PROCEEDINGS OF THE BALKAN SCIENTIFIC CONFERENCE OF BIOLOGY IN PLOVDIV (BULGARIA) FROM 19TH TILL 21ST OF MAY 2005 (EDS B. GRUEV, M. NIKOLOVA AND A. DONEV), 2005 (P. 303–314)

DIAGNOSIS OF MUSCULAR DYSTROPHIES BY WESTERN BLOT

Cristina Bordea*, R. Vatasescu, Georgiana Stoica, D. Miscalencu, Anca Dinischiotu, Marieta Costache, Elena Ionica

University of Bucharest, Faculty of Biology, Center of Molecular Biology, Splaiul Independentei, 91-95, Sect 5, Bucharest, Romania, Tel/Fax +4021.411.99.01, Contact person: Elena Ionica: incln @bio.bio.unibuc.ro

ABSTRACT. The aim of our studies is to improve the diagnosis of muscular dystrophies by immunoblot evaluation of sarcolemmal protein expression.

Muscle biopsies of quadriceps muscle were taken from 15 patients, suspected for muscular dystrophies, as part of the routine procedure. Muscle samples from normal control subjects were obtained with consent, from legs amputated. The biopsies samples were frozen in isopentan cooled in liquid nitrogen. Conventional histological techniques (hematoxylin & eosin) and immunohistochemistry were performed on 7μm – transverse cryosections.

In our studies we used Western blot methods with a biphasic polyacrylamide gel system [1]. This method was optimized in order to separate the large proteins, more then 200 kDa (e.g. dystrophin), in the top of the gel, and the small proteins, under 150 kDa (e.g. calpain 3, sarcoglicans) in the bottom phase.

The monoclonal primary antibodies were applied on the same gel and the immunoreactive bands on blot were visualized by using a chromogen method with Western Breeze .

Dystrophin analysis by Western blot revealed a complete lack of this protein in 73,3% (11) of patients which is compatible with Duchenne muscular dystrophy (DMD) diagnosis. In 26,6% (4) of patients is detected an abnormal dystrophin immunolabelling and molecular weight which sustain Becker muscular dystrophy (BMD) diagnosis.

This method is particularly usefull in BMD patients because bands of abnormal size and abundance can be easily detected.

Definitive diagnosis of patients with inherited muscle disorders are essential for the provision of accurate prognostic and genetic counseling.

KEY WORDS. muscular dystrophies; dystrophin;Western blotting.

INTRODUCTION

Striated muscle fibbers interact with a specialized extracellular matrix, the basement membrane, upon which they depend for survival and function [2, 3, 4, 5].

This interaction is mediated primarily by a large oligomeric dystrophin - associated protein (DAP) complex as well as by integrin receptors. Based on their biochemical properties, the DAP complex can be subdivided into three subcomplexes as follows: the dystroglycan complex (α/β) the sarcoglycan (α/β/χ/δ/ε)-sarcospan complex, and the cytoplasmic complex (dystrophin, syntrophin, and dystrobrevin) [6].

Dystrophin associated proteins (DAP) complex has a great significance because it establish a transmembrane link between subsarcolemmal citoskeleton (F-actin) and extracellular matrix (merosin) (7, 8, 9, 10) The presence of these proteins is presumed to be required for the maintenance of the cytoarchitecture of myofibers as well as for their anchorage and viability.

Several of the muscular dystrophies characterized to date, are caused by mutations in the genes encoding for components of the dystrophin–associated complex (DAP) (3,4,5,10). The mutation in the genes of α-, β-, γ-sarcoglycans proteins lead to a group of muscular dystrophies with autosomal recessive transmission called limb girdle muscular dystrophy (LGMD).

In fact, the most prevalent form of muscle disorder, Duchenne muscular dystrophy (DMD), and its milder version Becker muscular dystrophy (BMD) is caused by mutations in the dystrophin gene. Dystrophin is an extremely large protein (427kDa), which in normal skeletal muscle is located to the inner face of sarcolemma, (11,12,13) being absent in Duchenne muscular dystrophy (12,14,15). Becker muscular dystrophy generally, results from an altered (14) or reduced level of dystrophin protein (16,17).The concomitant reduction of the DAP complex with dystrophin in striated muscles of Duchenne muscular dystrophy patients suggests a critical role for the DAP complex in the maintenance of membrane integrity (18,19).

The gene encoding dystrophin is the largest identified in humans, occupying approximately 1% of the X chromosome. It extends over 2300 Kb and comprises 79 exons (20). Approximately 60-70% from the patients which were diagnoses with DMD/DMB have a deletions in the dystrophin gene. The remaining 30% are assumed to results from micro deletions, point mutations or partial duplications (11).

