

Exon Skipping-Mediated Dystrophin Reading Frame Restoration for Small Mutations

Pietro Spitali,^{1†} Paola Rimessi,^{1†} Marina Fabris,¹ Daniela Perrone,^{2,3} Sofia Falzarano,¹ Matteo Bovolenta,¹ Cecilia Trabanelli,¹ Lara Mari,² Elena Bassi,¹ Sylvie Tuffery,⁴ Francesca Gualandi,¹ Nadir M. Maraldi,⁵ Patrizia Sabatelli-Giraud,⁵ Alessandro Medici,³ Luciano Merlini,¹ and Alessandra Ferlini^{1*}

¹Department of Experimental and Diagnostic Medicine, Section of Medical Genetics, University of Ferrara, Italy; ²Department of Chemistry, University of Ferrara, Ferrara, Italy; ³Department of Biology and Evolution, University of Ferrara, Ferrara, Italy; ⁴Université Montpellier 1, Faculté de Médecine and Inserm, U827, Montpellier, F-34000, France; ⁵Institute of Anatomy, University of Bologna and IGM-CNR, Unit of Bologna, c/o IOR, Bologna, Italy

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ABSTRACT: Exon skipping using antisense oligonucleotides (AONs) has successfully been used to reframe the mRNA in various Duchenne muscular dystrophy patients carrying deletions in the *DMD* gene. In this study we tested the feasibility of the exon skipping approach for patients with small mutations in in-frame exons. We first identified 54 disease-causing point mutations. We selected five patients with nonsense or frameshifting mutations in exons 10, 16, 26, 33, and 34. Wild-type and mutation specific 2'OMePS AONs were tested in cell-free splicing assays and in cultured cells derived from the selected patients. The obtained results confirm cell-free splicing assay as an alternative system to test exon skipping propensity when patients' cells are unavailable. In myogenic cells, similar levels of exon skipping were observed for wild-type and mutation specific AONs for exons 16, 26, and 33, whereas for exon 10 and exon 34 the efficacy of the AONs was significantly different. Interestingly, in some cases skipping efficiencies for mutated exons were quite dissimilar when compared with previous reports on the respective wild-type exons. This behavior may be related to the effect of the mutations on exon skipping propensity, and highlights the complexity of identifying optimal AONs for skipping exons with small mutations.

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KEY WORDS: exon skipping; antisense oligonucleotides; dystrophin; *DMD*

Introduction

Nonsense or frame-shifting mutations in the *DMD* gene (MIM# 310377) lead to Duchenne muscular dystrophy (DMD; MIM# 310200), a severe X-linked neuromuscular disorder due to the complete absence of functional dystrophin protein in males. By contrast, the milder allelic Becker muscular dystrophy (BMD; MIM# 310376) is caused by in-frame mutations that give rise to a smaller but functional protein [Hoffman et al., 1987; Monaco, 1989]. The interruption or maintenance of the dystrophin reading frame by the gene mutations explains the phenotypic differences observed in approximately 92% of the BMD/DMD cases [Aartsma-Rus et al., 2006; Koenig et al., 1989]. Although the majority of the mutations in the *DMD* gene consists of large rearrangements, an increasing number of both nonsense and small frame-shifting mutations has been widely reported, due to the improvement of the diagnostic systems [Bennett et al., 2001; Buzin et al., 2005; Flanigan et al., 2003; Hofstra et al., 2004; Roberts et al., 1993, 1994; Tuffery-Giraud et al., 2004; Whittock et al., 1997]. It is now estimated that point mutations account for nearly 30% of dystrophin mutations [Aartsma-Rus et al., 2006; Deburgrave et al., 2007].

Due to the approach of mutation-specific clinical trials, dystrophin mutation characterization meanwhile has become mandatory. For instance, only patients carrying nonsense mutations are eligible for participation in the phase I/IIa trial held by PTC Therapeutics, based on a stop codon read-through strategy [Welch et al., 2007]. Another mutation-specific approach is antisense-induced exon skipping aimed at the reframing of dystrophin transcripts. Among the various antisense types or modifications, the 2'OMePS antisense oligonucleotides (AONs) targeting exon 51 have been recently used in a pilot study in four DMD patients with very encouraging results [van Deutekom et al., 2007].

Because the majority of DMD-causing mutations are out-of-frame deletions clustered within the two major hotspot regions, the exon skipping approach has been focused on this type of rearrangements. However, it has recently been estimated that, in theory, single and double exon skipping would be applicable to 79% of deletions, 91% of small mutations, and 73% of duplications, amounting to 83% of all *DMD* mutations [Aartsma-Rus et al., 2009]. Indeed, only two nonsense mutations were approached in vitro by 2'OMePS AONs designed on the wild-type exon sequence [Aartsma-Rus et al., 2003, 2004].

Additional Supporting Information may be found in the online version of this article.

[†]The first two authors contributed equally to this article.

