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Antisense oligonucleotide-mediated exon skipping of CHRNA1 pre-mRNA as potential therapy for Congenital Myasthenic Syndromes

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ABSTRACT

CHRNA1 encodes the α subunit of nicotinic acetylcholine receptors (nAChRs) and is expressed at the neuromuscular junction. Moreover, it is one of the causative genes of Congenital Myasthenic Syndromes (CMS). *CHRNA1* undergoes alternative splicing to produce two splice variants: P3A(−), without exon P3A, and P3A(+), with the exon P3A. Only P3A(−) forms functional nAChR. Aberrant alternative splicing caused by intronic or exonic point mutations in patients leads to an extraordinary increase in P3A(+) and a concomitant decrease in P3A(−). Consequently this resulted in a shortage of functional receptors. Aiming to restore the imbalance between the two splice products, antisense oligonucleotides (AONs) were employed to induce exon P3A skipping. Three AON sequences were designed to sterically block the putative binding sequences for splicing factors necessary for exon recognition. Herein, we show that AON complementary to the 5′ splice site of the exon was the most effective at exon skipping of the minigene with causative mutations, as well as endogenous wild-type *CHRNA1*. We conclude that single administration of the AON against the 5′ splice site is a promising therapeutic approach for patients based on the dose-dependent effect of the AON and the additive effect of combined AONs. This conclusion is favorable to patients with inherited diseases of uncertain etiology that arise from aberrant splicing leading to a subsequent loss of functional translation products because our findings encourage the option of AON treatment as a therapeutic for these prospectively identified diseases.

1. Introduction

The neurotransmitter, acetylcholine (Ach), performs a major role in transmitting excitement of motor neurons to muscles at neuromuscular junctions (NMJs) connecting motor neurons to muscle fibers. Congenital Myasthenic Syndromes (CMSs) are caused by mutations in genes responsible for the formation, maintenance and regulation of NMJs, thereby impairing acetylcholine signaling in the muscle [1,2]. Characteristics of CMSs are muscle weakness and easy fatigability. No definitive treatment is available for CMS patients, and responsiveness to treatment varies widely according to the individual and CMS subtype [3]. Therefore, subtype-specific etiological therapy is recommended, especially for those of which the mainstays of therapy have showed little or no benefit.

The nicotinic acetylcholine receptor (nAChR), which is expressed at the post-synaptic NMJ membrane, is a neurotransmitter-gated ion channel that mediates synaptic transmission initiated by the binding of Ach to the receptor, whose signaling, in turn, processes lead to muscle contraction. Five transmembrane subunits form the nAChR, a pentameric complex consisting of 2α, β, δ and ε (in fetal muscle, γ replaces ε). The *CHRNA1* gene encodes the α subunit and undergoes alternative splicing to produce two splice variants: P3A(−), without exon P3A, and P3A(+), with exon P3A [4]. Only P3A(−) forms functional nAChR [5]. The exon P3A, located between exons 3 and 4, is a 75-bp inframe exon containing no stop codon and is conserved only in hominoids [6]. However, the functional significance of P3A(+) has not been described to date, even though mRNA levels for the two isoforms were found in equal proportions in total RNA obtained from human skeletal muscle [4]. Disruption of the extracellular domain of the α subunit by the exon P3A insertion is predicted to result in a blockade of the formation of the functional pentameric nAChRs; this is because the domain is essential for the initiation of subunit assembly [7].

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It has been reported that the two point mutations within *CHRNA1* individually identified in CMS patients alters the proportion of the two variants, leading to an extraordinary increase in the P3A(+) transcript and a concomitant decrease in the P3A(−) transcript [8,9]. The first identified point mutation, IVS3-8G>A, is located within intron 3, 8-bp upstream of exon P3A. The other substitution, αP3A23′G>A, is located at the 23rd nucleotide of exon P3A. Each mutation causes a disruption in the intronic or exonic splicing silencer that results in altered interactions of splicing factors PTB, hnRNP L and hnRNP LL with their splicing regulatory elements of *CHRNA1* pre-mRNA [8–10]. It was also reported that these patients possess compound heterozygous missense mutations that lead to down-regulation of *CHRNA1* expression on the other allele. These mutations contribute to a deficiency of the functional nAChRs and ultimately cause CMS.

