Research Article • DOI: 10.2478/v10134-010-0034-7 • Translational Neuroscience • 1(3) • 2010 • 233-237

Translational Neuroscience

VERSITA

EXON-SPECIFIC DYSTROPHIN ANTIBODIES FOR STUDIES OF DUCHENNE MUSCULAR DYSTROPHY

Le Thanh Lam[1],
Giang H. Nguyen[1,*],
Nguyen thi Man[1],
Caroline A. Sewry [1,2],
Glenn E. Morris [1,3]

[1]*Wolfson Centre for Inherited Neuromuscular Disease, RJAH Orthopaedic Hospital, Oswestry, SY10 7AG, UK*

[2]*Dubowitz Neuromuscular Centre, Institute of Child Health, 30 Guilford Street London, WC1 1EH*

[3]*Institute of Science and Technology in Medicine, Keele University, ST4 7QB, UK*

**Present address: Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC 27709, U.S.A*

The first three authors contributed equally to this study.

Received 11 August 2010
accepted 15 September 2010

Abstract

Exon-specific anti-dystrophin antibodies are used to monitor the success of treatments for Duchenne muscular dystrophy that aim to restore the missing dystrophin protein. Dystrophin is a large cytoskeletal protein encoded by 79 exons and expressed mainly in muscle. Most cases of Duchenne and Becker muscular dystrophies are caused by genetic deletion of one or more exons. In-frame deletions permit some synthesis of internally-deleted dystrophin and cause the milder Becker form, while out-of-frame deletions in the severe Duchenne form result in early stop-codons and no functional dystrophin synthesis. In this study, we describe the production of ten new monoclonal antibodies against a rod region encoded by exons 55-59 and their mapping to specific dystrophin exons, thus filling a major gap in the spectrum of available antibodies. The antibodies have already been applied in a published clinical trial of a drug treatment for Duchenne muscular dystrophy.

Keywords

Dystrophin • Muscular dystrophy • Monoclonal antibody • Epitope mapping • Gene therapy • Cell therapy

1. Introduction

The aim of this study, which has been in progress for over ten years, has been to produce monoclonal antibodies (mAbs) that recognise specific amino-acid sequences spread throughout the dystrophin molecule (3,684 amino-acids). The 150 mAbs that we have produced so far recognise sequences encoded by 34 of the 79 exons of dystrophin, ranging from exon 1 to exon 77. We have paid particular attention to the exons that are commonly deleted in Duchenne muscular dystrophy and have shown that they can be used to characterise mutant Becker dystrophins at the protein level in muscle biopsies [1]. The antibodies have been used to characterise the various short forms of dystrophin, Dp71 [2-4], Dp116, Dp140 [5] and Dp260 [6], and were often used in the first descriptions of these short forms. One of the most important applications of the mAbs has been in the characterisation of revertant fibres in Duchenne patients [7] and mdx mice [8]. The dystrophin in revertant fibres always has missing exons (in-frame deletions) whereas dystrophin supplied in gene or cell therapy trials is usually full-length. Since some early apparent successes with cell therapy were shown to be mistakenly due to revertant fibres [9], the mAbs have become almost essential for demonstrating successful dystrophin replacement. For example, there is no way that a Duchenne patient with an exon 45 deletion can produce any form of dystrophin, revertant or otherwise, containing exon 45. MAbs specific for exon 45 can therefore be used to identify unequivocally successful dystrophin expression in muscle biopsies from treated patients. A good example of this is the evidence that dystrophin-positive fibres in a Duchenne patient can arise from stem cells in bone marrow transplants given several years earlier [10].

2. Experimental Procedures

2.1 Recombinant proteins

A fragment of human dystrophin cDNA that included the whole of exons 52 to 59 inclusive was amplified from a plasmid construct containing full-length dystrophin cDNA (pDMD1, kindly provided by Prof. George Dickson, University of London) using PCR primers 5′-gcggatcctctggtgacacaacctgtgg and 5′-gcttcgaaatacggtgagagctgaatgcc. The PCR product (1714 bp) was purified (QIAquick, Qiagen) and digested with BamHI and HindIII to give a 1256bp product. This was ligated with T4 ligase into pET21c plasmid that had been digested with the same restriction enzymes and treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli* strain BL21(DE3) made competent by heat-shock. Ampicillin-resistant colonies were screened by PCR, using primers within the pET21 sequence, for a PCR product of the expected size. Positive colonies were further screened by induction with IPTG and analysis of recombinant protein expression on Coomassie Blue-stained SDS-PAGE gels. After transformation of *E. coli* BL21(DE3) and induction with IPTG, bacterial pellets were washed by sonication in TNE buffer and recombinant protein was extracted by sequential extraction with 2M, 4M, 6M and 8M urea in PBS.

