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Immunohistochemical Study of Muscular Dystrophies in Egyptian Pediatric Population: A Hospital Based Study

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Abstract: *Context* muscular dystrophy is a group of muscle diseases that weaken the musculoskeletal system without primary abnormality in the lower motor neuron. In Egypt, consanguineous marriage is a major social and cultural event leading to uniquely common diseases characterized by single gene defect. *Aims* studying incidence of different types of muscular dystrophies in Egyptian pediatric patients and comparing the clinicopathological features among Egyptian patients included with registries of other countries. *Settings and design* this is a prospective study, from patients diagnosed clinically as muscular dystrophy patients attending pediatric clinics in Abou-Elreesh Hospital during the period of March 2013 till February 2015. Analysis of 45 frozen muscle biopsies was done. *Materials and Methods* each specimen was stained with H&E, Masson's Trichrome and PAS. Immunohistochemistry was done to classify different types of dystrophy using the following antibodies against: Spectrin, Dystrophin I & II, Sarcoglycans α and β and Merosin. *Results* out of the 45 cases studied, dystrophinopathies are the most common type (69%). The mean age is 5.2 years. Males constituted the majority of cases (84%). The most common motor power presentation is Gower's maneuver. *Conclusions* diagnosis of subtypes of muscular dystrophy is important for prognosis and genetic counseling for parents and recommended to be done in all patients.

Key words: Muscular Dystrophy · Dystrophin · Duchene Muscular Dystrophy · Limb Girdle Muscular Dystrophy

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy in children, it is a severe X-linked form with an incidence of 1/3,500 male births [1].

It causes progressive skeletal muscle weakness & without treatment patients rarely survive beyond their teens, as they will suffer from progressive respiratory muscle weakness & respiratory failure [2]. It is caused by the absence of the membrane cytoskeletal protein dystrophin [3].

DMD is allelic with Becker muscular dystrophy (BMD), a less severe form of myopathy with nearly similar clinical features affecting 1/30,000 males [4].

The limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of diseases which are clinically characterized by progressive weakness of the shoulder & pelvic girdle muscles [5].

Congenital muscular dystrophies are a group of infants presenting with marked hypotonic & weakness since birth or early in life. A primary deficiency of lamimin alpha2 (Merosin) is the most common form [6]. There is an integral role for muscle biopsy in evaluation of the patient with neuromuscular disease. It is an essential element in the assessment of a patient with suspected myopathy with occasional exceptions [7].

MATERIALS AND METHODS

This study is approved by the department ethical committee. This is a prospective study included 53 patients clinically suspected to have muscular dystrophy after correlating the pathological findings, immunohisto chemical results with the clinical, electrophysiological and laboratory findings, 45 cases were confirmed histopathological & immunohisto chemically to be muscular dystrophy. For all Patients

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clinical and laboratory data were collected including; Age, Sex, Clinical history (Including onset of disease, motor milestones, motor power, cardiac or brain involvement), Family history, Laboratory findings (CK level), Electrophysiological findings (EMG), Genetic study (If available).

Muscle Biopsy: Muscle biopsy from the Quadriceps muscle was performed under local anesthesia in all patients. The muscle portion was cut in a transverse section, frozen in liquid nitrogen then frozen sections were performed. Slides were prepared from frozen section then stained with hematoxylin/eosin, Masson's trichrome (To assess degree of endomysial & perimysial fibrosis) and periodic acid-Schiff (PAS) (Detection of glycogen) as a routine.

Immunostaining: Additional sections were prepared from the frozen section and immunostaining with monoclonal antibodies to Spectrin (To ensure integrity of the sarcolemma), Dystrophin I & II, Sarcoglycans α and β, Merosin (Laminin Alpha 2 Chain) (Novacastra, New Castle, Uk) as a primary was done.

Statistical presentation and analysis was conducted, using the mean ± SD and median (range) for quantitative variables and number for qualititave variables.

RESULTS

Among the 53 studied cases, 45 cases proved to be muscular dystrophy. The most common subtype of muscular dystrophies was dystrophinopathy 31 cases (69%) followed by congenital muscular dystrophies (20%) and the least common was Limb girdle muscular dystrophy (Figure 1).