Given the size of the dystrophin gene and the possibility of point mutations (which are not always identified by genetic analysis) diagnosis of dystrophynopathies may be more quickly achieved by using Western blotting. The method presented allow us simultaneously analysis of current muscular dystrophy proteins.

MATERIAL AND METHODS

Patients

Muscle biopsies (usually from gastrocnemius) were taken from patients as a part of the routine diagnostic procedure. Muscle samples from normal control subjects were also obtained, with consent, from legs amputated at the knee (gastrocnemius). The biopsies samples were frozen in isopentan cooled in liquid nitrogen.

Immunohistochemistry

Diagnosis by conventional histological techniques (hematoxylin & eosin) and indirect immunohistochemistry was primarily undertaken in the Department of Neurosciences, Institute of Medicine and Neurosciences, Clinical Hospital Colentina, Bucharest.

Polyacrilamide gel electrophoresis and Western blottting

BioRad TRANS-BLOT SD equipment was used to cast one 16-cm gel, 1,5 thick.

The resolving gel was poured in two phases: the first (bottom phase) of 7% polyacrilamide gel. The second gel of 5,5% was poured after the bottom part polymerize. After the resolving gel was set, the stacking gel of 3% acrylamide was poured above the resolving gel and allowed to polymerize around the comb (20 x 1,5cm).The gel polimerize overnight. The next day the samples were prepared while the gels were setting. Frozen muscle samples (60mg) were quickly weighed and homogenized for 15 minutes in a Poter homogenizer with extraction buffer (0,125mol/L Tris/HCl buffer, pH 6,4, 10%glicerol, 4% SDS, 4mol/L urea, 10% mercaptoethanol, and 0,001% bromphenol blue) pH 6,8. The samples were placed in boiling water for 3 minutes and centrifuged at 8000 x g for 15 minutes before 30µl aliquots of the supernatant were applied to each lane. The gel were run at 200V, one hour in a tank buffer containing 1,44 % glycine, 0,3% Tris, and 0,2% SDS.

After electrophoresis the gel was blotted with a current of a constant amperage calculated with respect to BioRad instructions (mA=L gel x l gel x 3), for 1 hour. We use a nitrocellulose membrane and Towbin buffer as a transfer buffer.

After blotting a part of the gel was fixed in 20% tricloracetic acid, stained in 0,115% Coomasie brilliant blue in 25%ethanol/10%acetic acid and unstained in ethanol: and acid acetic.

Immunolabeling of blots

The non-specific sites from the blot were blocked by incubations in Blocking solutions from the kit (Western breeze kit, Invitrogen), on a rocking table, overnight, at 4°C. The primary mouse monoclonal antibodies were added individual or together in a cocktail (**Table 1**).

After incubation in blocking solution overnight, the blot was washed in Wash solutions for 3 times, 2 min./wash. After the final wash, the blots are incubates with Second antibody solution for 1 hour. After 3 times, 2 min./wash, the immunoreactive bands on the blot were visualized by exposure to Chromogen solutions. The blots can

be scanned and the images stored on optical disc for future densiometric analysis or for illustrations by VILBER LOURMAT SYSTEM (Bio-Profil).

RESULTS

Immunofluorescence analyses

An absence or marked deficiency of dystrophin immunostain was observed in DMD. Immunostain for utrophin is extended from the neuromuscular junction (NMJ) at surface of whole sarcolemma in all the analysed cases (data not show). Glycoproteins of sarcoglycan complex are expressed but sometimes they are lower expressed.

In BMD patients immunofluorescence revealed a variable reduction of dystrophin expression (Fig.2). Expression of utrophin on whole surface of sarcolemma suport diagnosis of Becker muscular dystrophy (data not show).

Western blot analyses

Fig1. shows a blot labelled for the Dys1 and Dys2, which was obtained after western blot analyses of five patients. Comparatively with lane M which illustrates several bands representing proteins of molecular marker weight, samples from normal control subjects, (lanes labeled C), demonstrate a clear separation of the bands representing dystrophin. A single band (427kDa) is detected with the C-terminal antibody, while the antibody to the rod domain typicaly recognises a doublet (427 kDa and 400 kDa) plus lower molecular mass metabolites (11).

In the lanes 1A and 1B we can see a reduction in the amount of normal dystrophin protein. By using rod domain antibody (Fig.1, lane 1A) we obtained a double band, and a single band was detected by using C-terminal antibody (Fig.1, lane 1B). For these bands the signal was at less intensity, which can be associated with a lower amount of protein, comparatively with normal control samples. These data lead to Becker muscular dystrophy diagnosis. Our results were well correlated by immunofluorescence studies which show a variation in dystrophin labelling intensity (Fig.2).