*Correspondence to: Alessandra Ferlini, Department of Experimental and Diagnostic Medicine, Section of Medical Genetics, University of Ferrara, 74 44100 Ferrara, Italy. E-mail: fla@unife.it

Although single exon skipping for small mutations can be applied for mutations lying within in-frame exons only, many patients are still eligible for such a treatment (11% of all DMD patients; Aartsma-Rus et al., 2009). Therefore, the general aim of this study was to confirm the applicability of single exon skipping to small mutations. We also intended to evaluate whether “universal” or “private,” mutation-specific AONs were more effective in inducing the exon skipping of mutated dystrophin exons, effectively impacting the design of future therapies for these patients. It is, in fact, known that small mutations can disrupt exon splicing enhancers (ESEs) and silencers (ESSs), splicing regulatory sequences devoted to the exon recognition process [Cartegni et al., 2003]. Mutations located in ESE and ESS elements may affect splicing. Consequently, a significant proportion of exonic point mutations could exert an effect completely different as would be predicted from the genetic code. Positive and negative regulatory elements play opposite and fundamental roles in a finely tuned exon recognition process [Nielsen et al., 2007], which need to be taken into account when interfering with the splicing process. Because mutations lying within ESEs may strongly affect the splicing process [Solis et al., 2008], we used this double approach to point out this relevant topic.

We have identified 54 unrelated DMD patients carrying small mutations in the *DMD* gene, of which 32 are novel. Among 24 patients carrying a mutation in an in-frame exon, we selected 5 mutations in 5 different exons to be modulated (listed in Table 1). We induced the specific exon skipping using AONs designed either on the wild-type or on the mutated sequence both in cell-free splicing assays (four exons) and myogenic cell cultures (all five exons). The predictive value of a cell-free splicing assay on exon skipping propensity was assessed to have an alternative assay in case cells from the patients were not available. The AONs design was performed on the basis of well established in silico parameters (sites for SC35 and SF2/ASF binding sites) [Aartsma-Rus et al., 2005, 2008]. This approach follows the concept of targeting exon sequences containing ESEs. It has, in fact, already been established that AONs-mediated exon skipping occurs by blocking the access of the splicing machinery to ESE sites in the exon [Aartsma-Rus et al., 2006]. Exon-internal AONs have proven to be more effective than AONs lying on the canonical splice sites [Aartsma-Rus et al., 2008]. They also reduced the possible risk of off-targeting of nonspecific sequences [Aartsma-Rus et al., 2004].

We showed that skipping efficiencies for certain mutated exons were significantly different from what has been previously reported for the respective wild-type exons. This is the first report describing AONs modulation of dystrophin exons containing point mutations through efficacy comparison of the wild-type versus the mutation-specific “private” AONs.

Material and Methods

Mutation Analysis

DNA from 54 unrelated DMD patients was extracted by QIAGEN BioRobot (Universal System 8000) from whole blood, after informed consent and for diagnostic purposes. All these patients were negative for *DMD* deletions/duplications as assessed by MLPA.

We designed a set of 79 primer pairs for single-exon amplification. Oligonucleotide sequences and amplification conditions are available upon request. The amplicons were sequenced using a 3130 automated sequencer (Applied Biosystem, Bedford, MA)

Table 1. Patients with Dystrophin Small Mutations Analyzed and Characteristics of the AONs Used in the Present Study

Patient	Mutation	n. exon	AON type	AONs sequence	AON length	% GC	Cell-free assays skipping %	SF2/ASF	SC35	SRp40	SRp55	Maximum ESE Finder values over threshold				Cloned intron boundaries
												Distance from 3'ss	Distance from 5'ss	5' Intron length	3' Intron length	
3	c.1132_1135dupCAGT	10	WT	guccacagcagaagaagacc	20	55.00	14.83	4.62 (2)	3.20 (2)	3.88	2.82	100 nt	73 nt	52,717 bp	650 bp	nt 961–196; nt 1,149+98
7	c.1912delC	10	MS	guuugaacucagcugucuuu	20	40.00	25.63	2.94	3.28 (3)	/	2.86	166 nt	7 nt	52,717 bp	650 bp	nt 961–196; nt 1,149+98
		16	WT	ucuunucuaugaucgcuuuaa	22	31.82	5.10	2.24	2.46	3.87	3.07	3 nt	154 nt	7,648 bp	20,367 bp	nt 1,813–293; nt 1,992+97
18	c.3447_3448GG>TT	16	MS	uucugguacacugacuuuuucag	25	36.00	5.10	3.82 (3)	3.23 (3)	3.67 (2)	3.39	78 nt	76 nt	7,648 bp	20,367 bp	nt 1,813–293; nt 1,992+97
		26	WT	ucuguaaucaucugagguu	20	35.00	–8.2	3.82	3.08	3.63	3.74	131 nt	20 nt	8,606 bp	6,023 bp	nt 3,433–211; nt 3,603+206
21	c.4565delT	26	MS	cuccuccuaagccuauuuuc	21	47.62	–13.5	3.36	/	/	/	10 nt	140 nt	8,606 bp	6,023 bp	nt 3,433–211; nt 3,603+206
		33	WT	cgcucucuunucuuuacua	20	45.00	50.72	5.62	/	/	3.74	67 nt	68 nt	3,035 bp	5,629 bp	nt 4,519–227; nt 4,674+120
		33	MS	cuuuuaccuuuuuuuuuuuuuu	22	40.91	19.93	2.34 (2)	2.44	4.21 (2)	/	31 nt	102 nt	3,035 bp	5,629 bp	nt 4,519–227; nt 4,674+120
23	c.4780delTins37	34	WT	uccauucugucagcagcag	20	50.00	9.19	/	3.31 (3)	3.27	3.74 (2)	84 nt	67 nt	5,629 bp	15,310 bp	nt 4,675–231; nt 4,845+298
		34	MS	gaucauaugacgaaugau	20	30.00	85.55	/	/	/	/	108 nt	29 nt	5,629 bp	15,310 bp	nt 4,675–231; nt 4,845+298

