

MUSCULAR DYSTROPHIES AND THE ROLE OF DYSTROPHIN IN THEIR DIAGNOSIS

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ABSTRACT

Introduction: Muscular dystrophies (MD) traditionally refer to a group of genetically determined, progressive, degenerative disorders of the skeletal muscle. The most common disease manifestations being Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD). This descriptive study was carried out on 40 patients of muscular dystrophies, selected on clinical grounds and subjected to biochemical, morphological and immunohistochemical analysis.

Materials and Methods: Muscle biopsies were taken from patients by an open method and formalin fixed paraffin embedded blocks were made. Haematoxylin and Eosin stain, PAS and Gomori's trichrome and immunohistochemical stains were conducted on the sections from these blocks. Dystrophin and beta – Spectrin antibodies were used for immunohistochemistry.

Results: Among the 40 cases of muscular dystrophy the above investigations were correlated with clinical findings to reach the final diagnosis in each case. In Pakistan the diagnosis of muscular dystrophies is still based on clinical grounds and CPK values only, however the present study has provided us an opportunity to combine clinical, biochemical, morphological and immunohistochemical evaluations of the patients with muscular dystrophies. It was concluded from this study that although muscular dystrophy can be diagnosed using clinical parameters and CPK levels, histochemistry and IHC can confirm and differentiate the various types of muscular dystrophy and make it possible to identify the female patients of DMD and BMD.

Key Words: Dystrophin, Immunohistochemistry (IHC), Muscular Dystrophies.

INTRODUCTION

Dystrophin is a very large protein having 3685 amino acids. It has four domains i.e. aminoterminal domain, Central – rod domain, Cysteine – rich domain, and the Carboxy – terminal domain.^{1,2} This DAPC is composed of many important proteins like dystroglycan (α , β , γ and δ sub-units), syntrophin, dystrobrevin and caveolin etc.^{1,3} Dystrophin and the DAPC are thought to play a very crucial role in maintenance of the integrity and normal functioning of skeletal muscle membrane during cycles of contraction and relaxation.^{4,5} According to researchers full protection against contraction generated stress requires proper functioning of dystrophin as well as DAPC.^{4,5} When there is a genetic absence of any of these proteins the muscle fibers become prone to tearing during contraction and the affected person develops one of the many forms of muscular dystrophies.^{6,7}

“Muscular dystrophy” traditionally refers to a group of genetically determined, progressive, degenerative disorders of the muscle.⁸ Duchenne and Becker dystrophies are now called dystrophinopathies because they are caused by the mutations of the dystrophin gene.⁷ The dystrophin gene is the

largest gene in the human genome spanning more than 3Mb (almost 0.1% of the entire human genome) and approximately 1.5% of the X-chromosome.² This large size makes it prone to rearrangements and recombinations that result in mutations.⁹ About 70% of the DMD and BMD are inherited as X-linked recessive disorders and therefore the disease affects mainly the male gender, and runs in families.¹⁰ About 2/3rd of the mothers of the affected boys are thought to be carriers.¹¹

Although 90% females in dystrophinopathies are asymptomatic carriers, in some instances they may even present with the disease.¹² Different pathogenetic mechanisms for the females to be the patients of dystrophinopathies have increasingly been identified, for example skewed X-inactivation, X-autosome translocation and females with Turner's syndrome. Recently a case report showed unipaternal disomy of entire X-chromosome to be the pathogenetic mechanism in such females.^{12,13} These females showed myopathic features on biopsy. Based on them they were grouped under LGMD before the use of dystrophin IHC that showed a mosaic pattern, after which they were diagnosed as females with dystrophinopathies.¹²

Diagnosis of muscular dystrophy usually begins with the clinical suspicion that has proven to be very sensitive and has high positive predictive value.⁸ The usual clinical features of myopathy include predominantly proximal muscle weakness that is symmetrical, increases progressively; there is exercise intolerance, muscle hypotonia, muscle hyporeflexia and calf – muscle hypertrophy. The characteristic Gower sign is positive and there may be contractures and dysmorphic facial appearance.¹⁴ Highly elevated levels of enzyme CK > 1000 IU/L almost always indicates a muscle disease.⁷ Muscle biopsy with IHC for dystrophins is the gold – standard for distinguishing muscular dystrophies.¹⁰ The constellation of histological features characteristic of myopathic process include, variability of fiber size, centralisation / internalisation of nuclei, nuclear chains, opaque – fibers, increased endomysial fibrosis, rounded fibers and split fibers etc.⁷⁻¹⁵

