Iranian Journal of Pathology (2010)5 (1), 2-8

Original Article

The Diagnostic Value of Utrophin in Mild Dystrophinopathy (Becker Muscular Dystrophy)

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ABSTRACT

Background and Objective: **Becker Muscular Dystrophy (BMD) is a subtype of dystrophinopathies and designated as "mild form of dystrophinopathy". The frequency rate of the disease is 1:18000 to 1:30000 in different populations and the symptoms are presented at about 5-6 years of age. The diagnostic panel composed of Serum Ceratin Kinase (SCK) measurement, Electromyography (EMG), and as a major component, muscle biopsy and immunohistochemistry for dystrophines; finally the results should be confirmed by Western blot (WB) analysis, which is a sensitive method for protein detection. The aim of this study was using utrophin, an autosomal homologue for dystrophin, which is upregulated in dystrophinopathies, parallel to WB in order to evaluate its diagnostic value.**

Materials & Methods: **In this case-control study, fifteen clinically suspected cases of BMD were examined from 2006 to 2008. After muscle biopsy and dystrophin IHC, the muscle samples were immunostained for utrophin and the tissue extract were analyzed for protein components.**

Results: **In all of the cases, the results revealed partial staining for utrophin in the sarcolemma and pale or distorted band of dystrophin in WBA.**

Conclusion: **Utrophin immunostain could be considered as an important component of BMD diagnostic panel and may be substituted for WBA, which is an expensive and time-consuming method.**

Keywords: **Utrophin, Dystrophin, Becker Muscular Dystrophy**

Introduction

Becker muscular dystrophy (BMD) is a subtype of muscular dystrophies which like Duchenne Muscular dystrophy (DMD) belongs to the family of dystrophinopathies. The disorders are related to dystrophin protein defect in muscle sarcolemma and are really allelic forms of an unique genetic disease. The pattern of inheritance is X-linked recessive and the frequency rate for BMD is 1/18000 to 1/30000 live births (1). The responsible gene for the disease has been localized on chromosome Xp21, in 1980 (2);

Received: 7 March 2009
Accepted: 17 June 2009
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and the gene product, dystrophin protein, diagnosed as a component of protein complex in muscle membrane, which connect intracellular actin to extracellular matrix (3, 4). The clinical signs of BMD are progressive muscle weakness & atrophy which begin in the age of 5-6 yr without any sensory defects. Serum ceratin kinase (SCK) is always increased about 5 folds and electromyography (EMG) (5) shows myopathic pattern. Since the signs are not specific to the disease and a physician could realize other forms of muscular dystrophies such as limb girdle muscular dystrophies (LGMD) with the same manifestations, definite diagnosis is very important for the patients' management, genetic counseling for the family and prenatal diagnosis.

Although the molecular techniques are useful for the diagnosis, they are not capable to detect the Xp21 mutations in 30-40% of cases, so muscle biopsy has been introduced to play a golden role in the laboratory diagnosis of BMD, if followed by immunohistochemistry of dystrophin on frozen muscle samples which reveals deficit of dystrophin protein in the sarcolemma manifesting by irregular or on/off pattern of immunostain. If such protocol were combined with immunoblothing techniques (5), would have a higher specificity for the diagnosis. In recent years, utrophin protein, an autosomal homologue for dystrophin which composed of n-terminal, central core & C-terminal components (as dystrophin) has been recognized (6), this protein is the product of a dystrophin homologue gene on chromosome 6p42 (7). Utrophin is normally expressed in neuromuscular junction and is in connection with dystrophin related proteins (DRP). Although the exact role of utrophin is not clear, this protein has a similar function as dystrophin and can be functionally replaced in its situation in mdx (dystrophin deficient) mice (8). Recent studies show that administration of adenoviral vectors with zinc finger motifs (ZFP), which are able to activate the utrophin promoter, significantly improve the muscle functions (9). Despite the widespread efforts on the treatment of dystrophinopathies, in Iran we should first focus on the diagnosis. Therefore, the aim of this study was the evaluation of the diagnostic value of utrophin in comparison with western blot (WB) for BMD.