List of DMD patients carrying small mutations within dystrophin gene, who were selected for AONs targeting on MyoD transfected fibroblasts. Mutations were annotated on GenBank reference sequence NM_004006.1. Characteristics of the AONs used in the present study are shown. For each AON the highest value is given for each of the SR proteins; the number of nucleotides between the AONs and the 5' and 3' splice sites (SS) is also shown. The distance to the 3' and 5' splice sites is determined from the first (3' splice site) or last (5' splice site) nucleotide in the target sequence. Last column shows the nucleotide composition of the cloned exons and adjacent intron boundaries used for the cell-free in vitro splicing assay. ^aMutation in patient 23 has been previously described [Tuffery-Giraud et al., 2004]. AONs = antisense oligonucleotides.

following the manufacturer's recommendations. Supp. Table S1 lists the mutations identified. Mutation numbering system is based on cDNA sequence (GenBank reference sequence NM_004006.1) in which nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Constructs

The five selected mutated exons and relative intron boundaries (shown in Table 1) were amplified from the patients' genomic DNAs using in-house protocols (available upon request) and cloned in the previously described pBG vector [Gualandi et al., 2003]. Forward primers for each amplicon were designed upstream of the branch site consensus, whereas reverse primers were located at least 100 nucleotides downstream of the donor splice site. The correct nucleotide composition of all constructs was confirmed by sequencing.

AONs Design and Synthesis

Wild-type AON design was based on the highest ESE-Finder value [Cartegni et al., 2003] (http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese_finder.cgi?process=home) and on the scores for SF2/ASF and SC35, paying attention to the absolute distance to the 3' splice site as previously reported [Aartsma-Rus et al., 2005]. AONs sequences, characteristics, and chosen parameters are shown in Table 1.

The AONs were synthesized with a full-length phosphorothioate backbone and 2'-O-methyl modified ribose molecules. Oligonucleotide synthesis was carried out on an ÄKTA™ oligopilot plus 10 DNA/RNA synthesizer (GE Healthcare, Piscataway, NJ) using its trityl-on mode. The sequence was synthesized on a 2- μ mol scale using Primer Support 200 loaded at 80 μ mol/g (Amersham Biosciences, Piscataway, NJ) as previously described [Rimessi et al., 2009]. The final oligonucleotide was dissolved in water and filtered through a short column of Dowex 50WX8 (Na⁺ form, 100–200 mesh) to afford after lyophilization 0.8 μ mol (40%) of target compound. The purity of the full-length desired product was confirmed by MALDI-TOF MS, ³¹P-NMR and RP-HPLC analyses.

In Vitro Cell-Free Splicing Assay

Five in-frame mutated exons cloned into the pBG splicing vector, were transcribed in vitro using T7 RNA polymerase and α^{32} -rGTP, and purified from 5% acrylamide gel to perform in vitro cell-free splicing assays in HeLa nuclear extracts, as previously described [Gualandi et al., 2003]. All the splicing products were reverse transcribed using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were amplified using the Ex2/Ex3 oligos designed on the rabbit β -globin sequences [Gualandi et al., 2003], cloned in the pCRII vector (TA Cloning Invitrogen, Carlsbad, CA), and sequenced on ABI Prism 3130 (Applied Biosystems) to confirm the accuracy of the exon splicing process (amplification conditions and primer sequences are available upon request).

To induce exon skipping in the cell-free in vitro system, 2'OMePS AONs were used in concentrations ranging from 100 to 500 nM. The incubation lasted 3 hours. To semiquantify the amount of the skipped transcript we performed quantification on images from scanned autoradiographs. The fragments were

quantified by Quantity One software (BioRad, Hercules, CA). We compared the amount of spliced products with the total amount product in the presence as well as in the absence of AON. We assumed that the total of the two transcripts (unspliced/spliced) accounts for 100%.

Myogenic Cell Cultures and AONs Transfection

Primary human fibroblasts from five patients (3, 7, 18, 21, and 23 in Supp. Table S1 and listed also in Table 1) were isolated from skin biopsies (obtained after informed consent for research purposes, Ethical Approval N. 9/2005). Cells were cultivated in high-glucose DMEM (GIBCO, Gaithersburg, MD), supplemented with 20% fetal bovine serum (FBS; GIBCO) and antibiotic/antimycotic solution (Sigma, St. Louis, MO). Myogenesis was induced by infection with an Ad5-derived, EA1-deleted adenoviral vector carrying the MyoD gene as previously described [Aartsma-Rus et al., 2003; Havenga et al., 2002; Roest et al., 1996]. All experiments were carried out using cells with a maximum of four passages. Myotubes obtained after 10–14 days of culture in differentiation medium (2% FBS) were transfected with AONs (100 nM) in the presence of polyethylenimine (ExGen500, MBI Fermentas) (2 μ l per μ g of AON) as transfection reagent, as previously described [Van Deutekom et al., 2007].

Myogenic Cultures RNA Studies

Forty-eight hours posttransfection, total RNA was isolated from myotube cultures (RNeasy Kit, Qiagen, Chatsworth, CA) and reverse transcribed into cDNA using random primers and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was performed on β -actin to verify cDNA synthesis and on dystrophin (primers sequence available upon request) using primers situated on several flanking exons to exclude spontaneous multi-exon skipping. For exons 26 and 34 a nested PCR was necessary to detect specific exon skipping.