Other two patients diagnosed with Becker muscular dystrophy, showed bands of abnormal size and abundance, because they had a dystrophin molecule of abnormal molecular weight:

- at one patient (Fig.1, lanes 3A and 3B) we obtained a weakly signal (almost undetectable) for the antibody against the rod domain (Fig.1 3A) of dystrophin and a complete absence of immunolabelling (or nondetectable dystrophin) when we used C-terminal antibody (Fig.1, lane 3B).
- at the second patients (Lanes 4A and 4B) of Fig1 is from the patient with Becker muscular dystrophy and demonstrates a double bands of abnormal size when we use the antibody for the rod domain of dystrophin (Fig. lane 4A). A band of abnormal size is also detected with C-terminal antibody (Fig.1, lane 4B).

Each of the patiens with Duchenne muscular dystrophy shows a complete absence of dystrophin labeling on blots (for both rod domain and C-terminus antibodies (Fig.1, Patient 2 lanes 2A ,2B, Patient 5 lanes 5A, 5B).

We examined expression of dystrophin in DMD/BMD patients by Western blot and immunofluorescence studies. The combination of these techniques allowed us to demonstrate a variable dystrophin expresion and the presence of two different dystrophins, normal-sized or reduced-sized in muscle fibers of DMB patients. Dystrophin analysis by Western blot revealed a complete lack of this protein in 73,3% (11) of patients which is compatible with Duchenne muscular dystrophy (DMD) diagnosis. In 26,6% (4) of patients is detected an abnormal dystrophin immunolabelling and molecular weight which sustain Becker muscular dystrophy (BMD) diagnosis (Table 2). Our results obtained by Western blot (Table 2) are confirmed by those from immunofluorescence.

DISCUSSION

Patients for this study was diagnosed as dystrophinopathies (DMD/DMB) by immunohistochemical analysis of dystrophin. Because, this technique was not relevant, for a clear diagnosis, we tried to confirm the diagnosis, using Western blot analyses. The Western blot system is a multiplex in which many of the current muscular dystrophy proteins could be analyzed simultaneously on one pair of blots.

By Western blots, the value of analysing dystrophin of skeletal muscle for differential diagnosis of Xp21 muscular dystrophies, was fairly well establish by Nicholson LV, 1989 (21).

Western blot analysis confirms the diagnosis of Duchenne muscular dystrophy for both cases analysed. Each of the patiens with Becker muscular dystrophy express reduced levels or truncated dystrophin isoforms.

On Western blots of our DMD patients no dystrophin labelling was detected using antibodies against COOH terminal end, and a negative immunolabeling for dystrophin was observed using antibodies against rod domain. These result are in concordance with the dates from the literature (11,22).

The majority of DMD patients have frame-shifting mutations at the dystrophin gene, that could be the result of no dystrophin protein produced beyond the position of the new STOP codon. In fact, complete lack of dystrophin (the absence of dystrophin signal) is patognomonic for DMD. (11,23) although faint bands in various places might be indicate particular mutations (24,25).

By other studies (26,27) Western blot analysis demonstrates that little or no dystrophin protein can be detected using antibodies against COOH-terminal end of the protein, and in some patients, by using antibodies against NH_2-terminal end of the protein could be detected a truncated protein (26,27). It could suppose that dystrophines which lack the C-terminus are unstable and rapidly degraded by the cell.

Dystrophin of normal size and amount is inconsistent with Duchenne muscular dystrophy, and instead implies an autosomal recessive limb girdle muscular dystrophy (28)

In contrast, the majority of BMD mutations maintain the reading frame (in-frame mutations) of the dystrophin transcript, and result in the production of the dystrophin protein which is internally deleted. Indeed, most BMD patients have detectable dystrophin of reduced size on Western blot analysis of muscle biopsy

samples using antibodies against both COOH- and NH2-terminal (26,27,28) Some of BMD patients revealed by Western blot analysis a normal-sized but reduced abundance of dystrophin (11,29,30).

Our BMD patients have detectable dystrophin of reduced size and abundance on Western blot analysis by using antibodies against both COOH- and NH2-terminal. One of three BMD cases had weakly (almost undetectable) bands lower then the bands in the normal control samples when using antibody to the rod domain of dystrophin while a complete absence of immunolabelling was demonstrated by using C-terminus antibody. This patern of dystrophin expression is associated with a severe BMD phenotype. It is known that BMD covers a wide range of presenting symptoms.

The occurence of additional band is not always an underlying genetic cause. These unidentified bands appears to be due to degradation products probably induced by the transport of the samples from Hospital Colentina to our laboratory.