Material and Methods

Fifteen patients with clinical signs of BMD were selected from the referred myopathic patients to Genetics Research Center between the years 2006 & 2008 . After physical exam, drawing pedigree and ethics considerations, which were confirmed by Ethics Committee of Genetics Research Center, blood sample were taken for CK measurement. (In order to remove false positive results of serum ceratin kinase, the patient should not been injected two weeks before the sampling).

All of the patients were screened for Xp21 mutation and the results were negative. Electromyography, which was performed for all of the patients, revealed myopathic pattern. After all of the above steps, becker muscular dystrophy suspected patients were selected for muscle biopsy.

Muscle biopsy was taken from deltoid muscle through longitudinal axis and the sample transported to 10% formaldheyed (Merck KGaA, Darmstadt, Germany), with tissue orientation maintenance, and a few pieces of tissue immediately transferred into previously chilled (-80ºC) Isopanthene (Merck, Darmstadt, Germany) for the performance of immunohistochemistry and WB.

After tissue processing of formalin-fixed specimens and preparing paraffin blocks, the 4-5 µm thickness sections were undergone Hematoxilin (Padtan teb , Iran) & Eosin (Merck, Darmstadt, Germany), staining (step1).

For the frozen tissue, which was stored in -80ºC freezer, tissue sections with 4-5µm thickness were prepared by cryotome (LeicaCM 1850) and the sections were undergone immunostaining for dystrophin 1 (against rod domain), dystrophin 2 (c-terminus) ,dystrophin 3 (n-terminus) (step2) and utrophin antibody (step3) prepared from Novocastra company , Newcastle ,U.K.

Small pieces of frozen muscle were undergone protein extraction as follows: 1 part crashed tissue with 20 parts of extraction buffer [Tris HCl (SIGMA, Steinhem, Germany), DDT (Applichem, Darmstadt, Germany), SDS, Glycerol, Boromophenol blue (Merck, Darmstadt, Germany), were inserted into boiling water for 3min. and then centrifuged [rpm 11000 (eppendorf 5415 D centrifuge) for 3 min.

The supernatants were used for loading. The loaded specimens were undergone electrophoresis and then transferred into membrane once for dystrophin (step4) and then for utrophin (step5) antibodies using BIO-RAD protocols (10, 11).

Results

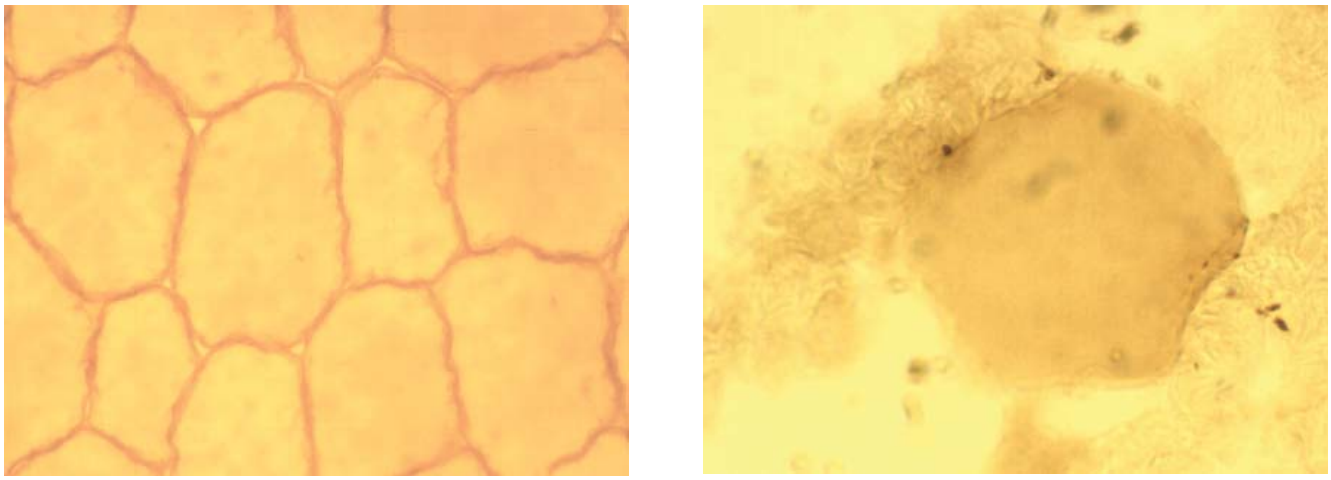
Results of step1- [Hematoxillin & Eosin (H&E) stain on muscle samples]:

