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Research Article

A Short Morpholino CAG15 Corrects Aberrant Alternative Splicing of *Clcn1* and *Serca1 In Vivo*

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Abstract

Myotonic dystrophy type 1 (DM1) is a hereditary disease associated with multisystemic disorders including myotonia, muscle weakness, and cataracts. DM1 cells express DMPK mRNA harboring expanded CUG repeats, which are responsible for sequestering MBNL1, an RNA-binding protein. MBNL1 regulates alternative splicing of several genes, and sequestration of MBNL1 in the nucleus possibly causes DM-specific symptoms such as myotonia. Therefore, it has been suggested that blocking the interaction between MBNL1 and the RNA CUG repeats would be a promising therapeutic strategy for DM1. A CAG-repeat morpholino oligonucleotide (MO) is expected to bind to expanded CUG repeats in RNA, thus preventing MBNL1 sequestration. In the present report, we optimized the lengths of relevant MOs. We introduced 15-mer, 20-mer, and 25-mer MOs into Neuro 2a cells, which express CUG repeat-containing RNA. We also injected MOs into mice with DM1 (the HSA^{LR} line). Such work showed that the 15-mer MO most effectively corrected aberrant splicing of *Clcn1* and *Serca1*. To increase MO permeability, a 15-mer vivo-MO was administered intravenously and resulted in corrected aberrant *Clcn1* splicing in the quadriceps of HSA^{LR} mice. These results suggest that the short 15-mer MO most efficiently corrects aberrant splicing.

Abbreviations

DM1: Myotonic Dystrophy Type 1;

MBNL: Muscleblind-Like Protein 1;

DMPK: Myotonic Dystrophy Protein Kinase;

CELF: CUG-BP, and ETR-3-like factor, embryonic lethal abnormal vision-type RNA-binding protein 3-like factor;

MO: Morpholino Oligonucleotide;

TA muscle: Tibialis Anterior Muscle

Keywords: Myotonic dystrophy (DM1); Antisense; *Clcn1*; *Serca1*; Alternative Splicing

Introduction

Myotonic dystrophy (DM), an autosomal-dominant disorder, is the most common type of adult muscular dystrophy. There are two types of DM: DM1 and DM2. We focused on DM1, which is caused by expansion of CTG repeats in the 3′ untranslated region (UTR) of the *DMPK* gene [1]. The symptoms of DM1 patients include myotonia, muscle weakness, cataracts, insulin-resistance, and cognitive impairment [2]. The cells of DM1 patients express DMPK mRNA with expanded CUG repeat sequences; such RNA sequesters MBNL1, an RNA-binding protein [3, 4]. Protein dysfunction causes aberrant splicing of several genes and is thought to trigger certain symptoms such as myotonia. Some abnormal splicings are evident in DM1 patients. Abnormal splicing of *CLCN1* is thought to cause electrical discharge in muscles [5]. Abnormal splicing of *SERCA1* may affect intracellular Ca^{2+} homeostasis (Zhao et al., Biochim.Biophys. Acta, in press submitted). *MBNL* exon 5 is highly conserved between birds and mammals, and its regulation is thought to be important for modulation of MBNL1 function during embryonic development [6].

Both antisense materials and various small molecules [7,8] have been investigated as DM1 therapies. Efforts have been made to optimize the lengths of antisense materials. The presence of a minimum of five CAG triplets was required when a 2′-O-methyl antisense oligonucleotide was used to correct aberrant splicing in cell models of DM [9]. Very short 8- or 10-unit antisense-locked nucleic acids also resulted in corrected aberrant alternative splicing [10].

In the present study, morpholino oligonucleotides (MOs), which are thought to be safer than 2′-O-methyl oligonucleotides, were administered to mice with DM1. We optimized MO length. To increase the permeability of antisense materials, we used a 15-mer vivo-MO, which was previously shown to yield promising results [11-13]; vivo-MOs carry cell-penetrating octaguanidium dendrimers, and their uptake is thought to be high. We also intravenously injected a vivo-MO into HSA^{LR} mice and found a beneficial effect thereof on splicing.

Materials and Methods

Morpholino oligonucleotides (MOs) and vivo-MOs

MOs and vivo-MOs were purchased from Gene Tools (Philomath, OR, USA). The oligonucleotide sequences were (5′to3′) as follows: CAG15, CAGCAGCAGCAGCAG; CAG20, CAGCAGCAGCAGCAGCAGCA; and CAG25, CAGCAGCAGCAGCAGCAGCAGCAGC. All MOs were dissolved in water.

Constructs

The *Clcn1* construct has been described previously [14]. The DM480 construct contains a fragment of the 3′ region of DMPK with interrupted CTG480 repeats and has also been described previously [15].

