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DUCHENNE MUSCULAR DYSTROPHY

α-dystroglycan immunoexpression in skeletal muscle and cognitive performance

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ABSTRACT - The Duchenne muscular systrophy (DMD) is a muscular dystrophy with cognitive impairment present in 20-30% of the cases. In the present study, in order to study the relationship between the α-dystroglycan (α-DG) immunostaining in skeletal muscle and cognitive performance in DMD patients, 19 were assessed. Twelve patients performed the intelligence quotient (IQ) below the average. Among the 19 patients, two were assessed by the Stanford-Binet test and 17 by Wechsler Intelligence Scale for Children-III (WISC-III). Nine patients performed a verbal IQ below the average, only three patients performed an average verbal IQ. The muscle biopsies immunostained with antibodies to α-DG showed that 17 patients presented a low expression, below 25% of the total fibers. Two patients presented α-DG immunostaining above 40% and an IQ within the average. No significant statistical relationship was demonstrated among total IQ, verbal IQ and execution IQ and α-DG immunostaining at these patients muscle samples.

KEY WORDS: Duchenne muscular dystrophy, α-dystroglycan, cognitive impairment, dystrophin.

Distrofia muscular de Duchenne: imunoexpressão da α-distroglicana em musculatura esquelética e performance cognitiva

RESUMO - A distrofia muscular de Duchenne (DMD) é uma distrofia muscular com comprometimento cognitivo presente em 20-30% dos casos. No presente estudo, com a finalidade de estudar a relação entre a imunoexpressão da α-distroglicana (α-DG) em musculatura esquelética e a performance cognitiva em pacientes com DMD, foram avaliadas 19 crianças. Doze pacientes apresentaram o quociente de inteligência (QI) abaixo da média. Entre os 19 pacientes, dois foram avaliados pelo teste de Stanford-Binet e 17 pelo Wechsler Intelligence Scale para crianças-III (WISC-III). Nove apresentaram QI verbal abaixo da média, e apenas três QI verbal na média. As biopsias musculares com os anticorpos para α-DG mostraram que 17 pacientes apresentaram baixa expressão, abaixo de 25% do total de fibras. Dois pacientes apresentaram a imunoexpressão da α-DG acima de 40% e QI dentro da média. Não foi demonstrada relação estatisticamente significante entre o QI total, QI verbal e QI de execução e a imunoexpressão da α-DG .

PALAVRAS-CHAVE: distrofia muscular de Duchenne, α-distroglicana, déficit cognitivo, distrofina.

The Duchenne muscular dystrophy (DMD) is defined as an X linked recessive disorder, and the main features being progressive muscular weakness related to dystrophin protein deficiency in the skeletal muscle[1]. There is a wide range of symptoms in DMD, which delay diagnosis, because the initial observations are non-specific and the age when boys present the first symptoms is variable[2]. In the majority of boys, the common symptoms begin, before four years old, and are commonly characterized by abnormal gait, walking with difficulty and frequent fall overs. Other symptoms include delays in motor milestones and awkward gait[1].

The DMD is characterized by the absence of dystrophin the skeletal muscle[3]. The dystrophin is a protein that is also located in other cells including the brain in the cortical postsynaptic membrane and in the Purkinje cells. These findings suggest the important role of the dystrophin in the brain, where it is probably involved at the synapses architecture and function[4].

The cerebellum expresses a dystrophin isoform, that differs in a few amino-acids, the P-dystrophin, near the cerebellar Purkinje cells. Another dys-

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trophin isoform, the C-dystrophin is expressed in the pyramidal cells of the cortex and the cellular body and dendrites in the hyppocampus[5-7]. The dystrophin expression was found in three patients who died with a non neuromuscular cause, in the cellular body, dendrites of the brain cortex and Purkinje cells, and was absent in three patiens with DMD[8]. In the muscle, the dystrophin is linked to a proteic complex called, the dystrophin-glycoprotein complex (DGC). Several proteins of this complex are involved in a great variety of processes, including the synaptogenesis and microbial pathogenesis[9]. The DGC complex can be divided into three sub-complexes: the dystroglycan, the cytoplasmatic and the sarcoglycan. The dystroglycan complex, an extracelullar matrix component, is synthesized ubiquitously and is involved in several cellular processes. It binds to laminin 2 and laminin 4, agrin and perlecan. The β-dystroglycan (β-DG) binds directly to dystrophin and α-dystroglycan (α-DG)[9]. The α-DG is located in the extracellular peripheral membrane, while the (β-DG), α, β, γ and δ sarcoglycans and sarcospan are located across the membrane. The syntrophin, dystrobrevin, rapsyn, n-NOS and Grb2 are intracellular proteins[10]. A rupture in the link among DGC proteins occur in the dystrophinopathies (DMDuchenne/Becker), resulting in sarcolemma instability and secondary lesion from muscle contraction[11].