Immunohistochemistry detects the mutated proteins of muscular dystrophies that is a powerful tool in distinguishing different muscular dystrophies because of its speed, accuracy and an increasing availability of antibodies to dystrophin and its associated proteins.¹⁶ The evaluation of IHC staining pattern of dystrophin should be performed using beta spectrin that serves as an excellent control for muscle membrane integrity.¹⁷ This eliminates the chances of false negative dystrophin staining that could be caused by a damage to the muscle membrane during handling or processing of muscle biopsy.¹⁸ At present in majority of the centers, immunohistochemistry is used on muscle tissue for the diagnosis of muscular dystrophies on fresh frozen sections.⁷

As muscular dystrophies are complex and relatively uncommon disorders very little work has been done on them in Pakistan where none reported during the past over two decades. The present study was aimed to elaborate upon morphological findings in muscular dystrophies and to see, in particular, the utility of dystrophin and beta – spectrin IHC in their diagnosis, differential diagnosis, and if possible sub-categorisation and prognosis of muscular dystrophies.

MATERIALS AND METHODS

Patients

After approval from The Ethical Committee of University of Health Sciences, Lahore the study was conducted in the Department of Morbid Anatomy and Histopathology. This study was carried out on 40 patients who visited PSRD Hospital i.e. Pakistan Society of Rehabilitation of the Disabled. These patients of muscular dystrophies were selected on clinical grounds for this descriptive study. Those who fulfilled the clinical criteria and gave the infor-

med written consent (signed by the patient / guardian) were subjected to further investigations.

Biochemical Investigations

Serum CPK and aldolase levels were performed in the department of Allied Health Sciences.

Muscle Biopsy

Muscle biopsy was taken from each patient by an open method, it was immediately placed on a dry filter paper (Whatmann # 1) for immobilisation and then left in 10% neutral buffered formalin used as transport medium as well as preservative for muscle biopsy specimens. After overnight fixation formalin fixed paraffin embedded blocks were prepared. They were stained with haematoxyline and eosin, periodic acid Schiff's reaction (PAS), Gomori's trichrome and immunohistochemical stains to demonstrate β -spectrin and dystrophin antibodies.

IHC Staining

The paraffin blocks were used to cut 8 micro meter sections. After they were brought to water, peroxidase block was performed by putting 1 to 2 drops of 3% hydrogen peroxidase. For target retrieval, heat mediated antigen retrieval method was used. These target retrieval solutions were used, (1) citrate / EDTA buffer 10 mM citric acid / 2 mM EDTA at pH 6.2, (2) citrate buffer 10 mM, pH 6.0 and (3) EDTA buffer 1 mM at pH 8.0. Primary antibody incubation was performed using two primary antibodies from *Novocastra i.e. Spec – 1 and Dys – 2 (one is spectrin antibody and other is dystrophin antibody) on two separate sections from each block and incubated for two hours. Beta – spectrin was used as a positive control for the interpretation of dystrophin immunostaining. The method for detection of staining used was two steps Streptavidin – biotin – peroxidase using Novocastra universal detection kit and DAB was used as chromogen. The slides were counter stained with haematoxylin and mounted using DPX. Results of all the above investigations were correlated with clinical findings to reach the final diagnosis in each case.

**Disclosure*

None of the authors have any financial interests.

RESULTS

A total of 40 patients of primary muscular dystrophies were included in this descriptive study according to the inclusion criteria. In addition five normal skeletal muscle biopsies were taken as controls. Among the diseased 40 subjects, 27 (67.50%) were males and 13 (32.50%) were females. Their major clinical features were: predominantly proximal muscle weakness, symmetrically involved muscles, pro-

gressively increasing weakness, exercise intolerance, characteristic style of standing from sitting posture (Gower's sign), positive family history, no sensory loss, calf muscle hypertrophy, decreased muscle power and contractures. Their ages ranged from 4 years to 28 years. The biochemical markers studied were serum CK and serum aldolase, they were markedly elevated i.e. up to > 1000 and many folds among our patients, respectively (Table 1).