Cell culture and transfection

Neuro 2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37°C under 5% (v/v) CO_2. Neuro 2a is a mouse neuroblast line. It is suitable transfection host and has been used for studies on the mechanism of cellular machinery. The cells were transfected with 0.6 μg of the plasmids (ratio, *Clcn1* minigene:DM480 = 1:5) using FuGENE 6 (Madison, Wisconsin, USA, Promega). After 24 h, MOs were transfected (2 μM) using Endo-Porter (Gene Tools). The cells were harvested 48 h after transfection and total RNAs purified using a GenElute Mammalian Total Miniprep kit (Sigma-Aldrich). The cells were cultured in 12-well plates.

Animals

HSA^{LR} mice [16] were used. These animals express human skeletal actin mRNA with 250 CUG repeats in the 3′ UTR. All animal experiments were conducted in accordance with the Regulations for Animal Experimentation of the University of Tokyo (Tokyo, Japan).

Administration of MOs with bubble liposomes

We injected 5 μg of a 15-mer, 20-mer, or 25-mer MO into the tibialis anterior (TA) muscles of mice in combination with 30 μL bubble liposomes, prepared as described previously [17]. Ultrasonic energy (frequency 1 MHz; duty cycle 50%; intensity 2.0 W/cm^2; time 60 s) was delivered transdermally downstream of the injection site immediately after liposome administration, using a 6-mm diameter probe fitted with a Sonitron 1000 device (Rich-Mar, Chattanooga, TN, USA). Liposomes were administered once weekly for 3 weeks, and 2 weeks after the last administration, the TA muscles were harvested.

Administration of vivo-MO

Seven-week-old mice were injected with 6 mg/kg CAG15 vivo-MO once a week for 3 weeks. Two weeks after the last administration, the skeletal muscles were harvested and preserved at –80°C.

Identification of splice variants

Total muscle RNAs were extracted using TRIzol reagent (Life Technologies). Total RNA (1 μg) was reverse-transcribed using a PrimeScript first-strand cDNA synthesis kit (TaKaRa). PCR was performed using the following primer pairs: *Clcn1* minigene Forward (Fw): 5′-AAGTCCGGACTCAGATCTCG-3′, *Clcn1* minigene Reverse (Rv): 5′-CATCTCCATCTCCAGCTCCT-3′; *Clcn1* Fw: 5′-GTCCTCAGCAAGTTTATGTCC-3′, *Clcn1* Rv: 5′- GAATCCTCGCCAGTAATTCC-3′; *Serca1* Fw: 5′-GCTCATGGTCCTCAAGATCTCAC-3′, *Serca1* Rv: 5′-GGGTCAGTGCCTCAGCTTTG-3′; and *Mbnl1* Fw: 5′-GCTGCCCAATACCAGGTCAAC-3′, *Mbnl1* Rv: 5′-TG-

GTGGGAGAAATGCTGTATGC-3′. PCR products were resolved on 8% (w/v) polyacrylamide gels, stained with ethidium bromide, and detected using LAS-3000 (Fujifilm); band intensities were measured using the Multigauge system (Fujifilm). The ratio of exon 7A inclusion within *Clcn1* was calculated as (7A inclusion)/(7A inclusion + 7A exclusion) × 100. The ratio of exon 22 exclusion from *Serca1* was calculated as (22 exclusion)/(22 inclusion + 22 exclusion) × 100. The ratio of exon 5 inclusion in *Mbnl1* was calculated as (5 inclusion)/(5 inclusion + 5 exclusion) × 100.

Statistical analysis

Statistical analyses were calculated using the Prism (ver.4) statistical software.

Results

Splicing assay using Neuro2a cells

Neuro 2a cells were transfected with minigene and (CUG)480 repeat constructs, followed by a 15-mer (CAG15), 20-mer (CAG20), or 25-mer (CAG25) MO. CAG15 decreased the extent of abnormal splicing by around 14%. However, this difference was not significant, although shorter MOs tended to decrease the proportion of abnormal exon 7A splicing (Fig. 1).

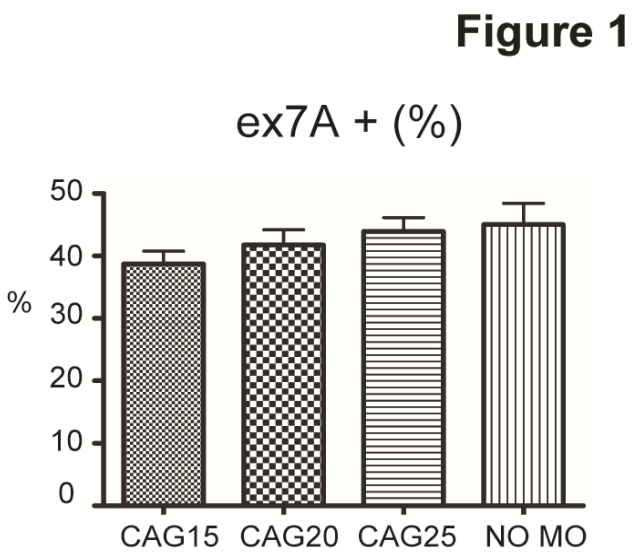

Figure 1. Effect of CAG MOs on splicing of *Clcn1* in Neuro 2a cells.

