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Dystrophin Immunohistochemistry in Muscular Dystrophies on Formalin Fixed Paraffin Embedded Tissue

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Abstract. Muscle biopsy with proper immunohistochemistry (IHC) is the gold-standard to distinguish muscular dystrophies (deadly degenerative disorders). Immunohistochemistry detects the mutated proteins of muscular dystrophies. Presently IHC is performed on fresh frozen muscle biopsy specimens. This study was performed on 32 children suffering from various types of muscular dystrophies. Among them 71.87% (n = 23) were diagnosed as DMD, whereas 31.25% (n = 10) were diagnosed as BMD and the remaining one case (3.12%) as LGMD. Nine of the 32 patients were females. It shows that IHC can also be performed on formalin fixed paraffin embedded muscle tissues. In this study comprising of 23 males and 9 females, we used paraffin blocks of skeletal muscle tissue and performed IHC using dystrophin and β-spectrin antibodies in the diagnosis of various types of muscular dystrophies. In Pakistan diagnosis of muscular dystrophies is still dependent on clinical features and creatine phosphokinase (CPK) values. This is the first study from the subcontinent performing immunohistochemistry successfully on formaline fixed paraffin embedded (FFPE) blocks for the diagnosis of muscular dystrophies.

Key Words: Ducheme muscular dystrophy, Becker's muscular dystrophy, dystrophin, frozen sections.

INTRODUCTION

The term "muscular dystrophy" traditionally refers to a group of genetically determined, progressive, degenerative disorders of the muscle that occurs during childhood (Shukla *et al*., 2004). 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 (Miura and Jasmin, 2006) .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 (Cohen and Muntoni, 2004; Simon *et al*., 2004). In addition it may be mentioned that although the disease primarily occurs in boys; it can be seen in females who are actually the carriers. When female children get the disease, they show all myopathic symptoms (Emery, 1998). Although genetic testing has revolutionised the evaluation of muscular dystrophies the diagnosis of many is still dependent on muscle biopsy. Muscle biopsy with proper immunohistochemistry (IHC) is the gold-standard and required to definitively distinguish muscular dystrophies (Freund *et al*., 2007; Lovitt *et al*., 2006).

IHC detects the mutated proteins of muscular dystrophies .It is seen that immunohistochemistry is more reliable than the genetic analysis that uses multiplex polymerase chain reaction. IHC is a powerful tool in distinguishing different muscular dystrophies because of its speed, accuracy and the increasing availability of antibodies to dystrophin and its associated proteins (Sheriffs *et al*., 2001). It is even better than EMG that some times gives mixed pattern and carries a low specificity (Shukla *et al*., 2004). Immunohistochemical examination has a major role in further classifying muscular dystrophies into different types, which is indispensable for prognostication and would be even more useful once effective therapeutic modalities are available (Shukla *et al*., 2004).

Presently in majority of the centers, immuno-histochemistry is used on muscle tissue for the diagnosis of muscular dystrophies using fresh frozen tissue section technique (http://www.emedicine.com/neuro/topic230.htm). The fresh frozen tissue needs vigilant expert care using expensive equipment like cryostat, for precise results and to avoid freezing artifacts. These facilities are not available in all the centers specially in developing countries. This limits the use of these valuable and significant investigations and consequently they can be carried

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out only in limited centers. It was therefore a necessity that led us to try these two primary antibodies *i.e*. β-spectrin and dystrophin (Novocastra) on formalin fixed paraffin embedded muscle biopsies in children suffering from muscular dystrophies such as Duchenne, Beckers and Limb Girdle types. Formalin fixation is the most common and inexpensive method of tissue preservation available in all histopathology centers. Unlike fresh frozen section it does not require sophisticated equipment and vigilant care. The paraffin embedded blocks instead are very easy to store for reuse.

MATERIALS AND METHODS

Patients and tissue processing

After getting approval from the Ethical Committee of University of Health Sciences, Lahore, 32 patients with strong clinical suspicion of muscular dystrophy (child, weakness of lower limbs and muscular hypertrophy) were recruited from Pakistan Society of Rehabilitation of the Disabled (PSRD), Lahore. The study was conducted in the Department of Morbid Anatomy & Histopathology, at University of Health Sciences, Lahore. For each patient an open surgical biopsy specimen (1x0.8x1 cm) from the quadriceps muscle was obtained under local anaesthesia. Muscle biopsy was immediately placed on a dry filter paper (Whatman #1) for immobilisation and then placed in 10 % neutral buffered formalin that was used as transport medium as well as preservative for muscle biopsy specimen. After overnight fixation in 10% neutral buffered formalin the muscle biopsies were processed further to get paraffin embedded blocks. This procedure can be applicable on muscle tissues that need to be studied for enzymes and IHC to know the status of sarcolemma. These tissues were also stained with haematoxylin and eosin (H & E); Periodic acid Schiff's reaction (PAS) and Gomori's trichrome stains. In addition to the 32 diseased tissues, 6 biopsies were taken as controls from age matched muscle tissues from children who came for operations for some other diseases.

