Tibialis anterior muscle needle biopsy and sensitive biomolecular methods: a useful tool in myotonic dystrophy type 1


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Abstract

Myotonic dystrophy type 1 (DM1) is a neuromuscular disorder caused by a CTG repeat expansion in 3’UTR of DMPK gene. This mutation causes accumulation of toxic RNA in nuclear foci leading to splicing misregulation of specific genes. In view of future clinical trials with antisense oligonucleotides in DM1 patients, it is important to set up sensitive and minimally-invasive tools to monitor the efficacy of treatments on skeletal muscle. A tibialis anterior (TA) muscle sample of about 60 mg was obtained from 5 DM1 patients and 5 healthy subjects through a needle biopsy. A fragment of about 40 mg was used for histological examination and a fragment of about 20 mg was used for biomolecular analysis. The TA fragments obtained with the minimally-invasive needle biopsy technique is enough to perform all the histopathological and biomolecular evaluations useful to monitor a clinical trial on DM1 patients.

Introduction

Myotonic dystrophy type 1 (DM1) is the most common muscular dystrophy in adults, affecting 1 in 6-10,000 live birth. Main clinical features of DMI are myotonia, weakness and atrophy of skeletal muscles. DM1 is caused by the expansion of a CTG repeat (from 30 to 3000 repeats) in the 3’ untranslated region of the Dystrophia Myotonica Protein Kinase (DMPK) gene. This mutation gives rise to toxic RNAs containing repetitive CUG repeat that accumulate in cell nuclei as ribonuclear inclusions causing changes of alternative splicing for a specific group of transcripts. Expanded CUG repeats interact with RNA-binding proteins such as Muscleblind-like (MBNL) family protein, causing their nuclear sequestration and subsequently resulting in reduced protein activity in several cellular processes. Another pathogenic effect of DMPK mutation is the induction of post-transcriptional upregulation of another RNA binding protein, CUG-binding protein 1 (CUGBP1). MBNLs and CUGBP1 are antagonistic regulators of alternative splicing, and their functional imbalance leads to embryonic abnormalities for several MBNL-sensitive exons. MBNLs are necessary for the correct alternative splicing and protein synthesis in DM1 muscle.

Currently, there are no disease-modifying therapies for DM1 patients. DM1 treatments are only to manage symptoms. To date, two main experimental therapeutic strategies of targeting expanded repeat RNA in DM1 have been described: i) antisense oligomer-induced degradation of toxic CUG-containing RNA and ii) inhibition of toxic RNA-binding proteins. The expected effects of these treatments are the prevention of MBNLs sequestration and/or a significant reduction of nuclear foci formation. These results should lead to the correction of alternative splicing abnormalities for several MBNL-sensitive exons. Indeed, alternative splicing events have good potential to function as biomarkers of DM1 severity and therapeutic response since the splicing misregulation is directly connected to RNA toxicity and proteins sequestrations. Moreover, many splicing defects are correlated to muscle weakness and histopathology and some of them directly implicated in symptoms of DM1. However, at skeletal muscle level, there is no a definitive mechanistic explanation for the histopathological features of this disease. Therefore, the study of splicing regulation in skeletal muscle of DM1 patients is crucial to understand and elucidate the mechanisms involved in DM1 pathogenesis.
ularly indicated for the diagnosis of neuromuscular disease in infancy and childhood or in inflammatory myopathy and to evaluate the disease progression and the response to treatment. The sample size obtained by this technique is smaller than that obtained with an open biopsy and some limitations are the impossibility to directly visualize the site of sampling and the difficulty to identify the right orientation of the fibres of the obtained sample. However, a recent study on more than 13,000 patients demonstrated that using needle biopsy it is possible to obtain an appropriate sample for the analyses required in >99% of cases.31

In this work we show that, taking advantage of both the minimal invasiveness of needle biopsy and the suitability of TA muscle for the analysis of DMI biomarkers, the small muscle fragment obtained from a TA needle biopsy is enough to perform an histopathological and biomolecular characterization of skeletal muscle in DMI patients. This muscle characterization will be used to monitor a DMI clinical trial by comparing the obtained results before and after the therapeutic treatment.