Here, we explored the feasibility of targeted exon skipping to correct the aberrant splicing of *CHRNA1* with the mutations identified in CMS patients. Antisense oligonucleotides (AONs) were employed to sterically block putative binding sequences of splicing factors necessary for exon recognition, as well as consequently reduce the inclusion of exon P3A [11]. A minigene construct harboring exons 2–4 of *CHRNA1* and AONs were transfected into HEK293 cells. The effect of AONs on the splicing pattern of the minigene was then evaluated. Semi-quantitative and quantitative analyses revealed that all AONs tested enhanced exon P3A skipping in the minigene system. Furthermore, the most effective AON for the minigene also promoted exon skipping of the endogenous transcript in a cell line expressing wild-type *CHRNA1*, suggesting its potential beneficial effect on endogenous mutated *CHRNA1*.

2. Material and methods

2.1. Minigene and antisense oligonucleotide (AON)

The pRBG4 minigene contains a genomic fragment spanning exons 2–4 of human *CHRNA1* with partial deletion of intron 3 [8,9]. The two previously reported point mutations were introduced into the minigene by site-directed mutagenesis. Primer sequences used are shown in Table S1.

Phosphorothioate 2′-*O*-methyl RNA oligonucleotides were obtained from FASMAC (Kanagawa, Japan). AON sequences used were as follows: 5′-CAGAAAAGGAGAAAGACCUA-3′ (AON-PPT), 5′-CUACCAUGUCACCCUGUCCA-3′ (AON-3ss), 5′-GGUGAUUACUGACCUCAUUC-3′ (AON-5ss), and 5′-AGGACUGCGUGAGAUGGUAG-3′ (AON-control).

2.2. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells and human rhabdomyosarcoma (RD) cells were cultured in Dulbecco's Modified Eagle Media (Sigma, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

Minigene constructs (0.5 μg) and AONs were transfected into HEK293 cells in 6-well plates with 4 μL/well Lipofectamine 2000 (Life Technologies, CA, USA) according to the manufacturer's protocol. Transfection of AON into RD cells was performed with 6 μL/well Lipofectamine 3000 (Life Technologies) following the manufacturer's instructions.

2.3. RNA isolation and reverse transcription

48 h after transfection, total RNA of harvested cells was extracted with GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich, MO, USA) without DNase I treatment.

First-stranded cDNA was synthesized from total RNA of each sample using PrimeScript 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan) with oligo(dT) primer.

2.4. Polymerase chain reaction (PCR)

Alternatively processed transcripts derived from the minigenes and those from endogenous *CHRNA1* in RD cells were selectively amplified by PCR using ExTaq HS (Takara). Both primer sets for minigenes and the endogenous gene flanked exon P3A. PCR products were resolved by 8% polyacrylamide gel electrophoresis. DNA fragments were stained with ethidium bromide and visualized using ImageQuant LAS 4000mini System (GE Health Care, Uppsala, Sweden). Sequences and annealing regions of primers are shown in Table S1 and Fig. S1.

StepOnePlus Real-Time PCR Systems (Applied Biosystems, CA, USA) were used for quantitative analysis with *Power* SYBR Green PCR Master Mix (Life Technologies). We confirmed that the primers used for real-time PCR specifically amplified target transcripts from the minigenes and endogenous *CHRNA1*. Expression levels of each transcript were normalized to *RPL13a*, a housekeeping gene, and calculated according to the comparative Ct method. Sequences and annealing regions of primers are shown in Table S1 and Fig. S1.

