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Antisense Oligonucleotide Induced *Dystrophin* Exon 45 Skipping at a Low Half-Maximal Effective Concentration in a Cell-Free Splicing System

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Antisense oligonucleotides (AOs) can facilitate the expression of internally deleted dystrophin in dystrophin-deficient Duchenne muscular dystrophy (DMD) by correcting the reading frame of the pre-mRNA with AO-mediated exon skipping. An antisense 18-mer 2′-O-methyl RNA/ethylene-bridged nucleic acid chimera AO targeting exon 45 of the *dystrophin* gene, *AO85*, can induce exon 45 skipping efficiently in cultured cells. AO85 is expected to facilitate dystrophin expression in 8%–9% of all DMD patients. Here, we examined the kinetics of AO85-mediated exon 45 skipping in a cell-free splicing system. *In vitro* transcribed pre-mRNAs containing *dystrophin* exon 45 and part of its flanking introns within a hybrid minigene were incubated with HeLa cell nuclear extract, and the resultant mRNAs were amplified by semiquantitative reverse transcriptase–polymerase chain reaction. Time-course analysis revealed that the splicing process fitted well to first order kinetics. Addition of AO85 produced an extra spliced product, deleting exon 45 (Δexon 45), indicating AO85-mediated exon 45 skipping. Production of Δexon 45 increased linearly with increasing concentrations of AO85, reaching a maximum of nearly 80% of the transcripts. The half-maximal effective concentration (EC_{50}) of AO85 was 58.0 nM. The percentage of Δexon 45 among the transcripts decreased inversely with the pre-mRNA concentration; Lineweaver-Burk plotting revealed a competitive fashion of AO85 action. The low EC_{50} indicates high potential of AO85 for clinical application.

Introduction

ANTISENSE OLIGONUCLEOTIDES (AOs) are traditionally employed to destroy the target RNA by RNAase H-mediated digestion (Pan and Clawson, 2006). AO action is dependent on binding to specific mRNA sequences through Watson-Crick base pair hybridization, before digestion of the bound fragment by RNAase H. AOs have been applied to the treatment of infectious diseases or cancers by degrading target mRNAs (Van Aerschot, 2006; Bennett and Swayze, 2010), and their kinetics have been studied (Duan et al., 2008). Currently, AOs are attracting much attention as modulators of splicing that can generate new gene transcripts (Perez et al., 2010; Wood et al., 2010).

Duchenne muscular dystrophy (DMD) is a rapidly progressive muscle wasting disease that usually results in death during the third decade. DMD is characterized by a dystrophin deficiency that usually arises from out-of-frame deletion mutations in the *dystrophin* gene that create a premature stop codon in the *dystrophin* mRNA. AO-mediated exon skipping therapy has been proposed for the treatment of DMD, producing in-frame *dystrophin* mRNA from the out-of-frame mRNA by inducing exon skipping. The newly generated in-frame *dystrophin* mRNA is expected to produce semifunctional, internally deleted dystrophin protein (Takeshima et al., 1995). The production of internally deleted dystrophin has been clinically demonstrated in some patients who showed natural skipping of an exon encoding a nonsense mutation (Shiga et al., 1997; Flanigan et al., 2011). Compared with gene replacement therapy, the benefits of exon skipping with AOs include the ability to use the endogenous gene and easy chemical synthesis of AOs. Currently, induction of exon skipping with AOs is considered one of the most plausible treatments for DMD (Aartsma-Rus, 2010; Lu et al., 2011). Remarkably, systemic administration of 2′-O-methyl phosphorothioate AO (PRO051) showed a promising result of a modest improvement in the 6 minutes walk test after 12 weeks of the treatment (Goemans et al., 2011).

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AOs with phosphorothioate backbones directed against the splicing enhancer sequence within human *dystrophin* exon 19 provided the first demonstration of exon skipping in human cells (Pramono et al., 1996). Since then, intravenous infusion of AOs against exon 19 has been reported to induce exon 19 skipping and the expression of internally deleted dystrophin in an exon 20-deleted DMD patient (Takeshima et al., 2006). Several chemical modifications of the first-generation phosphorothioate AOs have been introduced over the past decade to improve the efficiency of antisense therapeutics. Morpholino AOs have been shown to induce *dystrophin* exon 51 skipping and promote dystrophin expression in DMD muscle cells (Partridge, 2010).

A modified nucleic acid, 2′-O,4′-C-ethylene-bridged nucleic acid (ENA), has high binding affinity for the complementary RNA strand and more nuclease resistance than unmodified nucleic acid (Morita et al., 2001; Veedu and Wengel, 2010). One antisense 2′-O-methyl RNA/ENA (RNA/ENA) chimera was shown to be 40 times more effective than the conventional phosphorothioate backbone oligonucleotides in inducing exon 19 skipping (Yagi et al., 2004). Furthermore, an RNA/ENA chimera against *dystrophin* exon 41 encoding a nonsense mutation has been shown to induce efficient skipping of a mutated exon 41 (Surono et al., 2004). Considering that skipping of exon 45 is expected to express internally deleted dystrophin in 8%–9% of DMD patients (Aartsma-Rus et al., 2009a; Takeshima et al., 2010), AOs that induce exon 45 skipping have been proposed as one of the most important AOs for DMD treatment. In our previous studies, we demonstrated that an 18-mer RNA/ENA chimera, AO85, strongly induced exon 45 skipping in cultured myocytes (Takagi et al., 2004a; Takeshima et al., 2011). However, its kinetics, including the half-maximal effective concentration (EC50), are still unknown, as for other AOs designed for DMD treatment (Aartsma-Rus, 2010).

To facilitate the clinical application of AO85, here we examined AO-mediated exon 45 skipping in a cell-free splicing system and determined the splicing parameters of AO85, revealing a low EC_{50}.

Materials and Methods

AO85

An 18-mer RNA/ENA chimera (5′-CgCTgcCCaaTgCCatCC-3′; upper and lower case letters represent ENA and 2′-O-methyl RNA, respectively), AO85, complementary to the 5′ region of *dystrophin* exon 45 (Takeshima et al., 2011), was synthesized by KNC Laboratories Co. (Kobe, Japan) and dissolved in water.

In vitro *transcription*

The region encompassing *dystrophin* exon 45 and part of its flanking introns was amplified from human genomic DNA by polymerase chain reaction (PCR) using a set of primers containing *Nhe*I and *Bam*HI restriction enzyme recognition sites (forward primer, Int44F*Nhe*I 5′-GCGGCTAGCGCTAACCGAGAGGGTGCTTTT-3′; reverse primer, Int45R*Bam*HI 5′-GCGGGATCCGCAGAAAACCACTAACTAGCCACA-3′; restriction sites underlined). The amplified product was digested with *Nhe*I and *Bam*HI (New England Biolabs, Hitchen, UK) and inserted between the 2 exons A and B of the predigested expression vector H492 (Habara et al., 2008) to generate a hybrid minigene (Fig. 1a). The sequence of the hybrid minigene was confirmed by DNA sequencing using an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). A region of the hybrid minigene including exon A, *dystrophin* exon 45 and exon B was PCR amplified using forward (T7(19), 5′-TAATACGACTCACTATAGG-3′) and reverse (YH304, 5′-CTCGAGCAGCCAGTTAAGTCTCTCAC-3′) primers and then purified using a MinElute PCR Purification kit (QIAGEN, Hilden, Germany). The purified template DNAs were subjected to *in vitro* transcription using an mMESSAGE mMACHINE High Yield Capped RNA Transcription kit (Ambion, Austin, TX). After treatment of the transcription product with DNase (Ambion), the resultant RNAs were purified on a G-50 Quick Spin Column (Roche Applied Science, Indianapolis, IN) and used as a substrate for the splicing reaction. The concentration and size of the

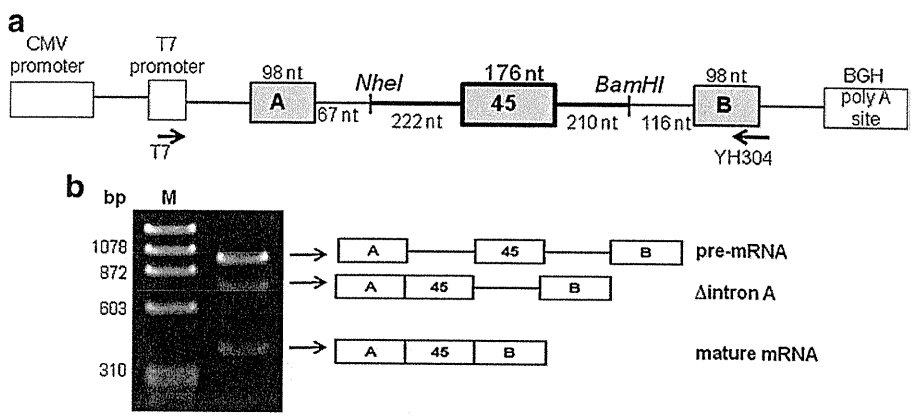

FIG. 1. Cell-free splicing reaction. **(a)** Schematic description of the hybrid-minigene. Pre-mRNA was *in vitro* transcribed from template DNA that was obtained by PCR amplification of the hybrid-minigene with primers T7 and YH304. *Shaded boxes* and *bars* indicate exons and introns, respectively. *Dystrophin* exon 45 (176 nt) and its flanking introns (222 nt upstream and 210 nt downstream; *bold lines*) were inserted into the multicloning site of the pre-constructed expression vector H492 that contains exons A and B, a cytomegalovirus (CMV) enhancer-promoter, a T7 promoter, and the bovine growth hormone gene (*BGH*) polyadenylation signal (*boxes*). **(b)** Products of the cell-free splicing reaction. The synthesized pre-mRNA was incubated with HeLa cell nuclear extract for 2 hours at 30°C. The reaction products were RT-PCR amplified and separated by agarose gel electrophoresis (indicated). The structure of each product is described schematically on the right. *Boxes* and *bars* indicate exons and introns, respectively. RT-PCR, reverse transcriptase–polymerase chain reaction.

purified RNAs were determined using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and an Agilent 2100 Bioanalyzer with an RNA Nano kit (Agilent Technologies, Santa Clara, CA), respectively.