Materials and Methods
Patients and TA muscle biopsy

TA muscle biopsies were taken under sterile conditions from 5 DMI patients and from 5 healthy subjects and were used for this study after receiving written informed consent from the patients. The site is prepped with betadine. Local anaesthesia is performed by 2% mepivacaine injection (7-8 cc) in the subcutaneous tissue and intramuscular. A 1 cm transverse skin incision is performed at the junction between the proximal and middle third of the anterior side of the leg, 2 cm laterally to the tibial crest. Minimal blunt dissection is performed to visualize the deep fascia, which is then incised with a blade for 5 mm. An UCH tibialis anterior cylinder is then pushed home and a sample obtained with the guillotine action of the cutting edge. The biopsy is performed perpendicular to the longitudinal orientation of the myofibers as opposed to open biopsies, and repeated with a 45° angle to the skin, almost parallel to the orientation of the myofibers, similar to open biopsies. If necessary, more muscle may be obtained by subsequent reinsertion of the needle through the same skin incision. After withdrawal of the needle firm pressure is applied to the thigh for 5 min to prevent any hematoma. Since wound tension is critical in lower limbs, the skin edges are closed with 5-0 monofilament absorbable subcutaneous stitches. Compressive dressing is applied for 5 days.

One sample of TA muscle of about 60 mg was taken. One fragment of about 40 mg was used for histological examination and one fragment of about 20 mg was used for biomolecular analysis. The diagnosis of DMI was based upon the clinical diagnostic criteria set by the International Consortium for Myotonic Dystrophy.32 DMI genotyping has been performed on genomic DNA extracted from peripheral blood leukocytes as described.33

Clinical data on patients used in these study are reported in Table 1.

This study was authorized by the Institutional Ethics Committee (ASL MI2, Melegnano, MI, Italy) and was conducted according to the principles expressed in the Declaration of Helsinki, the institutional regulation and Italian laws and guidelines.

Muscle histopathology and immunohistochemistry

To identify the right orientation of the fibers, a muscle sample of about 40 mg was dissected under a stereomicroscope. Muscle tissue was fresh-frozen in isopentane cooled in liquid nitrogen. Histopathological analyses were performed on serial sections (8 µm) processed for routine histological or histochemical stainings. A standard myofibrillar ATPase staining protocol was used before preincubation at pH 4.3, 4.6, and 10.4.35 The most typical alterations, such as nuclear clump fibers (i.e., aggregates of myonuclei with a thin rim of cytoplasm), nuclear centralization and fiber size variability, were analyzed on serial muscle sections. Central nuclei and nuclear clumps were classified as absent or present. The evaluation of the area of the total transverse section was made with Image J (NIH, MD, US) on a digital image of the entire muscle cross-sectional area obtained by tiling slightly overlapping images of the transverse muscle section.

For immunohistochemistry, sections 6 µm thick were air-dried and rehydrated in phosphate buffer pH 7.4 (PBS). Non-specific binding sites were blocked with normal goat serum (NGS; Dako, Denmark) at a dilution 1:20 in PBS containing 2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature (RT). Mouse monoclonal primary antibodies against two different myosin heavy chain (MHC) isoforms were used at the following dilutions: MHCfast, 1:400 in PBS + 2% BSA; MHCslow, 1:400 in PBS + 2% BSA (Sigma-Aldrich). Each antibody was applied for 1 h at RT. After washing in PBS 3 times for 5 min, sections were incubated with goat anti-mouse biotinylated secondary antibody diluted 1:300 in PBS + 2% BSA for 1 h at RT. After PBS washing (3x5 min), sections were incubated with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for 30 min and then exposed to the 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) chromogen reaction solution for 10 min. Nuclei were counterstained with Mayer’s haematoxylin. Quantitative evaluation

Table 1. Clinical data of DM1 patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age at biopsy</th>
<th>Age at onset</th>
<th>CTG expansion</th>
<th>MRC</th>
<th>MIRS</th>
<th>Myotonia</th>
<th>Arrhythmia</th>
<th>CPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM1-1</td>
<td>33</td>
<td>20</td>
<td>360</td>
<td>129</td>
<td>2</td>
<td>Present</td>
<td>Not present</td>
<td></td>
</tr>
<tr>
<td>DM1-2</td>
<td>21</td>
<td>15</td>
<td>460</td>
<td>105.22</td>
<td>2</td>
<td>Present</td>
<td>Present</td>
<td>203 U/L</td>
</tr>
<tr>
<td>DM1-3</td>
<td>27</td>
<td>21</td>
<td>890</td>
<td>105</td>
<td>3</td>
<td>Present</td>
<td>Not present</td>
<td></td>
</tr>
<tr>
<td>DM1-4</td>
<td>42</td>
<td>18</td>
<td>190</td>
<td>130</td>
<td>2</td>
<td>Not present</td>
<td>Not present</td>
<td></td>
</tr>
<tr>
<td>DM1-5</td>
<td>26</td>
<td>13</td>
<td>360</td>
<td>111.33</td>
<td>3</td>
<td>Present</td>
<td>Not present</td>
<td>531 U/L</td>
</tr>
</tbody>
</table>

