Design of Bifunctional Antisense Oligonucleotides for Exon Inclusion

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Abstract

Bifunctional antisense oligonucleotide (AON) is a specially designed AON to regulate pre-messenger RNA (pre-mRNA) splicing of a target gene. It is composed of two domains. The antisense domain contains sequences complementary to the target gene. The tail domain includes RNA sequences that recruit RNA binding proteins which may act positively or negatively in pre-mRNA splicing. This approach can be designed as targeted oligonucleotide enhancers of splicing, named TOES, for exon inclusion; or as targeted oligonucleotide silencers of splicing, named TOSS, for exon skipping. Here, we provide detailed methods for the design of TOES for exon inclusion, using *SMN2* exon 7 splicing as an example. A number of annealing sites and the tail sequences previously published are listed. We also present methodology of assessing the effects of TOES on exon inclusion in fibroblasts cultured from a SMA patient. The effects of TOES on *SMN2* exon 7 splicing were validated at RNA level by PCR and quantitative real-time PCR, and at protein level by western blotting.

Key words Antisense oligonucleotide, Bifunctional antisense, Pre-mRNA splicing, TOES, Splice switching, Exon inclusion, Exon skipping

1 Introduction

Harnessing antisense oligonucleotides (AONs) to redirect the altered pre-messenger RNA (pre-mRNA) splicing and modulate target gene expression is an efficient therapeutic strategy for genetic disorders associated with alternative splicing. A number of AON approaches have been investigated on redirecting pre-mRNA splicing. The original strategy is to use AONs complementary to a cryptic splice site to prevent its use and favored selection of the authentic site [1]. This approach has been used regularly to alter the proportion of splice isoforms produced from mutated genes or alternative splicing units. In addition to blocking the splice sites, alternative splicing events are often controlled by regulatory proteins bound to exonic and intronic elements located beyond the alternative splice sites. A valid approach is to use AONs to directly

target exonic or intronic elements by blocking the binding of regulatory proteins to these elements that are involved in pre-mRNA splicing. This strategy has been successfully used to augment the exon 7 inclusion in *SMN2* gene by using a short AON to target an intronic splicing silencer (ISS) within the gene [2–5]. Nusinersen, an 18-mer AON annealing to the ISS-N1 element in *SMN2* intron 7 is the first antisense drug approved by the US Food and Drug Administration (FDA) for treatment of any types of spinal muscular atrophy (SMA) [6–8]. This strategy has also been proved to be very effective in Duchene muscular dystrophy (DMD) by promoting the skipping of an exon in the *DMD* gene to restore the interrupted reading frame hence partial rescue of the functional dystrophin protein [9, 10]. Three AON drugs, eteplirsen for exon 51 skipping and golodirsen and viltolarsen for exon 53 skipping in the *DMD* gene, have been approved by the FDA for treatment of DMD [11–13].

The other splice switching approach is the use of bifunctional oligonucleotides to increase the number of positively or negatively acting signals in an exon or intron and to regulate the alternative splicing. The oligonucleotides were designed with one domain (the antisense domain) annealing to the target exon or intron, and another domain (the tail domain) containing a sequence that either recruits RNA binding proteins involved in pre-mRNA splicing [14] or is made of a synthetic protein domain covalently linked to the antisense domain [15]. This approach may be designed as targeted oligonucleotide enhancers of splicing (TOES) for exon inclusion [14, 16], or as targeted oligonucleotide silencers of splicing (TOSS) for exon skipping [17].

The effectiveness of TOES as a potential therapy for SMA by augmenting exon 7 splicing in *SMN2* gene has been approved both in vitro in cellular model [14, 16] and in vivo in mouse model [18–20]. A bifunctional oligonucleotide targeted to *SMN2* exon 7 was expressed in transgenic mice within a modified U7 snRNA gene. Expression of the TOES-U7 RNA in a mouse model of SMA produced a substantial improvement in function and lifespan [20]. Two other bifunctional oligonucleotides targeting the intronic splicing silencers in *SMN2* intron 6 and intron 7, which have the dual effects of blocking the silencer and recruiting activator proteins, also showed the potential therapeutic effects in the transgenic mouse models of SMA [18, 19].