The H&E stained slides prepared from paraffin-embedded tissue blocks show the following points in all of the patients with a few differences of severity in individuals as internalized nuclei, fiber splitting, atrophic & angulated fibers.

Positive controls (muscle samples from healthy individuals who were admitted due to non-myopathic causes) and negative controls (muscle samples of DMD patients) were examined at the same time.

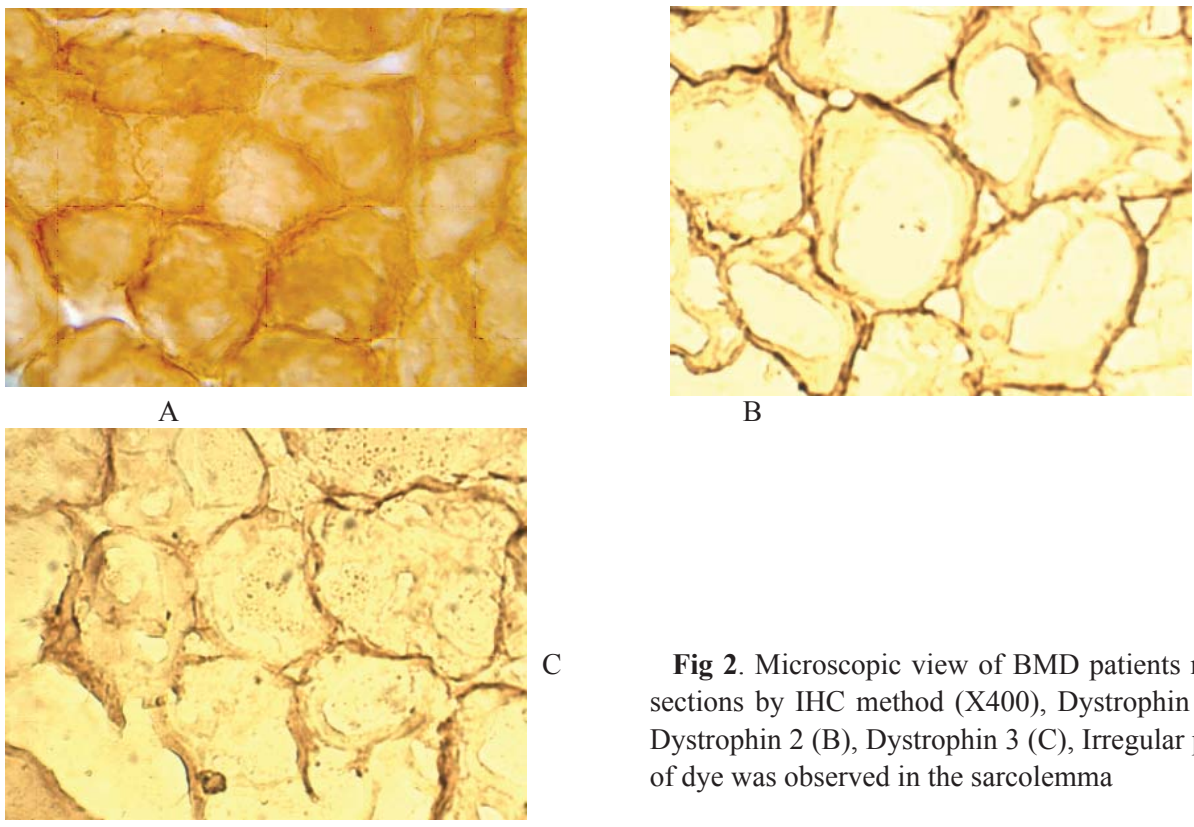
Results of step 2-(Dystrophin immunostaining on patients' muscle samples):

Immunostained muscles were studied in association with positive & negative controls. The positive controls revealed complete rings around the muscle fibers while the negative controls (DMD patients) showed no rings (Fig.1). Spectrin antibody also applied on the sections which despite high homology of this molecule with dystrophin revealed the sarcolemma integrity; all of the subtypes of dystrophin [1-3], showed on & off patterns of staining (Fig.2).