MRC, Medical Research Council, scale for muscle strength; scale (0-5 grade) on 15 muscles at both side in the upper and lower limbs for a total of 150 maximum score; MIRS, muscle impairment rating scale, stage of the disease for myotonic dystrophy type 1 (DM1) patients; CPK, creatine phosphokinase blood levels.
of fiber diameter was made as described previously by Vihola et al. with Image J (Scion Corp.) on images taken with a light microscope (160x, original magnification). The size of muscle fibers was assessed by measuring the smallest fiber diameter.

All data were elaborated using Microcal Origin (Microcal Software Inc., Northampton, MA, USA). The metahistograms were normalized to normal mean diameter for men and women.

Fluorescence in situ hybridization combined with MBNL1 immunofluorescence

The fluorescence in situ hybridization (FISH) was performed on DM1 muscle frozen sections using a (CAG)6-CA probe (IDT, Coralville, IA, USA) as previously reported by Cardani et al. Briefly, 6 μm thick transverse cryostatic sections were air dried for 30 min and fixed with 2% paraformaldehyde for 30 min at 4°C. The sections were then washed in PBS and permeabilized for 5 min in 2% acetone in PBS, prechilled at −20°C. After washing in PBS, sections were incubated in 40% formamide and 2x saline solution citrate (SSC) for 10 min at RT and hybridized for 2 h at 37°C. With 1 ng/μL (CAG)6-CATexas red labeled probe in 30% formamide, 2xSSC. The sections were then washed first in 40% formamide and 2xSSC at 45°C for 30 min, then in 1xSSC at 45°C for 15 min and another 1xSSC wash at RT. The FISH labeled sections were then processed for the immunofluorescence detection of MBNL1, as follows. The sections were pre-incubated for 20 min at RT with 5% NGS in PBS+2% BSA and then incubated overnight at 4°C with the rabbit polyclonal antibody A2764 for MBNL1 (1:1000 in PBS+2% BSA). The sections were washed in PBS and then incubated for 1 h at RT with a goat anti-rabbit Alexa488-labeled antibody (Molecular Probes, Life Technologies, Milan, Italy), diluted 1:200 in PBS+2% BSA, counterstained with DAPI (Sigma-Aldrich), and mounted with Mowiol (Calbiochem, Milan, Italy).

cDNA synthesis

Frozen TA samples (20 mg) were practiced for the extraction of total RNA using TRIzol reagent (Life Technologies) and 20 ng of RNA were reverse transcribed using the WT-Ovation RNA Amplification System (NuGEN, San Carlos, CA, USA) according to the manufacturer’s instructions. The resulting cDNAs were used to perform both quantitative real time-PCR (qRT-PCR) and classical PCR.

Quantitative RT-PCR

The expression level of the DMPK gene was measured by Quantitative RT-PCR (qRT-PCR) using the HS01094329_m1 Assay-on-demand gene expression product, labeled with FAM dye. GAPDH was chosen as the housekeeping, internal control and its expression level was measured using the HS0278991_g1 Assay-on-demand gene expression product, labeled with FAM dye. Each PCR reaction was performed in triplicate using the TaqMan Universal PCR Master Mix - No AmpErase UNG (Applied Biosystems, Life Technologies) and the 7900HT Fast Real Time PCR System (Applied Biosystem, Life Technologies).