Splicing efficiencies were calculated as described in Methods. Statistical significance was assessed using the aid of Dunnett's multiple comparison test (p>0.05, n=5).

MO injection into HSA^{LR} mice

After the first injection of MOs and bubble liposomes into the TA muscles of HSA^{LR} mice, the 15-mer (p<0.01) and 20-mer (p<0.05) MOs significantly reduced the extent of exon 7A inclusion within *Clcn1*, and the 15-mer MO significantly (p<0.05) reduced the extent of exon 22 exclusion from *Serca1,* compared with the splicing patterns in saline-injected mice (data not shown).

Next, we injected MOs twice more. The CAG15 MO significantly reduced the extent of exon 7A inclusion within *Clcn1*. Moreover, the CAG15, CAG20, and CAG25 MOs significantly reduced the extent of exon 22 exclusion from *Serca1*. CAG MOs did not affect splicing of *Mbnl1* (Fig. 2).

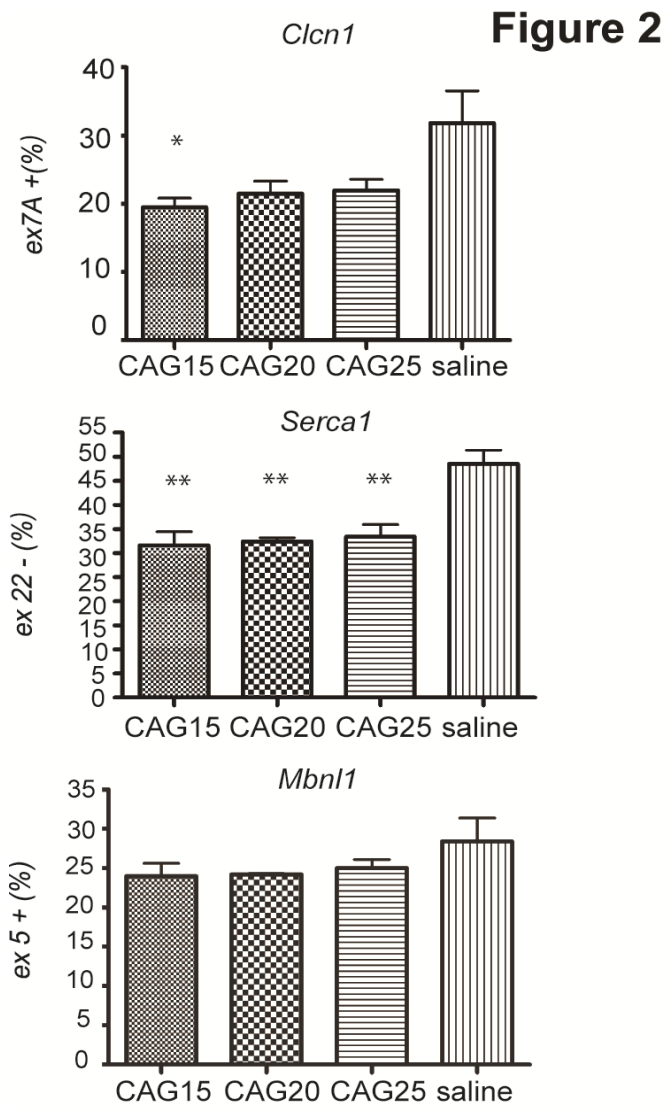

Figure 2. Effect of CAG MOs on the splicing levels of several genes in muscles of HSA^{LR} mice.

A 15-mer MO (CAG15) significantly corrected abnormal splicing of *Clcn1*. Also, 15-mer (CAG15), 20-mer (CAG20), and 25-mer (CAG25)

MOs significantly reduced abnormal splicing of *Serca1*. Abnormal splicing of *Mbnl1* was not affected. Statistical significance was assessed using the aid of Dunnett's multiple comparison test (**p<0.01, *p<0.05, n=3).

Administration of a vivo-MO to HSA^{LR} mice

Next, a 15 mer vivo-MO (or saline) was injected intravenously into HSA^{LR} mice. The vivo-MO significantly reduced exon 7A inclusion in the quadriceps (Fig. 3). No differences were seen among the TA, gastrocnemius, or diaphragm muscles (data not shown).