In vitro *splicing reaction*

The *in vitro* splicing reaction was carried out at 30°C for 2 hours in a volume of 20 μL containing 30 ng pre-mRNA, 50% (v/v) HeLa cell nuclear extract (Lot No. 4168HNE; Computer Cell Culture Center, Mons, Belgium), 1.6 mM $MgCl_2$, 0.5 mM ATP, 20 mM creatine phosphate, and 20 U of RNaseOUT (Invitrogen, Carlsbad, CA) (Habara et al., 2008). In some experiments, the indicated amounts of AO85 were added directly to the reaction mixture. The incubation temperature or time was varied to study the temperature or time dependency of the *in vitro* splicing.

After the designated incubation time, reaction mixtures were treated with Proteinase K (Sigma-Aldrich, St. Louis, MO) and the resulting RNAs were purified by phenol–chloroform extraction and ethanol precipitation as previously described (Habara et al., 2008). Purified total RNA (750 ng) was reverse transcribed using random hexamer primers with M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol and then PCR amplified using a forward primer from exon A (YH307, 5′-ATTACTCGCTCA GAAGCTGTGTTGC-3′) and a reverse primer from exon B (YH308, 5′-AAGTCTCTCACTTAGCAACTGGCAG-3′) (Habara et al., 2008). PCRs were performed in a volume of 20 μL containing 4 μL of cDNA, 1× Ex Taq Buffer, 250 nM dNTPs (Takara Bio, Inc., Kyoto, Japan), 10 pmol of each primer, and 1 U of Ex Taq Polymerase (Takara Bio, Inc.). The PCR cycling conditions were as follows: initial denaturation at 94°C for 2 minutes followed by 16 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes. PCR products were analyzed by agarose gel electrophoresis. Each amplified product was semiquantified by measuring the peak areas of capillary electrophoresis using an Agilent 2100 Bioanalyzer with a DNA1000 kit (Agilent Technologies). The sequences of all the detected bands were confirmed by subcloning and sequencing as previously described (Tran et al., 2006). As an internal standard, the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was also amplified by reverse transcriptase (RT)-PCR from cDNA, using primers spanning exons 3–6 of the *GAPDH* cDNA (Zhu et al., 2007).

The percentage of spliced products among the total transcripts was calculated by the following formula: splicing efficiency (%) = [spliced/(spliced + unspliced pre-mRNAs)] × 100 (Zheng and Baker, 2000). The percentage of the skipped exon among the total transcripts was defined as [Δexon45/(normally spliced product + Δexon45)] × 100. The ratio of normally spliced mRNA to the internal standard *GAPDH* was calculated and its relationship with the pre-mRNA was analyzed.

Statistical analysis

All analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). The mean ± standard deviation was derived from 2 or more independent experiments. Both nonlinear and linear regressions were used to determine the mechanism of AO85-induced exon 45 skipping.

Results

Cell-free splicing of pre-mRNA

Synthesized pre-mRNA was incubated with HeLa cell nuclear extract and the resulting products were analyzed by RT-PCR of total RNA extracted from the reaction mixture. Amplification of a fragment extending from exons A to B revealed 3 products on agarose gel electrophoresis (Fig. 1b). The largest product corresponded to the substrate pre-mRNA that maintained the 3 exons (exons A, 45, and B) and 2 introns (introns A and 45). The middle-sized product lacked the upstream intron A from the pre-mRNA (Δintron A), indicating a splicing intermediate. The smallest band lacked both introns (introns A and 45), corresponding to the mature mRNA. All 3 products showed the expected exon/exon junction sequences and there were no unexpected splicing products caused by cryptic splice site activation. Mature mRNA was obtained most abundantly at 30°C compared with at 37°C or 40°C (data not shown), as reported previously (Habara et al., 2009).

We then monitored the time-course of the splicing reaction by semiquantitative RT-PCR (Fig. 2a). With an incubation time of zero, only the pre-mRNA was detected. At 15 mins, Δintron A was also detected. This indicates that the 5′ intron,

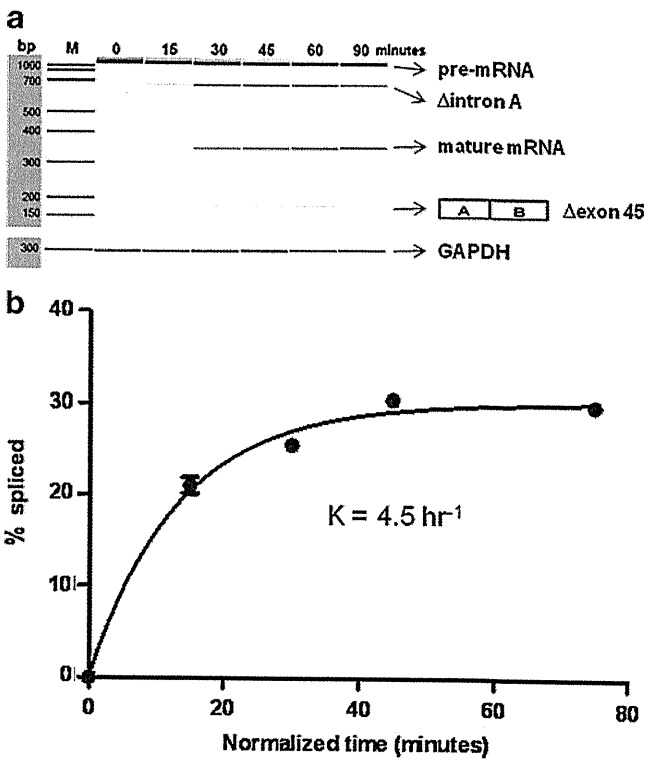

FIG. 2. Time-course of the cell-free splicing reaction. **(a)** Capillary gel electrophoretic patterns of RT-PCR products. At 15 minutes, Δintron A only was visible. From 30 minutes onwards, 3 spliced products were detected, as indicated schematically on the *right*. *Boxes* and *bars* indicate exons and introns, respectively. The *GAPDH* RT-PCR products are shown at the *bottom*. **(b)** When the 15-minute lag period was taken into consideration (corrected time), the percentage of spliced product fitted a pseudo first-order model, with a rate constant (K) of 4.5 $hour^{-1}$. Values represent the means for 3 measurements. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

intron A, was spliced first in this cell-free splicing system. At 30 minutes' incubation, the amount of Δintron A increased and the mature mRNA was detected. Trace amounts of Δexon 45 were also detected (Fig. 2a).

We then analyzed the percentage of spliced product. Because the Δintron A splicing product appeared at 15 minutes (Fig. 2a), the first 15 minutes was considered an initial lag period (Hicks et al., 2005). When the 15-min lag period was taken into consideration, the appearance of spliced products followed a profile characteristic of a first order reaction (Fig. 2b). The product appearance data fitted the pseudo-first order rate description $Y = C \times (1-e^{-kt})$, where Y is the fraction spliced, C is the fraction spliced at the end-point of the reaction, k is the apparent rate constant, and t is the time (Hicks et al., 2005). The observed rate constant was 4.5 ± 0.42 hour^{-1}, fitting well to the 1-phase association model with $R^2 = 0.9905$ (Fig. 2b) and confirming that this cell-free splicing reaction is time dependent. In the following experiments, the incubation time was shortened to 30 minutes.

Induction of exon 45 skipping with AO85

To examine whether AO85 was able to induce exon 45 skipping in the cell-free splicing system, 0–160 nM AO85 was added to the incubation mixture, and the resulting RNAs were analyzed by RT-PCR. In the absence of AO85, 3 products, corresponding to pre-mRNA, Δintron A, and mature mRNA, were clearly observed (Fig. 3a). At 20 nM AO85, the percentage of Δexon 45 increased, whereas both Δintron A and the mature mRNA decreased. At 160 nM AO85, almost 80% of the total product was Δexon 45. When the percentage of Δexon 45 was plotted against AO85 concentration, the percentage increased linearly (Fig. 3b). The data fitted well to a dose–response model, with $R^2 = 0.9990$. From this, the EC_{50} was calculated as 58.0 nM.

Competitive fashion of AO85 action

We next analyzed action patterns of AO85 in a total splicing reaction by measuring the production of mature mRNA with different concentrations of AO85 and pre-mRNA. The amount of pre-mRNA ranged from 0 to 10.5 nM with 3 different AO85 concentrations (0, 20, and 40 nM). The amount of mature mRNA was normalized to that of *GAPDH*. On a Lineweaver-Burk plot (Fig. 4a), the data showed that AO85 inhibited the production of mature mRNA in a dose-dependent manner and that 3 lines linking spots met together at the Y axis, indicating a competitive fashion of AO85 action. The *Ki* of the inhibition was found 10.1 nM (Fig. 4b).