We describe here the details in design of bifunctional oligonucleotide for exon inclusion by correcting *SMN2* exon 7 splicing as an example (Fig. 1). TOES oligonucleotides are designed to contain two domains, an antisense domain complementary to sequences of *SMN2* gene and a tail domain comprising sequences known as binding moieties for splicing activator proteins. The following design principles for TOES oligonucleotides are followed: (1) the antisense sequence may anneal to the potential

2 Materials

1. Online software to identify splicing motifs, e.g., Human Splicing Finder (<http://www.umd.be/HSF/HSF.shtml>).
2. Online software to predict the secondary structures of the target gene and AONs (<http://rna.urmc.rochester.edu/RNAstructure.html>).
3. Online software to calculate oligonucleotide properties on annealing temperature, GC content, and self-complementary (<http://biotools.nubic.northwestern.edu/OligoCalc.html>).

1. Oligonucleotides are synthesized commercially by Eurogentec Ltd. (www.eurogentec.com).
2. RNase and DNase-free distilled water (*see* **Note 1**).
3. RNase and DNase-free 1.5 mL Eppendorf tubes.

2.3 Culture of Skin Fibroblasts from SMA Patient

1. Growth medium: Dulbecco's modified eagle medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Glutamax.
2. Trypsin-EDTA.
3. Phosphate-buffered saline (PBS).
4. Incubator set at 37 °C and 5% CO₂.

2.4 Fibroblast Transfection

1. Transfection reagent (e.g. Lipofectamine 2000).
2. Reduced serum medium for transfection (e.g. Opti-MEM).
3. 6 well plate or 35 mm diameter culture dish.
4. Sterile 1.5 mL Eppendorf tubes.

2.5 RNA Extraction

1. RNA isolation kit.
2. β-mercaptoethanol.
3. 70% ethanol (molecular grade).
4. RNase-free water.
5. 1.5 mL RNase-free Eppendorf tubes.
6. NanoDrop spectrophotometer.

2.6 cDNA Synthesis

1. cDNA synthesis kit.
2. Thermocycler.
3. 0.2 mL PCR tubes.

2.7 Polymerase Chain Reaction (PCR)

1. cDNA template from Subheading 2.6.
2. Taq Polymerase.
3. Primers (10 μM forward primer and 10 μM reverse primer). Primers sequences are shown in Table 1.
4. PCR buffer: 10× PCR buffer, 10 mM dNTPs, 50 mM MgCl₂, Taq DNA polymerase (5 U/μL), and nuclease-free water.
5. 0.2 mL PCR tubes.
6. Thermocycler.
7. Tris–Borate–EDTA 1× (TBE) buffer.
8. Agarose.
9. DNA gel stain.
10. Loading buffer.
11. DNA ladder.
12. Gel imaging system.

2.8 Quantitative Real-Time PCR

1. cDNA template from Subheading 2.6.
2. qPCR primers (10 pmol/μL each). Sequences are shown in Table 1.

Table 1**Sequences of primers used for exon 7 inclusion quantification by PCR and quantitative real-time PCR**

Assay	Products	Sequences (5'–3')	Annealing T _m (°C)
PCR	Full-length SMN2 (505 bp)	F: CTC CCA TAT GTC CAG ATT CTC TT	55
	Δ7 SMN2 (451 bp)	R: CTA CAA CAC CCT TCT CAC AG	
qRT-PCR	Full-length SMN2 (133 bp)	F: ATA CTG GCT ATT ATA TGG GTT TT	60
		R: TCC AGA TCT GTC TGA TCG TTT C	
	Δ7 SMN2 (125 bp)	F: TGG ACC ACC AAT AAT TCC CC	
		R: ATG CCA GCA TTT CCA TAT AAT AGC C	

3. Universal SYBR Green Master Mix.
4. 96-well real-time PCR plate.
5. Sealing film.
6. Real-Time PCR Thermal Cycler.

2.9 Western Blotting

1. Protein extraction buffer: 0.25% SDS, 75 mM Tris–HCl (pH 6.8), or RIPA buffer.
2. Protease inhibitor cocktail tablets.
3. Pierce BCA Protein Assay Kit.
4. PBST washing buffer (PBS, pH 7.4, 0.1% Tween 20).
5. Mini gel tank and blot transfer set.
6. NuPAGE 10% Bis-Tris precast gels, LDS sample buffer (4×), SDS running buffer (20×), antioxidant, sample reducing buffer, transfer buffer (20×), methanol.
7. Protein molecular weight ladder.
8. PVDF membrane.
9. Odyssey blocking buffer for PVDF membrane blocking.
10. Antibodies: mouse anti-SMN monoclonal antibody (BD Transduction Laboratories), mouse anti-β-tubulin monoclonal antibody (Sigma), IRDye 800CW-conjugated goat anti-mouse secondary antibody (Li-Cor).
11. Odyssey imaging instrument to quantify western blot signals.