A wide range of abnormalities have been found in 30 cases of DMD patients' brains assessed by Yoshioka[12]: subtle brain atrophy, mild dilated ventriculum and cortical atrophy in and others[13,9]. In another study, an increased incidence of neuronal loss and gliosis, as well as, abnormal dendritic branching and arborization of cortical piramidal neuron was found[14]. 20-30 % of DMD children have a cognitive impairment. They are mildly mentally retarded, and their intelligence quotient (IQ) is shifted approximately one standard deviation curve to the left of the population[9]. The average IQ is 85, and 30% presented an IQ below 70[13]. The poor IQ performance has been attributed to the verbal language[9]. This was a remarkable finding by Karagan and Zellweger, in 53 DMD patients[15]. In the brains of mice, using a α-DG antibody, the protein was found in neurons of the cerebral cortex, olfactory bulb, hippocampus, basal ganglia, thalamus, hypothalamus, brainstem and cerebellum. Its immunostaining was also found in granule cells of the hippocampus dentate gyrus and some thalamus and hypothalamus neurons where the dystrophin was absent, but the utrophin and dystrophin isoforms were present[16]. The α-DG binds to two surface proteins at the celullar membrane, agrin and neurexin, both involved in synaptogenesis. The fact that α-DG is present in GABAergic synapses and is binding to these proteins, suggest its role in synaptogenesis[17]. The common sites of expression, between dystrophin and α-DG are the pyramidal cell neurons, few cerebral cortical neurons, and cerebellum Purkinje cells and CA1-CA3 hyppocampus neurons[16]. Although the α-DG immunostaining has already been demonstrated in mdx muscle fiber[18], few studies demonstrated this protein in DMD patients muscle fibers, like the ones described by Cullen et al. and Ohlendieck et al.[19,20]. In these studies, severe α-DG reduction was obtained in DMD patients muscle fibers, and the remaining labelling was found in the same position as normal, but in a patchy form[19].

The purpose of this study was to demonstrate the role of α-DG in the cognitive performance of DMD patients, as there are common sites of expression, between dystrophin and α-DG in the brain and cerebellum, and also evidence of the importance of α-DG in synaptogenesis.

METHOD

Patients – The study was carried out on 19 DMD patients, aged 6 to 16 years old, with a consent form signed by their parents. The design of this work has been approved by the local Ethical Committee at UNIFESP. Clinical features and laboratory data of the patients are shown in Table 1.

The diagnosis of DMD was made on the basis of clinical and muscle biopsy findings, including variation in fiber size, focal areas of degeneration, rounded opaque fibers, internal nuclei, splitting fibers, proliferation of connective and adipose tissue, and type 1 fiber predominance. Immunostaining by the monoclonal antibody (Novocastra, UK) against dystrophin (C, N, and R fractions) was absent in the muscle specimens of all the patients.

Histochemistry – Muscle biopsies were performed from the deltoid muscle in all patients. Transverse serial frozen 10-μm sections were stained with hematoxylin-eosin (HE), modified Gomori trichrome, cytochrome c oxidase (COX), NADH-tetrazolium reductase (NADH-TR), oil red O, PAS, succinate dehydrogenase (SDH) and adenosine triphosphatasse (ATPase) at pH 9.4, 4.6 and 4.3.

Immunohistochemistry – Indirect immunofluorescence staining was performed on 6 μm frozen serial sections. The sections were air-dried for two hours and fixed in 100% ice-cold acetone for 10 minutes and incubated with buffers containing 2% bovine serum albumine (BSA) in a phosphate buffer saline (PBS) for 20 min at room temper-

Table 1. Clinical pictures and laboratory data of 19 patients with Duchenne muscular dystrophy.