Alternative splicing analysis

The RT-PCR splicing analysis for the INSR (insulin receptor) was performed using Platinum Taq Polymerase (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. Briefly, the reaction was conducted in a mix containing 150 ng of cDNA, Taq MgCl2-free buffer, 2 mM MgCl2, 400 nM dNTPs, 800 μM forward primer and 800 μM reverse primer. After 2 min at 94°C, 35 cycles of amplification were performed, each consisting of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. The RT-PCR splicing analysis for CAMK2G (calcium/calmodulin-dependent protein kinase II gamma), CAMK2B (calcium/calmodulin-dependent protein kinase II beta), CACNA1S (dihydropyridine receptor), CLCN1 (skeletal muscle chloride channel, voltage-sensitive 1), NFIX (Nuclear Factor IX), PDLIM3 (PDZ and LIM domain 3) and LDB3 (LIM Domain Binding 3) were performed using My Taq Red Mix (Bioline), according to manufacturer’s protocol. The PCR cycling conditions were: 1 min at 95°C followed by 35 cycles of 15 s at 95°C, 15 s at primer specific Tm, 10 s at 72°C. The primers used for all PCR reactions were listed in Table 2 with their respective temperatures of melting (Tm). Total PCR products were electrophoretically resolved on 2% agarose gel for INSR, CAMK2G, CAMK2B, CACNA1S, LDB3, NFIX and PDLIM3 genes and 3.5% agarose gel for CLCN1.

Qualitative analysis of the amplified products was performed using EtBr-stained gels (Sigma-Aldrich) scanned on a ChemiDoc Universal Hood (Biorad). The quantitative analysis was performed quantifying the intensity of each band with Image J software densitometry and calculating the fraction of abnormally spliced (AS) isoform respect to the total amount of isoforms (AS isoform/total). The expression level of GAPDH was used as the housekeeping, internal control.

Results

One aim of this work was to verify if a muscle fragment of about 40 mg obtained from TA needle biopsy of DM1 patients was adequate to evaluate the histopathological alterations and to analyze in situ biomolecular markers. The mean area size of transverse muscle sections obtained from 10 TA needle biopsies resulted to be 1.75±0.74 mm², about 13 fold smaller than the area size of transverse sections obtained from an open biopsy of biceps brachii (data not shown). Nevertheless, routine stainings performed on TA transverse sections permit to evaluate the presence of the histological alterations commonly observable in DM1 skeletal muscle such as nuclear clump fibers, nuclear centralization and fiber size variability (Figure 1). The results of histopathological analysis of DM1 TA are reported in Table 3. Moreover, the immunohistochemical staining of MHC slow and fast myosin and the subse-
quent analysis of type 1 and type 2 fiber diameters allow to obtain metahistograms of fiber diameter distribution (Figure 2) and to verify the dystrophic changes. The results of the analysis of immunostained sections were reported in Table 3.

To date, targeting the mutant RNA appears to hold some of the greatest prospect for therapeutic intervention in DM1. The reduction of CUZexp RNA would be expected to release sequestered MBNL1 protein and to improve its splicing regulatory activity. Fluorescent in situ hybridization (FISH) in combination with MBNL1 immunofluorescence performed on muscle sections is a useful method to reveal if a reduction in the average number of foci of CUZexp RNA and of MBNL1 before and after the ASO treatment is evident. This reduction can be evaluated on sections obtained from the small muscle sample of TA needle biopsy since the number of myonuclei that can be screened (388±56 per mm²) is enough to obtain significant results on changes in the number and size of ribonuclear inclusions or MBNL1 foci. All DM1 patients used in this study showed ribonuclear inclusions colocalizing with MBNL1 foci (Figure 3).

Consistent with MBNL1 activity restoration, alternative splicing of MBNL1-dependent exons should be examined in order to verify the efficacy of the therapeutic treatment. To do this it is necessary to use an efficient, sensitive and reliable RNA-to-DNA conversion step since the available muscle sample is limited. NuGen’s Ovation qPCR System is a highly efficient system that provides a fast and simple method for preparing amplified cDNA from a very small input of total RNA. Moreover, 20% more genes are detected using the amplified cDNAs obtained with the Ovation qPCR System than with those obtained with classical RT-PCR techniques. This system amplifies the