The salient common morphological features were : fiber size variability, hypertrophic fibers, increased internalisation of nuclei, opaque fibers, increased endomysial fibrosis, fat metaplasia, inflammation around the necrotic fibers, fiber splitting, regenerative myofibers, rounding of fibers, nuclear chains, myophagocytosis etc. (Fig. 1, 2).

The special stains used were Periodic acid Schiff's reaction (PAS) and Gomori's trichrome stains. The PAS stain showed indistinct checker board pattern in which muscle fibers are deeply and lightly stained positive (Fig. 3). The Gomori's trichrome was performed to assess the amount of fibrosis (Fig. 4). β -Spectrin immunostaining acted as positive control in all biopsies thereby ruling out false negative results of dystrophin (Fig. 5). Four patterns of dystrophin immunostaining were observed : there was complete absence of membranous staining (Fig. 6), broken and interrupted membrane staining, mosaic pattern of immunostaining and complete presence of membrane staining that was of very weak intensity than normal.

Final diagnosis of the patients

The diagnosis was established after correlating the clinical, biochemical, morphological and immunohistochemical findings in each case. In a total of 40 patients $n = 27$ (67.50%) were finally diagnosed as DMD, whereas $n=12$ (30%) were diagnosed as BMD and the remaining one case (2.5%) was labelled as LGMD.

The 13 female patients showed typical features of muscular dystrophies including progressive proximal muscle weakness, frequent falls, muscle wasting etc as in the male counterparts. Similarly serum CK and aldolase levels were also raised in them though lower than in the males. Muscle biopsies revealed typical dystrophic features whereas dystrophin IHC revealed the diagnostic mosaic pattern of immunostaining in 12 of the 13 females thereby diagnosed as DMD / BMD. The remaining one patient had complete presence of membranous staining but of weak intensity hence diagnosed as LGMD.

Table 1: Shows the serum CK and Aldolase values in 40 patients.

Biochemical Parameters (units)	Minimum	Maximum	Mean	Std. Deviation
CK (IU/L)	279	8538	3257.27	2559.481
Aldolase (IU/L)	1.80	36.16	10.9120	6.76638

DISCUSSION

Muscular dystrophies encompass a diverse category of disease conditions with a broad spectrum of pathological outcomes, the most common disease manifestations being Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). These originate from deleterious mutations in the dystrophin gene, leading to a loss of the protein product.¹⁹ In turn, the absence of dystrophin protein results in segmental necrosis of muscle fibers, leading to severe skeletal muscle wasting and death in early adulthood.^{2,20} Diagnosis of a muscular dystrophy usually begins with the clinical suspicion and it has proven to be very sensitive possessing a very high positive predictive value.⁸

These features are also reflected in the results of the present study in which we selected 40 patients on clinical grounds including history and physical examination, as the patients of muscular dystrophies and later the specific investigations like biochemistry, morphology and most importantly IHC confirmed that, all (100%) of them suffered from muscular dystrophies. The salient clinical features include history of progressive symmetrical proximal muscle weakness, calf muscle hypertrophy, decreased muscle power, muscle wasting and contractures etc. These findings are in complete harmony with most of the earlier studies in this region.^{7,8,21} Use of a single haematoxylin and eosin (H&E) stain reveals the key features of muscular dystrophies like variation in fiber size and shape, internal nuclei, fiber splits, necrosis, myophagocytosis and fibrofatty metaplasia giving more than 50% information about the biopsy.⁷