PCRs were performed by five cycles of 94°C (30 sec), 63°C (30 sec), and 72°C (30 sec) plus 30 cycles of 94°C (30 sec), 62°C (30 sec), and 72°C (30 sec). PCR products were analyzed on 1.5% agarose gels. All amplified fragments were sequenced on ABI Prism 3130 (Applied Biosystems). We did not perform semi-quantitative densitometric analysis on RT-PCR products, because we adopted the Real-time quantitative assay.

Exon-Specific Real-Time Assay (ESRA)

To precisely quantify the percentage of exon skipping we set up eight novel ESRA detecting human dystrophin exons 6, 10, 16, 26, 33, 34, 40, and 70. These exons were chosen because they are not involved in spontaneous alternative splicing events in humans. We performed the ESRA on exons 6, 10, 16, 26, 40, and 70, considered as references, to quantify the amount of dystrophin transcript both in patients' and in control myotubes. In addition, the same ESRA were used to measure the level of physiological exon skipping and the percentage of induced exon skipping in treated cells compared to untreated cells (internal reference). These data were confirmed using an external reference gene (β -actin gene) on cDNA samples from each myotube culture (treated and control cells). All ESRA are based on TaqMan MGB technology, and have been designed by PrimerExpress Applied Biosystems software (primer and probe sequences are available upon request). The amount of the target sequences in respect to internal references (represented by a nonmutated dystrophin

exon) and to an appropriate endogenous control (β -actin gene) was evaluated by the comparative CT method in respect to the untreated control ($\Delta\Delta$ Ct Method) (Applied Biosystems User Bulletin #2).

Immunofluorescence Analysis

Treated myotube cultures were grown onto coverslips and fixed in -20°C methanol at 2–6 days posttransfection, depending on the survival rate of the myotubes. Samples were incubated for 30 min in phosphate-buffered saline (PBS) additioned with 4% bovine serum albumin. All samples were labeled with a polyclonal antidyostrophin antibody (H300 Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:40, washed with PBS, and revealed with a TRITC-conjugated antirabbit secondary antibody; all samples were double labeled with mouse monoclonal antibodies against desmin or developmental myosin heavy chain (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), diluted 1:10 and 1:60, respectively. After several washings with PBS, all samples were incubated with FITC-conjugated antimouse secondary antibodies (Dako, Carpinteria, CA). The slides were mounted with antifade mounting medium (Molecular Probes, Eugene, OR) and analyzed using Nikon Eclipse 80i fluorescence microscope.

Results

Small Mutations Identification

A series of 54 patients with no deletions or duplications were screened for small mutations. Identified mutations are reported in Supp. Table S1. Among the 54 mutations, 32 are novel. The mutation in patient 23 was previously described [Tuffery-Giraud et al., 2004] and has been included in Table 1 (and Supp. Table S1), as the fibroblasts of this patient were used for AON modulation.

Pathogenic mutations were identified for each patient. Mutations include splicing, frame shifting, and nonsense mutations, and 24 DMD patients show small mutations in 16 different in-frame exons. To confirm the applicability of exon skipping for small mutations occurring within in-frame exons we selected five patients to be investigated (number 3 [NM_004006.1:c.1132_1135dup in exon 10], 7 [NM_004006.1:c.1912_1912delC in exon 16], 18 [NM_004006.1:c.3447_3448delGGinsTT in exon 26], 21 [NM_004006.1:c.4565delT in exon 33], and 23 [NM_004006.1:c.4780delTins37 in exon 34]).

AONs Design

For each exon we designed AONs specific for the mutation (mutation specific, msAONs) and AONs not covering the mutations (wild-type, wtAONs) (see Table 1). AONs targeting exons 16 and 26 have been previously designed targeting the acceptor splice sites [Wilton et al., 2007]. We decided to design and synthesize novel exon 16 and 26 AONs targeting exon-internal sequences. The wtAON against exon 33 is 5 nucleotides shorter than the one reported previously [Wilton et al., 2007]. AON on exon 34 was already designed and tested, but because it did not produce any skipping [Wilton et al., 2007], we designed a novel AON closer to the 3' splice site.

ESE-finder software showed that the mutations in exon 10 and 34 did not result in differences in predicted SR protein binding sites. In contrast, the mutated exon 16 shows two additional SR proteins binding sites caused by the mutation (among these, the highest score was for SC35 protein). In exon 33, the mutation

causes the formation of a binding site for the SC35 protein. The exon 26 mutation is responsible for the loss of two SR protein binding sites.

Taking these data into account and considering that our scope was to also include mutation-specific AONs, we were able to design msAONs for exons 10, 16, 26, and 33 according to the rules described above (see Materials and Methods section). The mutation in exon 34 consists of the insertion of a novel stretch of 37 nucleotides, and we decided to design the msAON to target the inserted sequence. This stretch does not result in significant changes in SR protein binding sites as predicted by ESE-finder analysis.