3. Results

3.1. The effect of AONs on minigene splicing

The aim of this study was to correct excessive inclusion of exon P3A caused by IVS3-8G>A and αP3A23′G>A point mutations within the *CHRNA11* gene in CMS patients. The desirable outcome is increased expression of P3A(−) encoding functional the α subunit of nAChRs. AONs were applied to prevent binding of splicing factors necessary for exon recognition, thus aiming to skip exon P3A. To determine promising target sequences on the *CHRNA1* pre-mRNA, 20-mer 2′-*O*-methyl phosphorothioate AONs targeting the polypyrimidine tract (PPT) in intron 3 (AON-PPT), the 3′ splice site (AON-3′ss) and 5′ splice site (AON-5′ss) of exon P3A (Fig. 1B) were designed. A previous study showed that the binding of the splicing factor PTB to the PPT inhibits associations of $U2AF^{65}$ to the PPT and U1 snRNP to the 5′ splice site, both of which are required for exon P3A definition [9,10]. The suppressed interactions block exon P3A recognition. Therefore, AON designed to sterically block PPT was predicted to prevent exon recognition in a similar way as that of PTB. Furthermore, AON-3′ss and AON-5′ss were presumed to promote exon skipping by blocking consensus sequences defining each exon boundary, and consequently provoking utilization of the splice site of the adjacent exons. A minigene system was employed to recapitulate alternative splicing of *CHRNA1* in patients and control subjects. These minigenes harbor exons 2–4 of *CHRNA1* located downstream of the CMV promoter (Fig. 1B). The two point mutations identified in CMS patients were individually introduced into the wild-type minigene to induce exclusive inclusion of exon P3A as observed in patients (Fig. 1A and B). The minigenes and AONs were co-transfected into HEK293 cells, and the effect of each AON was assessed by quantification of transcripts with real-time PCR analysis.

HEK293 cells transfected with the wild-type minigene expressed both P3A(−) and P3A(+), whereas cells transfected with each of the mutated minigenes (IVS3-8 G>A, aP3A23′ G>A) (Fig. 1C) showed a weak P3A(−) band and a strong P3A(+) band. Sequences of both PCR products were confirmed by Sanger sequencing. An additional band observed slightly below the P3A(+) band represents an artifact from the minigenes. Therefore, primer sets for quantitative PCR were carefully designed so that quantification of