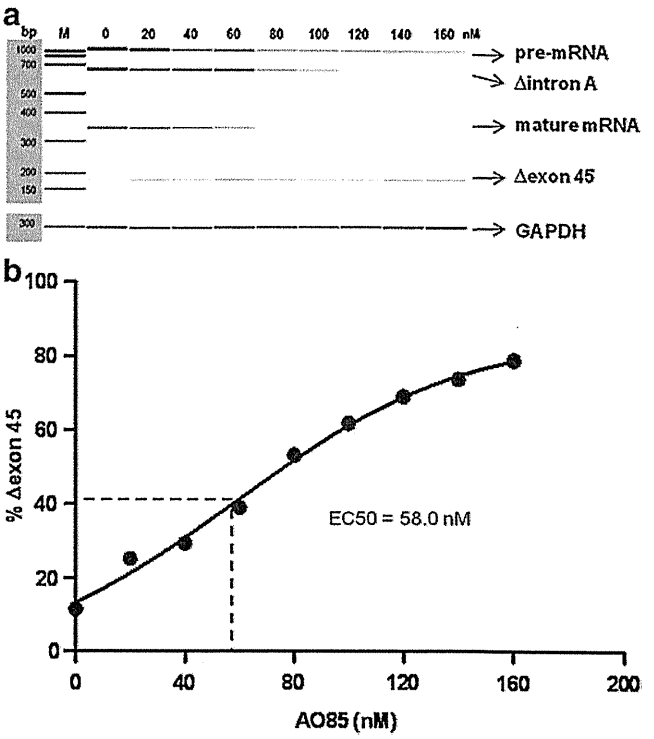

FIG. 3. Dose dependency of exon 45 skipping. **(a)** Capillary gel electrophoretic patterns of RT-PCR products. The indicated concentration (0–160 nM) of AO85 was added to the *in vitro* splicing system. The intensity of Δexon 45 increased with increasing concentration of AO85, in contrast to the intensity of the normally spliced band, as indicated schematically on the *right*. *Boxes* and *bars* indicate exons and introns, respectively. The *GAPDH* RT-PCR products are shown at the *bottom*. **(b)** The data fitted well to a linear dose–response model. The EC50 was calculated as 57.96 nM. Values represent the means for 3 measurements. AO, antisense oligonucleotide; EC50, half-maximal effective concentration.

Discussion

We showed that AO85 induced exon 45 skipping in a cell-free splicing system that contained substrate pre-mRNA and HeLa cell nuclear extract, indicating that exon skipping does not require intact cells. Regardless of the shortened introns (Fig. 1) the pre-mRNA was subjected to exon skipping. The *in vitro* splicing assay has been shown useful to examine antisense-mediated exon skipping, although there are limitations in applying the results in understanding *in vivo* splicing (Spitali et al., 2009). One of the most important limitations is that *in vitro* splicing requires the use of relatively short transcripts (approximately 1,000–2,000 bp) that usually lack the complexity and length of natural pre-mRNA, and thus may not identify the rate limiting steps for expression *in vivo* (Nasim et al., 2002). In our *in vitro* splicing system total amount of RNA decreased at the high concentration of AO85 (Fig. 3a). This may be due to tight connection of AO85 to the target sequence, hampering PCR amplification or unknown RNAse activation.

The cell-free splicing reaction had an initial lag period of up to 15 minutes (Fig. 2). Regulatory elements located within introns and exons guide the splicing complex, the spliceosome, and auxiliary RNA-binding proteins to the correct sites for intron removal and exon joining (Pandya-Jones and Black, 2009; Ward and Cooper, 2010). Presumably, exogenously added pre-mRNAs are initially coated with heteronuclear ribonucleoproteins (hnRNPs) that are abundant in nuclear extracts, and the competing association and dissociation between hnRNPs and components of the spliceosome requires time (Hicks et al., 2005). We assume that the observed lag period corresponds to the spliceosome association. After this initial phase, the splicing reaction proceeded linearly.

AO85, which was identified through trial-and-error, is considered to bind to an exon splicing enhancer (Takeshima et al., 2011). Nuclear proteins bound to the enhancer sequence

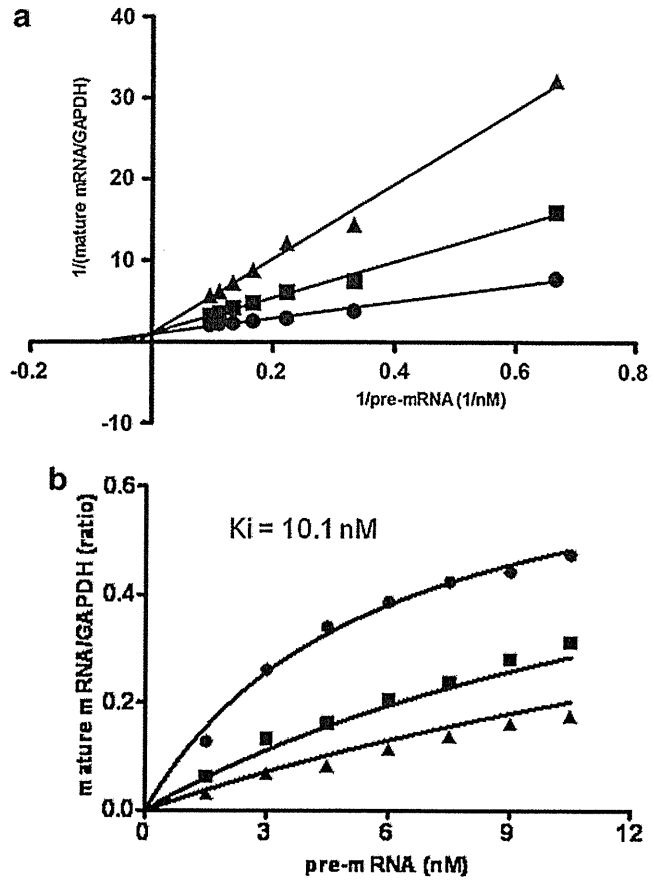

FIG. 4. Competitive fashion of of AO85 action. **(a)** Lineweaver-Burk plot for the production of mature mRNA for 3 concentrations of AO85 and various concentration of pre-mRNA. The ratio of normally spliced mRNA to *GAPDH* was plotted against 1/pre-mRNA concentration (0–10.5 nM). The concentrations of AO85 were 0, 20, or 40 nM (*circles*, *squares*, and *triangles*, respectively). The results showed competitive inhibition of AO85. Values represent the means of 3 experiments. **(b)** Nonlinear regression of production of mature mRNA in 3 concentration of AO85 and various concentration of pre-mRNA. The ratio of normally spliced mRNA was plotted against the substrate pre-mRNA concentration (0–10.5 nM). The concentration of AO85 was 0, 20, and 40 nM (*circle*, *box*, and *triangles*, respectively). The *Ki* value was calculated as 10.1 nM. Values represent means from 3 experiments.

can promote exon definition by directly recruiting other splicing factors and/or by antagonizing the action of nearby silencer elements. Thus, failure of nuclear proteins to bind to the enhancer sequence would prohibit recognition of the exon by the splicing machinery and cause exon skipping (Takeshima et al., 1995; Pramono et al., 1996; Freier and Altmann, 1997; Stahel and Zangemeister-Wittke, 2003; Aartsma-Rus et al., 2009b; Popplewell et al., 2009). AO85 is expected to bind to a region targeted by 2 SR proteins, SRp30c and SRp40 (Mathews et al., 1999), suggesting that AO85 competes with these 2 SR proteins for binding the pre-mRNA. The binding site for SRp30c is partially located where the pre-mRNA adopts an open secondary structure, whereas the predicted binding site of SRp40 is located within a closed structure (Mathews et al., 1999; Zuker, 2003). AO85 probably competes with SRp30c, but this needs further examination. Our results showing a competitive fashion of AO85 action (Fig. 4a) support the idea of competition with nuclear proteins.

Our study of the kinetics of AO85-mediated exon skipping in *in vitro* splicing revealed that (1) AO85 induced exon 45 skipping by inhibiting the production of mature mRNA, with a *Ki* of 10.1 nM. This relatively low *Ki* value indicates that AO85 is a potent competitor in the recognition of exon 45, and (2) the EC_{50} of AO85 was 58 nM. The IC_{50} of AOs in gene knockdown has been determined to be between 70 and 220 nM (Grunweller et al., 2003), indicating that AO85 works efficiently in our *in vitro* splicing system. At 160 nM AO85, exon skipping was induced in approximately 80% of transcripts. In comparison with this, at most 70% of exon skipping has been induced by 500 nM 2′-O-methyl phosphorothioate AOs in another cell-free splicing reaction (Spitali et al., 2009). The recent efforts to improve exon skipping efficiency by optimizing the AO sequence, backbone chemistry, and additional modifications (Kurreck et al., 2002) are worthwhile. Considering that AO85 has low EC_{50} (58.0 nM) (Fig. 3b), high exon skipping efficiency (80%) (Fig. 3b), and ability to induce exon 45 skipping in cultured human muscle cells (Takagi et al., 2004b), we believe that AO85 has high potential for clinical use. However, further studies are necessary before clinical use.

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Author Disclosure Statement

No competing financial interests exist.

References

AARTSMA-RUS, A. (2010). Antisense-mediated modulation of splicing: therapeutic implications for duchenne muscular dystrophy. RNA Biol. **7,** 453–461.

AARTSMA-RUS, A., FOKKEMA, I., VERSCHUUREN, J., GINJAAR, I., VAN DEUTEKOM, J., VAN OMMEN, G.J., and DEN DUNNEN, J.T. (2009a). Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. Hum. Mutat. **30,** 293–299.

AARTSMA-RUS, A., VAN VLIET, L., HIRSCHI, M., JANSON, A.A., HEEMSKERK, H., DE WINTER, C.L., DE KIMPE, S., VAN DEUTEKOM, J.C., T HOEN, P.A., and VAN OMMEN, G.J. (2009b). Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. Mol. Ther. **17,** 548–553.

BENNETT, C.F., and SWAYZE, E.E. (2010). RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Ann. Rev. Pharmacol. Toxicol. **50,** 259–293.

DUAN, M., ZHOU, Z., LIN, R.X., YANG, J., XIA, X.Z., and WANG, S.Q. (2008). *In vitro* and *in vivo* protection against the

highly pathogenic H5N1 influenza virus by an antisense phosphorothioate oligonucleotide. Antivir. Ther. **13,** 109–114.

FLANIGAN, K.M., DUNN, D.M., VON NIEDERHAUSERN, A., SOLTANZADEH, P., HOWARD, M.T., SAMPSON, J.B., SWOBODA, K.J., BROMBERG, M.B., MENDELL, J.R., TAYLOR, L., et al. (2011). Nonsense mutation-associated Becker muscular dystrophy: interplay between exon definition and splicing regulatory elements within the DMD gene. Hum. Mutat. **32,** 299–308.

