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Splicing of human chloride channel 1

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ABSTRACT

Expression of *chloride channel 1* (*CLCN1*/*ClC-1*) in skeletal muscle is driven by alternative splicing, a process regulated in part by RNA-binding protein families MBNL and CELF. Aberrant splicing of *CLCN1* produces many mRNAs, which were translated into inactive proteins, resulting in myotonia in myotonic dystrophy (DM), a genetic disorder caused by the expansion of a CTG or CCTG repeat. This increase in abnormal splicing variants containing exons 6B, 7A or the insertion of a TAG stop codon just before exon 7 leads to a decrease in expression of the normal splice pattern. The majority of studies examining splicing in *CLCN1* have been performed using mouse *Clcn1*, as have investigations into the activation and suppression of normal splicing variant expression by *MBNL1-3* and *CELF3–6*, respectively. In contrast, examinations of human *CLCN1* have been less common due to the greater complexity of splicing patterns. Here, we constructed a minigene containing *CLCN1* exons 5–7 and established a novel assay system to quantify the expression of the normal splicing variant of *CLCN1* using real-time RT-PCR. Antisense oligonucleotides could promote normal *CLCN1* alternative splicing but the effective sequence was different from that of *Clcn1*. This result differs from previous reports using *Clcn1*, highlighting the effect of differences in splicing patterns between mice and humans.

1. Introduction

Myotonic dystrophy (*dystrophia myotonica*, DM) is an autosomal dominant disorder and the most common form of inherited muscular dystrophy in adults [1]. DM is characterised by a wide range of symptoms, including myotonia, progressive muscle loss, cataracts, cardiac conduction defects, insulin resistance and cognitive impairments [2]. Two forms of DM, DM1 and DM2, have been described to date. In DM1, disease symptoms result from an aberrant expansion of the CTG trinucleotide repeat in the 3′ untranslated region (UTR) in myotonic dystrophy protein kinase (*DMPK*) on chromosome 19 [3–5]. In DM2, disease is caused by an expansion of a CCTG tetranucleotide repeat in intron 1 of the CCHC-type zinc finger, nucleic acid binding protein (*CNBP*) gene on chromosome 3 [6]. Among patients with DM, several lines of evidence have suggested a relationship between transcribed RNA CUG or CCUG repeats and disease symptoms. First, the number of CUG repeats correlates with the severity of symptoms [5]. Second, in cells derived from patients with DM, expanded CUG and CCUG repeats accumulate in the nucleus [6–8]. Third, HSA^{LR} transgenic mice expressing an expanded CUG repeat inserted in the human skeletal muscle actin (*HSA*) gene manifest myotonia and abnormal muscle histology [9].

In addition to sequence repeats, abnormalities in RNA metabolism have also been found in patients with DM. Aberrant splicing has been reported in multiple genes, including chloride channel 1 (*CLCN1*), insulin receptor (*INSR*), bridging integrator 1 (*BIN1*), myomesin 1 (*MYOM1*) and actin-binding LIM protein 1 (*ABLIM1*), among others [10–14], leading to a variety of symptoms in these patients. This aberrant splicing is thought to be driven by two families of splicing factors, muscleblind-like (*MBNL*) and CUG binding protein/ELAV-like family (CELF). MBNL proteins MBNL1–3 bind CHG/CHHG (H: A, C and U) sequences of RNA and co-localise with mRNAs containing CUG expanded repeats [15]. This process leads to a decrease in the intracellular concentrations of functionally available MBNL proteins. Alternatively, CELF proteins, especially CELF1 and CELF2, are activated by CUG repeats, although the pathways regulating this process have not been fully elucidated [16]. This imbalance of MBNL and CELF in turn leads to abnormal splicing of downstream genes, further exacerbating DM symptoms.

Here, we examine the effects of MBNL1-3 and CELF1-6 on DM. *CLCN1* is thought to be responsible for myotonia, the most characteristic symptom in DM1 [17]. Many studies have been performed using mouse *Clcn1*. In these models, a frameshift occurs following

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the insertion of exon 7A (79 bp) between exon 6 and 7 in *Clcn1*; these immature mRNA transcripts are then degraded by the nonsense-mediated mRNA decay (NMD) machinery, resulting in lower steady state levels of CLCN1 protein. Correction of the abnormal splicing patterns in HSA^{LR} transgenic mice has been shown to rescue this phenotype, leading to recovery from myotonia [18]. Moreover, in *Clcn1*, MBNL1–3 decrease the insertion of exon 7A, whereas CELF3–6 increase it [19]. However, chloride channelopathy in DM1 has been reported to be due to downregulation of CLCN1 transcription [20]. So, the mechanism of myotonia in DM1 is controversial.

Far less is known regarding the function of human *CLCN1*, as mouse and human *CLCN1* exhibit distinctly different splicing patterns. *CLCN1* encodes for two additional exons, 6B (55 bp) not present in the mouse orthologue and 7A (79 bp) between exons 6 and 7; insertion of either or both of these exons into *Clcn1* results in a frameshift mutation. Alternative splicing of these exons has been shown to produce numerous variants in the skeletal muscle of patients with DM, including variants such as 5–6B–7A–7–8, 5–6–6B–7A–7–8 and 5–8 [17]. Beyond these two additional exons, another splicing pattern characterised by a three base pair (TAG triplet) extension of exon 7 has also been detected [10]. This inserted TAG sequence is thought to act as a stop codon, resulting in the production of the immature mRNA.