In Vitro Cell-Free Splicing Assays

The five mutated exons (10, 16, 26, 33, and 34 from patients 3, 7, 18, 21, and 23 in Table 1) were cloned into the pBG splicing vector [Gualandi et al., 2003] and analyzed using in vitro splicing assays. Splice variants were characterized and all the exon-exon junctions were sequenced (data not shown). In all cases the sequencing of the cloned splicing products demonstrated that the dystrophin mutated exons were correctly spliced between exons 2 and 3 of the rabbit β -globin gene (Fig. 1A). Antisense modulation using 2'OMePS msAONs and wtAONs was able to induce exon skipping in four out of five constructs in cell-free splicing assays at the scaled AONs concentration used (Fig. 1B). The semiquantitative method we adopted is based on the quantification of the amount of the spliced transcript (included exon) compared to the total amount of product (spliced and unspliced) in the presence of AON relative to the same ratio in the absence of AON. In a major part of the in vitro assays the exon-skipping percentage did not depend on the AON concentration (Fig. 1C). We therefore calculated the average value of the skipping percentages (Fig. 1D), which could be more easily compared to the AONs efficacy in patient-derived cells. We observed that msAON was more efficient for skipping exon 34 (85.55% msAON vs. 9.19% wtAON) and exon 10 (25.63% msAON vs. 14.83% wtAON), whereas wtAON was more efficacious for exon 33 skipping (50.72% wtAON vs. 19.93% msAON). For exon 16 we did not observe any differences in skipping efficacy between wt and msAON (about 5%), whereas we were not able to skip exon 26 in vitro (Fig. 1C and D).

Myogenic Cell AONs Transfection and Transcription Analysis

The same wt- and ms-AONs tested in the in vitro splicing system were used in cell cultures. Using both AON types we were able to detect two products representing the unskipped and skipped transcripts by both primary (exons 16 and 33) and nested RT-PCR (exon 26 and 34) in patient-derived cell cultures (Fig. 2A). In contrast, no exon 10 skipping was observed in either primary nor nested PCR (Fig. 2A), in accordance with previously published results obtained with the universal AON [Wilton et al., 2007]. These experiments were highly reproducible.

RT-PCR of both untreated and treated cells did not show any unspecific splicing product, excluding the cells carrying the exon 10 mutation, which always showed a low level of physiological exon 9 skipping. This event has also been observed in untreated control muscle cells [Aartsma-Rus et al., 2005; Reiss and Rininsland, 1994].

To precisely quantify the specific level of exon skipping as well as to detect very low skipping amounts, we used an ESRA approach for all patient-derived treated and untreated cells. The

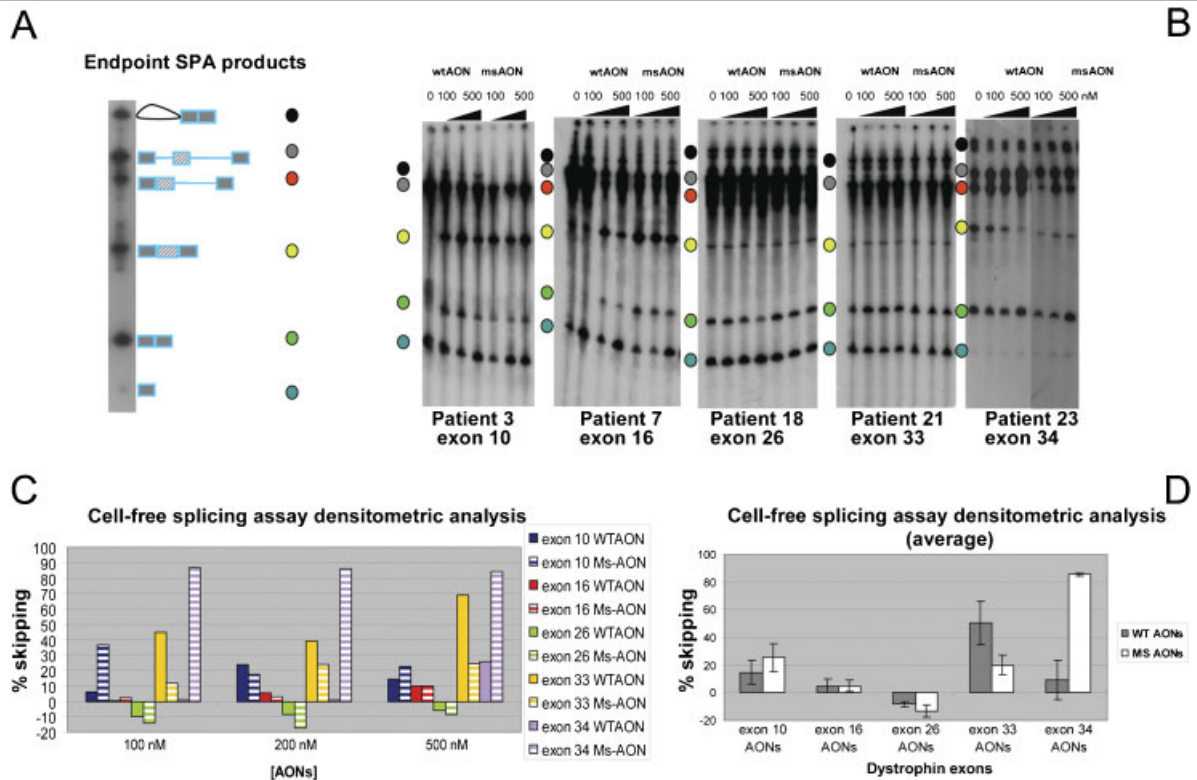


Figure 1. **A:** In vitro cell-free splicing assay endpoint (incubation time of 3 hours), with a scheme of all possible products. Colored dots represent the different products: black: lariat molecule; gray: unspliced template; red: splicing intermediate; yellow: exogenous exon spliced between β -globin exons 2 and 3; green: exogenous exon skipped transcript (β -globin exons 2 and 3); blue: β -globin exon 2. **B:** Specific exon skipping using 2'OMePS AONs in the in vitro cell-free splicing assays. wtAONs were designed on a wild-type sequence, msAONs were designed on mutated sequences. The five mutated exons (patients 3, 7, 18, 21, and 23) were in vitro spliced in the presence of scaled AONs concentrations (100, 200, and 500 nM). **C:** Graphic representation of the densitometric analysis of the exon skipping percentages obtained using the 100, 200, and 500 nM concentrations of all the different AONs. The colors and bars differentiate the skipping efficacy of each mutated exon with wt or msAONs. **D:** Graphic representation of the skipping average value considering the results obtained using all the scaled AONs concentration.