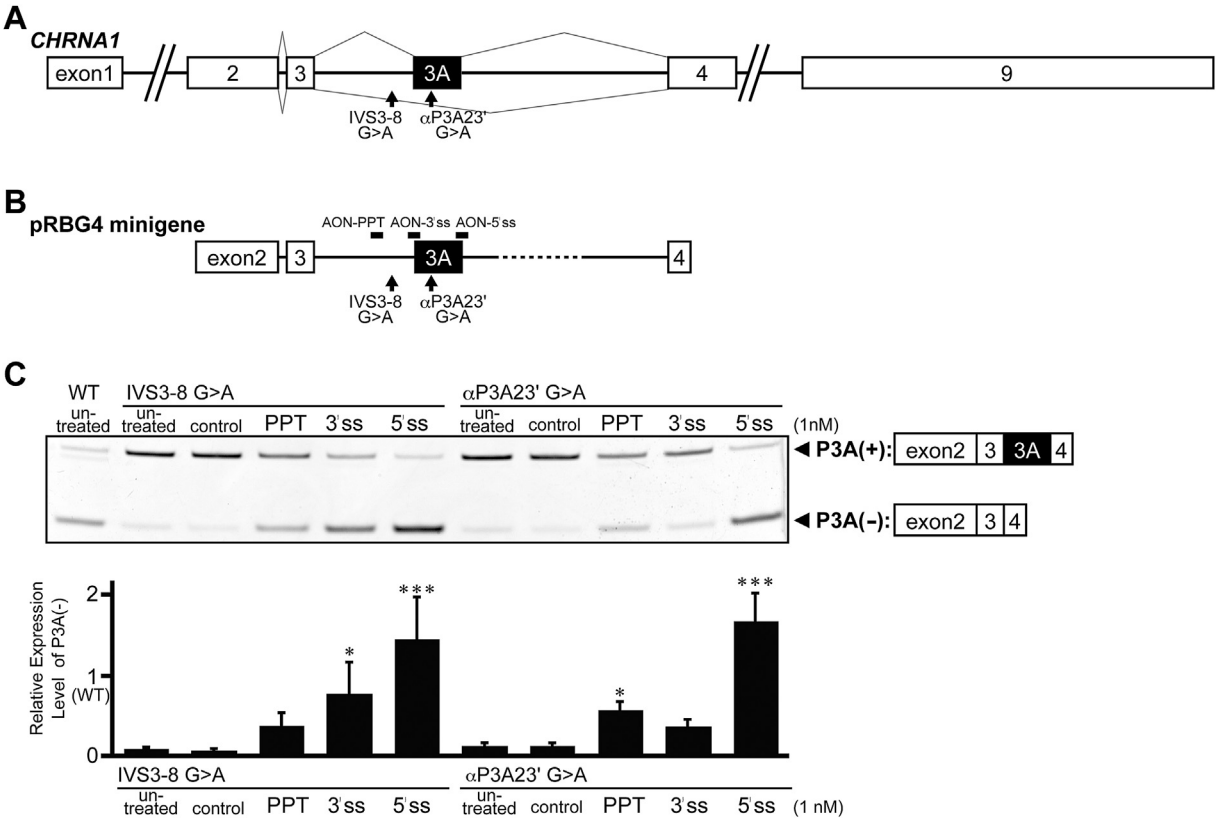

Fig. 1. Comparative efficiency of AON-mediated exon P3A skipping in a minigene system. (A) Schematic representation of the *CHRNA1* gene and two point mutations (IVS3-8 G>A and αP3A23′ G>A) identified in CMS patients. (B) Schematic representation of the pRBG4 minigene and positions of AONs targeting the pre-mRNA. The minigene harbors exons 2–4 of *CHRNA1* with partial deletion of intron 3 (dots). The two point mutations were individually introduced into the wild-type minigene (arrows). AON sequences complementary to the polypyrimidine tract (AON-PPT), 3′ splice site (AON-3′ss) and 5′ splice site (AON-5′ss) of exon P3A were designed not to include the mutations (wide bars). (C) RT-PCR analysis of the effect of AONs on wild-type and mutated minigenes splicing. Each minigene containing the CMS mutations recapitulated the abnormal splicing patterns in HEK293 cells, exhibiting exclusive exon P3A inclusion in untreated HEK293 cells. Treatment with each AON induced exon skipping, resulting in increased expression of the P3A(−) transcript and decreased P3A(+) transcript expression. Quantitative analysis of the P3A(−) transcript with single AON treatment by real-time PCR is shown in the lower panel. Relative expression levels of P3A(−) were calculated by dividing the relative amount of P3A(−) by that of all minigene transcripts. The ratio of wild-type transcript was calculated to be 1. AON-5′ss was the most effective at increasing P3A(−) for both mutations at a concentration of 1 nM. Data are expressed as means ± SD ($n = 4$) (Dunnett's test compared to control, $^{*}p < 0.05$, $^{***}p < 0.001$).

the alternative splicing pattern of minigenes in HEK293 cells was not interfered with by the existence of artificial transcripts.

All AONs employed herein increased exon P3A skipping relative to untreated condition or AON-control cells (Fig. 1C). Quantitative PCR revealed that the ratio of the splice variants was shifted to favor the production of P3A(−) at various degrees in the presence of each AON (except AON-control). We demonstrated that AON-5′ss was the most effective at increasing P3A(−) for both mutations when used at a concentration of 1 nM. In addition, the exclusion of exon P3A occurred in an AON sequence-specific manner because no effect on splicing was detected using the AON-control, which consists of random sequence without any complementary sequence in the endogenous genes or minigenes.

3.2. Further evaluation of the effect of AON-5′ss on minigene splicing

Since AON-5′ss showed the highest efficacy in correction of the aberrant splicing caused by both point mutations, we attempted to investigate the effect profile of AON-5′ss in greater detail. The efficiency of AON-mediated exon skipping was further assessed by titration of 0.25–4 nM AON-5′ss to determine whether this AON inhibits exon recognition in a dose-dependent manner. Increasing AON-5′ss concentrations resulted in an elevated amount of P3A(−) relative to the entire transcript from the minigene (Fig. 2A). Importantly, only 0.5 nM AON-5′ss was sufficient to achieve the wild-type P3A(−) expression level with respect to both mutations. These results indicated a dose-dependent effect of AON-5′ss on exon P3A skipping.