Table 2. List of PCR primers used for alternative splicing analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INSR</td>
<td>5’-CCAAAGACAGACTCTCAGAT-3’</td>
<td>5’-AACATCGCCAAGGACCTGC-3’</td>
<td>60</td>
</tr>
<tr>
<td>CAMK2G ex17</td>
<td>5’-CACCCCTAAGGAGATGGA-3’</td>
<td>5’-AAAGCCTCCAATTGTTGAC-3’</td>
<td>60</td>
</tr>
<tr>
<td>CAMK2G ex18</td>
<td>5’-CTCAGGAGATGACCTGAAAC-3’</td>
<td>5’-CCAGGTCGAAGGGAGTGAAG-3’</td>
<td>60</td>
</tr>
<tr>
<td>CAMK2B</td>
<td>5’-CAGGAGCTGAGTGGGTCTCG-3’</td>
<td>5’-ACCCTTTCAGCTTTGCTGGA-3’</td>
<td>62</td>
</tr>
<tr>
<td>NFIX</td>
<td>5’-GCTCCTCTGAGAGACCCCTC-3’</td>
<td>5’-AGGAGGTCGCACTCTTCTG-3’</td>
<td>60</td>
</tr>
<tr>
<td>LDB3 ex11</td>
<td>5’-GACCCCTGAGAGACCCCTC-3’</td>
<td>5’-CTCACTCTTTCAGCTTTGGAAC-3’</td>
<td>62</td>
</tr>
<tr>
<td>LDB3 ex5</td>
<td>5’-GAGGCTGCACAGACAGCATCATGCC-3’</td>
<td>5’-TCCACCCCTGACGGATGTC-3’</td>
<td>62</td>
</tr>
<tr>
<td>PDLIM3</td>
<td>5’-AGCCACCTTTCATCAAAATAC-3’</td>
<td>5’-AGAGGCTGCTCACCACATTGAC-3’</td>
<td>58</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-GCTCTCCCGCTTCCCTTCT-3’</td>
<td>5’-GCTACCATGCCCCACCTGGA-3’</td>
<td>60</td>
</tr>
</tbody>
</table>

Tm, melting temperature.

Table 3. Histopathological features of tibialis anterior needle biopsy in DM1 patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Central nuclei</th>
<th>Nuclear clumps</th>
<th>AFast</th>
<th>HFast</th>
<th>ASlow</th>
<th>HSlow</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM1-1</td>
<td>Rare</td>
<td>Rare</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>DM1-2</td>
<td>Absent</td>
<td>Absent</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>DM1-3</td>
<td>Present</td>
<td>Absent</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DM1-4</td>
<td>Absent</td>
<td>Present</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>DM1-5</td>
<td>Rare</td>
<td>Absent</td>
<td>-</td>
<td>++</td>
<td>±</td>
<td>+</td>
</tr>
</tbody>
</table>

AFast, fast fiber atrophy; HFast, fast fiber hypertrophy; ASlow, slow fiber atrophy; HSlow, slow fiber hypertrophy; ±, absent; ±, modest; +, present; ++, prominent (grade based on fiber diameter metahistograms).
entire transcriptome of 5-50 ng total RNA into micrograms of cDNA across a wide range of transcript abundance.

In our study, the total RNA extracted from a TA frozen sample of about 20 mg was around 8 µg. This quantity of RNA is sufficient to perform 400 RT-PCR using the Ovation qPCR System starting from 20 ng of RNA for each reaction. The total cDNA amount obtained was around 2 mg. This quantity is enough to perform serial molecular analysis, such as qRT-PCR and classical PCR, in order to evaluate the expression levels of DMPK and the splicing pattern of several genes involved in the multisystemic phenotype of DM1 patients respectively. The analysis of the expression levels of DMPK gene by qRT-PCR in muscle biopsy showed that while in healthy patients the expression levels of the gene are quite homogeneous, in DM1 patients an evident variability in the amount of DMPK mRNA was observable. Indeed in 3/5 DM1 patients the DMPK expression is lower than that observed in healthy subjects, while in two patients DMPK expression is respectively 4 and 7 fold higher than the media levels observed in healthy controls and 7 and 11 fold higher than media levels observed in the other DM1 patients (Figure 4 A). Alternative splicing of several genes involved in the multi-systemic phenotype of DM1 was analyzed by RT-PCR. For each splice event, the fraction of splice products that included or skipped the alternative exon(s) was compared for five healthy controls and five DM1 subjects. We observed a splicing alteration of CAMK2G exon 18, LDB3 exon 11 and LDB3 exon 5 in 60% of DM1 patients and a misregulation of INSR exon 11, CAMK2G exon 17, CAMK2B exon 13, CACN2S1 exon 29, CLCN1 exon 7a isoforms, NFIX exon 7 and PDLIM3 exon 5 in 80% of DM1 patients (Figure 4 B,C).