3 Methods

3.1 Design of Bifunctional Oligonucleotides

1. Predict the potential binding motifs of the negative splicing regulator heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) in the target intron or exon sequences, using *Human Splicing Finder* online software.

2. Other splicing repressors, such as intronic splicing silencers and exonic splicing silencers, may also be identified in the literature. A number of annealing sites in intron 6, exon 7, and intron 7 have been reported to augment *SMN2* exon 7 splicing by bifunctional AONs (Table 2) (*see Note 2*).
3. AONs, of 15–20 mer in length, are designed to anneal to the potential binding sites of hnRNP A1 or other splicing silencers.
4. The GC content of each AON sequence should be 40–65%, with an ideal content of approximately 60%.
5. Avoid four consecutive “G,” strong secondary structure or self-complementary sequences, and self-dimers.
6. Chemical modifications, e.g., 2'-*O*-methyl and locked nucleic acid (LNA), may be applied to the antisense sequence to improve stability and increase binding affinity.
7. Select the tail domain. Examples are listed in Table 2.
8. No chemical modifications are recommended in the tail domain except the cap sequence (Fig. 1) [16] (*see Note 3*).

3.2 Transfection of SMA Fibroblasts

1. Seed the cells in a 6-well plate at a concentration of 2×10^5 cells per well, which gives 80% confluence on the next day.
2. Cells are cultured in 2 mL of growth medium for 24 h.
3. 24 h later, change the growth medium to 1 mL Opti-MEM and leave the cells in the incubator during the preparation of transfection mixes.
4. Prepare the transfection reagent mixes in sterile 1.5 mL tubes. For each sample, prepare two mixes: the first mix (Mix A) contains 100 μ L Opti-MEM and 1 μ L AON at desired concentration (e.g. 1 μ L AON at 100 μ M to get a 100 nM final concentration). While for the mock control add only 100 μ L Opti-MEM. The second mix (Mix B) contains 100 μ L Opti-MEM and 5 μ L Lipofectamine 2000.
5. Mix the AON-containing tube (Mix A) with the lipofectamine-containing tube (Mix B) at a ratio of 1:1 (100 μ L + 100 μ L).
6. Incubate the transfection mix for 20 min at room temperature (RT).
7. Add 800 μ L Opti-MEM in the transfection mix to top it up to a final volume of 1 mL.
8. Remove Opti-MEM from the 6 well plate and replace with 1 mL transfection mix in each well.
9. Incubate the plate for at least 6 h at 37 °C with 5% CO₂ (*see Note 4*).

Table 2
The reported bi-functional AONs designed for SMN2 exon 7 inclusion

Intron/ exon	Antisense binding domain	Sequence of the antisense domain (5'–3')	Sequence of the tail domain (5'–3')	Recruiting protein	Reference
Exon 7	hnRNP A1 (ESS)	GAUUUUGUCUAAAAC	ACAGGAGGCAGGAGGAGGA	SRSF1	[5, 7]
Intron 7	hnRNP A1 binding site (ISS-N1)	GAUUCACUUUUCAUAAUUGCUGG	CACACGACACACGACACACGA	SF2/ASF	[10]
Intron 7	hnRNP A1 binding site (ISS-N1)	GAUUCACUUUUCAUAAUUGCUGG	GAAGGAGGGAAGGAGGGAAGGAGG	hTra2β1	[10]
Intron 6	Element 1 (E1)	CUAUUAUAGAUAGUUA UUCAACAAA	CACACGACACACGACACACGA	SF2/ASF	[9]
Intron 6	Element 1 (E1)	CUAUUAUAGAUAGUUA UUCAACAAA	GAAGGAGGGAAGGAGGGAAGGAGG	hTra2β1	[9]
Intron 7	hnRNP A1 binding site (ISS-N1)	AGUAAGAUUCACUUU	UGUGUGUGUGUGUGUGUG	TDP-43	[8]