CONCLUSIONS

The results of Western blot were compatible with those from imohistochemistry but difference were also observed, e.g. one patient diagnosis with Becker muscular dystrophy by immunhistochemical analysis shows no dystrophin labeling on blots which is according to Duchenne muscular dystrophy diagnosis.

Dystrophin immunolabeling on blots is particulay useful in BMD patients because bands of abnormal size and abundance are easily detected. Analysis of muscular proteins on Western blot are necessary in directing further genetic analysis.

Definitive diagnosis of patients with inherited muscle disorderes are essential for

The provision of accurate prognostic and genetic counseling (31).

ACKNOWLEDGMENTS

We thank to Dr. Biol. Emilia Manole and Dr. Marilena Alexianu for the muscle biopsy kindly provided to us.We also thank them to the results of immunohistochemical studies which was very important for our successful studies. This work was done using the National Programme of Research and Development, Viasan, grant no 238/2003.

REFERENCES

ANDERSON, L.V.B., K. DAVISON, 1999. Multiplex Western Bloting System for the Analysis of Muscular Dystrophies Proteins. Am. J. Pathol., 154 (4),1017-1022.

MAYNE, R., R.D. SANDERSON, 1985. The extracellular matrix of skeletal muscle. Collagen Relat. Res.,5, 449-468.

CAMPBELL, K.B., 1995. Three muscular dystrophies loss of cytoskeleton extracellular matrix linkage. Cell,80, 675-679.

CAMPBELL, K.P., R.H. CROSBIE , 1996. Utrophin to the rescue. Nature, 384,1996, 308-309.

WEWER, U.M, E.ENGVALL, 1996. Merosin/laminin2 and muscular dystrophy. Neuromuscul. Disord. 6, 409-418

COTE´, P.D., H. MOUKHLES, S. CARBONETTO, 2002. Dystroglycan Is Not Required for Localization of Dystrophin, Syntrophin, and Neuronal Nitric-oxide Synthase at the Sarcolemma but Regulates Integrin α7B Expression and Caveolin-3 Distribution, J. Biol. Chem. Vol. 277, No. 7, 4672–4679

TINSLEY, J.M., D.J. BLAKE, R.A. ZUELLIG, K C.A., SERNETTS.W., CAMPBELL K.P.,.E. DAVIES, 1994. Increasing complexity of the dystrophin associated complex. Proc.Natl.Acad. Sci. USA, 91, 8307-8313.

IBRAGHIMOV-BERSKROVNAYA, O., ERVASTI J.M., LEVEILLE C.J SLAUGHTER, 1992. Primary structure of dystrophin–associated glycoproteins linking dystrophin to the extracellular matrix, Nature, 355, 696-702.

ERVAST,I J.M., CAMPBELL, K.P., 1991. Membrane Organization of the Dystrophin Glycoprotein Complex, Cell, 66, 1121-1131.

VACHON, P.H., HONG XU, LING LIU, FROSTY LOECHEL,YUKIKO HAYASHI, KIICHI ARAHATA, JOHN C. REED, ULLA M. WEWER, EVA ENGVALL, 1997 Integrins (*a7b*1) in Muscle Function and Survival Disrupted Expression in Merosin-deficient Congenital Muscular Dystrophy, J. Clin. Invest., Volume 100, Number 7, October, 1870–1881

ANDERSON, L.V.B., 2002. Diseases associated with sarcolemmal and extracellular matrix defects, in Structural and Molecular Basisi of Skeletal Muscle Diseases, Ed By G. Carpati, International Society of neuropathology

HOFFMAN, E.P., BROWN, R.H.J., KUNKEL, L.M., 1987. Dystrophin: The protein product of the Duchenne muscukar dystrophy locus, Cell, 51, 919-928.

ZUBRZYCKA-GAARN, E.E., BULMAN, D.E., KARPATI, G., BURGHES, A.H.M., BELFALL B., KLAMUT, H.J. TALBOT, J., WORTON, R.G., 1988. The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. Nature, 333, 466-469.

HOFFMAN, E.P., FISCHBECK, K.H., BROWN, R.H., JOHNSON, M., MEDORI, R., LOIKE, J.D., HARRIS, J.B., WATERSTON, R., BROOKE, M., SPECHT, L., KUPSKY, W., CHAMBERLAIN, J., CASKEY, CT., SHAPIRO, F., KUNKEL, L.M., 1988. Dystrophin characterization in the muscle biopsies from Duchenne and Becker muscular patients. New.Engl.J. Med., 318, 1363-1368.