* *E-mail: glenn.morris@rjah.nhs.uk*

2.2 Subcloning for epitope mapping

Overlapping dystrophin protein fragments were generated by PCR of dystrophin cDNA exons 52-55, 52-56, 52-57, 52-58, 54-59, 55-59, 56-59 and 57-59, using the following primers (restriction sites in boldface):

Exon 55 reverse cg**ctcgag**ttcaccttggaggtcttgc
Exon 56 reverse cg**ctcgag**actggtcagaactggcttcc
Exon 56 for gg**gaattc**taaaagagctgatgaaacaatg
Exon 57 forward cg**gaattc**ttaggtcccatttggaagcc
Exon 54 for gg**gaattc**tcacagaaaccaagcagttgg
Exon 55 for cg**gaattc**cttcataaaagggtgagtgagcg
Exon 57 reverse cc**ctcgag**caattccctcttgaaggccc
Exon 58 reverse gg**ctcgag**aagccgagtgacattctgg.

The PCR products were purified (QIAquick, Qiagen) and digested with EcoRI and XhoI for ligation with T4 ligase into pET21c plasmid digested with the same restriction enzymes and treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli* strain BL21(DE3) for dystrophin protein fragment expression. For mapping purposes, the total SDS extract from induced *E. coli* cells can be applied to the gel, without the need for protein purification.

2.3 Hybridoma production

We have described the method in great detail elsewhere [11]. Sp2/O myeloma cells were fused with the spleen cells from a single mouse hyperimmunized with the partially-purified recombinant fragment of the dystrophin helical rod region encoded by exons 52-59. Culture supernatants were screened by ELISA for reaction with the recombinant immunogen and then by western blotting and immunohistochemistry of human muscle before selecting hybridomas for two rounds of limiting dilution cloning to establish permanent hybridoma cell lines. All monoclonal antibodies produced in the present study were of the IgG1 subtype.

2.4 Western blotting

SDS-PAGE on 15% polyacrylamide gels and Western blotting on nitrocellulose membranes were carried out essentially as described elsewhere [12]. Antibody reacting bands were visualized following development with peroxidase-labelled horse anti-[mouse Ig] (1/1000; Vector Labs) and diaminobenzidine as substrate.

2.5 Immunohistochemistry

Culture supernatants containing monoclonal antibodies were diluted 1:3 in PBS and incubated on 5 mm frozen human muscle sections for 1 hour at 25oC. Primary antibody was then removed by washing four times with PBS. Sections were then incubated with 5µg/ml goat anti-mouse ALEXA 488 (Molecular Probes, Eugene, Oregon, USA) secondary antibody diluted in PBS containing 1% horse serum, 1% fetal bovine serum and 0.1% BSA, for 1 hour before washing and mounting in Hydromount (Merck). Images of muscle sections were obtained with Leica fluorescence photomicroscope with 40x objective.

3. Results

3.1 Ten new mAbs against dystrophin encoded by exons 55/56, 58 and 59 and their characterization by epitope mapping

Ten mAbs were produced by fusing Sp2/O myeloma cells with the spleen cells from a single mouse hyperimmunized with a partially-purified recombinant fragment of the dystrophin helical rod region encoded by exons 52-59.

The mAbs were mapped using PCR-generated subfragments also expressed in *E. coli*. Figure 1a shows a stained gel of the original immunogen and 8 sub-fragments produced from it. Identical western blots were developed using each of the ten mAbs separately. Four mAbs gave the staining pattern shown in Figure 1b and this shows

