

# Treatment of Duchenne Muscular Dystrophy with Oligonucleotides against an Exonic Splicing Enhancer Sequence

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## Abstract

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease and so far, the treatment of DMD has not yet been established. We have proposed a novel treatment for DMD whereby the correction of the translational reading frame from out-of-frame to in-frame transforms severe phenotype into the milder phenotype. Based on the molecular analysis of dystrophin Kobe where the presence of an intra-exon deletion caused exon skipping during splicing, a part of the exon 19 sequence of the dystrophin gene was found to function as a splicing enhancer sequence, a sequence necessary for proper splicing. When 31-mer phosphorothioate oligonucleotides complementary to this sequence were added to the culture medium of normal lymphoblastoid cells, exon 19 skipping was specifically induced. The possibility to transform out-of-frame to in-frame using the oligonucleotide was examined in myocytes from a DMD case who has an out-of-frame deletion of exon 20. The transfection of the oligonucleotide induced exon 19 skipping in a proportion of dystrophin transcripts, thereby creating an in-frame mRNA lacking both exons 19 (88 nt) and 20 (242 nt). Markedly, more than 15 % of myocytes was stained positive for dystrophin concomitant with the appearance of an in-frame transcript, while no dystrophin-positive myocytes were identified without transfection. These results pave a novel way to treat DMD patients by administering oligonucleotides against an exonic splicing enhancer. One natural example of this transformation is described.

**Key words:** dystrophin, splicing, splicing enhancer sequence, antisense oligonucleotide, Duchenne muscular dystrophy, treatment.

**Abbreviations:** DMD (Duchenne muscular dystrophy)  
BMD (Becker muscular dystrophy)

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## Introduction

Duchenne muscular dystrophy (DMD) is a rapid, progressive muscle wasting disease that usually results in death at around the age of 20, while Becker muscular dystrophy (BMD) is a clinically less-severe form of the disease that often has only slight debilitating effects. Deletion mutations in the dystrophin gene have been identified in two thirds of DMD/BMD cases, and the clinical progression of DMD or BMD patients can be predicted from whether the deletion disrupts or maintains the translational reading frame of the mRNA (reading-frame rule) [12].

The frame-shift mutations in DMD patients result in the complete absence of dystrophin in their skeletal muscle, whereas muscle tissue from BMD patients

contains truncated dystrophin translated from the in-frame mRNA. DMD treatment could be achieved by changing an out-of-frame mutation causing DMD into an in-frame mutation characteristic of BMD by modifying the dystrophin mRNA [8,13].

Here we report that transfection of oligonucleotide against an exonic splicing enhancer sequence successfully induced exon skipping and led production of truncated dystrophin in myocytes from DMD. These results point to a novel way to treat DMD patients with administering an oligonucleotide against an exonic splicing enhancer.

## Oligonucleotide Treatment of Duchenne Muscular Dystrophy

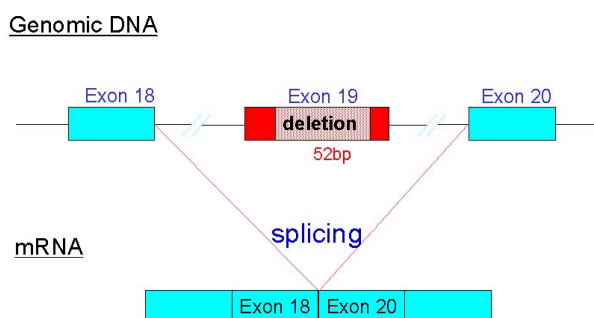


Fig. 1. Schematic representation of exon 19 of dystrophin Kobe.

In the pre-mRNA sequence (upper line), 52 bp of the 88 bp of exon 19 of the dystrophin gene are deleted in dystrophin Kobe. This truncated exon 19 is spliced out together with the intron to produce mature mRNA lacking all of exon 19 (lower line).