INFANTE, J.P., HUSZAGH, V.A., 1999. Mechanisms of resistance to pathogenesis in muscular dystrophies. Mol.Cell. Biochem.,195, 155-167.

BLAKE, D.J., TINSLEY, J.M., DAVIES, K.E., 1996. Utrophin: A Structural and Functional Comparision to Dystrophin. Brain Pathol., 6, 37-47.

ARAHATA, K, ISHIURA, S., TSUNEO, I., TSUKAHARA, T., SUHARA, Y, EGUCHI, C., ISHIHARA, T, NONAKA, I, OZAWA, `E., HIDEO, S., 1988. Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy peptide, Nature,333, 861-863.

P.H. VACHON, HONG XU, LING LIU, FROSTY LOECHEL,YUKIKO HAYASHI, KIICHI ARAHATA, JOHN C. REED, ULLA M. WEWER, EVA ENGVALL, 1997. Integrins (*a7b*1) in Muscle Function and Survival Disrupted Expression in Merosin-deficient Congenital Muscular Dystrophy, J. Clin. Invest., Volume 100, Number 7, October, 1870–1881

WEWER, U.M., M.E. DURKIN, X. ZHANG, H. LAURSE, N.H. NIELSEN J., TOWFIGHI, E. ENGVALL, R. ALBRECHTSEN, 1995. Laminin β2 chain and adhalin deficiency in the skeletal muscle of Walker-Warburg syndrome, Neurology, 45, 2099-2101.

NOBILE, C., MARCHI, J., NIGRO, V., ROBERTS, R.G., DANIELI, G.A., 1997. Exon-intron organization of the human dystrophin gene. Genomics, 45, 421-424.

NICHOLSON, L.V., DAVISON, K., FALKOUS, G., HARWOOD, C., O' DONNELL, E., SLATERE, C.R., HARRIS, J.B., 1989. Dystrophin, in skeletal muscle.I. Western blot analysis using monoclonal antibody J.Neurol. Sci. 94(1-3):125-36).

SADOULET-PUCCIO, H.M., KUNKEL, L.M., 1996. Dystrophin and its isoforms. Brain Pathol., 6, 25-35.

VOIT, T., STUETTGEN, P., CREMER. M., GOEBEL. H.H., 1991. Dystrophin as a diagnostic marker in Duchenne and Becker muscular dystrophy. (Correlation of immunofluorescence and western blot). Neuropediatrics, 22(3):152-6).

BONEMANN. A., ANDERSON. L.V., 2000. Diagnostic protein expression in human muscle biopsies. Brain Pathol., 10, 193-214.

BULMAN. D.E., GANGOPADHYAY. S.B., BEBCHUCK. K.G., WORTON. R.G., RAY. P.N., 1991. Point mutations in the human dystrophin gene: identificatio through western blot analysis. Genomics, 10, 457-461.

BULMAN. D.E., MURPHY. E.G., ZUBRZYCKA-GAARN. E.E., WORTON. R.G., RAY. P.N., 1991. Differentiation of Duchenne and becker muscular dystrophy phenotypes with amino- and carboxy-termiinal antisera specific for dystrophin. Am.J. Hum. Genet., 48, 295-304.

HOFMAN. E.P., KUNKEL. L.M., ANGELINI. C., CLARKE. A., JOHNSON. M., HARRIS, J.B., 1989. Improved diagnosis of becker muscular dystrophy by dystrophin testing. Neurology, 39, 1011-7.

HOFFMAN, E.P., BRAIN PATHOL, 1996. 6, 49-61 Clinical and Histopathological Features of Abnormalities of the Dystrophin-Based Membrane Cytoskeleton.

CHEVRON, M.P., TUFFERY, S., ECHENNE, B., DEMAILLE, J., CLAUSTRES, M., BECKER, 1992. Muscular dystrophy: demonstration of the carrier status of a female by immunoblotting and immunostaining. Neuromusc Disord, 2(1):47-50.

MONGINI, T., DORRIGUZZI, C., PALMUCCI, L., CHIADO-PIAT, L., 1996. Quantitative and qualitative alterations of dystrophin are expressed in muscle cell cultures of Xp21 muscular dystrophy patients (Duchenne and Becker type). Eur.J.Clin. Invest., 26(4):322-4)

COOPER, S.T., HARRIET, P LO., KATHRYN N. NORTH, 2003. Single section Western blot. Improving the molecular diagnosis of the muscular dystrophies. Neurology, 61, 1-.3.