Previous studies have reported that a combination of multiple AONs (so-called “cocktail AON”) can exhibit striking exon skipping capacity [12,13]. Therefore, we tested whether various combinations of AONs could correct predominant inclusion of exon P3A with a higher efficiency than a single AON. Minigenes and cocktails of various AON pairs (0.5 nM each, total 1 nM) were co-transfected into HEK293 cells. All AON combinations exhibited potent effects on exon P3A skipping regardless of the two point mutations (Fig. 2B). However, the effectiveness appeared additive and no drastic change in exon skipping efficiency attributed to AON combinations was observed. Although the combination of AON-3′ss (0.5 nM) and AON-5′ss (0.5 nM) exerted a significant restoration effect, the efficiency was considered intermediate between that of AON-3′ss (1 nM) and AON-5′ss (1 nM). Therefore, treatment with the single AON-5′ss is predicted to show better potency than with any combination of AONs. Taken together, we propose that treatment with the single AON-5′ss is a simple and promising method of

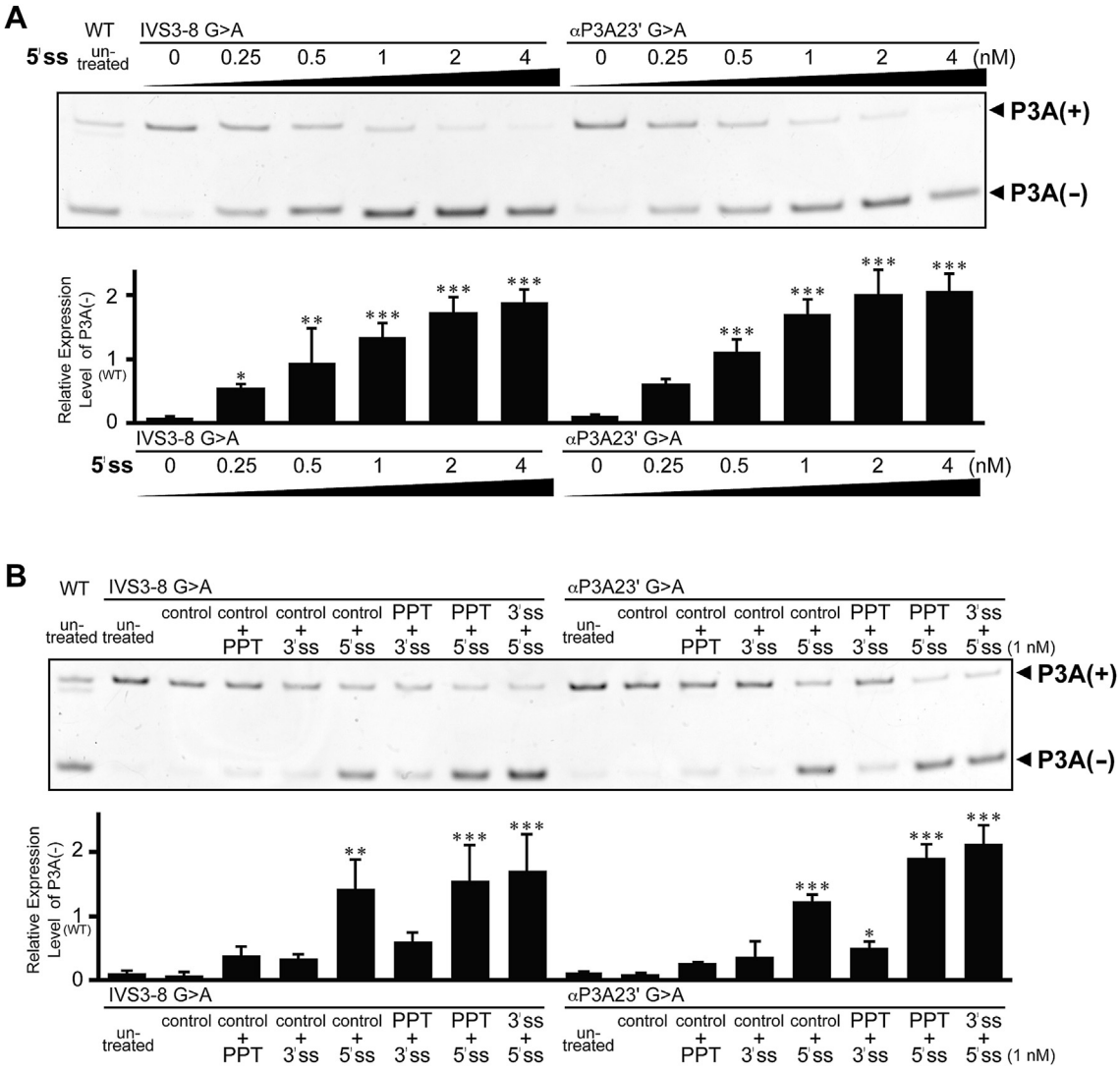

Fig. 2. The effect of AON-5′ss is dose-dependent and additive. (A) RT-PCR analysis of the effect of AON-5′ss titration on mutated minigene splicing. Increasing AON concentrations (0–4 nM) resulted in an elevated amount of P3A(−) and a reduced amount of P3A(+) in a dose-dependent manner. Quantitative analysis of the P3A(−) transcript in the presence of AON-5′ss by real-time PCR is shown in the lower panel. The 0.5 nM of AON-5′ss was sufficient to correct the aberrant splicing and restore P3A(−) expression levels to normal on both mutations. Data are expressed as means ± SD ($n = 4$) (Dunnett's test compared to control, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (B) RT-PCR analysis of the effect of AON combination treatment on mutated minigene splicing. AON cocktails containing AON-5′ss were the most effective at shifting splice variants from P3A(+) to P3A(−) in both point mutations. Quantitative analysis of the P3A(−) transcript by real-time PCR is shown in the lower panel. Combinatory AON treatment induced an additive effect on exon P3A skipping. Data are expressed as means ± SD ($n = 3$) (Dunnett's test compared to control, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

correcting aberrant splicing of exon P3A in *CHRNA1* and can be predicted to exhibit fewer off-target effects compared with the cocktails.