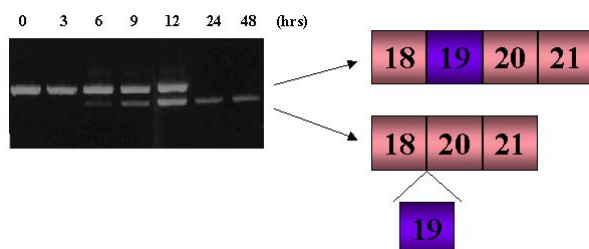


Fig. 2. Induction of exon 19 skipping

For transcription to proceed, lymphoblastoid cells were incubated for the indicated time. A region encompassing exons 18 to 20 was then amplified by RT nested PCR from the dystrophin mRNA extracted from the cells. Only one amplified product corresponding to the normal cDNA was amplified after 0 or 3 hours of incubation. A new product lacking exon 19 started to be produced after 6 hours of incubation, and the relative abundance of this product increased thereafter. All products were derived from the product with exon 19 skipping after 24 hours of incubation or longer. The exon component of the amplified product was schematically described on the right and numbers on the top represent the incubation time (hours).

### Reading-frame rule

Duchenne muscular dystrophy (DMD) is a common inherited disease with a worldwide incidence of 1 in 3,500 male births. DMD patients appear normal until the age of 3-5 years, after which they begin to experience difficulty in rising from the floor, climbing stairs, and other activities involving the large proximal skeletal muscles. The muscular weakness is characteristically progressive. Affected individuals are wheelchair-bound by the age of 12 and succumb to cardiac or respiratory failure in their mid to late 20s. Interestingly, Becker

muscular dystrophy (BMD) a milder form of X-linked muscular dystrophy, is distinguished from DMD by its delayed onset, later dependence on wheel-chair support and longer life span: affected boys remain ambulatory beyond the age of 16 years and a few may lead near-normal lives [4].

Although both DMD and BMD patients have been shown to have deletion mutations in the dystrophin gene, the extent of the deletion does not always correlate with the severity of the disease: some BMD patients with mild symptoms have deletions encompassing numerous exons whereas some DMD patients with severe symptoms lack only a few exons. In some cases, long deletions resulting in BMD and short deletions resulting in DMD may even overlap. The reading-frame rule explains the difference as follows [12]; BMD patients with long deletions may be able to produce a dystrophin mRNA that would still direct the production of an internally truncated semi-functional protein. Shorter deletions harbored by severe DMD patients, on the other hand, would bring together exons that, when spliced, could change the translational reading frame in the mRNA such that a premature stop codon is created. This rule predicts that milder BMD patients would produce a smaller semi-functional protein while DMD patients would either produce a severely truncated form lacking the entire C-terminal region or would not produce a protein at all. Subsequent gene analyses have shown that over 90% of deletion mutations that cause BMD maintain the dystrophin mRNA reading frame, whereas those causing DMD are usually frame shifts [2]. As expected from the reading-frame rule, this protein is completely missing in boys with DMD, whereas muscle tissue from BMD patients contains reduced amounts of dystrophin. Thus, DMD and BMD represent examples of allelic heterogeneity differing so much in its clinical symptoms.

Dystrophin Kobe In one particular dystrophin gene mutation named dystrophin Kobe [9], we found that exon skipping during splicing was induced by the presence of an intra-exon deletion mutation in the genome although all of the consensus sequences known to be required for splicing were unaffected [10]. The deletion detected by PCR analysis revealed that the product amplified from exon 19 encompassing region of the dystrophin gene of the DMD case in question was smaller than normal. This result suggested the presence of a novel mutation within the amplified region. Sequence analysis confirmed that this was the case by showing that 52 bp out of 88 bp of exon 19 were deleted at 2-3 bp upstream from the splice donor site (Fig. 1). This 52 bp deletion was considered to result in a frame-shift mutation that would cause DMD [9].

The dystrophin mRNA of dystrophin Kobe was then analyzed using reverse-transcription PCR (RT-PCR) [10]. Surprisingly, the product amplified from the region extending from exon 18 to 20 was smaller than the predicted one according to the results of the genomic

## Oligonucleotide Treatment of Duchenne Muscular Dystrophy

DNA analysis. Sequence analysis indicated that the whole of exon 19 was missing from the dystrophin cDNA, indicating complete skipping of exon 19 (Fig. 1). This showed that the deletion mutation within an exon sequence could induce a splicing error during the maturation of messenger RNA even though the known consensus sequences at the 5' and 3' splicing sites of exon 19 were maintained [10]. Dystrophin Kobe suggests that the deleted sequence of exon 19 may function as a cis-acting element for exact splicing for the upstream and downstream introns. An *in vitro* splicing system using artificial dystrophin pre-mRNAs disclosed that splicing of intron 18 was almost completely abolished when the wild-type exon 19 was replaced by the dystrophin Kobe exon 19 [15].

