, and end stage chronic myopathy in 2 (Table 2). The diagnosis was suggested by histology-histochemistry in 16 cases (72.7%).

The immunohistochemistry study for dystrophin was carried out in Curitiba at the Neuromuscular Laboratory of the Hospital de Clínicas da Universidade Federal do Paraná, having been used a four micron-thick frozen section incubated with polyclonal antibodies. These antibodies were raised in sheep against fusion proteins of amminoacids 407-815 (60 kDa) diluted 1:1000 in phosphate-buffered saline, using biotinylated anti-sheep and streptavidin-fluorescein and having been examined by a photo-microscope equipped with epi-illumination [5,23]. All patients had a control section incubated with non-immune serum and cases with other diseases underwent the same procedure such as control for dystrophin stain. Previous works have demonstrated a good correlation of this immunofluorescence technique for dystrophin and immunoblot analysis in several diseases [1].

Table 1. Clinical and laboratory data.

Case number	Sex	Age at investigation	Age at first symptom	Vignos Scale	CK	LDH	Aldolase	SGOT	SGPT	Electromyography
1	F	0.08	Birth		1.00	0.00	2.00	0.00	0.00	Myopathic
2	F	0.10	Birth		27.00	3.00	2.00			Neuromyopathic
3	M	0.20	Birth		20.00	1.00	10.00	0.00	0.00	
4	M	3.00	0.08		62.00	5.85	19.32			
5	F	0.25	0.08		12.57	0.30	3.51	0.00	0.09	
6	F	0.30	Birth		13.00	1.00				
7	F	0.70	0.20		1.00	0.00	0.00			
8	M	0.80	Birth		10.00	0.00	3.00	0.00	0.00	
9	M	0.87	Birth		31.60	0.69	0.00	3.66	2.50	Myopathic
10	F	0.90	Birth		11.00	1.00				Myopathic
11	M	1.00	Birth		3.24	0.00	0.00	0.67	0.00	Myopathic
12	M	1.10	0.50		5.00	0.00	2.00	0.00	0.00	Myopathic
13*	M	2.00	0.30		5.00	2.00	3.00	0.00	0.00	Myopathic
14	M	3.25	Birth	7	7.05	0.26	0.93			Myopathic
15	F	3.25	1.32	3	1.00	0.00	0.00	0.00	0.00	
16	M	3.30	Birth			0.00	0.00	0.00	0.00	
17	M	7.00	Birth	2	0.00	0.00	0.00	0.00	0.00	Myopathic
18	M	7.00	Birth	10	2.00	1.00	0.00	0.00	0.00	
19	F	8.90	0.10	5	6.00	1.00	1.00	0.00	0.00	Myopathic
20	M	9.00	Birth	8	0.00	0.00	0.00	0.00	0.00	
21	M	11.00	0.08	7	0.35	0.00	0.00	0.00	0.00	Myopathic
22	F	17.00	Birth	3	0.00	0.00	0.00	0.00	0.00	Myopathic

* Fukuyama congenital muscular dystrophy. M, male; F, female. Age at investigation and first symptoms in years. CK, creatinekinase; LDH, lactic dehidrogenase; SGOT, aspartate aminotransferase; SGPT, alanine aminotransferase. All enzymes are expressed in number of times increased above their normal values.

Table 2. Histological alterations in muscle biopsy (Number of cases with abnormalities).