Dystrophinopathies were further sub classified into; DMD 22 (71%) cases, BMD 9 (29%) cases (Figure 2).

Congenital Muscular Dystrophies Comprised: Merosin deficient CMD five (56%) cases and merosin positive CMD four (44%) cases.

Mean age at onset was 5.2 years. Age ranged between one to 12 years. Most of the cases were first presented between 3 and 6 years (40%). While Mean age at diagnosis was 8.2 years. Age ranged between 1.5 & 18 years. Most of them (28.8%) were found between 9 years & 12 years. The mean age delay between onset of symptoms and time of diagnosis was 3 years.

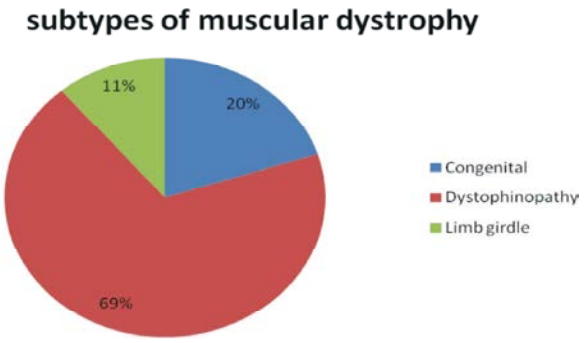

Fig. 1: Frequency of various subtypes of muscular dystrophy

Table 1: Clinical & radiological findings (In all 45 studied cases)

Clinical finding	Number
Inability to walk	11
Hypotonia	9
Gower's maneuver	28
Frequent falls	12
Pseudo-hypertrophy	23
Heart problems "cardiomyopathy" (In dystrophinopathies)	5
Waddling gait	14
Tip toes walking	4
Delayed motor milestones	17
MRI demyelinating changes (in congenital merosin negative muscular dystrophy)	2

Table 2: Genetic study results in Dystrophinopathy cases

Genetics	Number
Deletion mutation	2
No deletion mutation	4
Genetic study not done	25
Total	31

There was significantly higher male predominance, 38 cases (84%), while females were only 7 cases (16%). In dystrophinopathy group, all cases were males.

Variable proximal muscle weakness was presented in all cases, the commonest presentation was Gower's manoeuver and most of the cases had more than one clinical finding. All cases showed myopathic picture on EMG whether patchy or diffuse (Table 1).

Most of the dystrophy cases showed negative family history (62.2%), while only 37.8 % of cases showed positive family history. The congenital group was the highest to have positive family history (66.6%) of cases, while in dystrophinopathy group only 32% have positive family history.

The mean creatine kinase (CK) level in congenital dystrophy group was 1474.00 ± 1573.88, in the dystrophinopathy group 10194.77 ± 7782.39 and in the limb girdle dystrophy group was 10481.20 ± 13975.67.