It was next investigated whether antisense oligonucleotides against the deleted sequence modulates splicing. An antisense 31 mer 2'-O-methylribonucleotide was designed added to *in vitro* splicing reaction. It inhibited splicing of wild-type pre-mRNA in a dose- and time-dependent manner [15]. These results indicated that the deleted region is a splicing enhancer sequence that is necessary for proper splicing of intron 18 even in the presence of splicing consensus sequences and that the antisense oligonucleotide functions as a inhibitor of splicing enhancer sequence.

### Induction of exon 19 skipping

Since the aforementioned result suggested a possibility of artificial induction of exon 19 skipping, the antisense oligonucleotide against the splicing enhancer sequence was then transfected to normal lymphoblastoid cells. With this transfection, remarkably, skipping of exon 19 started to appear after 6 hours of incubation and complete skipping was observed after 24 hours of incubation (Fig. 2) [13]. None of the other 78 dystrophin exons were skipped and exon 19 skipping could not be induced by the sense oligonucleotide. These results showed that antisense oligonucleotides against a splicing enhancer sequence can induce exon skipping even in living cells.

### Production of dystrophin in DMD derived muscle cells

We subsequently investigated whether the antisense nucleotide can be used to treat a DMD case with 242 nucleotide deletion of exon 20. If exon 19 (88 bp) skipping can be induced in this case, the translational reading frame of dystrophin mRNA will be restored. As a result, this modulation of splicing is expected to lead to the production of internally deleted dystrophin in muscle cells of the case. A Japanese DMD patient was identified to have a deletion of exon 20 of the dystrophin gene. Primary muscle culture cells established from his muscle were transfected with the 31-mer-phosphorothioate oligonucleotide

(5'-GCCTGAGCTGATCTGCTGGCATCTTGCAGTT-3') covering a splicing enhancer sequence of exon 19. Introduction of the oligonucleotide into the nuclei of

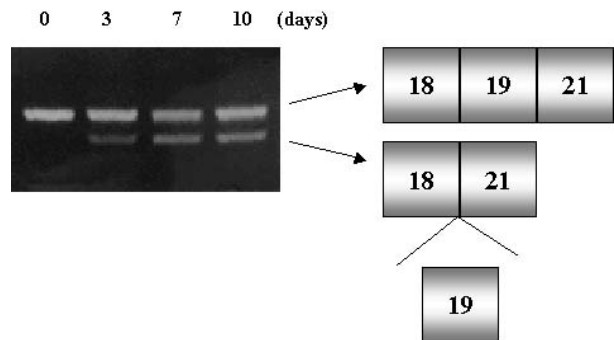
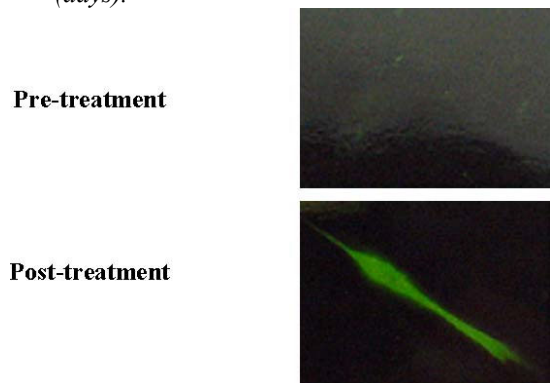


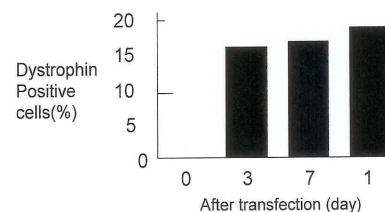
Fig. 3. Transfection of the oligonucleotide into myocytes  
a. dystrophin mRNA analysis

The region from exon 18 to exon 21 of dystrophin mRNA was amplified from cDNA obtained after 0, 3, 7, 10 days of culture following transfection. Only one amplified product corresponding to the exon 20 deletion-type cDNA was amplified before transfection (0). A second product lacking exon 19 started to be produced in extracts of cells after 3 days of incubation (3) and was still obtained with cell extracts after 7 and 10 days of incubation (7 and 10, respectively). The exon composition of the amplified product is represented schematically on the right and numbers at the top represent the incubation time (days).