FREIER, S.M., and ALTMANN, K.H. (1997). The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA:RNA duplexes. Nucleic Acids Res. **25,** 4429–4443.

GOEMANS, N.M., TULINIUS, M., VAN DEN AKKER, J.T., BURM, B.E., EKHART, P.F., HEUVELMANS, N., HOLLING, T., JANSON, A.A., PLATENBURG, G.J., SIPKENS, J.A., et al. (2011). Systemic administration of PRO051 in Duchenne's muscular dystrophy. N. Eng. J. Med. **364,** 1513–1522.

GRUNWELLER, A., WYSZKO, E., BIEBER, B., JAHNEL, R., and ERDMANN, V.A., KURRECK, J. (2003). Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2'-O-methyl RNA, phosphorothioates and small interfering RNA. Nucleic Acids Res. **31,** 3185–3193.

HABARA, Y., DOSHITA, M., HIROZAWA, S., YOKONO, Y., YAGI, M., TAKESHIMA, Y., and MATSUO, M. (2008). A strong exonic splicing enhancer in dystrophin exon 19 achieve proper splicing without an upstream polypyrimidine tract. J. Biochem. **143,** 303–310.

HABARA, Y., TAKESHIMA, Y., AWANO, H., OKIZUKA, Y., ZHANG, Z., SAIKI, K., YAGI, M., and MATSUO, M. (2009). *In vitro* splicing analysis showed that availability of a cryptic splice site is not a determinant for alternative splicing patterns caused by +1G—>A mutations in introns of the dystrophin gene. J. Med. Genet. **46,** 542–547.

HICKS, M.J., LAM, B.J., and HERTEL, K.J. (2005). Analyzing mechanisms of alternative pre-mRNA splicing using *in vitro* splicing assays. Methods **37,** 306–313.

KURRECK, J., WYSZKO, E., GILLEN, C., and ERDMANN, V.A. (2002). Design of antisense oligonucleotides stabilized by locked nucleic acids. Nucleic Acids Res. **30,** 1911–1918.

LU, Q.L., YOKOTA, T., TAKEDA, S., GARCIA, L., MUNTONI, F., and PARTRIDGE, T. (2011). The status of exon skipping as a therapeutic approach to duchenne muscular dystrophy. Mol. Ther. **19,** 9–15.

MATHEWS, D.H., SABINA, J., ZUKER, M., and TURNER, D.H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. **288,** 911–940.

MORITA, K., HASEGAWA, C., KANEKO, M., TSUTSUMI, S., SONE, J., ISHIKAWA, T., IMANISHI, T., and KOIZUMI, M. (2001). 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) with nuclease-resistance and high affinity for RNA. Nucleic Acids Res. Suppl. **1,** 241–242.

NASIM, M.T., CHOWDHURY, H.M., and EPERON, I.C. (2002). A double reporter assay for detecting changes in the ratio of spliced and unspliced mRNA in mammalian cells. Nucleic Acids Res. **30,** e109.

PAN, W.H., and CLAWSON, G.A. (2006). Antisense applications for biological control. J. Cell Biochem. **98,** 14–35.

PANDYA-JONES, A., and BLACK, D.L. (2009). Co-transcriptional splicing of constitutive and alternative exons. RNA **15,** 1896–1908.

PARTRIDGE, T. (2010). The potential of exon skipping for treatment for Duchenne muscular dystrophy. J Child Neurol. **25,** 1165–1170.

PEREZ, B., RODRIGUEZ-PASCAU, L., VILAGELIU, L., GRINBERG, D., UGARTE, M., and DESVIAT, L.R. (2010). Present and future of antisense therapy for splicing modulation in inherited metabolic disease. J. Inherit. Metab. Dis. **33,** 397–403.

POPPLEWELL, L.J., TROLLET, C., DICKSON, G., and GRAHAM, I.R. (2009). Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene. Mol. Ther. **17,** 554–561.

PRAMONO, Z.A., TAKESHIMA, Y., ALIMSARDJONO, H., ISHII, A., TAKEDA, S., and MATSUO, M. (1996). 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. **226,** 445–449.

SHIGA, N., TAKESHIMA, Y., SAKAMOTO, H., INOUE, K., YOKOTA, Y., YOKOYAMA, M., and MATSUO, M. (1997). 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. **100,** 2204–2210.

SPITALI, P., RIMESSI, P., FABRIS, M., PERRONE, D., FALZARANO, S., BOVOLENTA, M., TRABANELLI, C., MARI, L., BASSI, E., TUFFERY, S., et al. (2009). Exon skipping-mediated dystrophin reading frame restoration for small mutations. Hum. Mutat. **30,** 1527–1534.

STAHEL, R.A., and ZANGEMEISTER-WITTKE, U. (2003). Antisense oligonucleotides for cancer therapy-an overview. Lung Cancer **41 Suppl 1,** S81–S88.

SURONO, A., TRAN, V.K., TAKSHIMA, Y., WADA, H., YAGI, M., TAKAGI, M., KOIZUMI, M., and MATSUO, M. (2004). Chimeric RNA/ethylene bridged nucleic acids promote dystrophin expression in myocytes of Duchenne muscular dystrophy by inducing skipping of the nonsense-mutation-encoding exon. Hum. Gene Ther. **15,** 749–757.

TAKAGI, M., MORITA, K., NAKAI, D., NAKAGOMI, R., TOKUI, T., and KOIZUMI, M. (2004a). Enhancement of the inhibitory activity of oatp antisense oligonucleotides by incorporation of 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) without a loss of subtype selectivity. Biochemistry **43,** 4501–4510.

TAKAGI, M., YAGI, M., ISHIBASHI, K., TAKESHIMA, Y., SURONO, A., MATSUO, M., and KOIZUMI, M. (2004b). Design of 2'-O-Me RNA/ENA chimera oligonucleotides to induce exon skipping in dystrophin pre-mRNA. Nucleic Acids Symp. Ser. (Oxf), **48,** 297–298.

TAKESHIMA, Y., YAGI, M., and MATSUO, M. (2011). Optimizing RNA/ENA chimeric antisense oligonucleotides using *in vitro* splicing. In: Exon Skipping: Methods and Protocols: Methods in Molecular Biology. A. Aartsma-Rus, ed. (Humana Press, New Jersey) in press.

TAKESHIMA, Y., NISHIO, H., SAKAMOTO, H., NAKAMURA, H., and MATSUO, M. (1995). 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. **95,** 515–520.

TAKESHIMA, Y., YAGI, M., OKIZUKA, Y., AWANO, H., ZHANG, Z., YAMAUCHI, Y., NISHIO, H., and MATSUO, M. (2010). Mutation spectrum of the dystrophin gene in 442 Duchenne/Becker muscular dystrophy cases from one Japanese referral center. J. Hum. Genet. **55,** 379–388.

TAKESHIMA, Y., YAGI, M., WADA, H., ISHIBASHI, K., NISHIYAMA, A., KAKUMOTO, M., SAKAEDA, T., SAURA, R., OKUMURA, K., and MATSUO, M. (2006). Intravenous infusion of an antisense oligonucleotide results in exon skip-

ping in muscle dystrophin mRNA of Duchenne muscular dystrophy. Pediatr. Res. **59,** 690–694.

TRAN, V.K., TAKESHIMA, Y., ZHANG, Z., YAGI, M., NISHIYAMA, A., HABARA, Y., and MATSUO, M. (2006). Splicing analysis disclosed a determinant single nucleotide for exon skipping caused by a novel intra-exonic four-nucleotide deletion in the dystrophin gene. J. Med. Genet. **43,** 924–930.

VAN AERSCHOT, A. (2006). Oligonucleotides as antivirals: dream or realistic perspective? Antivir. Res. **71,** 307–316.

VEEDU, R.N., and WENGEL, J. (2010). Locked nucleic acids: promising nucleic acid analogs for therapeutic applications. Chem. Biodivers **7,** 536–542.

WARD, A.J., and COOPER, T.A. (2010). The pathobiology of splicing. J. Pathol. **220,** 152–163.

WOOD, M.J., GAIT, M.J., and YIN, H. (2010). RNA-targeted splice-correction therapy for neuromuscular disease. Brain **133,** 957–972.

YAGI, M., TAKESHIMA, Y., SURUNO, A., TAKAGI, M., KOIZUMI, M., and MATSUO, M. (2004). Chimeric RNA and 2′-O, 4′-C-ethylene-bridged nucleic acids have stronger activity than phosphorothioate oligodeoxynucleotides in induction of exon-19 skipping in dystropin mRNA. Oligonucleotides **14,** 33–40.

ZHENG, Z.M., and BAKER, C.C. (2000). Parameters that affect *in vitro* splicing of bovine papillomavirus type 1 late pre-mRNAs. J. Virol Methods **85,** 203–214.

ZHU, J., KREN, B.T., PARK, C.W., BILGIM, R., WONG, P.Y., and STEER, C.J. (2007). Erythroid-specific expression of beta-globin by the sleeping beauty transposon for Sickle cell disease. Biochemistry **46,** 6844–6858.

ZUKER, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31,** 3406–3415.

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Chemical treatment enhances skipping of a mutated exon in the *dystrophin* gene

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Duchenne muscular dystrophy (DMD) is a fatal muscle wasting disease caused by a loss of the dystrophin protein. Control of dystrophin mRNA splicing to convert severe DMD to a milder phenotype is attracting much attention. Here we report a dystrophinopathy patient who has a point mutation in exon 31 of the *dystrophin* gene. Although the mutation generates a stop codon, a small amount of internally deleted, but functional, dystrophin protein is produced in the patient cells. An analysis of the mRNA reveals that the mutation promotes exon skipping and restores the open reading frame of dystrophin. Presumably, the mutation disrupts an exonic splicing enhancer and creates an exonic splicing silencer. Therefore, we searched for small chemicals that enhance exon skipping, and found that TG003 promotes the skipping of exon 31 in the endogenous *dystrophin* gene in a dose-dependent manner and increases the production of the dystrophin protein in the patient's cells.