Type of abnormality	Intensity of abnormalities			
	Absent	Discrete	Moderate	Severe
Concective tissue proliferation		3	11	8
Proliferation of adipose tissue	4	4	9	5
Fiber size variation		2	4	16
Large group atrophy	20	1	1	
Small group atrophy	20	1	1	
Small angulated fibers	20	2		
Small rounded atrophic fibers		3	11	8
Hypertrofic fibers	8	8	6	
Internal nuclei	7	7	5	3
Necrotic fibers	6	7	6	3
Phagocytosis	6	8	5	3
Basophilic fibers	16	5	1	
Fiber splitting	10	5	4	3
Snake coil fibers	18	1	3	
Ring fibers	19		2	1
Endomysium inflammatory reaction	20	2		
Excessive inflammatory reaction	20	2		
Type 1 fibre predominance	13	9		
Type 2 fibre predominance	21	1		
Type 1 fibre hypertrophy	9	12		1
Type 2 fibre hypertrophy	8	14		
Type 1 fibre atrophy		17	5	
Type 2 fibre atrophy	4	15	3	
NADH-TR small dark angulated fibres	21	1		
NADH-TR moth eaten fibres	20	1		1
NADH-TR whorled fibres	12	5	5	
Inespecific esterase small dark angulated fibres	19	3		
Inespecific esterase macrophages	17	5		
Acid phosphatase focal increased fibres	9	6	6	1
Acid phosphatase positive fibres	9	7	6	
Acid phosphatase endomysium inflammatory cells	14	6	2	
Acid phosphatase macrophages (phagocytosis)	9	5	8	
Alkaline phosphatase positive fibres	11	5	3	3
Alkaline phosphatase endomysium cells	13	7	2	

No abnormality found in the oil red O, PAS, cresyl violet, succinic dehydrogenase and myophosphorylase.

RESULTS

All the 22 cases had an abnormal distribution of the immunofluorescence around the fibers (Figs 1, 2 and 3; Table 3): 17 cases had large patchy discontinuity of the fluorescence in the sarcolemma, 12 had small interruptions of the dystrophin immunofluorescence, and in 7 there was a combination of both, in the same or in different fibers (Figs 1, 2, 3). Fifteen cases had small gaps of fluorescence in the sarcolemma at regular intervals involving the whole fiber, like a rosary (Figs 2, 3). This rosary appearence was absent in 7 cases (possible Becker or childhood autosomal recessive muscular dystrophy). The abnormalities were: present in all fibers in 5 cases, very frequent in 8, occasional in 5, and rare in 4. Five cases had fibers with total absence of sacolemma fluorescence (Table 2).

COMMENTS

The congenital muscular dystrophies comprises a heterogeneous group of children, usually floppy babies, sometimes with arthrogryposis multiplex. They present a delay in the motor development, hypotonia, decreased muscle strength, progressive muscle atrophy and severe

Table 3. Types of abnormalities in the dystrophin immunefluorescence.

Case Number	Discontinuity			Frequency of fibers affected				Fibers without dystrophin
	Large	Small	Rosary	All	Frequent	Occasional	Rare	
1	+	+	+		+			
2	+		+	+				+
3	+		+	+				
4	+		+	+				+
5	+	+	+	+				
6	+		+	+				+
7		+	+				+	
8	+		+		+			
9	+	+	+			+		
10	+	+	+		+			
11		+				+		
12	+					+		
13	+		+		+			
14	+	+	+		+			+
15	+	+	+		+			
16	+		+		+			
17	+	+				+		
18		+				+		
19	+		+		+			+
20		+					+	
21		+					+	
22	+						+	
Total	17	12	15	5	8	5	4	5

+ Present.