quantification was made using both adjacent dystrophin exons and β -actin as reference transcripts and in comparison to nontreated cells. The skipping percentages are shown in Fig. 2B. Comparing the Ct values obtained with proximal and distal dystrophin exons (β -actin as reference), a 5'-3' transcriptional unbalance emerged, with 5' transcripts more abundant in respect to the 3' transcripts (data not shown). We therefore quantified the skipping percentage of the target exons using adjacent exons as references.

In patient 3 (c.1132_1135dup in exon 10) ESRA assay using exons 6 and 16 as references showed exon 10 skipping levels of 10.9% with msAONs, but only 2.4% of skipping with the wtAON. In contrast, patient 23 (c.4780delTins37 in exon 34) revealed skipping of 6.5% with wtAON and only 1.6% with msAON, using exons 26 and 40 as references. For patients 7 (c.1912delC in exon 16), 18 (c.3447_3448delinsTT in exon 26) and 21 (c.4565delT in exon 33) exon skipping levels were comparable for wt and ms AONs. Exon 33 skipping levels were very high: 58.8% (wtAON) and 58.3% (msAON) using exons 10 and 26 as references, exon 16 skipping levels were lower: 13.2% (wtAON) and 15.8% (msAON), using exons 10 and 20 as a reference, and skipping levels for exon 26 were 1.6% (wtAON) and 2.3% (msAON), with exon 10 and 16 as reference exons. ESRA analysis, assessing the skipping level through comparison with many references, provided robustness to the data, allowed us to detect the exon 10 skipping not visible by

RT-PCR, and identified a low level (less than 0.5%) of spontaneous skipping of the mutated exons in all untreated cells.

In conclusion, the difference in skipping efficiency is significant for exons 10 ($p = 0.0002$) and for exon 34 ($p = 0.0017$), because msAON induced higher levels of exon 10 skipping, and wtAON was more efficient for skipping exon 34 (Fig. 2B). In contrast, the skipping efficiencies for exons 16 and 26 did not vary significantly.

Immunofluorescence Analysis

Immunofluorescence analysis of desmin (data not shown) and developmental myosin heavy chain (Fig. 3A) was performed to define the differentiation stage of the Myo-D transformed fibroblasts. Double-labeling with antidystrophin antibodies revealed the rescue of the protein in all patient-derived cells treated with wtAONs or msAONs. Blinded observation as to the treatment state was performed to quantify the cytoplasmic fluorescence counting fluorescent cells/number of nuclei. The number of dystrophin positive myotubes varied significantly from 5 to 15%. Lowest levels (5%) were observed for cells with the mutation in exon 10 and treated with the wtAON, for exon 26 treated with both wt and msAONs, and for exon 34 treated with msAON. This corresponds to the lower levels of exon skipping observed for these AONs (Fig. 2B). Dystrophin localized at the sarcolemma in all transfected myotubes (Fig. 3A and B).