3.3 Splicing Assay of Bifunctional AONs on SMN2 Exon 7 Inclusion at RNA Level

1. Extract RNA from SMA fibroblasts using RNeasy Mini Kit according to manufacturer's instruction.
2. Reverse transcription: the cDNA is synthesized from 500 ng RNA using cDNA Synthesis kit according to manufacturer's instruction.
3. PCR of *SMN2* transcripts: Use 1 μ L cDNA in a 25 μ L PCR reaction with 500 pmol of each primer (Table 1), 200 μ M of dNTPs, 1.5 mM $MgCl_2$, 2.5 units of Taq polymerase and 1 $\times$ PCR buffer. The PCR amplification program is as follows: 1 cycle with 3 min at 94 $^{\circ}$ C (initial denaturation), 25–30 subsequent cycles of 30 s at 94 $^{\circ}$ C (denaturation), 30 s at 55 $^{\circ}$ C (annealing), and 30 s at 72 $^{\circ}$ C (extension), followed by a final 10-min extension at 72 $^{\circ}$ C. Check an aliquot of the PCR product (5–10 μ L) in 1.5% agarose gel electrophoresis and SYBR safe DNA stain using an UV transilluminator. The top band is the full-length *SMN2* product (505 bp). The lower band is the product without exon 7 ($\Delta 7$ *SMN2*, 451 bp).
4. Quantitative real-time PCR of full-length and $\Delta 7$ *SMN2* transcripts: product specific primers (Table 1), cDNA and 1 $\times$ PCR Master mix are mixed in a 20 μ L PCR reaction. The program includes activation at 95 $^{\circ}$ C for 3 min, 40 cycles of 95 $^{\circ}$ C for 10 s, and 60 $^{\circ}$ C for 1 min. The cycle at which the amount of fluorescence is above the threshold (C_t) is detected. For quantification, it is possible to use the standard curve method produced from serial dilutions of cDNA from untreated SMA fibroblasts, or the $\Delta\Delta C_t$ method. Normalize the ratios of full-length *SMN2* and $\Delta 7$ *SMN2* to a housekeeping gene (e.g. *HPRT1* or *GAPDH*) (see Note 5).

3.4 Bifunctional AONs on Restoring SMN Protein Measured by Western Blotting

1. Remove culture medium from the well. Add 100 μ L ice-cold lysis buffer to the cells. Keep on ice for 5–10 min. Collect lysates using cell scrapers to fresh 1.5 mL Eppendorf tube and homogenize thoroughly with pipette.
2. Centrifuge at 12,000 $\times g$ and 4 $^{\circ}$ C for 10 min. Transfer the supernatant to a fresh tube.
3. Measure protein concentration by a NanoDrop spectrophotometer using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions.
4. Load 5 mg total protein into NuPAGE precast gels and then electrophorese.
5. Transfer electrophoretically separated proteins from the gel to a PVDF membrane.
6. Block the PVDF membrane for 1 h in blocking buffer.
7. Incubate the membrane with the primary antibodies at 4 $^{\circ}$ C overnight on a shaker.

8. Wash the PVDF membrane for 3×10 min in PBST buffer.
9. Incubate the PVDF membrane with fluorescence secondary antibody for 1 h at room temperature.
10. Wash for 3×10 min in PBST and detect bands using the Odyssey Imaging software (Image Studio).

4 Notes

1. DEPC-treated RNase-free water should be avoided to dissolve oligonucleotides. Dissolved AONs should be aliquoted and stored at -20°C and avoid repeated freeze-thaw.
2. For TOES design, the most efficient binding sites of the antisense domain will be the validated exonic or intronic splicing silencers. For exonic silencers, the binding site is favorable to the upstream of the exon.
3. If the antisense domain anneals to an exon, it should avoid inducing any potential exon skipping of the binding exon.
4. Chemical modification of all the RNA nucleotides through the entire tail domain may reduce the binding affinity to protein. However, chemical modification may be only added to the last five nucleotides at the 5'-end of the tail domain (cap, as shown in Fig. 1) to improve the stability while still keep its binding affinity.
5. The duration of transfection can be prolonged to overnight or 24 h. For cells less tolerant to lipofectamine transfection, shorter incubation period, e.g. 6 h, is recommended.
6. It is recommended at least two housekeeping genes are used in the quantitative real-time PCR assay.

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