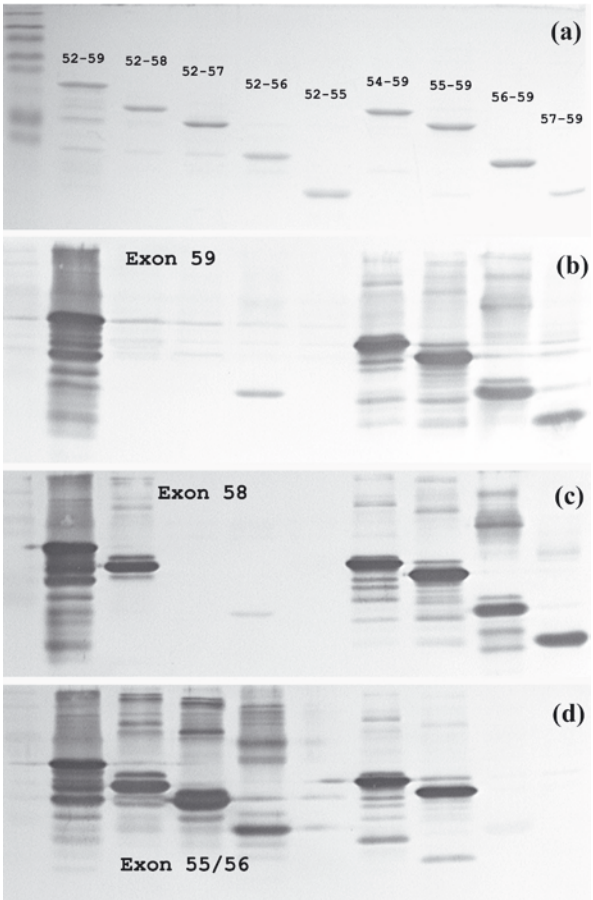

Figure 1. Epitope mapping using PCR-generated protein fragments. Recombinant fragments were separated on 15% acrylamide gels and either (a) stained with Coomassie Blue or (b-d) transferred to western blots and developed with each mAb. The pattern shown by (b) 4 mAbs that recognise exon 58 or (c) 4 mAbs that recognise exon 59 or (d) 2 mAbs that recognise exons 55/56 is illustrated (see Table 1).

that they require exon 59-encoded dystrophin sequences for binding. Similarly, another four mAb required exon 58 for binding, since they recognize the exon 52-58 fragment, but not exons 52-57 (Figure 1c). The last two mAbs gave more ambiguous mapping data, as shown in Figure 1d. They failed to bind exons 52-55 (lane 6), indicating a requirement for exon 56, but also failed to bind exons 56-59, indicating a requirement for exon 55. One interpretation of this result is that the epitope recognised by these mAbs includes dystrophin sequence from both exons 55 and 56, possibly at the exon-exon junction. Caution is required in using these mAbs since a positive result may indicate that either or both exons 55 and 56 are present, whereas a negative result would indicate that either or both are absent. The mapping results are summarised in Table 1.

3.2 All mAbs recognise endogenous dystrophin protein both on western blots of human muscle extracts and on immunohistochemistry of human muscle sections

To be of any translational value for clinical trials of treatments for muscular dystrophy, it is essential that anti-dystrophin mAbs work well on both western blots and especially on human muscle biopsy sections. This was ensured by the hybridoma screening process in which only those mAbs that worked in both applications were selected for cloning. However, the intensity of labelling and its specificity may vary between individual mAbs. Figure 2 shows that most of the ten mAbs give clear and intense staining of the sarcolemma in human muscle biopsy sections, indicating high specificity for the muscle membrane protein, dystrophin. On western blots, all ten mAbs detected a dystrophin band at 427kD in SDS extracts of human skeletal muscle (Figure 3). Many of the lower Mr bands were recognised by nearly all of the mAbs, suggesting that they may be degradation products of dystrophin which lack the N-terminus. We have shown elsewhere that dystrophin in vivo is degraded progressively from its N-terminus [13]. However, the possibility that one of the minor bands might be Dp116 has not been ruled out. These results are summarized in Table 2.

Table 1. Epitope mapping using PCR-generated sub-fragments.

Fragment \ mAb	EX58	EX59	EX 55-56
Ex54-59	+	+	+
Ex55-59	+	+	+
Ex56-59	+	+	-
Ex57-59	+	+	-
Ex54-55	-	-	-
Ex54-56	-	-	+
Ex54-57	-	-	+
Ex54-58	+	-	+

The theoretical patterns of binding to sub-fragments used in Figure 1 are shown and they match the actual binding patterns for 3 groups of mAb.