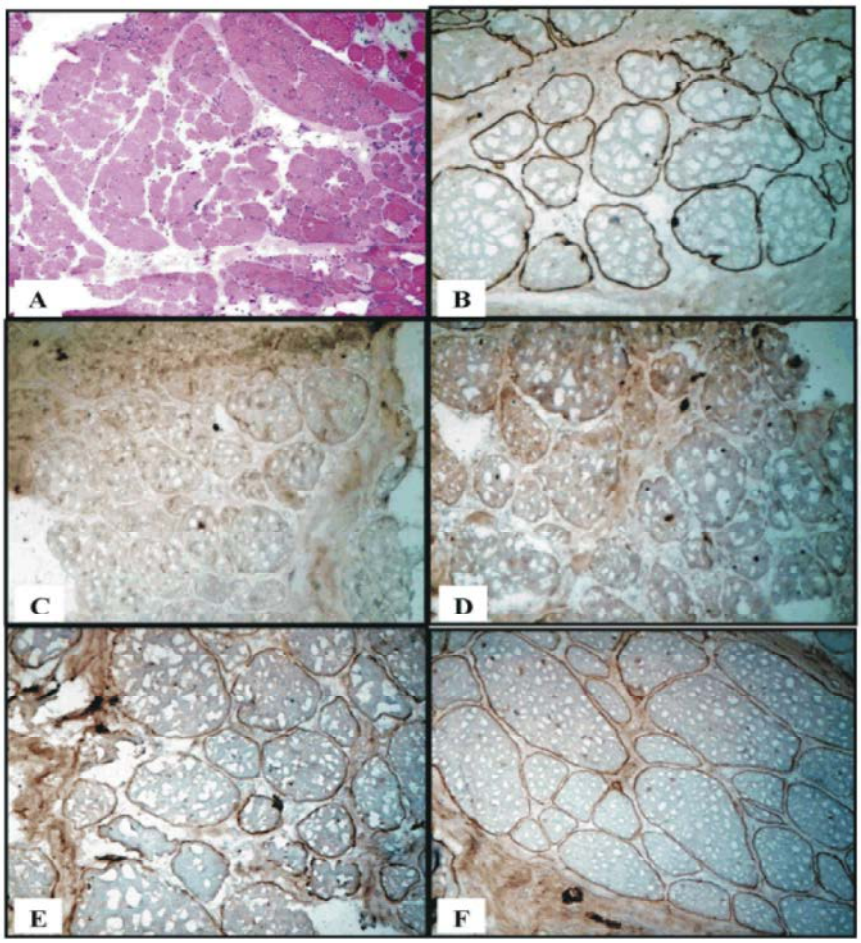

Fig. 2: A case of DMD. (A) Variations in fiber size, mild endomysial & perimysial fibrosis (H&E stained frozen section original x100). (B) Normal sarcolemmal staining for Spectrin. (C) & (D) Negative sarcolemmal staining for dystrophins I & II respectively, (E) & (F) normal staining of sarcolemma for sarcoglycans α & β respectively (immunoperoxidase original x400)

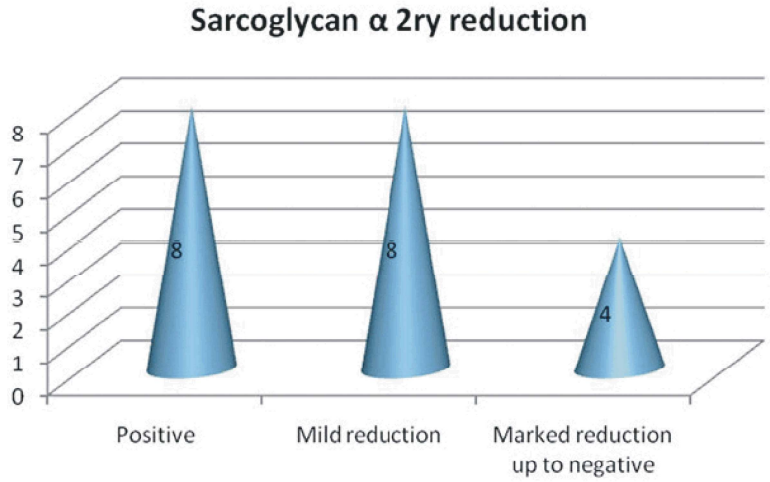

Fig. 3: Pattern of α Sarcoglycan staining in Dystrophinopathies

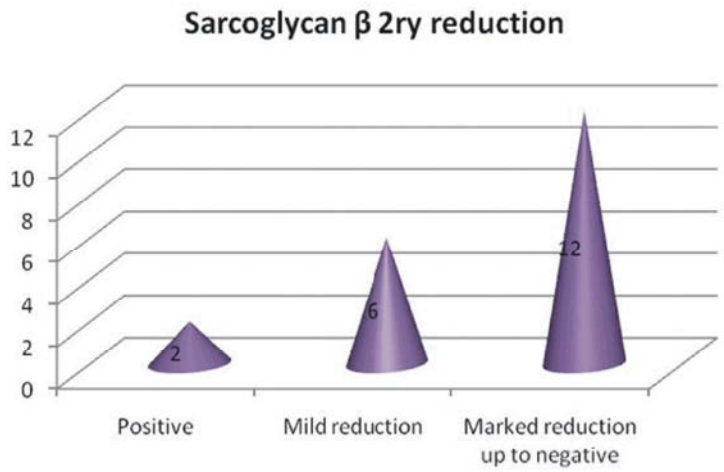

Fig. 4: Pattern of β Sarcoglycan staining in Dystrophinopathies

Out of the 6 dystrophinopathy cases underwent genetic study 2 cases showed genetic deletion in dystrophin gene (33.3%) Table 2.