Patient	IS	AB	FA	Milestones	Gait	%MRC	Score MA	CK
1	3y	5ya2m	None	Delayed	Loss at 7y	34,4	9	17X
2	2y	4y2M	None	Normal	Loss at 7y	81,8	7	15X
3	1y	10y2m	Uncle	Delayed	Loss at 10y	71,2	9	17X
4	2y	10y5m	Brother	Delayed	present	50	12	27X
5	5y	7y10m	None	Normal	Loss at 9y	58,7	12	26x
6	6y	8y	None	Normal	present	76	22	38X
7	6y	10y	None	Delayed	present	90	27	39X
8	2y	6y	None	Normal	present	92,5	31	64X
9	7y	9y5m	None	Normal	present	91,8	26	60X
10	5y	6y1m	None	Delayed	present	90	27	55X
11	4y	8y3m	None	Normal	present	96,2	22	59X
12	2y	11y	None	Delayed	present	80	27	40X
13	5y	8y	None	Delayed	present	87,5	23	26X
14	1y6m	6y	None	Delayed	present	87,5	30	57X
15	5y	8y4m	None	Normal	present	92,5	25	59X
16	7y	9y2m	None	Normal	present	91,2	30	52X
17	7y	9y1m	Brother/cousin	Normal	present	92,5	23	36X
18	6y	8y11m	None	Normal	present	98,7	38	79X
19	5y	7y9m	None	Delayed	present	93,7	38	53X

IS, Age of initials symptoms; Score MA, Motor Ability Score; AB, Age of biopsy; CK, creatine kinase: x times normal; FA, family antecedent: present or absent to DMD; Gait, present or age of loss; MRC, Medical Research Councill; MA, Motor Ability.

ature. Subsequently, the sections were incubated overnight at 4ºC with the primary monoclonal antibody IIH6 against the a-dystroglycan (generous gift of Kevin P. Campbell - Howard Hughes Medical Institute, Research Laboratories - University of Iowa, USA) [21], diluted 1:100 in PBS/BSA. Following incubation with the primary antibody the sections were washed three times with PBS and incubated at 37 celsius for 1 hour with a secondary antibody fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Vector Laboratories, CA, USA). These sections were examined by a fluorescent microscope (Olympus BX-60). Control specimens were obtained from age matched individuals with morphologically normal muscle tissue.

Quantitative analysis of immuno-positive fibers – The immunocytochemistry analysis with the α-dystroglycan antibody, IIH6, was done by counting the positive reagent fibers, complete or partially complete, related to the total number of the fibers of that field. An average of 2 to 3 fields, with 20x magnification, was expressed in a percentage of reagent fibers to the IIH6 antibody.

Motor ability and MRC score – The assessment of muscle strength was done manually using the MRC score, based on 32 muscle groups, according to Scott et al.[22] protocol regarding the quantitative method of measuring muscle function in DMD children[22].

Regarding motor ability (MA), the Hammersmith motor ability score was assessed through a group of 20 activities, graded on a three-point (0,1,2) scale (maximum score 40)[22].

Cognitive assessment – The cognitive assessment was obtained by a psychologist with experience in neuropsychological testing. The neuropsychological test WISC-III was used and adapted to the Brazilian population[23]. It was obtained through the full scale, verbal and the performance intelligence quotient (IQ). Two children were assessed by the Stanford-Binet Intelligence test, form L-M (normed in 1972), because their intelligence performance was very low, which made it impossible to be evaluated by the WISC-III. This test is applied by age groups[24], making the assessment possible for these patients. The interpretation of IQ was done according to the following scores[23]:

130 and above . . . . . .Very superior
120-129 . . . . . .Superior
110-119 . . . . . .High average
90-109 . . . . . .Average
80-89 . . . . . .Low average
70-79 . . . . . .Borderline
69 and below . . . . . .Extremely low

Data analysis – Statistics analysis was performed by the analysis of variance (ANOVA), between the cognitive assessment data and the α-dystroglycan immunostaining. A p value below 0.05 was considered significant.

RESULTS

Quantitative analysis of immuno-positive fibers – Seventeen (89%) had α-DG immunoexpression