Figure 3

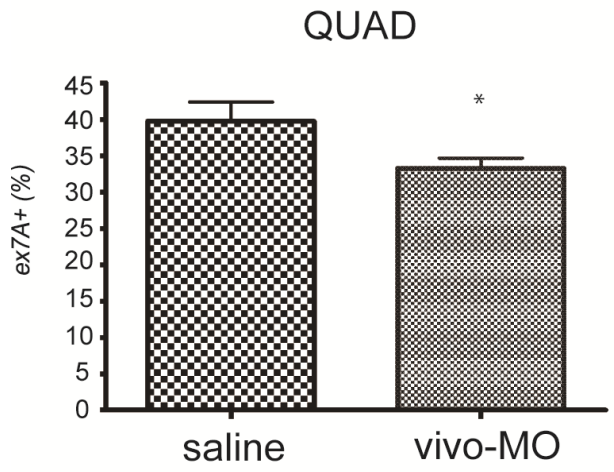

Figure 3. Effect of intravenous injection of vivo-MOs into HSA^{LR} mice.

Injection of a 15-mer vivo-MO corrected aberrant splicing of *Clcn1* to a significantly greater extent than did injection of saline. Statistical significance was assessed using Student's *t* test (*p<0.05, n=10) .

Discussion

Shorter MOs did not significantly reduce the degree of exon 7A inclusion within *Clcn1* in Neuro 2a cells. We explored other cell lines and varied the minigene amounts and transfection agents, but no significant effect was noted. One reason for this is that MOs are impermeable; we suggest that poor MO uptake affected the correction of aberrant splicing [18].

However, a 15-mer vivo-MO significantly corrected aberrant splicing of *Clcn1* and *Serca1*. Vivo-MOs are more permeable than are MOs but are also slightly toxic. An effective concentration devoid of side effects should be sought in the future.

Aberrant splicing of *Clcn1* and *Serca1* was corrected to significant extents by the 15-mer MO (Fig. 2) *in vivo*. However, neither the 15-mer MO nor the 15-mer vivo MO corrected aberrant splicing of *Mbnl1*. As *Mbnl1* knockout mice exhibit aberrant splicing of *Mbnl1* exon 5, such splicing may be regulated by MBNL1 per se [19]. Therefore, it was unexpected that the 15-mer MO did not correct the alternative splicing of *Mbnl1*. It is possible that MBNL1 differentially affects the alternative splicing of *Clcn1*, *Serca1,* and *Mbnl1*. Aberrant splicing of *Clcn1* and *Serca1* may be corrected by low levels of MBNL1; however, that of *Mbnl1* may require higher MBNL1 levels. Second, alternative splicing of *Mbnl1* may be regulated by other factors, rendering the 15-mer MO incapable of correcting the aberration. One such factor may be the RNA-binding protein CELF1 (CUGBP, Elav-like family 1). CELF1 is activated by RNA CUG repeat extension in the cells of DM1 patients, suggesting a close relationship between aberrant CELF1 splicing and DM1 [20]. However, activation of CELF family genes was not evident in DM1 mice, indicating that CELF1 may not regulate the splicing of MBNL1. Several RNA-binding proteins are reported to function abnormally in DM1 patients, but the protein(s) regulating *Mbnl1* splicing is/are unknown. Targeted therapy of DM1 would be feasible if the alternative splicing mechanism of *Mbnl1* was understood.

Vivo-MOs were more effective than were MOs when used to treat facioscapulohumeral muscular dystrophy (FSHD) [21]. Vivo-MOs were used to knock down PLTX1 (paired-like homeodomain transcription factor 1). Vivo-MOs were more permeable than were MOs but were not optimally effective when administered intravenously. In the cited work, intravenous injection of vivo-MOs corrected aberrant splicing of *Clcn1* in the quadriceps but not other muscles. We increased the concentration of vivo-MO administered to 12.5 mg/kg, but two of the six mice died. The expression levels of CUG vary among different muscles, explaining why the effectiveness of antisense oligonucleotides also varies. We injected vivo-MO directly into the mouse TA muscle and observed improved correction of aberrant splicing compared with that afforded by MOs. Intravenous administration of peptide-conjugated PMOs over 1 year triggered dystrophin expression in all skeletal muscles of a *mdx* mouse model [22]. If MOs could be modified (for example) by addition of penetrating peptides, splicing correction efficiencies would increase.

Methods facilitating delivery of antisense materials, and modifications thereof to improve permeability, are required.

Authors' contributions

K.N. and S.I. contributed to the concept, design, execution and analysis of the experiments. K.T. contributed to analysis of the experiments, and wrote the manuscript. M.K., K.O., N.O., Y.Z., and H.M. contributed to analysis of the experiments and provided advice and technical expertise. S.I. supervised the project and contributed to the concept and design of the experiments, provided funding, and wrote the manuscript.

Competing interest: The authors declare no competing financial interests.

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