### b. Immunohistochemistry of myocytes

Green corresponds to dystrophin reacting with antibody recognizing the C-terminal region of dystrophin. On the 3rd days after transfection, the cells are not fluorescent and appear dark against the faint background fluorescence, without transfection (-), while dystrophin was stained in cells



### c. Quantification of dystrophin-positive muscle cells

The data are expressed as mean of percentage of dystrophin positive cells per microscopic field.

## Oligonucleotide Treatment of Duchenne Muscular Dystrophy

### Normal splicing product



### Abnormal splicing product

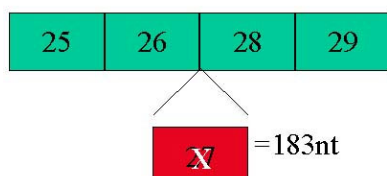


Fig. 4. Skipping of exon 27 that retains nonsense mutation

Two amplified products from dystrophin cDNA encompassing exons 25 to 30 were obtained from muscle cDNA of a BMD patient by RT-PCR. The one product consisted of normal exons (upper) whilst the other lacked exon 7 (lower). From the latter nonsense mutation encoded in exon 27 disappeared from dystrophin mRNA but the resulting mRNA maintained reading frame (lower).

cultured cells led to skipping of exon 19 in a proportion of total mRNA (Fig.3). The simultaneous disappearance of consecutive exons 19 and 20 from the dystrophin mRNA restored the translational reading frame, removed a downstream premature stop codon from the mRNA and caused the production of an in-frame mRNA. As expected, dystrophin-positive cells were identified (Fig.3a). The percentage of dystrophin positive cells was more than 15% at the 10th day after transfection (Fig.3b) [16].

### Natural example of transformation from DMD to BMD

We identified a natural example of a DMD to BMD conversion by a mechanism of exon skipping; a nonsense mutation of the dystrophin gene that was expected to result in a DMD phenotype was identified in a BMD case where skipping of the nonsense mutation containing exon produced in-frame dystrophin transcript [14]. The case had a nonsense mutation in exon 27 of the dystrophin gene. However, cDNA analysis from his skeletal muscle showed two kinds of transcripts; one had a normal exon content and the other transcript showed the skipping of exon 27 (Fig. 4). Since exon 27 encoded 183nt, the resulting dystrophin transcript maintained the translational reading frame thereby producing a truncated dystrophin. This result confirmed our hypothesis that DMD can be treated by the correction of the translational reading frame. In addition, another natural examples causing the conversion of DMD to

BMD was identified in a nonsense mutation of exons 29 and 72 [5,11].

### Treatment of a model mouse with oligonucleotide

Modification of dystrophin mRNA by oligonucleotides has been tried in *mdx* mouse, a DMD model mouse [3, 6, 18, 19]. As a result oligonucleotides that are complementary to the splicing consensus sequence was reported to induce splicing error of exon skipping, thereby resulting in synthesis of truncated dystrophin in *mdx* mouse [7, 17]. In these studies it was splicing consensus sequences at the exon/intron borders that were targeted to induce exon skipping, since sequence that are essential for intron removal had been limited to the exon/intron borders.

### Conclusion

Our innovative approach has several potential advantages over gene replacement; 1) oligonucleotides can be synthesized in large amounts; 2) oligonucleotides can be administered systemically by intravenous infusion [20]; 3) viral vectors, which are sometimes toxic or immunogenic, are not used; 4) genetic information transcribed naturally from the patient's dystrophin gene is modified at the splicing level. Therefore, this strategy seems to have high potential for clinical application. In accordance, strategy of the targeted exon skipping has been exemplified in exon 46 [1].