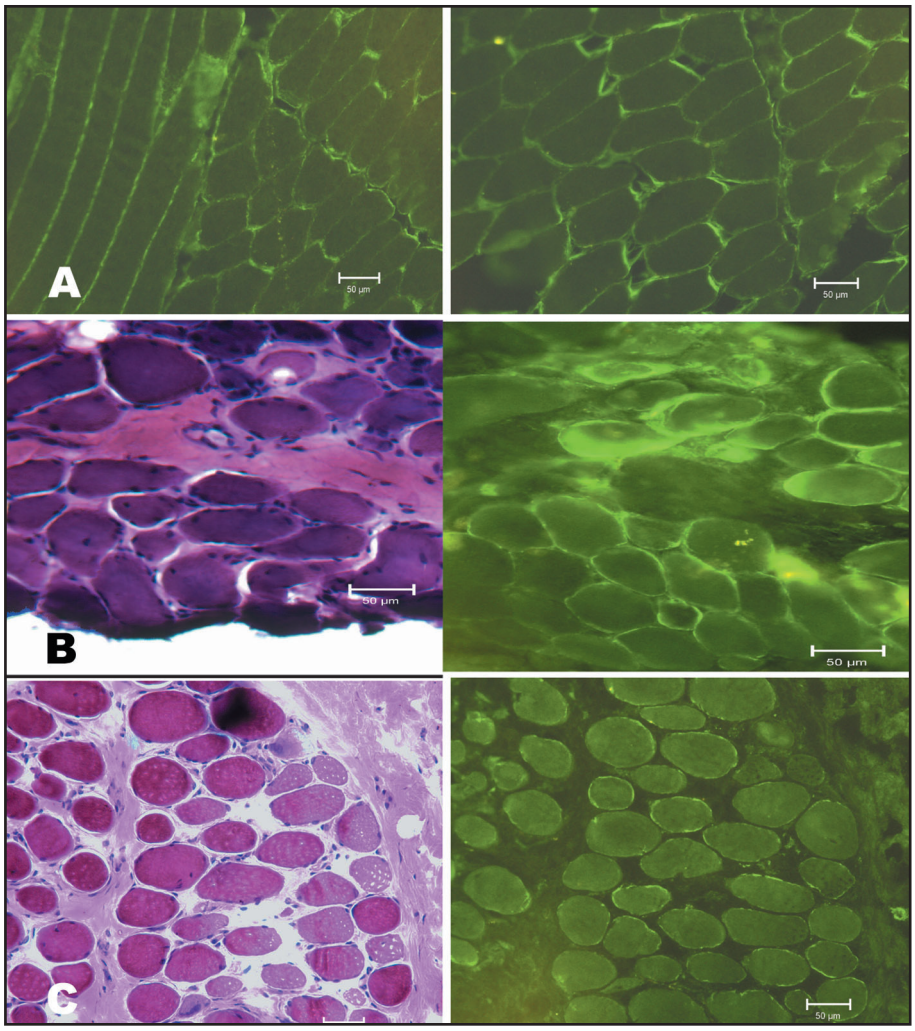

Fig 1. Immunoexpression with α-distroglican (α-DG) in control muscle tissues (A). Serial sections Haematoxylin and eosin (HE) and Immunoexpression with α-DG, patient 9: 70% muscle fibers immunoexperssion (B). Serial sections Haemetoxylin and eosin (HE) and Immunoexpression with α-DG, patient 6: 11% muscle fibers immunoexpression (C).

between 0 and 25%. Only two had 43% and 70% immunoexpression (Table 2). The data is demonstrated in Fig 1.

Cognitive assessment – None of the patients had an IQ similar or above 120. One patient demonstrated an IQ between 110-119. Three patients (16%) showed an average IQ of 90-109, and (63%) had shown an total IQ below average. Regarding verbal IQ, there was a high proportion of patients (nine) who scored an IQ below 80 and five scored below 70. Considering the performance IQ, there was a slight predominance (nine) of patients with average and low average IQ, and eight below average (Table 3).

Two patients (patients 5 and 15) were assessed by the Stanford-Binet test. Both performed extremely low deficient with a total IQ below 70. Patient 5 performed a total IQ of 26 and Patient 15, a total IQ of 56.

Data analysis – There was no statistical relationship between the α-DG immunoexpression and the total, verbal and performance IQ.

DISCUSSION

The muscular biopsies reported in this paper showed that seventeen patients had α-DG immunoexpression between 0 and 25% of total muscle fibers. Our findings are comparable to those found present in mdx mouse, the animal model for dystrophin deficiency, and in DMD patients. Several studies have already demonstrated that α-DG in muscle tissues is significantly reduced in mdx mouse, aro-

Table 2. The α-dystroglycan immunoreactivity in skeletal muscle.

Patients	α-diystroglycan / % total immunopositive fibers
1	13
2	2
3	13
4	8
5	6
6	11
7	43
8	10
9	70
10	1
11	18
12	14
13	3
14	4
15	16
16	4
17	5
18	0
19	2

Table 3. Intelligence quotient (IQ) by WISC-III performed in 17 DMD patients.

Patient	Total IQ	Verbal IQ	Performance IQ
1	54	56	60
2	113	116	106
3	63	67	64
4	71	83	63
6	84	92	77
7	82	97	85
8	85	87	86
9	90	92	88
10	63	64	68
11	68	65	76
12	68	78	63
13	53	57	57
14	78	75	85
16	74	70	83
17	78	78	81
18	105	115	95
19	91	87	98

und 85% 25, and also among other DGC proteins[26]. In the DMD muscle tissues, a severe reduction of α-DG was also revealed in muscle fibers[19,20].