To better understand the mechanism of splicing in *CLCN1*, we began by constructing a minigene spanning exons 5–7 of *CLCN1*, resulting in the synthetic *CLCN1* (5–7) minigene. We also established a new assay using real-time reverse transcription (RT)-PCR, which can distinguish between splicing variants based upon the presence of the TAG-inserted pattern. We found significant differences between the human and mouse orthologue, which may have important implications for the study of DM.

2. Materials and methods

2.1. Cell culture, transfection and RNA extraction

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and subcultured at 90–100% confluence. HEK293 cells were plated at 6.0×10^5 cells on 6-well plates 24 h before transfection. For RT-PCR analyses, the *CLCN1* (5–7) minigene (1.0 μg) was transfected with 3.0 μL FuGENE 6 (Promega). In the cellular splicing assay, minigenes (0.2 μg) and splicing factor constructs (0.8 μg) were transfected with 3.0 μL FuGENE 6. For antisense analysis, HEK293 cells were cultured in 12-well plates and transfected with minigenes (0.1 μg) and AONs (antisense oligonucleotides: phosphorothioate 2′ *O*-methyl RNA oligonucleotides (Coralville, IA) listed in Supplementary Table ST1, 50 pmol) using 4.0 μL Lipofectamine 2000 at 50–60% confluence and incubated for 48 h; total RNA was extracted using a GenElute Mammalian Total RNA Miniprep Kit with DNase treatment (Sigma-Aldrich), as described previously [13,14,21].

2.2. RT-PCR and sequence analysis

For cDNA synthesis, 1.0 μg of total RNA was reverse-transcribed with a PrimeScript 1st Strand cDNA synthesis Kit (TaKaRa Bio) in a total volume of 10 μL using oligo(dT) primers. cDNA samples were then diluted fivefold prior to use. PCR was performed using Ex Taq DNA polymerase (TaKaRa Bio) according to the manufacturer's protocol. For sequence analysis of the *CLCN1* (5–7) minigene, PCR was carried out using the following primer set: forward (5′-CATGGTCCTGCTGGAGTTCGTG-3′) and reverse (5′-CTCCAAGTGGTGTCCCAAAACAAC-3′). PCR conditions were as follows: an initial denaturation step at 96 °C for 2 min, 30 cycles at 96 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s, and a final extension step of 72 °C for 5 min. PCR products were then separated via 8% polyacrylamide gel electrophoresis and soaked in ethidium bromide solution (1 μg/ml); then the relevant bands were extracted from the gel. The spliced gel was shaken in solution buffer (0.5 M ammonium acetate and 1 mM EDTA in sterilised water) for 48 h, after which the products were washed in isopropyl alcohol followed by ethanol precipitation. Precipitates were then dissolved in a volume of 5 μL sterilised water. The purified DNA fragment was inserted into a pGEM-TEasy vector (Promega) using a Rapid DNA Ligation Kit (Roche) and sequenced to confirm proper insertion.

2.3. Real-time PCR

Expression of the normal splicing pattern variant of *CLCN1* was quantified using a relative standard curve method following amplification on a StepOnePlus Real-Time PCR System (Life Technologies). Reactions were performed using the Power SYBR Green PCR Master Mix (Life Technologies). PCR reaction was performed in a total volume of 10 μL using cDNA samples (1 μL). PCR conditions were as follows: an initial denaturation step at 96 °C for 20 s, followed by 40–55 cycles at 96 °C for 30 s and 67 °C for 30 s, and a final melt curve step at 96 °C for 30 s, 67 °C for 30 s and 96 °C for 30 s. Expression of the normal splicing pattern was compared against the expression of GFP in the *CLCN1* (5–7) minigene relative to the mock control. Each threshold cycle value (Ct-value) was calculated as an average of three replicates, with undetermined values treated as 0. Another mock sample (pcDNA3.1) was used as the control baseline for calculating relative quantification (RQ) value. The normal splicing pattern was amplified using the *CLCN1* (5–6–7) primer set: forward (5′-GTTCTGCGGGGTATATGAaCA-3′) and reverse (5′-CTCCAAGTGGTGTCCCAAAACAAC-3′); GFP was amplified using the following primers: forward (5′-AAGTTCAGCGTGTCCGG-3′) and reverse (5′-TGTCGCCCTCGAACTTC-3′). Each primer set was adjusted to a final concentration of 500 nM.

2.4. Standard curve

To draw the standard curve, initial templates were diluted in sterile water and supplemented with 0.2 pg/μL salmon sperm single strand DNA (D1626, Sigma), followed by a series of four ninefold dilutions. The pGEM-TEasy vector (Promega) containing the normal splicing pattern insert was used as the control template at a starting concentration of 1 ng/μL. GFP controls were amplified from mock control cDNA. Variance between replicates due to high annealing and extension temperatures was limited by using standard curves with an $R^2 > 0.95$.

2.5. Protein extraction

Transfected cells were harvested in sonication buffer (0.1% Triton X-100, 0.1% protease inhibitor mix in phosphate-buffered saline, PBS) and disrupted 10 times by sonication using Sonifier 450 disruptor (Branson) with an output of 3 and a duty cycle of 10%. After sonication, protein concentrations were quantified using a DC Protein Assay (Bio-Rad) according to the manufacturer's instructions. Briefly, each sample was diluted to 2 μg/μL with PBS and diluted twofold in 2 × sample buffer to a final concentration of 1 μg/μL. Samples were stored at −80 °C.

2.6. Western blotting

Samples were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore). The membranes were then blocked with 5% skim milk in PBS with 0.05% Tween 20 (PBST) for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies, anti-myc (1:5000, R950-25; Invitrogen)