Immunohistochemistry

For immunohistochemistry from the paraffin blocks, 8 micro meter sections were cut and tissues were taken on charged slides (poly lysine). The slides were dried at 60°C for 50 minutes in a hot air oven. The sections were de-waxed and brought to water. After the three steps in distilled water, peroxidase block was performed, by putting 1 to 2 drops of 3% hydrogen peroxidase block, enough to cover the section on the slides and incubated for 10 minutes. For target retrieval, heat mediated antigen retrieval method was applied. Three target retrieval solutions were used *i.e*. (1) citrate/ EDTA buffer (10mM citric acid/ 2mM EDTA) at pH 6.2. (2) Citrate buffer 10 mM, pH 6.0.and (3) EDTA buffer 1mM at pH 8.0. After antigen retrieval, sections were washed in PBS buffer (pH 7.6) for 5 minutes (3 washings). Primary antibody incubation was carried out using two primary antibodies (Novocastra) *i.e*. Spec-1 and Dys -2 (spectrin antibody and dystrophin antibody) on two separate sections from each block and incubated for two hours. Dilution for anti-Dystrophin was 1:20 and for anti-spectrin was 1:100. After primary incubation, sections were washed with PBS buffer (pH 7.6) for 5 minutes. The method of detection of staining used was two steps streptavidin-biotin-peroxidase method using Novocastra's universal detection kit. In the first step biotinylated link with secondary antibody was applied and incubated for 20 min. Slides were washed in PBS solution for 5 minutes (3 washings).

In the second step 1 to 2 drops of streptavidin peroxidase reagent enough to cover the section on the slides were applied and incubated for 20 min. The slides were washed in PBS solution for 5 minutes (3 washings). Applied 1 to 2 drops substrate chromogen solution (DAB) enough to cover the section on the slides and incubated for 2 to 5 min. Washed thoroughly with running tap water. Counter stained with haematoxylin**.** After dehydration slides were mounted using cover slips and DPX.

RESULTS

Among the 32 cases, 9 (28.12%) were females who showed the clinical, biochemical and morphological features of DMD (n = 5; 55.5%), BMD (n = 3; 33.3%) and LGMD (n = 1; 11.11%). The remaining 23 cases (71.87%) were males. The biopsies were studied to confirm the morphological diagnosis of muscular dystrophy using Haematoxylin

and Eosin, PAS and Gomori's trichrome stains. This was followed by IHC staining. β-spectrin immunostaining was positive in all biopsies and acted as control. It showed membrane staining of skeletal muscle fibers in all the 32 (100%) specimens, thereby ruling out false negative results of dystrophin immunostaining (Fig. 1A.). The normal controls stained with IHC for dystrophin and β-spectrin showed positive stain of the sarcolemmal membrane (Fig. 1B).

The dystrophin on the other hand showed four patterns of immunostaining. There was complete absence of membrane staining in (n=19) 59.37% biopsies (Fig. 1C). In others a broken and interrupted membrane staining in (n=6) 18.75% tissues (Fig. 1D), whereas mosaic pattern of immunostaining (some positive fibers and some negative fibers) was seen in (n=7) 21.87% biopsies (Fig. 1E). Only one (3.12%) specimen showed complete presence of membrane staining but it was of weaker intensity. The final diagnosis was made by correlating the clinical, biochemical, morphological and immunohistochemical findings in each case. In a total of 32 patients (biopsy specimens) (n=23) 71.87% were diagnosed as DMD, whereas (n=10) 31.25% were diagnosed as BMD and the remaining one case (3.12%) was of LGMD. In 32 patients, there were 09 cases in whom final categorisation was performed with the help of dystrophin immunostaining and in them the remaining investigations were insufficient to definitively distinguish between different muscular dystrophies.

DISCUSSION

As shown in the results of this study that among the 32 patients, 9 (28.12%) were females having muscular dystrophies of various types. This incidence of 28.12% is more than those already reported by other workers (Bakel *et al*., 1995; Boyd *et al*., 1986; Feroze and Aslamkhan, 1977; Giacalone and Francke, 1992; Pegoraro *et al*., 1994; Quan *et al*., 1997). The severity of symptoms was similarly variable as in males. This is supported by Quan *et al*. (1997). The initial morphological diagnosis of all the 32 cases includes 9 females, were carried out using routine H a E, PAS, and Masson's trichrome stains. This was followed by staining using IHC technique.