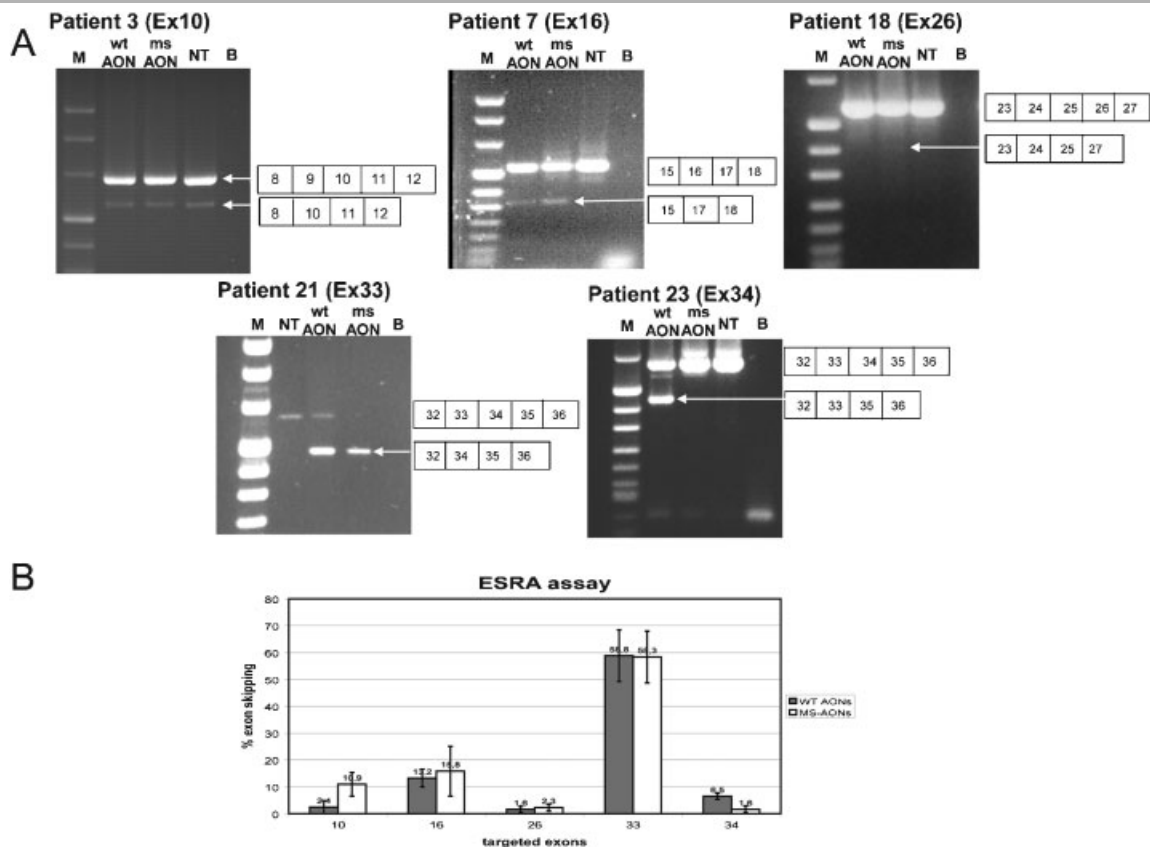


Figure 2. **A:** RT-PCRs of AONs-induced exon skipping in myogenic cells. Exons 10, 26, and 34 (patients 3, 18, and 23, respectively in Table 1) skipping was detected by nested RT-PCR, for the other two exons (30 and 33), primary RT-PCR only was performed. M is molecular weight marker VIII, B: blank sample. **B:** Graphic representation of dystrophin exons 10, 16, 26, 33, and 34 skipping quantitative differences in patient-derived AONs-treated Myo-D transformed fibroblasts in comparison with untreated cells calculated by Real-time assay (ESRA). Histograms represent the percentage of specific exon skipping using either wtAONs (gray bars) or msAONs (white bars) of treated cells with respect to untreated cells.

Discussion

The aim of our study was to induce AON-mediated exon skipping of in-frame exons containing small mutations. We tested 2'OMePS AONs targeting both wild-type and mutated sequences to evaluate the skipping performance and to compare the skipping efficiency between ms and wtAONs. We took advantage from a large cohort of patients we had identified to carry small mutations. We modulated both in cell-free and in patient-derived cells 5 dystrophin “in frame” exons (10, 16, 26, 33, and 34) containing five different small *DMD* mutations.

For the five selected dystrophin exons, the splicing was effectively reproduced in cell-free assays; however, AONs modulation was successful only for exons 10, 16, 33, and 34, whereas exon 26 could not be skipped. For exons 10 and 34 msAONs worked better, whereas for exons 16 and 33 the wtAONs produced higher skipping levels. These data suggest that cell free splicing assays might be used to test the exon skipping propensity. However, because the exon composition/length as well as the flanking intronic regions are crucial for the *in vitro* splicing [Eperon et al., 1988], peculiar mutations like the long stretch included in the mutated exon 34 could hamper the optimal AONs identification in the *in vitro* assay. Therefore, it cannot be considered a general tool for optimizing the design of the AONs.

The fact that our test produced quite different results compared to patient-derived myogenic cells underlines this. For the mutated

exons 16 and 33, we obtained similar levels of exon skipping with both wt and msAONs, comparable to what was previously described for wt exons [Wilton et al., 2007].

The mutation involving exon 10, not altering the splicing enhancer quality/distribution and therefore expected to be silent in term of skipping propensity, makes the mutated exon vulnerable to skipping with a 10.9% efficacy with msAON; slightly higher than described for the wt exon in myogenic cells testing [Wilton et al., 2007]. A possible explanation of this partially positive finding is that the mutation changes the exon 10 conformation. Although encouraging, the ESRA on exon 10 does not allow us to determine whether a contextual exon 9 skipping occurs [Wilton et al., 2007]. However, we underline that exon 9 skipping is a physiological, very common alternative splicing event, and it might therefore be very difficult to sort out its exclusion from the transcript. In addition, as exon 9 is in-frame, this exon skipping is unlikely to be detrimental.

Exon 26 was considered an exon difficult to skip (ratio <10%) [Wilton et al., 2007]. In our hands, both wtAON and msAON induced indeed a similar, very poor skipping level (1.6 and 2.3%, respectively). This finding does not seem to reflect the expected increase of exon 26 skipping propensity linked to the loss of the two SR protein binding sites due to the mutation, as predicted by the ESE finder program.

The insertion in exon 34 is difficult to analyze. Although it appears not to have any modifying influence on splicing factors