3.3. The effect of AONs on endogenous CHRNA1 in RD cells

We next tested whether the AONs also promote exclusion of exon P3A in the context of the endogenous transcript. Human rhabdomyosarcoma (RD) cells, which endogenously express wild-type *CHRNA1*, were utilized because primary myoblasts derived from CMS patients with the mutations were unavailable. The potency of AONs to endogenous splicing was evaluated using the wild-type *CHRNA1*. We deemed it reasonable to evaluate the potency of AONs on splicing of endogenously expressed wild-type *CHRNA1* in RD cells because AON treatment increased P3A(−) expression in HEK293 cells transfected with wild-type minigenes and AON-5′ss was the most effective, as seen in HEK293 cells with mutated minigenes (Fig. S2). These results suggest that splicing modulation with AON-5′ss is mutation-independent. A significant increase in the relative expression of endogenous P3A(−) was detected upon treatment with AON-PPT and AON-5′ss. AON-5′ss was the most effective in the endogenous system, similar to the minigene system (Fig. 3). Compared with the minigene system, the required AON concentrations were significantly higher and the expression changes were moderate due to the lower transfection efficiency of AONs into RD cells (data not shown).

4. Discussion

Herein, we showed that AON-5′ss complementary to the 5′ splice site of exon P3A of *CHRNA1* effectively reduced exon P3A inclusion into mature mRNA during splicing. Our results raise the possibility of clinical application of AON-mediated exon skipping for aberrant alternative splicing of *CHRNA1* in CMS patients. The

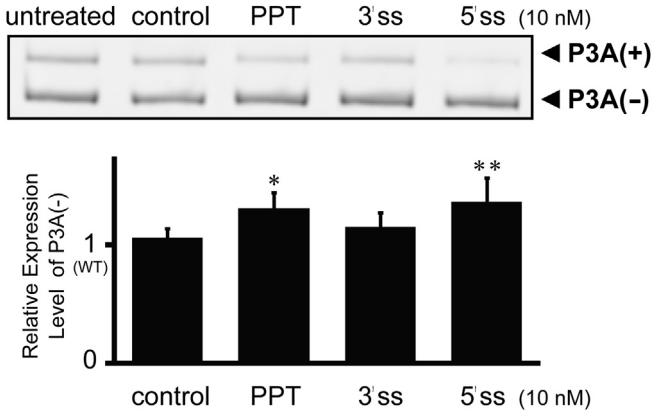

Fig. 3. Manipulation of endogenous wild-type *CHRNA1* splicing in RD cells by AON treatment. RT-PCR analysis of the effect of AONs on endogenous wild-type *CHRNA1* splicing in RD cells is shown in the upper panel. The P3A(+) transcript was diminished and the P3A(−) transcript slightly increased following AON treatment compared with untreated and AON-control treated cells. Quantitative analysis of the P3A(−) transcript from endogenous wild-type *CHRNA1* by real-time PCR is shown in the lower panel. A significant increase in the relative expression of P3A(−) was detected following treatment with AON-PPT and AON-5′ss. Data are expressed as means ± SD ($n = 5$) (Dunnett's test compared to control, $^{*}p < 0.05$, $^{**}p < 0.01$).