Immunohistochemical Findings in Dystrophinopathies: All cases of DMD were diagnosed with complete absence or marked reduction in Dystrophin I &/or II, cases of BMD were diagnosed when there was incomplete staining of dystrophin. However secondary changes in Sarcoglycans α and β were observed in most of the cases. Marked 2ry reduction in β Sarcoglycan was observed in 48.3% of the dystrophinopathy cases, while only 19.3% of α Sarcoglycan showed marked 2ry reduction (Fig. 3, 4).

DISCUSSION

The most important progress in the field of muscular diseases started in the 6th decade of the twentieth century when histochemical and histoenzymology techniques were applied to the muscular biopsy then followed by electronic microscopy, immunohistochemistry and Western blotting. For diagnosis of every muscular disease, it is necessary to corroborate the clinical data with the electrophysiological ones (Electromyography and nervous conduction data), the biochemical ones (Creatine kinase), complex morphological data and genetic studies [8].

In Egypt like other Middle East countries, consanguineous marriage is a major social and cultural event leading to uniquely common diseases characterized by single gene defect. The present study included 45 patients who were clinically diagnosed to have muscular dystrophy. The muscle biopsies were examined histologically, using ematoxylin/eosin, Masson's trichrome (To assess degree of endomysial & perimysial fibrosis) and periodic acid-Schiff (PAS) (Detection of glycogen) as a routine.

Histochemically and Immunohistochemically Using the Following Markers: Spectrin, Dystrophin I & II, Sarcoglycans α and β and merosin. Finally the diagnosis was as follows: 31 (69%) cases as dystrophinopathy, 9 (20%) cases as congenital muscular dystrophy and 5 (11%) cases as Limb girdle muscular dystrophy (Sarcoglycanopathies). In the present study, the dystrophinopathies were the most common form of dystrophy representing (69%) of the studied cases. This finding agrees with many other earlier studies [9].

In the present series DMD was the most common type of muscular dystrophies representing (71%) of dystrophinopathy cases studied and 48.9 of all studied cases. This finding agrees with Emery [10]. In the present series only 11% of muscular dystrophy patients were diagnosed as LGMD (Sarcoglycanopathies). This finding is fairly comparable with previous results Manzur and Muntoni [11].

In the present study 9 cases were diagnosed as Congenital muscular dystrophy representing (20%) of muscular dystrophies thus they were less common than dystrophinopathies. This percentage was higher than Northern England series [12]. As our study was targeting the pediatric age group only, however Norwood *et al*. [12] was targeting all age groups in a community based study, also we are in a country of high consanguinity marriage so we expect higher numbers of CMD.

All patients were of classic form (no apparent clinical involvement of the CNS). CMD with CNS manifestation were totally lacking in the present study as these

comprise muscle eye brain disease, almost exclusively reported in Finland [13], the Walker-Warburg syndrome which is also a rare disease but spread worldwide [14] and Fukuyama CMD which is associated with major brain abnormalities and is almost exclusively observed in Japan [15].

The 9 patients were further subgrouped according to the presence or absence of merosin on immunohistochemistry according to Tome *et al.* [14] merosin positive CMD (44%) were less common than merosin deficient (56%). Our merosin deficient were higher than that reported by Tome *et al.* [14]. As in the present series merosin positive (MP) CMD were the forms of CMD that showed dystrophic features and were not deficient in merosin, but could not be further classified as other immunohistochemical or molecular studies are not available. However results of the present study were in concordance with the results of Talim *et al.* [16].

As regards sex distribution, all dystrophinopathy cases were males; this is matching with Chelly *et al.* [17] as it is an X-linked disease and thus affects mainly males.

In the present study, the age at definite diagnosis ranged between 1.5 and 18 years, with mean age 8.2 years, while the mean age of onset of clinical presentation was 5.2 years, ranging between 1 and 12 years, there was a delay of about 3 years between onset of dystrophy symptoms and the time of definitive diagnosis. This pattern of age distribution was nearly matching with other studies Woodhead & Avril [18].