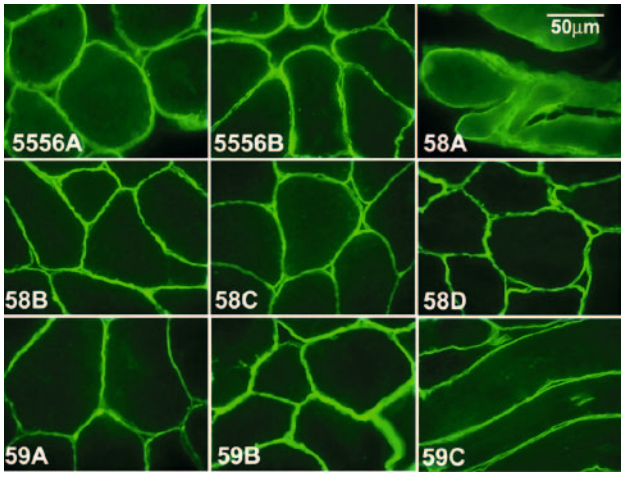

Figure 2. All mAbs recognise dystrophin at the sarcolemma of human skeletal muscle fibres. MANEX59D (not illustrated) gave a similar pattern.

4. Discussion

Several different approaches have been used in the attempt to replace the missing dystrophin protein in Duchenne muscle. Attempts have been made to introduce normal muscle-cell precursors into Duchenne muscle (myoblast cell therapy) [14]. Bone-marrow-derived stem cell transplantation can also give rise to dystrophin-positive fibres in Duchenne muscle [10]. Viral vectors or DNA alone have been used to introduce normal dystrophin-encoding DNA permanently into muscle cells (gene therapy) [15]. Drugs that cause "read-through" of nonsense mutations may be effective in those few cases with this type of mutation [16]. Oligonucleotide-based drugs have been used to cause targeted exon-skipping that restores the reading-frame when the dystrophin mRNA is produced from the primary RNA transcript [17]. Many of these approaches have potential problems with effective delivery and with the immune response (against cells, DNA and even dystrophin itself) [18]. In some, the aim is to "convert" the Duchenne phenotype into a Becker, rather than to affect a complete cure. Finally, drugs that further upregulate utrophin, the non-muscle homologue of dystrophin, in Duchenne muscle may have a beneficial

effect [19, 20], although it is unclear whether utrophin can effectively replace all dystrophin functions.

To assess whether any of these treatments has been effective in clinical trials, it is necessary to use antibodies against dystrophin to determine whether dystrophin has been produced during the therapy. Of course, improved muscle function is the ultimate aim, but it is important to confirm that such improvement is due to new dystrophin production, or, if no improvement was observed, to determine whether new dystrophin is present, though ineffective. Where treatments are performed by local injection, it is usual to take a muscle biopsy near the site of injection and to determine its dystrophin status by immunocytochemistry. This technique can detect even a small number of dystrophin-positive fibres in a biopsy, whereas western blotting may only work well when the proportion of positive fibres is quite high.

The production of ten new monoclonal antibodies against a rod region encoded by exons 55-59 has filled a major gap in the spectrum of available dystrophin antibodies (Figure 4). The ideal mAbs for immunohistochemistry are MANEX5556B, MANEX58B and MANEX59B (Table 2). The antibodies have already been used in a clinical trial of the read-through drug,

Table 2. All ten mAbs work in both western blots and immunohistochemistry.

Antibody	Clone	IMF	WB
MANEX5556A	2F8	+	+
MANEX5556B	2G12	++	+
MANEX58A	2D9	+	+
MANEX58B	1A5	+++	+
MANEX58C	2D4	++	+
MANEX58D	7G2	++	+
MANEX59A	3H9	++	+
MANEX59B	4E10	+++	++
MANEX59C	1H5	++	+
MANEX59D	1G8	++	+

The results from Figures 2 and 3 are summarised, with an indication of relative levels of staining intensity.

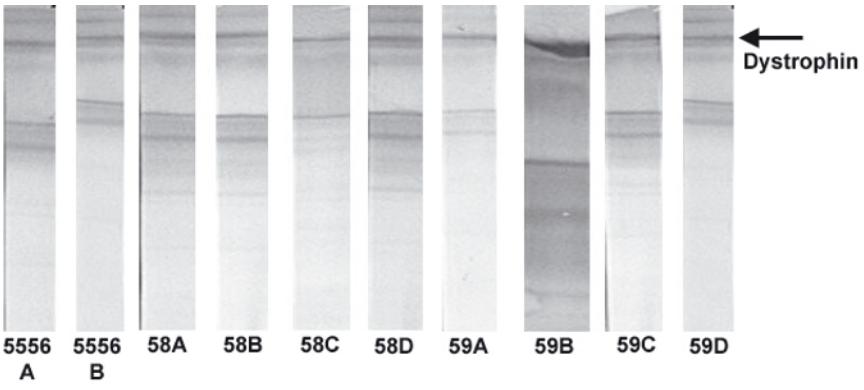

Figure 3. All mAbs recognise dystrophin on western blots.
A total protein extract of human skeletal muscle was separated on a 3-15% acrylamide gradient gel, transferred to nitrocellulose and cut into strips for reaction with different mAbs. The position of the dystrophin band at 427kD was identified using an established mAb against dystrophin (MANDYS1, not shown).