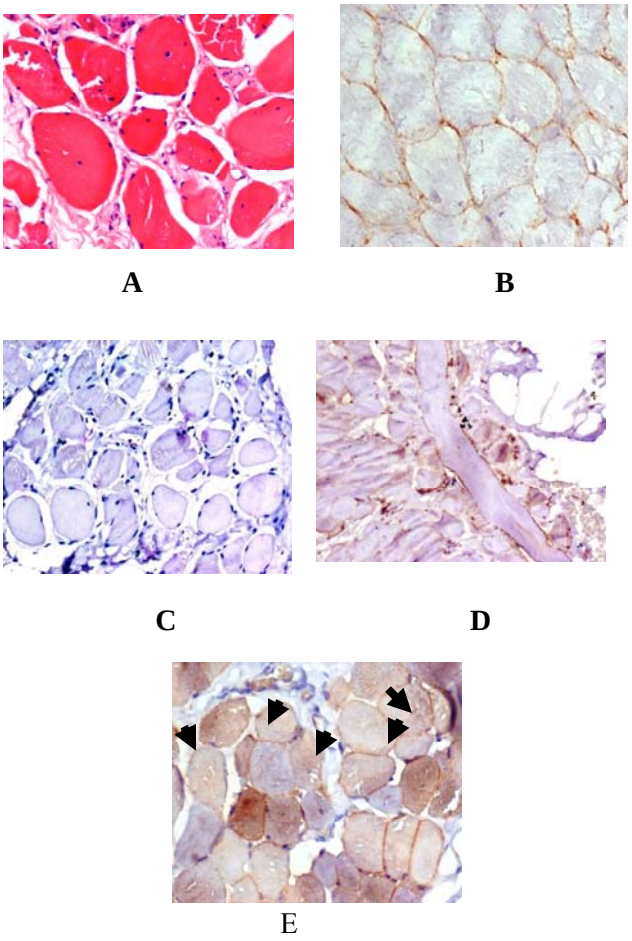

Fig. 1. Histological structure of quadriceps muscles of patients with strong clinical suspicion of muscular dystrophy. A, Muscle fibers showing bulkiness, nuclear internalisation and fibers splitting in a case of DMD. (H & E stained); B, Spectrin immunostaining on formalin fixed paraffin embedded (FFPE) muscle tissue (Positive control); C, Negative dystrophin staining on FFPE muscle biopsy specimen; D, Dystrophin IHC shows many unstained and stained muscle fibers giving a mosaic appearance. (arrow); E, showing the interrupted pattern of dystrophin in a biopsy from a BMD patient (arrow).

Immmunohistochemistry using dystrophin and related antibodies on muscle biopsy is a powerful tool in distinguishing various muscular dystrophies and it has proven better than genetic analysis using multiplex PCR because genetic

analysis may sometimes fail to detect small mutations or unusual mutations of the dystrophin gene (Cohen and Muntoni, 2004; Bridges, 2006). At present in majority of the centers, immunohistochemistry is used on muscle tissue for the diagnosis of muscular dystrophies on fresh frozen tissue sections (http://www.emedicine.com/neuro/topic230.htm). The present study has however shown that IHC using β-spectrin and dystrophin (Novocastra Spec-1 and Dys -2) were taken up very well on formalin fixed paraffin embedded (FFPE) muscle biopsy specimens. We used β-spectrin (Spec -1 by Novocastra) and dystrophin antibodies because spectrin is localised at the periphery of skeletal muscle fibers like dystrophin and labelling of this protein is thus an excellent control for documenting muscle membrane integrity in the fibers which are negative for dystrophin. This eliminates the chances of false negative dystrophin staining that could be caused by some damage to the muscle membrane during handling or processing of muscle biopsy (Nicholson *et al*., 1993).

This was in complete harmony with only one more study performed so far on formalin fixed paraffin embedded muscle tissue (Sheriffs *et al*., 2001) but was in contrast to the work reported by another group (Hoshino *et al*., 2000) who failed to get any immunostaining on formalin fixed paraffin embedded muscle biopsy sections by using the above method and they proposed CSAS (catalysed signal amplification system) for immunohistochemistry on formalin fixed tissue. The reason of failure to get any immunostaining by them was probably the very short incubation time given by them for the primary and secondary antibodies (15 minutes), whereas we used 2 hours incubation time for primary antibody and almost 50 minutes for steps of detection and got good staining with both the antibodies used.

The stored fresh frozen muscle tissue is prone to freeze drying, rendering them useless for further research whereas this is not the case with formalin fixed tissues that can be reused easily for subsequent research (Sheriffs *et al*., 2001).With the recent increase in the availability of different sarcolemmal antibodies there is a need to carry out retrospective studies on archieval muscle biopsies, which may help in solving the present mysteries about this group of diseases.

It is concluded that the diagnosis of muscular dystrophies can be established by performing immunoperoxidase staining for β-spectrin (as a control) and dystrophin, which is a gold standard for its diagnosis. Using this technique of formalin fixed paraffin sections we have been able to diagnose all our cases correctly. Formalin fixed paraffin embedded tissue has advantages over the fresh frozen tissues and does not require expensive equipment.

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