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### References

- [1] Aartsma-Rus A, Bremmer-Bout M, Janson A, den Dunnen J, van Ommen G, van Deutekom J: Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. *Neuromuscul. Disord.* 2002; 12 Suppl: S71-S77.
- [2] Baumbach LL, Chamberlain JS, Ward PA, Farwell NJ, Caskey CT: Molecular and clinical correlations of deletions leading to Duchenne and Becker muscular dystrophies. *Neurology* 1989; 39: 465-474.
- [3] Duncley MG, Manoharan M, Villiet P, Eperon IC, Dickson G: Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligoribonucleotides. *Hum. Mol. Genet.* 1998; 7: 1083-1090.
- [4] Emery AEH: Duchenne muscular dystrophy. Oxford University Press, Oxford 1993.
- [5] Ginjaar IB, Kneppers AL, v d Meulen JD, Anderson LV, Bremmer-Bout M, van Deutekom JC, Weegenaar J, den Dunnen JT, Bakker E:

## Oligonucleotide Treatment of Duchenne Muscular Dystrophy

- Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family. *Eur. J. Hum. Genet.* 2000; 8: 793-796.
- [6] Lu QL, Morris GE, Wilton SD, Ly T, Artem'yeva OV, Strong P, Partridge TA: Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *J. Cell Biol.* 2000; 148: 985-996.
- [7] Mann CJ, Honeyman K, Cheng AJ, Ly T, Lloyd F, Fletcher S, Morgan JE, Partridge TA, Wilton SD: Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc. Natl. Acad. Sci. U. S. A.* 2001; 98: 42-47.
- [8] Matsuo M: Duchenne/Becker muscular dystrophy: from molecular diagnosis to gene therapy. *Brain Dev.* 1996; 18: 167-172.
- [9] Matsuo M, Masumura T, Nakajima T, Kitoh Y, Takumi T, Nishio H, Koga J, Nakamura H: A very small frame-shifting deletion within exon 19 of the Duchenne muscular dystrophy gene. *Biochem. Biophys. Res. Commun.* 1990; 170: 963-967.
- [10] Matsuo M, Masumura T, Nishio H, Nakajima T, Kitoh Y, Takumi T, Koga J, Nakamura H: Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy Kobe. *J. Clin. Invest.* 1991; 87: 2127-2131.
- [11] Melis MA, Muntoni F, Cau M, Loi D, Puddu A, Boccone L, Mateddu A, Cianchetti C, Cao A: Novel nonsense mutation (C-->A nt 10512) in exon 72 of dystrophin gene leading to exon skipping in a patient with a mild dystrophinopathy. *Hum. Mutat.* 1998; Suppl 1: S137-S138
- [12] Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM: An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 1998; 2: 90-95.
- [13] Pramono ZA, Takeshima Y, Alimsardjono H, Ishii A, Takeda S, Matsuo M: Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence. *Biochem. Biophys. Res. Commun.* 1996; 226: 445-449.
- [14] Shiga N, Takeshima Y, Sakamoto H, Inoue K, Yokota Y, Yokoyama M, Matsuo M: Disruption of the splicing enhancer sequence within exon 27 of the dystrophin gene by a nonsense mutation induces partial skipping of the exon and is responsible for Becker muscular dystrophy. *J. Clin. Invest.* 1997; 100: 2204-2210.
- [15] Takeshima Y, Nishio H, Sakamoto H, Nakamura H, Matsuo M: Modulation of in vitro splicing of the upstream intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe. *J. Clin. Invest.* 1995; 95: 515-520.
- [16] Takeshima Y, Yagi M, Ishikawa Y, Ishikawa Y, Minami R, Nakamura H, Matsuo M: Oligonucleotides against a splicing enhancer sequence led to dystrophin production in muscle cells from a Duchenne muscular dystrophy patient. *Brain Dev.* 2001; 23: 788-798.
- [17] van Deutekom JCT, Bremmer-Bout M, Janson AAM, Ginjaar IB, Baas F, den Dunnen JT, van Ommen GJ: Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum. Mol. Genet.* 2001; 10: 1547-1554.
- [18] Wilton SD, Dye DE, Laing NG: Dystrophin gene transcripts skipping the mdx mutation. *Muscle Nerve* 1997; 20: 728-734.
- [19] Wilton SD, Lloyd F, Carville K, Fletcher S, Honeyman K, Agrawal S, Kole R: Specific removal of the nonsense mutation from the mdx dystrophin mRNA using antisense oligonucleotides. *Neuromuscul. Disord.* 1999; 9: 330-338.
- [20] Yacyshyn BR, Bowen-Yacyshyn MB, Jewell L, Tami JA, Bennett CF, Kisner DL, Shanahan WR, Jr.: A placebo-controlled trial of ICAM-1 antisense oligonucleotide in the treatment of Crohn's disease. *Gastroenterology* 1998; 114: 1133-1142.