A B

Fig1. Dystrophin staining by Immunohistochemistry method (×400), (A) Normal control, (B) DMD patient muscle sample (complete lack of ring around a single muscle fiber).



A B C

Fig 2. Microscopic view of BMD patients muscle sections by IHC method (X400), Dystrophin 1 (A), Dystrophin 2 (B), Dystrophin 3 (C), Irregular pattern of dye was observed in the sarcolemma

Results of step 3- (Utrophin immunostaining on patients' muscle samples):

Immunostained sections for utrophin revealed the similar pattern but reciprocal with dystrophin (Fig 3). Since utrophin gene is upregulated and utrophin synthesis is increased in dystrophinopathies, these findings have been predictable.

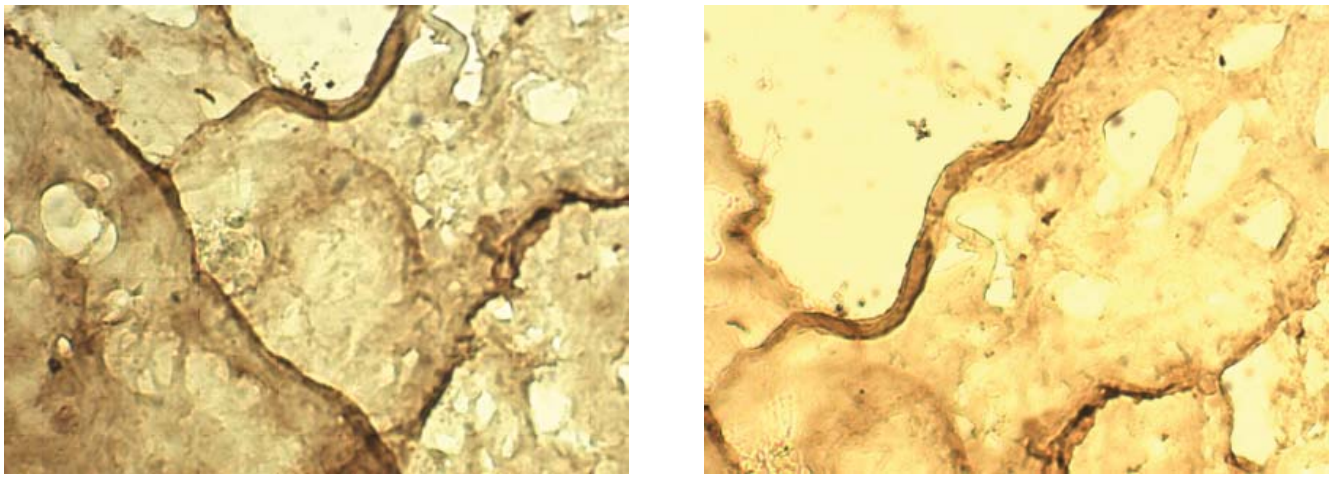


Fig 3. Microscopic view of BMD patients muscle tissue sections by IHC-utrophin immunostain- Irregular pattern of dye was observed in sarcolemma.(×400)

Results of step 4- (western blot analysis for dystrophin):

Muscle extracts were applied for protein analysis. After protein electrophoresis, membrane transferred bands could be visualized (Fig. 4).