AON effect was confirmed using the minigene system and endogenous *CHRNA1* in RD cells, in which treatment with AON-5′ss significantly increased P3A(−) transcript levels. We conclude that single-agent administration of AON-5′ss is superior to that of the other AONs based on the comparative efficacy of treatment with each AON on the minigenes and endogenous *CHRNA1*. The conclusion is also based on the fact that exon skipping efficiency of AON combinations was additive and did not surpass that of AON-5′ss only when used at the same final concentration. Furthermore, treatment with a single AON is predicted to have less off-target effects and is more cost-effective.

AONs are currently being employed to correct splicing abnormalities for a variety of diseases, including Duchenne muscular dystrophy [14,15] and spinal muscular atrophy [16,17], and have shown promising results both *in vitro* and *in vivo*. These AONs are believed to restore aberrations by sterically blocking putative cis-elements and physically interfering with the element recognition by the spliceosome [11]. Based on that premise, we designed three AON sequences against the PPT, 3′ splice site and 5′ splice site of exon P3A since these elements are necessary for formation of the exon-defined complex leading to the exon P3A definition [9,18]. Every AON worked well presumably with the expected mode of action showing varying degrees of efficiency without affecting transcription efficiency because no change was observed in the expression of total transcripts from the minigenes and endogenous *CHRNA1* (data not shown). This diversity in efficiency may be attributed to differential accessibilities of each AON to its complementary sequences on *CHRNA1* pre-mRNA arising from RNA conformation. Differences in contributions of each targeted cis-acting elements to formation of the defined complex could also explain the differential potencies.

Our results suggest that exclusive inclusion of exon P3A occurring in CMS patients can be restored by AON treatment, though lacking data on the effect of AONs on cells derived from CMS patients. This explanation is based on logical reasoning. Firstly, we postulate the same mechanism on minigene and endogenous gene based upon the agreement of data from the wild-type minigene and that from wild-type endogenous *CHRNA1*, despite a smaller change in RD cells that may have resulted from low transfection efficiency. Then, the same mechanism is also expected to underlie when AONs act on both the endogenous gene containing the mutations and mutated minigenes. Finally, due to the identical underlying mechanism, the desirable results obtained with mutated minigenes would be also seen with the endogenous gene with the causative mutations.

Use of pharmacologic therapy in CMS patients has been reported [1,3]. However, the selection of effective drugs requires an accurate diagnosis of CMS subtypes caused by different mutations, and the mis-prescription can be harmful. It was reported that the CMS patients with mutations studied here were less responsive to medical treatments currently available [8,9]. A previous study reported that tannic acid corrected abnormal splicing of *CHRNA1 in vitro* by up-regulating PTB [8]. In addition to therapeutic approaches with such chemical compounds, we propose AON as another promising option for CMS therapy, which has been described to directly act in the splicing process as a well-established method for exon skipping [19]. CMS is frequently misdiagnosed or undiagnosed due to its indistinguishability, unfamiliarity and requirement for specialized examination [2,3,20]. We believe that progress in medical treatment will encourage the identification of potential patients and promote accurate diagnoses.

Our results reinforce the usefulness of AONs for diseases caused by aberrant splicing, whether the etiologies are known or uncertain. It was estimated that mutations affecting splicing account for at least 20% of non-synonymous mutations [21,22], but researchers have sought to uncover pathologies focusing mainly on proteins with amino acid substitutions or expression level changes [23,24]. Splicing defects caused by genetic mutations are difficult to predict in practice due to the considerable sequence variation of splicing regulatory elements, with the exception of several conserved motifs such as the 3′- and 5′-splice sites, PPT and branch points. Moreover, these elements can be located in exons or introns or near to or distant from the splice sites. Despite these obstacles, *in silico* prediction of mutation-induced splicing outcomes has been developed based on accumulated knowledge [25–28]. It is expected that further genetic disorders caused by aberrant splicing will be identified thanks to the combination of *in silico* prediction and high-throughput DNA sequencing. We hope our finding will encourage the potential use of AON-based therapy for these unidentified diseases.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.035.

Transparency document

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