Table 1. Antibodies, Dilutions

Mouse primary antibodies	Secondary antibodies	Chromogen
Dys 1 (Dystrophin rod domain) 1:250 Novocastra Lab., Newcastle, UK	Western breeze (invitrogen)	Western breeze (invitrogen)
Dys 2 (Distrophin terminus) Novocastra Lab., Newcastle, UK	Western breeze (invitrogen)	Western breeze (invitrogen)

Table 2. The results of Western blot analyses DMD-Duchenne muscular dystrophy; DMB-Becker muscular dystrophy; ''-'' absence of immunolabeling for dystrophin; Dys1-antibody against rod domain of dystrophin; Dys2 –antibody against C-terminal end of dystrophin

No:	Disease	Dys 1	Dys 2
1	DMD	-	-
2	DMD	-	-
3	DMD	-	-
4	DMD	-	-
5	DMD	-	-
6	DMD	-	-
7	DMD	-	-
8	DMD	-	-
9	DMD	-	-
10	DMD	-	-
11	DMD	-	-
12	DMB	reduced	reduced
13	DMB	truncated	truncated
14	DMB	reduced, truncated	-
15	DMB	reduced	reduced

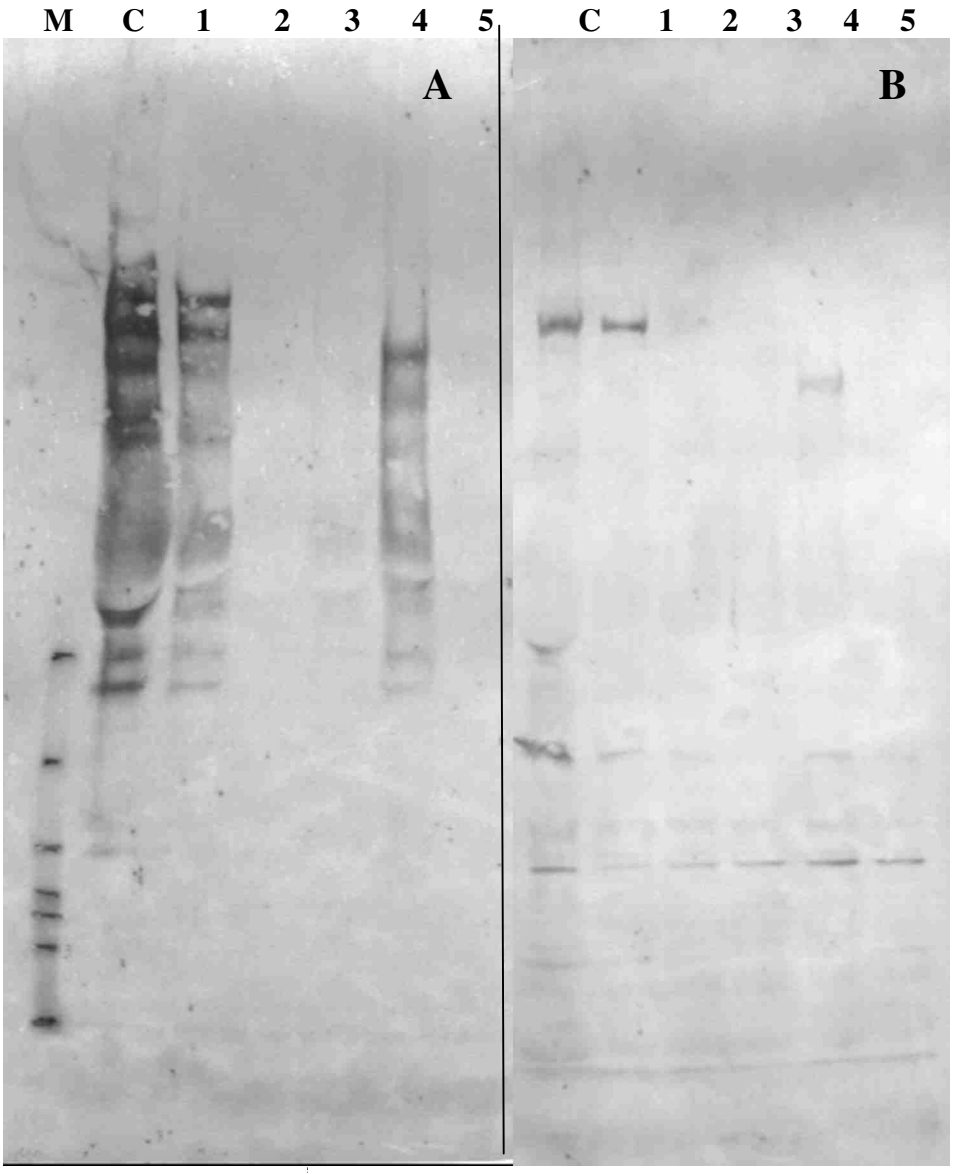
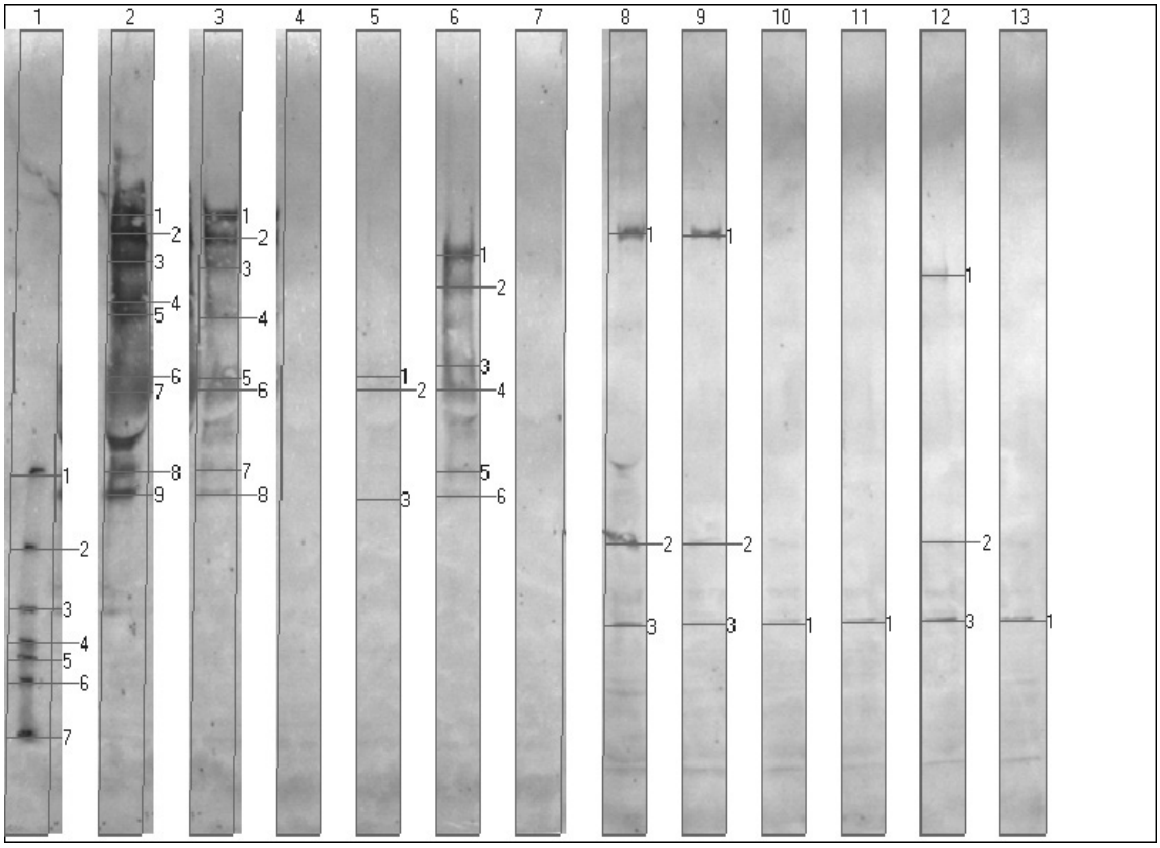