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Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease and is caused by a mutation in the *dystrophin* gene, the largest in the human genome, on the X chromosome[1]. Because of progressive muscle wasting, DMD patients usually succumb to cardiac or respiratory failure in their twenties. Becker muscular dystrophy (BMD) is a milder allelic variant of DMD, usually affecting adult males. A reading frame rule explains the difference between DMD and BMD. Nonsense mutations or deletions causing frame shifts in the dystrophin mRNA, both of which create premature termination codons (PTCs), usually result in a severe DMD phenotype, because of a lack of the dystrophin protein. In contrast, mutations/deletions that maintain the original reading frame in the mRNA cause the milder BMD phenotype, as a mutated, but still functional, dystrophin protein can be expressed from the mRNA[2]. However, in some mild BMD cases, the patients had nonsense mutations in exons but still produced novel in-frame dystrophin mRNAs by skipping the exons containing the nonsense codon[3–7]. Thus, internally deleted but partially functional dystrophin proteins can be produced from the exon-skipped mRNAs.

The current major therapeutic approach established by us, as well as by other groups, is to convert DMD to BMD phenotypes by restoring dystrophin protein expression by inducing exon skipping with antisense oligonucleotides (AONs)[8–11]. Several different AONs have been designed against either splice sites or splicing enhancer elements to induce exon skipping in cells of DMD patients. The AONs, which are designed to target those splicing elements, were demonstrated to restore the reading frame of dystrophin by causing skipping of the target exons. For example, the administration of an AON against an exonic splicing enhancer (ESE) in exon 19 promoted exon skipping in cells and increased production of an internally deleted dystrophin protein[12–14]. Another AON against exon 51 is currently under clinical trials[9,15,16]. However, considering the therapeutic cost and convenience, small chemical compounds have been highly awaited. A small compound PTC124 (refs 17 and 18), which induces read-through of the PTC, was reported to have the potential to treat some DMD patients who have nonsense mutations. Although a clinical trial of PTC124 for DMD patients who have nonsense mutations in the *dystrophin* gene was completed, no significant improvement of treated patients was observed (http://www.ptcbio.com/May_DBMD_Trial_Update.htm).

We have been interested in the role of phosphorylation of SR proteins in splicing regulation. SR proteins are heavily phosphorylated in cells and are involved in constitutive and alternative splicing[19,20]. By extensive screening of 100,000 chemical compounds in a chemical library using *in vitro* phosphorylation assay, we identified several synthetic chemical compounds that inhibit SR protein kinases specifically. We first identified a synthetic compound as a specific inhibitor of SR protein kinases and named it as SRPIN340 (ref. 21). Administration of SRPIN340 to mice retina changed the splicing pattern of vascular endothelial growth factor-A and suppressed vascular generation[22]. We next identified TG003, a kinase inhibitor specific for Cdc-like kinases (Clks)[22] that are also able to phosphorylate SR proteins. TG003 affected splicing both *in vitro* and *in vivo*[23,24]. Recently, we reported that spliceostatin, originally characterized as an antitumour drug, blocked splicing and promoted the export of unspliced pre-mRNAs[25,26].

Here we tested these compounds to determine whether splicing in this context in *ex vivo* myoblast cell culture could be modified, and found that TG003 enhanced exon skipping and produced an internally deleted dystrophin protein in the *in vitro*-formed myotubes of a dystrophinopathy patient who has a point mutation in exon 31 of the *dystrophin* gene.

Results

Point mutation causes skipping of exon 31 in a DMD patient. We have analysed and identified mutations in the *dystrophin* gene of more than 400 dystrophinopathy patients. We found that one patient (KUCG797) had a point mutation in exon 31. The mutation is a change from G to T (G to U on RNA) at position 4303 of the dystrophin cDNA (c.4303G>T, Fig. 1a). As this change replaced GAG for glutamate with the TAG for a stop codon (p.Glu1435X), the patient was not expected to produce dystrophin, resulting in severe DMD. The immunostaining of a biopsied skeletal muscle, however, showed patchy and discontinuous signals with antibodies recognizing N- or C-terminal dystrophin domains (Fig. 1e,f), which are comparable to BMD. Size and shape of muscle fibres were heterogeneous.

To explain the discrepancy between the genotype and the immunostaining pattern, we presumed that the nonsense mutation in exon 31 disrupted the ESE, which resulted in skipping of the mutated exon in the patient's muscle cells. To test this possibility, we analysed the *dystrophin* mRNA in skeletal muscle. The reverse transcription polymerase chain reaction (RT–PCR) amplification of the region extending from exon 27 to exon 32 showed two nearly equal amounts of two products: one of expected size with exons 27–32, including the TAG stop codon in exon 31, and the other a smaller product lacking exon 31 (Fig. 1h,i). However, the other introns of this *dystrophin* gene, which consists of 79 exons, seemed to be correctly spliced out (Supplementary Fig. S1, (−) lanes and Supplementary Table S1). The resulting dystrophin mRNA lacking the 111-bp-long exon 31 was in-frame to produce an internally deleted, but likely functional, dystrophin protein.

In the patient's muscle cells, the exon 31-containing mRNA should be lost by NMD, and only the internally deleted protein transcribed from the exon 31-skipped transcript can be expressed. Therefore, the dystrophin protein was immunostained with antibodies against the N- or C-terminal domains of dystrophin (Fig. 1b,c,e,f), but failed to be recognized by MANDYS1, a monoclonal antibody against the exon 31-encompassing region in the patient's cells (Fig. 1d,g).

Splicing regulators involved in exon 31 splicing. These results suggest that the point mutation in exon 31 not only produces a stop codon but also modifies an exonic splicing regulatory site. To test this hypothesis, we incorporated the wild-type and mutant *dystrophin* gene fragment that retains exon 31 and the flanking introns into the H492 vector, which has been used for splicing analysis in cells (Fig. 1j)[27–29], and transfected them into HeLa cells. Two PCR products were detected with the mutant plasmid (Fig. 1k), whereas only a single RT–PCR product was amplified from the wild-type plasmid. Sequencing analysis revealed that the smaller PCR product from the mutant does not include exon 31 (data not shown).

As mutant exon 31 with flanking introns cloned into the H492 vector is sufficient to promote skipping, we tried to identify the splicing regulatory factors that bind to the RNA portion. According to the SpliceAid program (http://www.introni.it/splicing.html)[30], exon 31 has the binding sequence of SRp30c/SRSF9 identified by SELEX (Supplementary Fig. S2b)[31]. The point mutation found in the patient disrupts the binding site of SRp30c/SRSF9, a member of the SR protein family (Supplementary Fig. S2a), which are often purine-rich and known to bind to ESEs. In addition, the mutation presumably generates an RNA sequence that has high homology to exonic splicing silencers (ESSs) recognized by the heterogeneous nuclear ribonucleoproteins (hnRNPs) A1 (Supplementary Fig. S2a,b)[32]. To test this notion, we first compared the binding to hnRNP A1 of the mutant RNA with that of the wild-type RNA in a gel mobility shift assay. The recombinant hnRNP A1 was prepared as a glutathione *S*-transferase (GST)-tagged protein (Supplementary Fig. S3b, lane 3) and mixed with either the wild-type or mutant dystrophin RNA, and the resultant complexes were analysed by native polyacrylamide gel electrophoresis. As shown in Figure 2a, two hnRNP A1–RNA complexes showed different patterns of migration on the gel