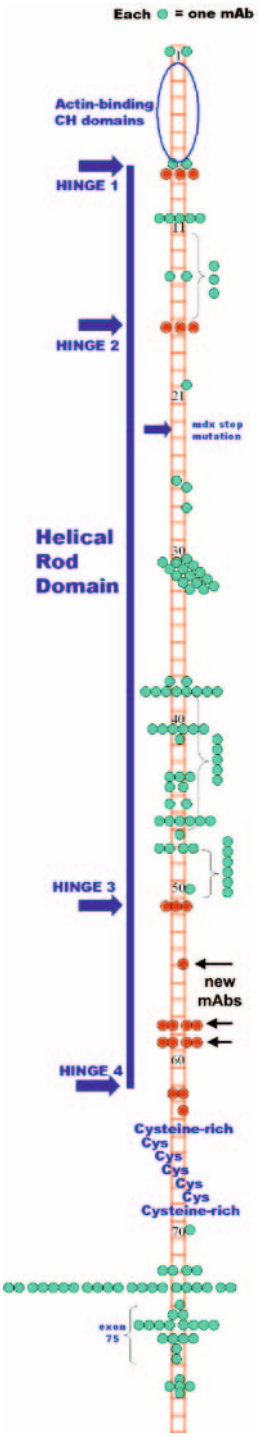

Figure 4. Graphical representation of the dystrophin molecule showing the positions of available dystrophin mAbs. The red dots with arrows show where the mAbs described in this paper fill the gap in exon coverage. Each dot represents an available anti-dystrophin mAb and green dots are previously-published mAbs.

gentamycin, as a treatment for the small proportion of Duchenne Muscular Dystrophy with a nonsense mutation in the dystrophin gene [21].

All mAbs described in this study are freely available for muscular dystrophy research from the MDA Monoclonal Antibody Resource: www.glennmorris.org.uk/mabs.htm

Acknowledgements.

We thank the Muscular Dystrophy Association (USA) for grant support. GHN was supported by a grant from Medical and Scientific Aid to VietNam, Laos and Cambodia.

References

[1] Le TT, Nguyen thi Man, Hori S, Sewry CA, Dubowitz V, Morris GE Characterization of genetic deletions in Becker Muscular Dystrophy using monoclonal antibodies against a deletion-prone region of dystrophin.Amer. J. Med. Genet. 58, 1995, 177-86

[2] Lederfein D, Levy N, Augier N, Leger J, Morris GE, Fuchs O, Yaffe D, Nudel U 71kD protein is a major product of the Duchenne Muscular Dystrophy gene in brain and other non-muscle tissues.Proc Natl Acad Sci.USA, 89, 1992, 5346-50.

[3] Hugnot JP, Gilgenkrantz H, Vincent N, Chafey P, Morris GE, Monaco T, Berwald-Netter Y, Koulakoff A, Kaplan JC, Kahn A, Chelly J. Novel products of the dystrophin gene: a distal transcript initiated from a unique alternative first exon encoding a 75 kDa protein widely distributed in non-muscle tissues.Proc Natl Acad Sci.USA, 89, 1992, 7506-10.

[4] Blake DJ, Love DR, Tinsley J, Morris GE, Turley H, Gatter K, Dickson G, Morgan J, Edwards YH, Davies KE Characterization of a 4.8kb transcript from the Duchenne muscular dystrophy locus expressed in Schwannoma cells.Hum. Mol. Genet. 1, 1992, 103-9.

[5] Morris GE, Simmons C, Nguyen thi Man, Apo-dystrophins (Dp140 and Dp71) and dystrophin splicing isoforms in developing brain. Biochem. Biophys. Res. Commun. 215, 1995, 361-7.

[6] d'Souza VN, Nguyen thi Man, Morris GE, Karges W, Pillers DM, Ray PN A novel dystrophin isoform is required for normal retinal electrophysiology Hum. Mol.. Genet. 4, 1995, 837-42.