Fig. 1. Immunoanalysis of muscle homogenates from five patients with DMD/DMB
A. *Blot labeled with antibodies to rod domain of dystrophin (400kDa)M-Molecular marker weight; C-normal control; lane 1-BMD, reduced dystrophin labelling; lane 2-DMD; lane 3-severe BMD, dystrophin immunolabeling almost undetectable ;lane 4-BMD, truncated dystrophin; lane 5-DMD* ***B.*** *Blot labeled with antibodies to carboxy terminus of dystrophin (400kDa) C-normal control; lane 1-BMD, reduced dystrophin labelling; lane 2-DMD; lane 3-severe BMD, absence of immunolabelling ;lane 4-BMD, truncated dystrophin; lane 5-DMD. Note that the complete loss of all bands in lane 2 StripA is evidence that the multitude bands seen in control lanes are dystrophin metabolites and not product of other genes.*



	MW-RF										
	L1	L2	L3	L5	L6	L8	L9	L10	L11	L12	L13
1	483.000	506.140	506.140	309.395	458.030	433.116	481.023	57.446	58.013	432.884	53.559
2	98.000	483.116	478.930	292.651	418.233	117.770	107.770			104.434	
3	62.000	440.020	441.250	155.191	321.053	50.054	57.440			50.553	
4	49.000	399.395	380.558		292.651						
5	38.000	384.744	307.302		192.136						
6	28.000	309.395	292.651		159.733						
7	17.000	290.558	194.279								
8		192.186	162.096								
9		162.096									