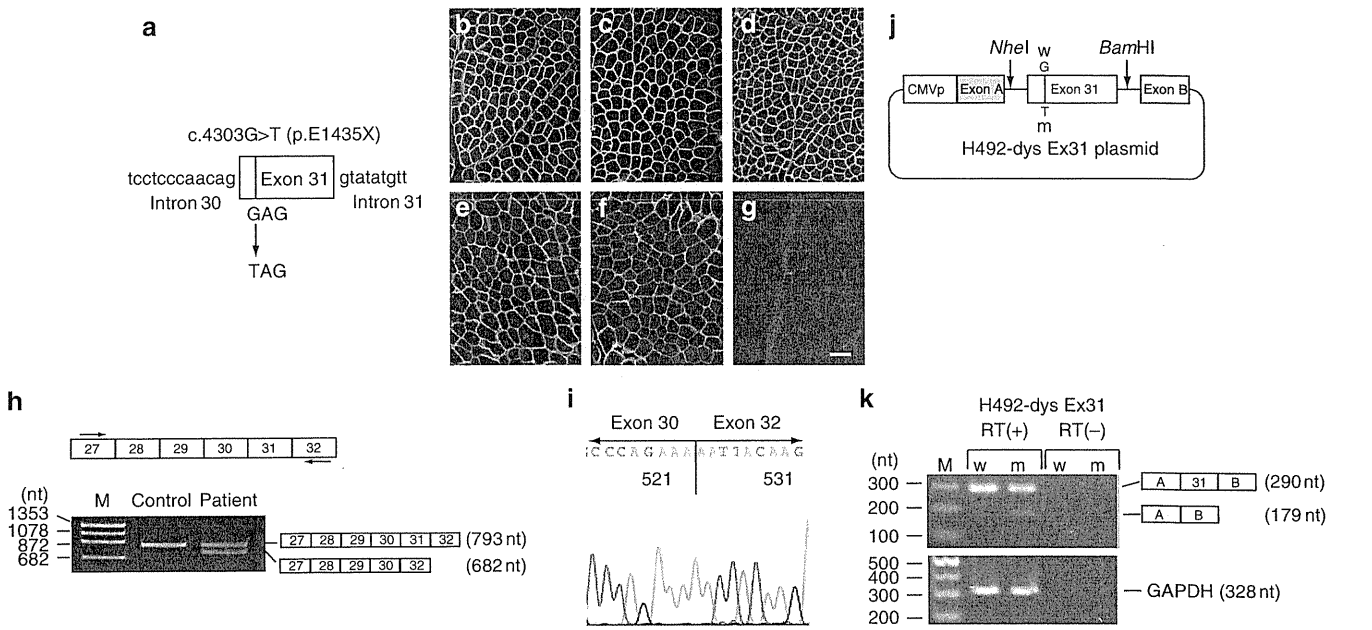

Figure 1 | Point mutation in exon 31 of the *dystrophin* gene causes exon skipping and restores the open reading frame for an internally deleted protein. (**a**) A point mutation that was found in the *dystrophin* gene of patient KUCG797. The position of c.4303G > T (p.Glu1435X) in exon 31 is indicated by the bar. (**b**–**g**) Dystrophin expression in the control (**b**–**d**) and in the patient's (**e**–**g**) muscles. Immunohistochemical examinations for C-terminal (DYS2), N-terminal (DYS3) and rod- (MANDYS1) domains were carried out. Immunoreactivity for DYS2 (exons 77–79 of dystrophin) and DYS3 (exons 10–12 of dystrophin) (**e**, **f**) was somewhat patchy and weaker in the patient's muscles as compared with the control cells (**b**, **c**). However, immunoreactivity for MANDYS1, which recognizes exon 31/32 of the dystrophin was defective only in the patient (compare panels **d**, **g**). Scale bar, 50 μm. (**h**) The RT-PCR products obtained from the control and patient were analysed on agarose gels. The RT-PCR products from the patient's RNA contained an additional shorter product. The DNA sequences of these bands were analysed, and the structure of each PCR product is shown schematically at the right of the panel. (**i**) Sequencing of the shorter product seen in (**c**) confirmed the skipping of exon 31 in the patient. (**j**) A schematic representation of the hybrid minigene plasmids that harbour either the wild-type (W) or mutated (m) exon 31 of the *dystrophin* gene. The minigene vector H492 encodes two cassette exons (A and B) and an intron sequence containing a multicloning site. The *dystrophin* gene region encompassing exon 31 with flanking introns was inserted into the *Nhe*I and *Bam*HI sites in the intron region of H492. These plasmids were transfected into HeLa cells and the pre-mRNAs were transcribed from the cytomegalovirus (CMV) promoter (CMVp). (**k**) The RT-PCR products of wild-type (W) and mutant (m) mRNA that were recovered from transfected HeLa cells were visualized on agarose gel. Two different PCR products were detected only with H492-dys Ex31m plasmid. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control for transfection efficiency. As negative controls, RT-PCR reactions without reverse transcriptase (RT −) were performed for both RNAs from the reporters and GAPDH.

(lanes 3–5 and 8–10). The faster-migrating complex contains one hnRNP A1 molecule on the RNA probe, whereas the slower-migrating complex includes more than two hnRNP A1 molecules[33]. The faster-migrating complex could be detected with both the wild-type and mutant RNA even at a low concentration of GST–hnRNP A1 (lanes 3 and 8), reflecting the efficiency of its formation with either RNA at the hnRNP A1-binding site conserved in exon 31 (ref. 5). In contrast, the slower-migrating complex was formed two times more efficiently with the mutant than with wild-type RNA at each concentration of GST–hnRNP A1 protein (compare lanes 3–5 and 8–10), and the difference was most obvious at the highest protein concentration (0.5 μM; lanes 5 and 10). As the estimated hnRNP A1 protein concentration in the HeLa nucleus is ~145 μM (hnRNP A1 6–7×10^7 molecules per cell[34,35], and 690 μm^3 for the volume of the HeLa cell nucleus[36]), the second binding site of the mutant exon 31 would be occupied by hnRNP A1 *in vivo*. As a control, GST alone was used for the assays and no binding was detected (Fig. 2a, lanes 2 and 7).

Consistent with the low binding score for SRp30c predicted by a Splice Aid analysis, weak binding of the SRp30c protein to the wild-type RNA was detected in the gel mobility shift assay (Supplementary Fig. S3a, lanes 3–5). However, the binding of SRp30c to the mutated RNA was 25–50% lower than that to the wild type (lane 8–10), indicating that the point mutation of exon 31 affects the recognition by SRp30c. All the results described above strongly suggested that the mutated exon 31 was not efficiently recognized as an exon in the context of splicing.

To confirm this, we carried out *in vitro* splicing assays. For the assays, we prepared pre-mRNA that contains either the wild-type or mutant exon 31 in the intronic region of chicken δ-crystallin (CDC) pre-mRNA[37] (Fig. 2b). The production of spliced mRNA containing the wild-type exon 31 (black circle) was detected with CDC-dys Ex31w pre-mRNA after a 60-min incubation (Fig. 2b, lanes 3–5). In contrast, the production of mRNA containing exon 31 from the CDC-dys Ex31m pre-mRNA was suppressed (Fig. 2b, lanes 8–10).

Next we investigated the effect of the overexpression of these RNA-binding proteins on the splicing pattern of a minigene in HeLa cells. As shown in Figure 1f previously, this minigene produced mRNA both with and without exon 31 (Fig. 2c, mock). When SRp30c/SRSF9 was overexpressed, the rate of exon skipping was reduced (Fig. 2c,d, SRp30c/SRSF9). In contrast, overexpression of hnRNP A1 promoted exon skipping (Fig. 2c,d, hnRNP A1, and Supplementary Fig. S4a). Although hnRNP A1 is an abundant protein in HeLa cells, as mentioned previously, we could detect similar amount of the exogenous protein to that of the endogenous protein (Supplementary Fig. S4b). As a control, SRp75/SRSF4, another SR protein[38], was used for the same assay, but failed to change the splicing pattern (Fig. 2c,d, SRp75/SRSF4). These results indicated that the skipping of the exon 31 in the *dystrophin* RNA was promoted

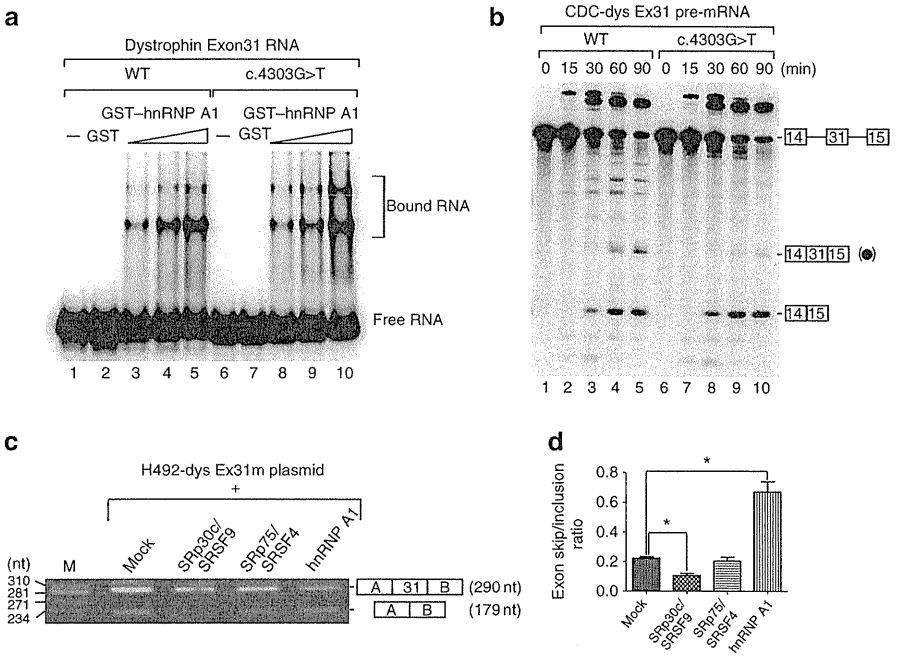

Figure 2 | The point mutation in exon 31 enhances binding to hnRNP A1 and exon skipping both *in vitro* and *in vivo*. (**a**) Gel mobility shift assays with GST-hnRNP A1 and dystrophin exon 31 RNA. ^{32}P-labelled dystrophin exon 31 RNA (wild type or mutant) was incubated with either GST alone (GST: lanes 2 and 7, 400 ng) or with GST-tagged hnRNP A1 (GST-hnRNP A1: lanes 3–5 and 8–10; 100, 200 and 400 ng, respectively), and the resultant complexes were subjected to 8% native polyacrylamide gel electrophoresis. Lanes 1 and 6 show where RNA itself migrates on the gel (marked as Free RNA on the right of the panel). Complexes of hnRNP A1 and RNA are also indicated as Bound RNA. All incubations were carried out at 20 °C for 30 min. (**b**) *In vitro* splicing assays with pre-mRNAs that contain exon 31. ^{32}P-labelled chicken δ-crystallin (CDC) pre-mRNA containing either wild-type (WT) or mutant (c.4303G > T) exon 31 in the intron was incubated with HeLa cell nuclear extracts at 30 °C for the time shown at the top of the panel. The RNA products were analysed by electrophoresis on 6% denaturing gel. The structures of the pre-mRNA and two different mRNAs are shown at the right of the panel. Boxes with numbers indicate exons, whereas the lines between boxes designate introns. The mRNA that includes exon 31 (closed circle) was produced more efficiently with CDC-dys Ex31 WT pre-mRNA than CDC-dys Ex31 c.4303G > T pre-mRNA. (**c**) Effect of overexpression of several RNA-binding proteins on the inclusion and skipping of the mutant exon 31. HeLa cells (3×10^6 cells) were cultured overnight and transfected with 150 ng of the mutant reporter plasmid used in Figure 1f in combination with 1.5 μg of the plasmid that expresses designated proteins with a Flag tag. RNAs were recovered and analysed by RT-PCR. The skipping of the mutated exon 31 was inhibited by SRp30c/SRSF9, but promoted by hnRNP A1. (**d**) Quantification of the ratio between exon skipping and inclusion for the RNA expressed from the mutant reporter plasmid in the presence of several RNA-binding proteins. Averages and standard deviations from three independent experiments are shown. *$P < 0.005$.

by disruption of the SRp30c/SRSF9-dependent ESE and production of an hnRNP A1-dependent ESS in exon 31.