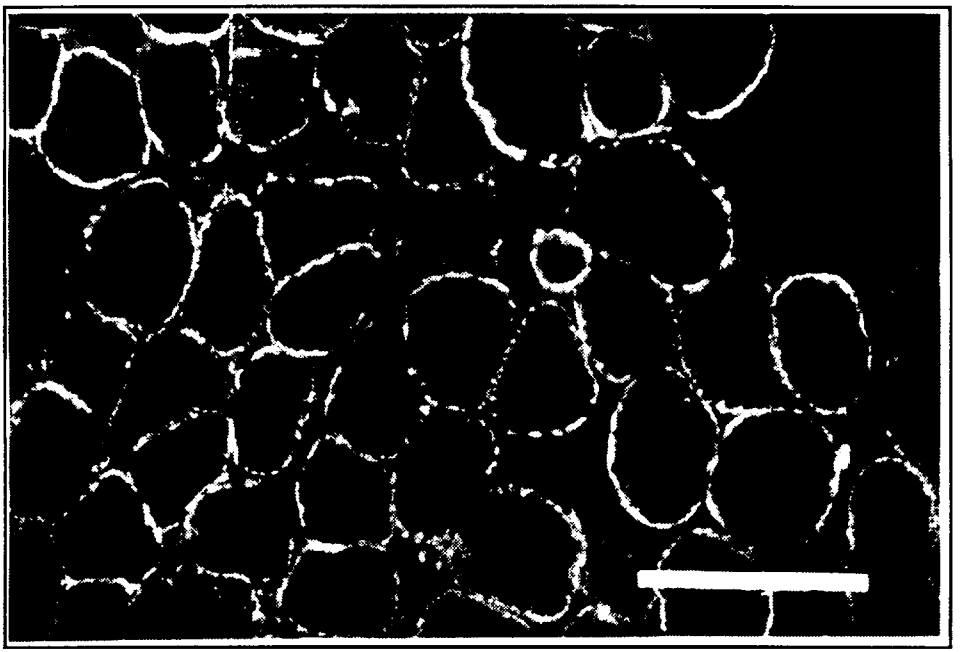

Fig 1. Dystrophin immunostaining. Most of the fibers with gaps at regular intervals, giving an appearance of rosary. Barr 50 μ.

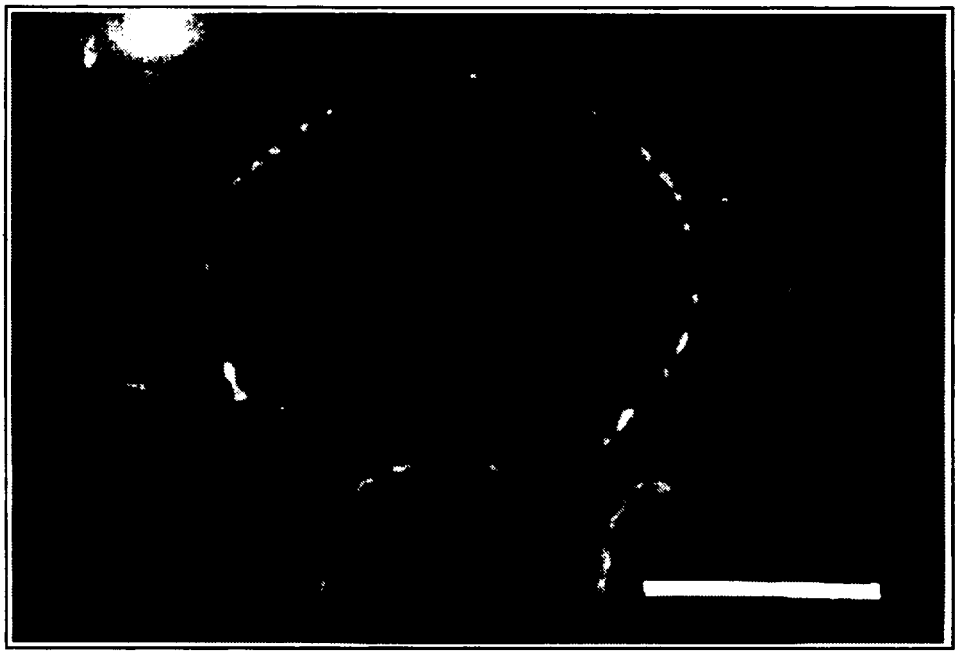

Fig 2. Dystrophin immunostaining. Close up of one fiber with a rosary appearance, with large and small interruptions. Barr 12.5 μ.

Fig 3. Dystrophin immunostaining. Fibers with large and small interruptions, fibers with minimal dystrophin staining (almost absent). Barr 12.5 μ.

contractures during the disease course (Type 1) [3,16,21]. Some had involvement of the central nervous system due to a defect in the migration of the neurons during their development, resulting in polymicrogyria and lisencephaly (Type 2 or Fukuyama type) [8,18]. In this study, the case number 13 was type 2 (Fukuyama) and the remaining, type 1 congenital muscular dystrophy.

The serum enzymes may be elevated and the electromyography usually suggests primary muscle involvement (myopathic pattern) [8,16,21]. The muscle biopsies show severe proliferation of the connective tissue, a variable degree of fatty infiltration, and a moderate variation of fiber size within the fascicles, with small and large diameter fibers. A variable degree of necrosis, phagocytosis and regeneration is found according to age. It was observed an involvement of all fiber types, a predominance of type 1, a decrease of type 2B fibers and an increased number of type 2C fibers as the disease progressed [3,8,16,18,21].