Despite the fact that DMD is not caused by defects of glycosyltransferases, the decreased expression of IIH6 (the antibody that recognizes a glycosylated epitope) can be interpreted as a secondary reduction, which has already been shown in congenital muscular dystrophies and mdx mouse, suggesting its efficiency in the demonstration of the α-DG expression in muscle fibers [27]. For further understanding of these findings it would be necessary to conduct research on other antibodies, including α-DG central antibodies (Core), other skeletal muscle proteins immunostaining and dystrophin mutations.

An unexpected finding in two patients was the high expression of the α-DG in the muscle fiber, one with 70% and the other with 43% of the total muscle fibers, an aspect that has not been seen yet.

Regarding the total IQ of this sample, no patient performed a superior IQ or a very superior the average. The majority of the patients (twelve) presented below the average (below 80) and 8 being intellectually extremely low (below 69). Only one of the patients performed an IQ in the superior average range. The association between mental deficiency and DMD has been long recognized.

IQ in relation total age, duration or gravity of the clinical stage, has not been revealed as a predictive factor of interference in the cognitive performance[28-30]. There is also no relationship to the intensity of motor involvement in our patients when we observe the percentage of MRC or MA, and the value of the total IQ. We had patients with 81.8% of MRC and 7 of MA with IQ values of 113. On the other hand, we had patients with 91.2% of MRC and 30 of MA with an IQ of 74.

We can speculate on the α-DG's role in the CNS, and in cognitive performance because it locates itself in neurons at the same distribution as dystrophin[9] which are found in the neurons of the pyramidal cells, cerebral cortex and in the Purkinje cells from the cerebellum and regions CA1-CA3 of hippocampus[16,31]; amongst other places such as the astrocytes found in the cerebral white matter and sensory neurons and motoneurons in the ventral and dorsal columns[32].

We have three patients (2, 18 and 19), with IQs within the average range and expression of the α-DG below 25% of the total muscle fibers. On the other hand, we have an intriguing finding of two children (patient 7 and 9) who presented a total IQ within the average range, and the immunoexpression of α-DG in the skeletal muscle as 43% and 70%. This could imply a relationship between the IQ and the expression of the α-DG, in relationship to the role of this antibody on the cognitive performance.

Out of the 17 patients evaluated by the WISC-III, 9 presented a verbal IQ below average range and five presented a marked decrease in verbal IQ (below 70). The ratio of patients with the average band of intelligence was 8 (47%). These findings are similar in the series of 53 DMD patients[15]. Although, in our patients, there was not a statistically significant link between α-DG and the verbal IQ, we can still discuss its role. The fact that one of the involved places in the expression of the α-DG in central nervous system (CNS) is the cerebellum within the Purkinje cells[16], we can speculate about the link between the verbal deficit in DMD children and dyslexia, because in dyslexia the cerebellum is a place of major structural disorder[33] and a place of expression of α-DG[16,31].

Some authors suggest a verbal deficit similar to dyslexic children is much more probable than a cognitive deficit[34]. Dyslexic and DMD children demonstrated difficulties in the phonological analysis and in the visual perception of the words[35,36].

Regarding the performance IQ of the 17 patients evaluated by the WISC-III, 8 (47%) presented an IQ below 80, rating in the category of intellectually deficient. The IQ within the average category and below average, was represented by nine (53%) of patients evaluated. Considering the remarkable motor involvement in our group, as referred to the data in Table 1 where motor ability (MA) and percentage of MRC are detailed, we observe that there is a large diversity of degree in motor disability among the individuals, what could reflect on the motor performance during the test assessment. In our sample we have individuals with a percentage of MRC of 34% (total 100%) and others with a percentage of MRC of 99%, as well as three have already lost their gait and are wheel-chair bound.

Little can be attributed to the α-DG, in the performance IQ, in our case study. However, we can consider that three of our patients have already lost their gait, and are wheelchair bound which inturn could interfere in the performance IQ tests, like two of them who presented the performance IQ below 70. The other three patients (10, 12, 13), had also presented an IQ below 70 but, a percentage of MRC between 87.5 and 90%. It seems that the performance IQ can be related to the ability of the individual in working quickly and presenting ready motor reflexes[37] that do not depend on the expression of the α-DG in the CNS. On the other hand, we could speculate that the verbal IQ, could be more directly related to the expression of the α-DG, as it is located in centers related to language, among them, the cerebellum[38].

The cognitive performance in DMD is a challenging and intriguing subject. As soon as we map all the muscle proteins, their function in muscle and other tissues like the brain, and know more about their relationship, we can understand the mechanisms involved in intelligence.

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