The age delay agree with a Malaysian study by Thong *et al.* [19] who studied 21 cases of dystrophinopathy and reported that there was a mean period of delay in the diagnosis of 3 years and Ciafaloni *et al.* [20] who reported there was a delay of about 2.5 years between onset of symptoms and the time of definitive diagnosis, this difference may relate to the lack of awareness, failure to recognize early delay in milestones, poverty & difficulty to reach health services in our community as other low socioeconomic communities.

The mean age in congenital muscular dystrophy group is 1.3 years, this agrees with Bertini *et al.* [21] who stated that this group is a heterogenous neuromuscular disorders with onset at birth or in infancy. Also agrees with Laila *et al.* [22] who studied congenital merosin deficient cases in Egyptian patients and found the mean age is 1.2 years (14 months). Results of the present study were also in concordance with the results of Talim *et al*. [16]. In Sarcoglycanopathies the age at onset ranged (4-10 years), the age findings are comparable with that reported by Sharma *et al*. [23], but much lower than the results of Ferreira *et al*. [24] where age at onset of the disease was 10- 40 years as our study was targeting pediatric patients only.

Our studied cases had variable presentations of proximal muscle weakness as delayed motor milestones, waddling gait, Gower's maneuver, tip toe walking, some cases were non ambulant, few patients presented with cardiomyopathy. Those presentations are presented in studied cases all over the world [18, 25, 26].

In our study 2 (40%) out of 5 congenital merosin deficient muscular dystrophy cases showed MRI demyelinating changes, this matches also with Hui *et al*. [27] Laila *et al*. [22].

In the present study out of the six cases of dystrophinopathy who underwent genetic study 2 cases (33.3%) showed deletion mutation in the dystrophin gene, while 4 cases (66.7%) showed no deletion mutation.

This result was lower than Egyptian study by Effat *et al*. [28]. The number of cases showing deletion mutation in genetic study are lower than the world study, this was stated by El-Sherief *et al*. [29] that although the dystrophin gene has been analyzed extensively all over the world, only a few studies have been reported on Egyptian patients. Also in our study only a small number of cases did genetic study, so this number is not representative as this test is too expensive in a country of low socioeconomic status. Large deletions and duplications can be detected using PCR primes, but point mutations remain undetected in most patients, because available techniques are expensive and the dystrophin gene is too large, so the available procedures are limited as new points emerge every few days [30]. So immunohistochemistry on muscle biopsy is important to detect absence of dystrophin protein in all cases even if the genetic study doesn't show any mutation.

The mean CK value of Dystrophinopathy cases reported in the present series was 10194 IU/L. This was matching CK values in other studies by Thong *et al.* [19], Yiu & Kornberg [26] and Swaminathan *et al.* [31] CK level is markedly raised, at least 10 to 20 times (Often 50 to 200 times) the upper limit of normal (N. up to 300 IU/L). In our study the mean CK value in Congenital muscular dystrophy group was 1474 IU/L, This result matches with Egyptian study by Laila *et al*. [22], & with Talim *et al*. [16].

Family history in dystrophinopathies was positive in 32% of patients. This result fairly match with another study reported by Rangel *et al*. [32], Malaysian study by Thong *et al.* [19].

In the present study the differentiation between DMD and BMD was by immuohistochemical examination for dystrophin which was completely absent in DMD except for very small proportion of revertant fibres in few cases and incomplete sarcolemmal immunostaining in BMD. This finding agrees with Barresi [33]. All DMD cases in our study showed negative staining of both dystrophin I (Against C-terminus domain) & II (Against N-terminus domain), Barresi [33] stated that due to large size of dystrophin it is important to use antibodies directed against several sites of the protein, to avoid false negative results due to deletion of specific epitopes. A panel of antibodies directed against N-terminal, C-terminal or rod domain is routinely used. Fifteen (48.3%) cases of dystrophinopathies showed a secondary marked reduction of β sarcoglycan immunostaining and 35.4% of cases showed only mild reduction and 19.3% of cases showed secondary marked reduction in α sarcoglycan immunostaining. This finding agrees with Barresi [33] who reported that reduced labelling for sarcoglycans is common in dystrophinopathies. This stresses the importance of the use of expanded panel of immunohistochemical antibodies for proper diagnosis.

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