[7] Le TT, Nguyen thi Man, Helliwell TR, Morris GE Characterization of revertant muscle fibres in Duchenne Muscular Dystrophy using exon-specific monoclonal antibodies against dystrophin. Amer. J. Hum. Genet. 56, 1995, 725-31.

[8] Lu QL, Morris GE,. Wilton SD, Ly T, Artem'yeva OV, Strong P, Partridge TA Massive Idiosyncratic Exon Skipping Corrects The Nonsense Mutation In Dystrophic Mouse Muscle And Produces Functional Revertant Fibres By Clonal Expansion J. Cell Biol. 148, 2000, 985-96.

[9] Partridge TA, Lu QL, Morris GE, Hoffmann EP Is myoblast transplantation effective?Nature Med. 4, 1998, 1208-9.

[10] Gussoni E, Bennett RR, Gilgoff I, Stein J, Chan Y, Muskiewicz KR, Lidov HG, Bönneman C, von Moers A, Morris GE, Chamberlain JS, Kunkel LM, Weinberg K Detection of donor nuclei in the muscles of a Duchenne muscular dystrophy patient 13 years following bone marrow transplantation J. Clin. Invest., 110, 2002, 807-14.

[11] Nguyen thi Man, Morris GE A rapid method for generating large numbers of high-affinity monoclonal antibodies from a single mouse, in "The Protein Protocols Handbook" 3rd Edn. (ed. Walker JM) Humana Press, Totowa, NJ (2009) pp 1961-74.

[12] Tunnah D., Sewry CA Vaux D, Schirmer EC, Morris GE The apparent absence of lamin B1 and emerin in many tissue nuclei is due to epitope masking. J. Mol. Histol., 36, 2005, 337-44.

[13] Hori S, Ohtani S, Nguyen thi Man, Morris GE The N-terminal half of dystrophin is protected from proteolysis in situ. Biochem. Biophys. Res. Commun., 209, 1995, 1062-7.

[14] Mendell JR, Kissel JT, Amato AA, King W, Signore L, Prior, TW et al. Myoblast transfer in the treatment of Duchenne's Muscular Dystrophy. New Engl. J. Med. 333, 1993, 832-8.

[15] Romero NB, Braun S, Benveniste O, Leturcq F, Hogrel JY, Morris GE. et al Phase I study of dystrophin plasmid-based gene therapy in Duchenne/Becker muscular dystrophy. Hum. Gene Therapy, 15, 2004, 1065-76.

[16] Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P et al. PTC124 targets genetic disorders caused by nonsense mutations. Nature 447, 2007, 87-91.

[17] Kinali M, Arechavala-Gomeza V, Feng L, Cirak S, Hunt D, Adkin C, Guglieri M, Ashton E, Abbs S, Nihoyannopoulos P, Garralda ME, Rutherford M, McCulley C, Popplewell L, Graham IR, Dickson G, Wood MJ, Wells DJ, Wilton SD, Kole R, Straub V, Bushby K, Sewry C, Morgan JE, Muntoni F Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. Lancet Neurol. 8, 2009, 918-28.

[18] Braun S, Thioudellet C, Rodriguez P, Ali-Hadji D, Perraud F, Accart N et al Immune rejection of human dystrophin following intramuscular injections of naked DNA in mdx mice. Gene Ther. 7, 2000, 1447-57.

[19] Nguyen thi Man, Ellis JM, Love DR, Davies KE, Gatter KC, Dickson G, Morris, GE Localization of the DMDL-gene-encoded dystrophin-related protein using a panel of 19 monoclonal antibodies. Presence at neuromuscular junctions, in the sarcolemma of dystrophic skeletal muscle, in vascular and other smooth muscles and in proliferating brain cell lines. J. Cell Biol. 115, 1991, 1695-700.

[20] Burton EA, Tinsley JM, Holzfeind PJ, Rodrigues NR, Davies KE A second promoter provides an alternative target for therapeutic up-regulation of utrophin in Duchenne muscular dystrophy. Proc Natl Acad Sci U S A. 96, 1999, 14025-30.

[21] Malik V, Rodino-Klapac LR, Viollet L, Wall C, King W, Al-Dahhak R, Lewis S, Shilling CJ, Kota J, Serrano-Munuera C, Hayes J, Mahan JD, Campbell KJ, Banwell B, Dasouki M, Watts V, Sivakumar K, Bien-Willner R, Flanigan KM, Sahenk Z, Barohn RJ, Walker CM, Mendell JR Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy. Ann Neurol. 67, 2010, 771-80.