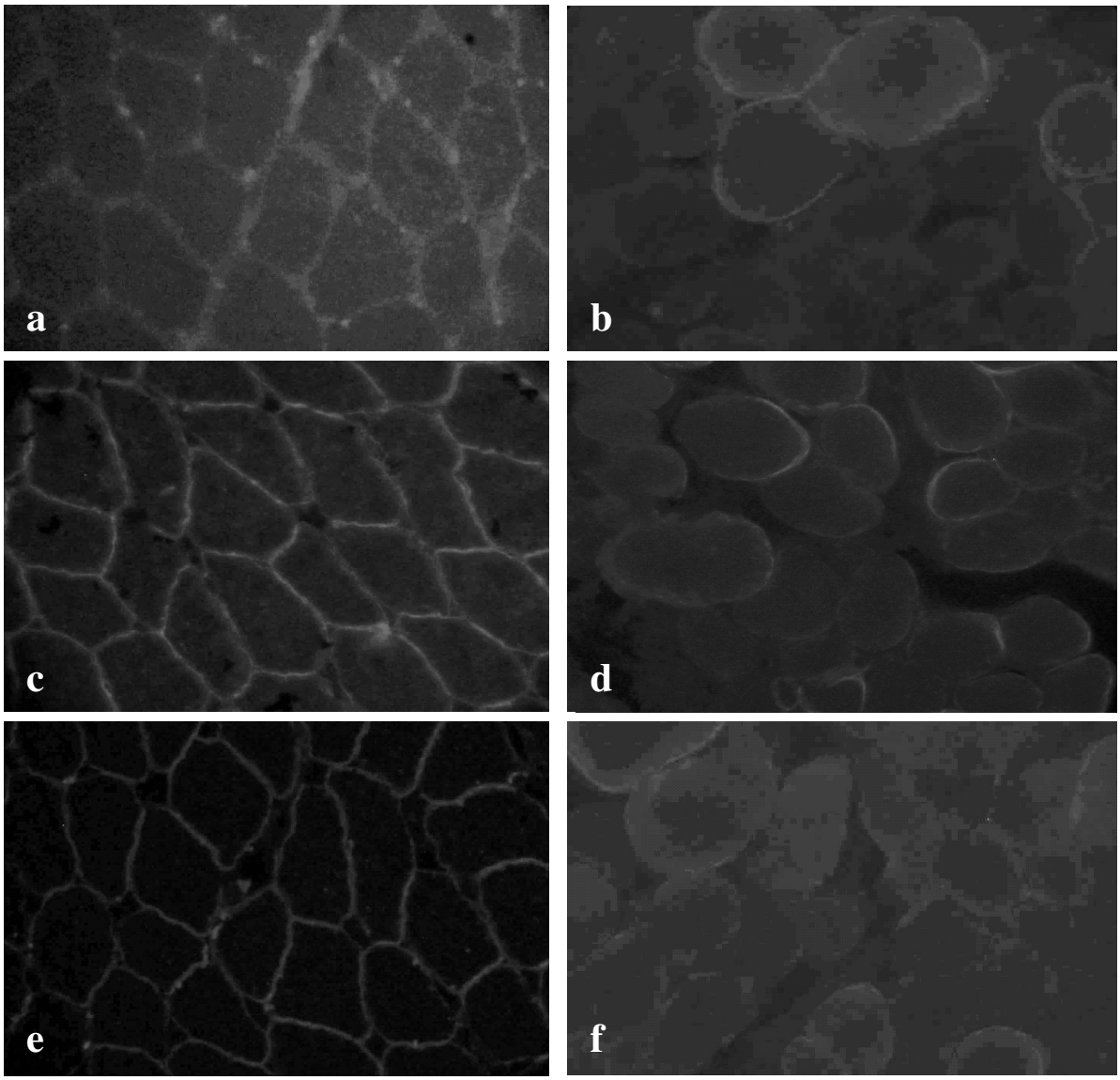


Fig 2. ***Immunofluorescence analysis of dystrophin expression in one patient with DMD missdiagnosed as DMB by immmunofluorescence and unaffected patient control. a****. Control for Dys1;* ***b.*** *Dys1 marked reduction of expression comparative with Control;* ***c.*** *Control for Dys2;* ***d.*** *marked reduction of expression comparative with Control* ***e.*** *Control for Dys3;* ***f.*** *very low immunolabeling for Dys 3 comparative with Control*