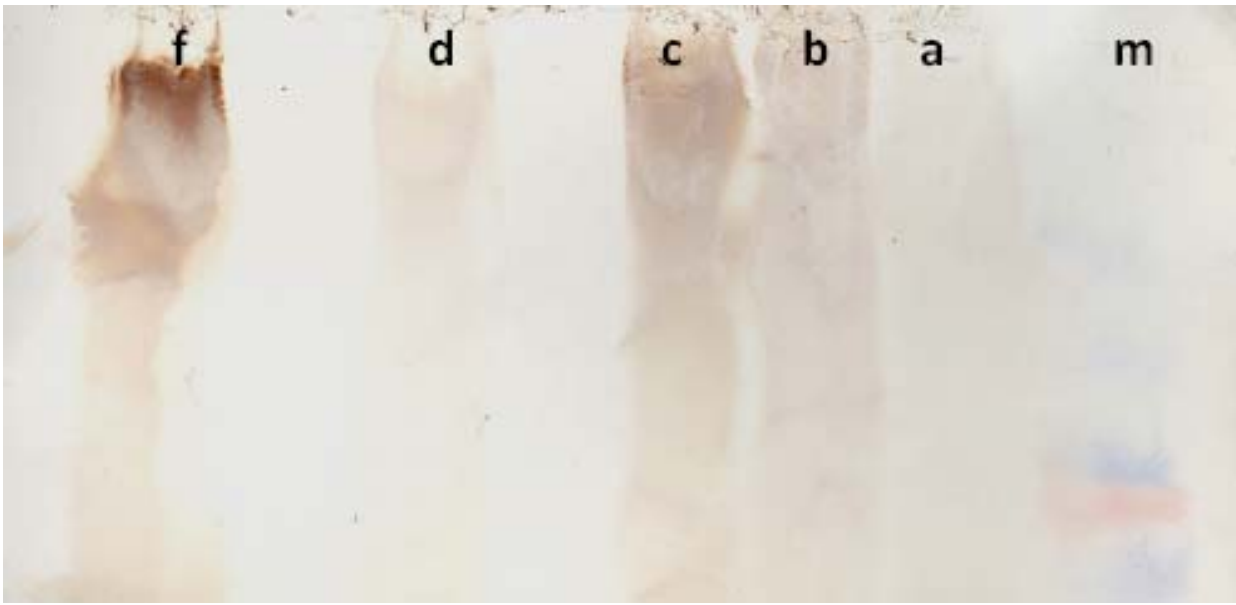


Fig 4. Membrane after transfer (dystrophin Ab), m: marker, a: DMD patient, b, c, d: BMD patients, f: normal control

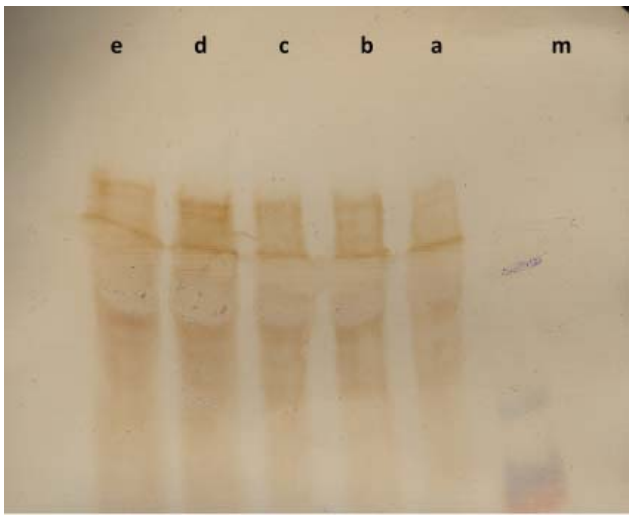


Fig. 5. Membrane after transfer (utrophin Ab), m: marker, a, b, d, e: BMD suspected patients, c: normal control

All of the patients revealed pale bands of dystrophin, which were reliable due to synchronous application of positive and negative controls.

Results of step 5- (Western blot analysis for utrophin):

Tissue extracts were examined for utrophin (Fig. 5). The results of the transfer revealed pale bands of utrophin. Since this protein could be normally found in muscle extract (not from sarcolemmal origin but from neuromuscular junctions and endothelial cells) its presence on gel & membrane could not help for the diagnosis of dystrophinopathic patients.