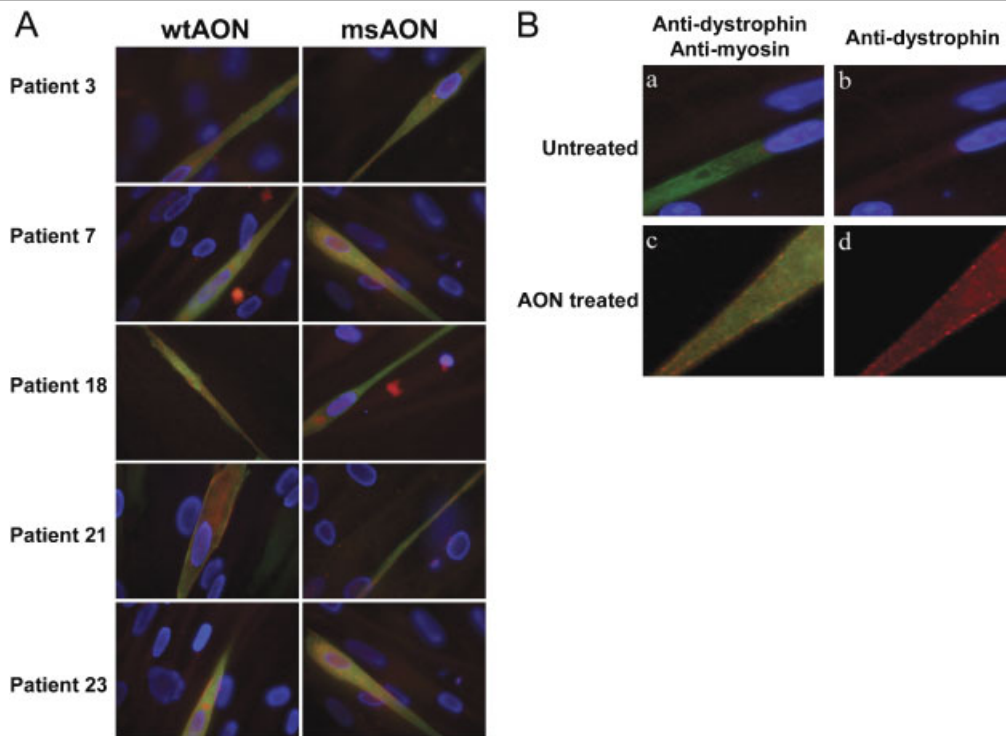


Figure 3. Immunofluorescence analysis of dystrophin in patients' cells. **A:** Double labeling with antimyosin heavy-chain (green) and dystrophin (red) antibodies clearly demonstrated the rescue of dystrophin expression in Myo-D transformed fibroblasts of all treated patient-derived cells. More than 50% of the AON-treated cells in all examined fields showed cytoplasmic green fluorescence, which was quantified as fluorescent cells/number of nuclei. Among these, 5–15% myotubes were dystrophin positive. High magnification (B, d) demonstrates the correct protein localization at the sarcolemma. wtAON indicates antisense oligonucleotide designed on the wild-type exon, msAON indicates antisense oligonucleotide designed on the mutation. **B:** Untreated and AON treated patient's cells stained with antimyosin heavy-chain and antidystrophin antibodies. No dystrophin signal is detected in untreated cells (a, b), whereas dystrophin membrane signal is observed upon the induced exon skipping in treated cells (c, d).

binding sites, obviously this long stretch may alter the exon skipping propensity in other ways. In fact, this mutation made the exon skipping propensity in myogenic cells increase, which is in contrast with previously published data [Wilton et al., 2007]. Indeed, we induced the skipping of the mutated exon 34 in 6.5% of the dystrophin transcript using the wtAON in patient-derived cells. A possible explanation is that the insertion/deletion mutation (insertion of 37 nucleotides and deletion of 1 nucleotide) enhances the exon 34 skipping propensity by enlarging the distance between motifs recognized by the SR proteins and other splicing proteins. Alternatively, the RNA secondary structure change could affect the recognition of the mutated exon, even though the accessibility of the splice sites is the same for wild-type and mutated exon 34 (data not shown).

In conclusion, we induced the specific exon skipping in four cell-free assays and in all five myogenic cells out of five selected mutated exons. When comparing the two different assays it was demonstrated that, in the absence of patient-derived cells availability, the cell-free assay could provide some information, but *in vitro* analysis in patient-derived cells is preferred. Furthermore, the results we obtained strongly suggest that some small mutations may unpredictably change the exon skipping propensity, implying that the exon skipping approach for in frame exons with small mutations deserves careful investigation. Furthermore, antisense targeting of small mutations might unravel splicing regulatory motifs, because flanking introns and exons are not involved in any rearrangement such as deletions/duplications and every

cis-regulatory motif like intronic splicing enhancers (ISEs) or silencers (ISSs) can play a role in the exon defining process.

Notably, AON-mediated skipping of these five exons was never accompanied by other unspecific splicing products, such as the induction of cryptic splicing. The only alternatively spliced product was exon 9 skipping that was also observed in control myotubes. It is plausible (and very interesting) that fusion introns, resulting from deletion or duplication junctions, may influence adjacent exon recognition and therefore splicing accuracy.

Despite the promising results obtained using the exon skipping approach, another crucial point should be considered: the possible effect of the lack of skipped exons on the dystrophin protein. Isolated deletions of exons 10 and 34 are described causing a BMD or only high CK phenotype [Fokkema et al., 2005]. An isolated deletion of exon 16 causes high CK only [Schwartz et al., 2007; and personal observation]. For exons 26 and 33, however, no isolated deletions are reported in the Leiden Open Variation Database LOVD database [Fokkema et al., 2005; White and den Dunnen, 2006]. Therefore, the skipping of these exons may be of low predictive value on the resulting phenotype(s). It might also be possible that such deletions have not been identified yet due to the fact that these deletions do not result in any dystrophic phenotype. However, they all belong to the spectrin-like region (exon 10 to the repeat 1, exon 16 to the repeat 3, exon 26 to the repeats 7–8, exon 33 and 34 to the repeats 11 and 12), and, according to the literature data, their skipping is expected to have mild/BMD-like consequences on the protein function.

The data presented here report the first attempt to modulate small dystrophin mutations by using both wild-type and “mutation-specific” AONs, and highlight both the peculiar splicing characteristics of mutated exons and the complexity of designing optimal AONs for exon skipping therapy toward personalized therapy.

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