Discussion

Clinical trials for a pharmacological treatment need a road map to patient-focused biomarker measures. Therefore, the study of disease mechanisms, pathogenesis and manifestations is required to select biomarker for the conceptualization of treatment benefits. Before treatment, it is also necessary to collect data from registry of patients in order to understand the natural history of muscle impairment through longitudinal study. So it will be easier to develop muscle impairment endpoints at clinical level, which are also very helpful and necessary during clinical trial in DM1. A phase I/II trial for the ASO ISIS-DMPKx was on-going in patients affected by myotonic dystrophy type 1 in order to evaluate the safety and tolerability of multiple doses of this compound (www.clinicaltrials.gov). ISIS-DMPKx was designed to reduce the production of toxic DMPK RNA in cells, including muscle cells. Therapeutic application of this strategy to DM1 patients is expected to cause a rapid knockdown of CUGexp RNA in skeletal muscle, correcting the physiological, histopathological and transcriptomic features of the disease. Published in vitro and in vivo preclinical studies demonstrated that various antisense oligomers targeting CUGexp repeats induce the degradation of mutant DMPK transcripts leading to a reduction of MBNL1 sequestration. Consequently, MBNL1 biological function is restored and the splicing alteration of several genes is corrected including CLCN1 alternative splicing leading to an improvement of myopathic changes.12-20

In view of a clinical trial on large scale, it appears evident the need to improve specific tools and to define biomolecular markers that can be used for monitoring the success of the therapy. In particular, it is necessary to validate tools characterized by feasibility and the test-retest reliability. The needle biopsy is a minimally invasive procedure that presents a low complication rate, causes a short scar and, therefore, it can be repeated on the same patient and on the same muscle over the time. These features make needle biopsy a good candidate as a tool to follow a clinical trial on DM1 patients. However, one limitation of this procedure is that the tissue samples obtained are...
very small. The main effort of this work was therefore to set the conditions in order to succeed in performing histopathological examination and biomolecular analysis on small muscle fragments obtained with needle biopsy. All the analysis were performed on TA muscle since it appears to be the best muscle to test therapeutic interventions in DM1 patients.38

The analysis performed on TA muscle section obtained from needle biopsy indicates that, despite the small fragment dimensions, the cross-section area obtained was enough to evaluate structural changes in muscle fibers, central nuclei frequency, muscle fiber diameter and presence of CUGexp RNA and MBNL1 foci. These parameters should be taken in consideration to verify if the therapeutic treatment can protect against structural changes (mainly a prevention of fiber atrophy) and can decrease toxic RNA and MBNL1 accumulation.

The use of specific reverse transcription and amplification systems allowed us to obtain a huge amount of cDNA starting from small amount of RNA. This permitted us to analyze DMPK expression levels in our TA muscle samples. As previously reported, our results show an inter-individual variability in DMPK expression in DM1.38 DM1 is associated with the misregulation of alternative splicing of many genes that can explain the multisystemic phenotype of the disease. For this reason, splicing events in skeletal muscle are useful biomarkers of DM1 therapeutic response. The amount of cDNA obtained also permit us to analyse the splicing alteration of a large number of genes such as INSR and CLCN1 that are involved in insulin resistance and myotonia respectively and CACNA1S whose misplacing may contribute to muscle degeneration. The obtained data confirmed that the percentage of splicing misregulation observed in our DM1 patients is similar to the percentage reported by Nakamori et al.39 However, it is reasonable to consider that in clinical trial only splice events of genes that correlate with skeletal muscle symptoms will be examined, on the assumption that the correction of these defects may lead to functional improvement.39

The main limitation of this study is that it does not extend our knowledge on DM1 pathomechanism beyond what is already known. Some data presented in this study were already discussed by Nakamory et al.39 who studied more muscle samples and more splicing events. However, the aim of our study was to assess if histological and biomolecular biomarkers might be evaluated on a small fragment of TA muscle to obtain the data necessary to evaluate the effects, positive as well as negative, of therapeutic treatment in DM1 patients. Indeed, by using the minimally invasive needle biopsies technique for sampling the small fragment of muscle, the procedure can be performed before and after the treatment on the same patient.

In conclusion, our analysis demonstrates that using a minimally invasive procedure as needle biopsy it is possible to obtain small TA fragments which are an adequate amount of muscle tissue to perform all the histopathological and biomolecular analysis useful to monitor the success of a clinical trial on DM1 patients.

References