All these dystrophic features were observed in all the biopsies in the present study. IHC using dystrophin and related antibodies on muscle biopsy is a very powerful tool in distinguishing various muscular dystrophies and it has proven better than genetic analysis using multiplex PCR that may sometimes fail to detect small or unusual mutations of the dystrophin gene.² IHC is more specific than EMG that carries low specificity; however it may not differentiate between myopathy and neuropathy.⁸ Western

blotting is another modality used for dystrophin analysis; however work on it shows that some important protein epitopes are lost during the long and strenuous process of Western blotting.²² The present study has however shown that IHC using two primary antibodies i.e. β -spectrin and dystrophin (by Novocatra Spec – 1 and Dys – 2) stained well the formalin fixed paraffin embedded (FFPE) muscle biopsy specimens.³² This was in complete agreement with the work performed on formalin fixed paraffin embedded muscle tissue by Sheriffs et al,¹⁶ on the other hand it was in contrast to the work reported by Sachiko et al,²³ who failed to get any immunostaining on formalin fixed paraffin embedded muscle biopsy by the above method.

As the DMD and BMD both are genetic disorders in which the defective gene is localised on the X-chromosome and the disease is transmitted in X-linked recessive manner thereby primarily affecting the males whereas females being the carriers of the disease are only rarely affected.¹² on the other hand, review of literature shows that Walton in 1956²⁴ reported the first female patient of DMD. She was a girl with Turner syndrome (45 XO) probably due to defective dystrophin gene present on the only X-chromosome; later in 1977 two more studies described girls with progressive muscular dystrophy and de novo translocations affecting the X – chromosome short arm.²⁴ From then onwards many studies reported females with X – autosome translocation.^{12,13,24} Addressing this issue that female patients can develop DMD and BMD, the mechanisms for a female to be a patient of DMD / BMD have been postulated by various workers.^{12,13,25,26}

However Benjamin and Becker stated that translocations may allow even the possibility of female presentations of BMD phenotype.²⁷ The increased use of dystrophin analysis on muscle biopsies for molecular diagnosis has uncovered many female patients with no previous family history of any neuromuscular disease to have a mosaic dystrophin immunostaining pattern on muscle biopsy.²⁸ Hence they were diagnosed as patients of dystrophinopathies (DMD / BMD). The staining pattern of dystrophin in them revealed the diagnostic mosaic appearance of dystrophinopathies.^{12,29} The above mentioned literature review supports the findings of the present study in which we diagnosed 13 (32.50%) female patients in a total of 40. Immunohistochemistry in all except one showed the typical mosaic pattern of dystrophin staining on muscle biopsies. Among the thirteen females with mosaic pattern of dystrophin IHC 07 were finally diagnosed as DMD and 05 were diagnosed as BMD patients after correlating the clinical, biochemical, morphological and IHC investigations. The only female who sho-

wed complete presence of dystrophin immunostaining of weak intensity was diagnosed as LGMD.

The present study shows an unusually high number (13 of 40 = 32.50%) of female patients of dystrophinopathies not mentioned in literature from Pakistan. It also signifies the utility and relevance of using dystrophin IHC for a correct diagnosis of various muscular dystrophies. The only one more morphological study reported from Pakistan, was about three decades ago by Ahmad,³⁰ who at that time had no facility for dystrophin marker. Later on there were only a few reports on biochemical changes³¹ and on the genetic aspects of DMD.^{9,26}

The present study, to our knowledge is the first detailed study, consisting of clinical, biochemical, morphological and immunohistochemical details of muscular dystrophies from this country. As there are many venues in muscular dystrophies that need to be explored, from pathogenesis to the management. Some of the future research that may be considered includes : large scale collaborative studies involving epidemiological, immunohistochemical as well as genetic analysis. They are required to find the exact face of muscular dystrophies in Pakistan, especially the details of female patients of DMD and BMD which have not been studied so far in our country; where according to the present study the number of such cases is rather high. In addition the genetic analysis of the mothers of patients to study the type of mutation is required, particularly in sporadic cases. One may also consider IHC using antibodies to different domains of dystrophin and to see their correlation with disease severity and prognosis. In addition the use of other sarcolemmal antibodies including domains of dystrophins, are to be studied for other types of dystrophies.

It was *concluded* from this study that although the routine morphology and some histochemical stains are very helpful in making the diagnosis of muscular dystrophies, the dystrophin IHC along with beta – spectrin control has become a gold standard in establishing the final diagnosis of muscular dystrophies. In addition to being carriers, females formed a significant number among the muscular dystrophy cases.

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