Both types of congenital muscular dystrophy have an autosomal recessive trait being both sexes equally involved, but type 1 sometimes appears sporadically [3,8,21]. For type 2 (Fukuyama) the gene frequency is $5.2\text{-}9.7 \times 10^{-3}$, suggesting a carrier rate population of one out of 52-97 persons and an incidence of the disease in the population of 6.9-11.9 per 100,000 people [8]. There is also a report of a family with congenital muscular dystrophy that has a relative with Duchenne muscular dystrophy [11] and a case of a 7 year-old girl who had small scattered groups of muscle fibers in the muscle biopsy with no dystrophin staining or with reduced and patched dystrophin staining similar to the cases described in this paper [1].

Due to the similarities between the histopathological changes of the congenital muscular dystrophies and early Duchenne muscular dystrophy, some studies undertook dystrophin analyses. Two papers reported absence of dystrophin in a few cases of the Fukuyama type congenital muscular dystrophy [1,4]. In another paper which studied 51 cases of congenital muscular dystrophies it was found that, in 36 cases of the Fukuyama type, there were occasional fibers with abnormal reaction which seemed confined to early degenerating or regenerating muscle fibers. Also in two patients there was total absence of dystrophin [2]. The total absence of dystrophin in some fibers (5 cases) may be due to fiber necrosis. The procedure to differentiate the real absence from the disappearance due to fiber necrosis is by staining for β-spectrin.

Possible explanations presented for the abnormal dystrophin in the Fukuyama congenital dystrophy cases are: a) mutation of the dystrophin gene results in more severe clinical presentation than the usual Duchenne cases; b) dystrophin deficiency associated to disorders unrelated to Duchenne dystrophy; c) a combination of Duchenne dystrophy and brain damage of unknown etiology with multiple genetic abnormalities involving the dystrophin gene or a possible interaction of both genes [1,4].

A report, which studied 28 patients with congenital muscular dystrophy, found abnormalities in the dystrophin distribution in 3 cases only, and suggested that the Duchenne muscular dystrophy gene has a wide variety of expressions. Some cases have very mild and others very severe phenotype, not commonly present at birth or even during the first year of life. At one end of the spectrum, the more severe is the congenital muscular dystrophy and at the other, mild, is the Becker muscular dystrophy [17]. Moreover, the report of a boy with a congenital myopathy, absence of dystrophin and deletion at the 5'end of the dystrophin gene, seems to corroborate to this hypothesis [20]. Also, most of our cases had the dystrophin immunofluorescence appearance similar to that one of the cases in a paper which reported the variability in carriers of Duchenne and Becker muscular dystrophy [7].

The report of the dystrophin-associated transmembrane glycoprotein (DAP), its severe reduction in the sarcolemma of Duchenne muscular dystrophy and the absence of the 50 KDa DAP in childhood autosomal recessive muscular dystrophy raised some questions as to if they are also abnormal in congenital muscle dystrophies [12,19]. Recently a reduced expression of the 43 KDa in the Fukuyama type of congenital muscular dystrophy with near normal expression of dystrophin was reported [13,15]. This suggested an interaction between the dystrophin and the gene of the Fukuyama

type of dystrophy, with the possibility of the 43 KDa dystroglycan gene as a possible candidate for mutation [13,15].

The present study suggests an abnormal expression of the dystrophin by immunoflorescence in this group of patients. It might have a similar or near-by genetic locus abnormality for dystrophin production. Also, the different types of antibodies used in other papers and the different thickness of the sections during the immunocytochemical preparation may explain the discrepancies in the incidence of the abnormalities reported. This study raises some questions upon the specificity of the immunofluorescence test of dystrophin alone in cases of children with early Becker muscular dystrophy due to the similarity of the abnormalities within the congenital muscular dystrophies. It also points to an abnormality of the dystrophin distribution in the sarcolemma in some cases of congenital muscular dystrophies. With the absence of the rosary dystrophin appearence it would not be possible for us to tell whether some of our cases are early Becker or childhood autosomal recessive muscular dystrophy. Further studies on immunobloting of the dystrophin as well as on the dystrophin-glycoprotein complex are necessary in order to better elucidate congenital muscular dystrophy pathogenesis

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