TG003 promotes skipping of a mutated exon 31 in HeLa cells. The fact that this patient can express an internally deleted but presumably partially functional dystrophin protein made us look for chemical compounds that promote the skipping of exon 31 for further production of the internally deleted protein to improve the patient's condition. We screened for specific inhibitors of a variety of kinases and found several compounds that affect alternative splicing[21]. As these chemicals were demonstrated to affect alternative splicing *in vitro* and *in vivo*, we investigated the effect of eight compounds on the mutated exon 31 using the H492 minigene vector (Supplementary Table S2). TG003 strongly induced the skipping of the mutated exon in comparison with SRPIN340 and the solvent alone (Fig. 3a,b, dimethylsulphoxide, TG003 and SRPIN340). To examine the effect of TG003 on the wild-type exon 31, we next incubated cells with different concentrations of the compound after transfection of the minigene plasmids. As shown in Figure 3c, TG003 promoted the skipping of the mutated exon 31 in a dose-dependent manner (Fig. 3c), but did not affect splicing of the wild type even at 50 μM (Fig. 3c). By quantitative RT–PCR analysis, we found that the ratio of skipped mRNA to total spliced mRNAs was increased by TG003 in a dose-dependent manner (Fig. 3d). We then looked for other patients who were sensitive to TG003, and found one patient whose mutated exon was skipped on the administration of TG003 (Supplementary Fig. S5). This dystrophy patient has a point mutation in exon 27 (c.3613delG (p.Glu1205LysfsX9)), which produces a premature termination codon via a frameshift. We cloned the *dystrophin* gene fragment containing the mutant exon 27 and flanking introns into the H492 vector and transfected the plasmid into HeLa cells. As shown in Supplementary Figure S5, exon 27-skipped mRNA production was promoted by TG003 in a dose-dependent manner with the mutant plasmid in HeLa cells, whereas splicing of the wild-type mRNA was not affected.

TG003 promotes *dystrophin* expression in the patient's cells. As the results obtained with a minigene construct strongly suggested that TG003 could induce exon 31-specific skipping, we next examined the effect of TG003 on splicing of the *dystrophin* gene in myoblasts. Muscle cells obtained from the patient were cultured on dishes. Equivalent amounts of dystrophin mRNA with and without exon 31 were detected by RT–PCR (Fig. 4a). When TG003 was added to these cells, it was shown by quantitative RT–PCR analysis that the ratio of exon 31-skipped mRNA to total mRNAs was increased in a concentration-dependent manner (Fig. 4b). To examine the effect of TG003 on the splicing of other *dystrophin* introns, we prepared a subset of primers to amplify all 79 exons of *dystrophin*. The results of RT–PCR with or without TG003 showed that all exons except exon 31 were included (Supplementary Fig. S1 and Supplementary

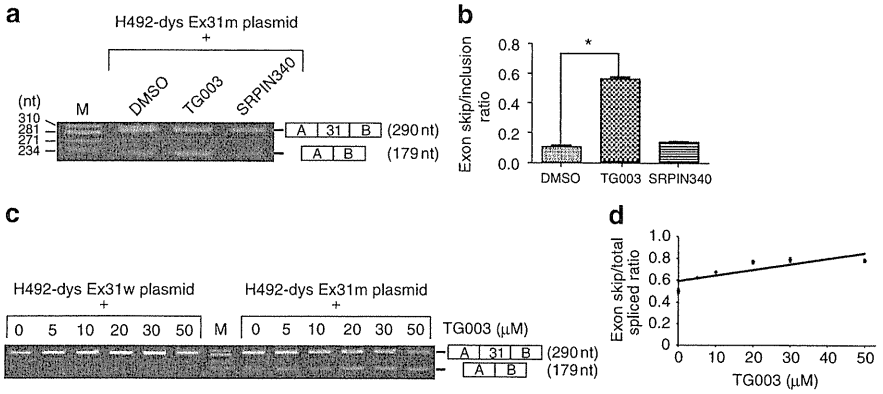

Figure 3 | TG003 promotes skipping of mutated exon 31 in HeLa cells in a dose-dependent manner. (**a**) Effect of chemical compounds on exon 31 skipping with a heterologous reporter construct in HeLa cells. After transfection of the reporter plasmid (H492-dys Ex31m) as given in Figure 2c, HeLa cells were incubated in the presence of the chemical compounds at 30 μM for 24 h. Dimethylsulphoxide (DMSO) was used as a negative control as it was used as a solvent of compounds. RNAs were recovered and analysed by RT-PCR. TG003, but not SRPIN340, promoted skipping of the mutated exon 31. (**b**) Quantification of the ratio between exon skipping and inclusion for the RNA products expressed from the mutant reporter plasmid in the presence of several RNA-binding proteins. Averages and standard deviations from three independent experiments are shown. *$P < 0.0001$. (**c**) RT-PCR of the RNA recovered from the reporter plasmid-transfected HeLa cells cultured in the presence of several concentrations of TG003. (**d**) Quantification of the ratio of exon skipping to total spliced for the RNA products expressed from the mutant reporter plasmid in the presence of several concentrations of TG003 by quantitative RT-PCR. Averages and standard deviations from three independent experiments are shown.

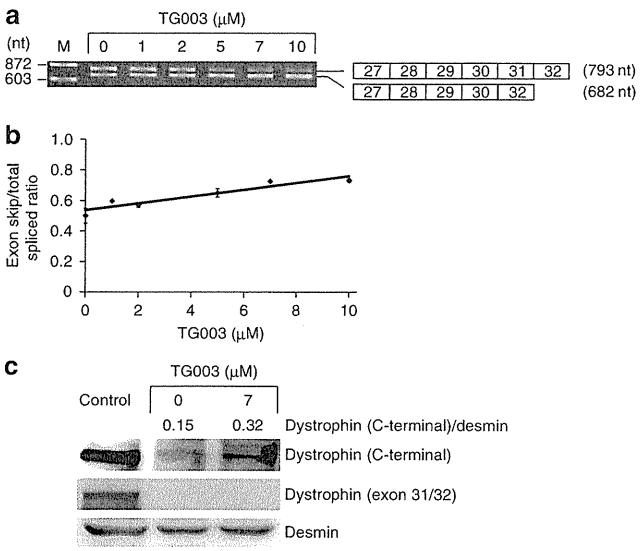

Figure 4 | TG003 induces not only exon 31 skipping but also Δexon 31 dystrophin protein expression in patient cells. (**a**) RT-PCR with RNA recovered from primary cultured muscle cells treated with different amounts of TG003. (**b**) Quantification of the ratio of exon skipping to total spliced for the RNA products expressed from the patient's endogenous *dystrophin* gene by quantitative RT-PCR. Averages and standard deviations from three independent experiments are shown. (**c**) Western blotting of dystrophin protein expression in TG003-treated cells. Proteins were detected with antibodies recognizing either the C terminus of dystrophin or the peptide encoded by exon 31. The antibody against desmin indicated that equivalent numbers of the cells were used for this assay.

Table S1), indicating that TG003 selectively promotes skipping of the mutated exon 31 in the patient's cells (or a mutated exon 27 in another patient).

Therefore, we next examine whether TG003 really increases the expression of the internally deleted, but functional, dystrophin protein in the patient's cells. Western blotting with an antibody that specifically recognizes the C terminus of dystrophin showed that the administration of TG003 (7 μM) increased the protein expression (Fig. 4c). The ratio of dystrophin (C terminal) signal to desmin signal was increased more than double by TG003. The antibody specific for the peptide with exon 31 did not detect it, indicating that the dystrophin protein lacks an exon 31-coding peptide (Fig. 4c). Therefore, TG003 promoted the expression of an internally deleted dystrophin protein by inducing exon 31 skipping in cells derived carrying the c.4303G > T mutation.

Discussion

This paper describes a patient with dystrophinopathy who had a point mutation in exon 31 of the *dystrophin* gene. As the mutation creates an in-frame stop codon, the mRNA was not expected to produce a protein. However, RT–PCR demonstrated that exon 31 was skipped and the dystrophin mRNA was naturally generated in this patient, and an internally deleted dystrophin protein was produced (Fig. 1e–g). We found that the point mutation in exon 31 disrupted the SRp30c-mediated ESE, and simultaneously created an ESS bound to hnRNP A1. This case is similar to that of the *survival of motor neuron 2* (*SMN2*) gene[39–42]. The *SMN2* gene has a C/T transition compared with the *SMN1* gene, which results in prevention of efficient exon 7 splicing. In these cases, either mutations or polymorphisms in exons can affect splicing by disrupting a positive element and creating a negative element[39–42]. In addition to affecting ESE, the c.4303G > T mutation increased the number of hnRNP A1-binding sites in exon 31 of the *dystrophin* gene. There was already one hnRNP A1-binding site in exon 31, but this mutation added another. The hnRNP A1 protein was shown to bind RNA cooperatively[43], and this would be facilitated by two high-affinity binding sites[44]. In our case, the hnRNP A1-cooperative binding seems to be facilitated by the c.4303G > T mutation, affecting the recognition and skipping of exon 31, as shown in Figure 2. It is very likely that there are more diseases and mutant phenotypes caused by the disruption/creation of splicing elements, as alternative splicing is maintained by a balance of positive and negative *cis*-elements in conjunction with trans factors.