Totally, in all of the cases the results reveal partial staining for utrophin in the muscle cell membrane and pale or distorted bands of dystrophin in WB.

Discussion

Our study showed that patients who were suspected for "BMD" in the first steps of diagnostic panel composed of (physical exam, SCK measurement, EMG, H&E staining, dystrophin 1, 2, 3 immunostain), were confirmed after tissue immunostaining for utrophin and showing abnormal expression (irregular staining of sarcolemma). These results were compatible with WB results for dystrophin, so utrophin immunostain on frozen tissue samples was as similar as WB for dystrophin in our study.

WB analysis on tissue extract of the patients for utrophin did not have additional data as so could be excluded from the panel.

The results of other studies confirm that utrophin expression is seen in the muscle of dystrophinopathic patients. In a study on 80 dystrophinopathic & other neuromuscular disorders, abnormal expression of utrophin was evident in all dystrophinopathies, while normal control samples and the other muscle disorders did not show utrophin in sarcolemma (12) .Of course this abnormal expression is also evident in inflammatory myopathies in association with abnormal expression of dystrophin. This shows the importance of concomitant using of spectrin antibody for controlling the integrity of sarcolemma. According to the above study, utrophin is very important for tissue interpretation and differential diagnosis of muscle disorders related to dystrophin (12).

Another study reports the diagnostic benefits of using utrophin in association with dystrophin for DMD carriers. In this study, mosaic pattern of utrophin is seen in the carriers with reciprocal evidence of dystrophin (13).

Since there is no exact treatment for dystrophinopathies till now (either for mild or for severe forms); and the only maintenance management such as corticosteroids, physiotheraphy, respiratory supports and surgery of special forms are used; the definite diagnosis for this group of patients is very important (14) for the future possible treatment, and the present study is looking for a way for the definite diagnosis of the Iranian patients through easier and more inexpensive routes.

According to the results of our study, substitution of utrophin immunostaining in place of WB is possible in the diagnostic panel of suspected BMD patients.

In severe forms of dystrophinopathies (DMD) utrophin immunostain is not necessary for the diagnosis, because monoclonal antibodies designed for all three parts of dystrophin molecule (D1, D2, D3) are the most sensitive & specific techniques for the diagnosis and are used when there is no detected mutation in Xp21 which in different studies composed of 30-40% of the patients (15).

The best situation of using monoclonal antibodies as used in our study, is frozen tissue sections, but if it is not possible to store the muscle samples in frozen form (-80ºC) there are a few studies on the results of successful immunostaining on formalin fixed paraffin blocked tissue of course in the presence of "Catalysed Signal Amplification" (16). The latter technique has been also used for Duchenne carriers,

which show weak immunologic binding of antibody to the sarcolemma.

Since dystrophin expression could be normally seen in other tissues (such as normal brain), in mdx mice, utrophin expression has been examined in both muscle & brain and the results showed that utrophin upregulation is only seen in muscle tissue (17).

Because utrophin in normally seen in neuromuscular junctions (NMJ), peripheral nerves, vascular tissue and regenerated fibers, choroid plexus & caudate putamen (18, 19) it seems that using muscle extract for WB analysis could not release additional data for BMD diagnosis. This was shown in our study with the similar utrophin bands in BMD patients and normal controls.

Studies show the competition of the sites of attachment of dystrophin & utrophin, so utrophin upregulation as a cure solution for DMD, severe forms of BMD & Duchenne outliers is highly appreciated (20).

Conclusion

For the definite diagnosis of mild form of dystrophinopathies utrophin immunostaining could be substituted for the expensive and time-consuming technique, western blot analysis. Using this technique in the diagnostic panel of dystrophinopathies, (mild form-BMD) could confirm the diagnosis in order to have the benefit of possible future treatments, genetic counseling, prenatal diagnosis, and carrier detection.

Acknowledgements

The study was supported by Genetics Reseach Center, grant No 801/G/H/201.

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