Our results demonstrated that TG003 promoted exon skipping only in the mutant exon 31, but not in the wild type. TG003 is a specific inhibitor for Clks[23], and Clks have been shown to phosphorylate SR proteins[24,45–48]. SRp30c/SRSF9 is likely involved in the recognition of exon 31 for splicing (Fig. 2 and Supplementary Figs S2 and S3) and can be a target of TG003. However, TG003 had no effect

on the splicing of the wild-type exon 31, in which SRp30c/SRSF9 is involved (Fig. 3c). We assume that SRp30c contributes to the inclusion of the exon, but it is not phosphorylated by Clk in the nucleus. Therefore, TG003 had no effect on WT exon 31. Moreover, we have currently demonstrated that the overexpression of Clks results in the specific phosphorylation of SRp75/SRSF4 among SR proteins *in vivo*[24]. This means that not all SR proteins are phosphorylated by Clks. It has been reported that the roles of SR proteins in splicing are not always the same. Although it is generally assumed that SR proteins promote the inclusion of exons, some SR proteins can cause the skipping of exons in several alternative splicing events[19,20,49]. Thus, it is possible that some SR protein(s), which is phosphorylated by Clks, remains dephosphorylated by TG003, and this protein cooperates with hnRNP A1 as a coinhibitor of the recognition of exon 31 during splicing. Another possibility is that hnRNP A1 is a specific target of Clks, although there has been no evidence for this. Further experiments are required to examine these possible scenarios.

In this patient, skipping of the mutated exon 31 restores the open reading frame. Therefore, it is likely that an internally deleted, but functional, dystrophin protein is expressed (Fig. 1e–g). This strongly suggests that better clinical features could be expected if the skipping is induced more efficiently to produce more of the internally deleted protein. We found that TG003, reported as a Clk-specific inhibitor, stimulated skipping of the exon in both a minigene construct and the endogenous pre-mRNA in the patient's cells. When we administered 10 μM of TG003 into HeLa and COS cells, no morphological changes were observed and growth rate was not affected up to 3 days[23]. We also confirmed that TG003 had no cytotoxicity in HeLa cells at the dosage used in Figure 3c (data not shown), and even in mice at up to 100 mg kg^{-1} per day *per os* (Onogi H. and Hagiwara M., unpublished data). As shown in Figure 3c, TG003 induced the skipping of the mutated exon 31, but did not affect the splicing of the wild type. The selectivity of the effect of TG003 may explain the weak toxicity of the compound. TG003 was recently shown to inhibit the replication of the influenza virus by modifying the processing of viral RNA in cells[50]. Although more preclinical studies with animal models are needed, TG003 is the first chemical compound that is verified to improve dystrophin production in *in vitro* patient-derived myotubes. We thus started to look for patients sensitive to TG003, and found another dystrophy patient whose mutated exon was skipped (Supplementary Fig. S5). We hope that our findings will contribute towards the development of a therapy of Duchenne muscular dystrophy.

Methods

Case. The proband (KUCG 797) was a 5-year-old boy born to healthy Japanese parents without any family history of muscle disease. He started to walk independently at 1 year and 4 months of age and his motor development was normal. At the age of 2 years, his serum creatine kinase level was found to be 2,567 IU l^{-1} (normal < 169 IU l^{-1}) in a routine blood examination conducted on admission to hospital. He was referred to Kobe University Hospital to have his *dystrophin* gene examined for mutations. The mild creatine kinase elevation persisted (1,331–4,740 IU l^{-1}). No muscle weakness or abnormal gait was observed. At the age of 5 years, a muscle biopsy was performed. Informed consent for all study aspects including genetic testing was obtained from the parents, and these studies were approved by our ethics committees.

Mutation analysis. DNA was isolated from blood samples by standard phenol–chloroform extraction methods. Total RNA was isolated from peripheral lymphocytes that were collected from whole blood using Ficoll–Paque density gradients (Amersham Biosciences AB) or from thin-sliced muscle sections of frozen muscle samples. RT–PCR and RT-nested PCR were used to analyse the dystrophin mRNA expressed in skeletal muscle. A region encompassing exons 27–32 was amplified using an inner set of primers (forward c27f and reverse 2F, Supplementary Table S1). The products were purified and sequenced either directly or after subcloning into the vector pT7 Blue-T (Novagen). The DNA sequences were determined using an automated DNA sequencer (model 310; Applied Biosystems).

Plasmid construction. To construct H492-dys Ex31w and H492-dys Ex31m, the fragments encompassing exon 31 and flanking intronic regions were amplified from the genomic DNA of both the control and the patient by PCR. The primers used were Int 30f-*Nhe*I: 5′-GCGGCTAGCGTGATCCACCTGCCTCGAC-3′ and Int 31r-*Bam*HI: 5′-GCGGGATCCTCAAATCCAATCTTGCCAAT-3′. The amplified products were digested with *Nhe*I and *Bam*HI (New England Biolabs), and inserted into the H492 that had been digested with the same restriction enzymes. For the construction of Flag-SRp30c and Flag-SRp75, PCR-amplified human SRp30c and mouse SRp75 cDNAs were inserted between the *Bam*HI site and *Xho*I site of Flag-pCDNA3 (ref. 51), respectively. Human hnRNP A1 cDNA was amplified by PCR and inserted between the *Bam*HI and *Not*I sites of Flag-pCDNA3 (ref. 52) to construct Flag-hnRNP A1. To construct template plasmids for gel mobility shift assays, PCR-amplified wild-type and mutant *dystrophin* exon 31 were cloned into the *Hin*dIII and *Xho*I sites of pCDNA3 (Invitrogen). The resultant plasmids were designated as pCDNA3–dys Ex31w and pCDNA3–dys Ex31m. For making pCDC–dys Ex31w and pCDC–dys Ex31m, the wild-type or mutant exon 31 of the *dystrophin* gene was amplified by PCR and inserted between *Sac*I and *Sty*I sites of pCDC[37]. For protein expression in *Escherichia coli*, cDNAs for hnRNP A1 and SRp30c/SRSF9 lacking their termination codons were PCR-amplified and cloned between the *Bam*HI and *Xho*I sites of pET-GSTII[53]. All plasmids described above were verified by sequencing.

Cell culture and transfection. HeLa cells were cultured as described previously[37]. Transfection of the plasmids was carried out by using Lipofectamine 2000 as recommended by the manufacturer. After 24 h of transfection, RNAs were recovered from the transfected cells. The chemical compounds were incubated for 24 h.

Primary culture of the DMD patient's muscle cells. The patient's muscle cells were cultured in DMEM (Sigma) supplemented with 20% fetal bovine serum (Gibco), 4% Ultrocer G (PALL) and 1% antibiotic-antimycotic (Gibco) in a six-well plate (gelatin-coated micro plate (six-well) with lid, IWAKI) until confluent. To induce their differentiation into myotubes, the cells were cultured in DMEM (Sigma) supplemented with 2% horse serum (Gibco) and 1% antibiotic-antimycotic (Gibco) for 2 weeks with or without TG003. The medium and TG003 were refreshed every 2 days.

Immunonohistochemical analyses of skeletal muscle. Skeletal muscle samples obtained from the rectus muscle of the thigh by biopsy were flash-frozen with isopentane cooled in liquid nitrogen. Serial 10-μm-thick frozen sections were analysed with histochemical staining. The procedure for the immunohistochemistry was as follows: serial 10-μm-thick frozen muscle sections were fixed in cold acetone for 5 min. After blocking with normal goat serum, sections were incubated with primary antibodies overnight at 4 °C. The antibodies used were antidystrophin (DYS2: recognizing an epitope in exons 77–79, and DYS3: recognizing an epitope in exons 10–12, Novocastra) and antidystrophin (MANDYS1: recognizing an epitope in exon 31/32, a gift from Professor Dr Glenn E. Morris). After six rinses with PBS, sections were incubated with secondary antibodies, Alexa Fluor 488-labelled goat anti-mouse or anti-rabbit antibodies, at room temperature for 90 min.

Isolation of RNA and RT–PCR. RNA was isolated and analysed by RT–PCR, as described previously[4]. Primers used for amplification of *dystrophin* and human *glyceraldehyde-3-phosphate dehydrogenase* mRNAs are demonstrated in Supplementary Table S1. PCR products were analysed on 2% agarose gels in Tris–borate/EDTA buffer. Skipping efficiencies were determined from gel images by comparing the shortened dystrophin mRNAs to the intact transcript of full length in a densitometric analysis with Image J software (for patient samples) or by quantifying the skipped products with a DNA 1000 LabChip Kit on an Agilent 2100 bioanalyzer (Agilent Technologies; for hDMD mouse samples). Exon skip/inclusion ratios were calculated as the amount of skipped transcript relative to the full-length transcript. Where appropriate, a two-tailed Student's *t*-test was used to determine the statistical significance of the skipping. As DNA size markers, ϕX174-*Hae*III digest (TAKARA) or 2-Log DNA ladder (New England Biolab) was used for agarose gel electrophoresis.

Quantitative real-time RT–PCR. Real-time RT–PCR amplification was performed using a 7500 fast real-time PCR system (Applied Biosystems Inc.). Real-time PCR assays were carried out in a final volume of 20 μl consisting of 10 μl of TaqMan Fast Advanced Master Mix (Applied Biosystems Inc.), 18 pmol each of 5′ and 3′ primer, 5 pmol of specific probe and 10 ng of the sample cDNA. The real-time PCR conditions were as follows: 1 cycle for 20 s at 96 °C, followed by 40 cycles of 2 s at 96 °C for denaturation, 15 s at 60 °C for annealing and 15 s at 72 °C for extension. Spectral data were captured and analysed using 7500 Real-Time Analysis Software version 2.0.3 (Applied Biosystems Inc.). All samples were run in triplicate. β-Actin and desmin was analysed as an endogenous RNA reference gene for normalization of H492-dys 31-derived mRNA and endogenous dystrophin mRNA from the patient, respectively. Exon skip/total ratios were calculated as the proportion of skipped transcript relative to both the full-length and the skipped transcripts.

The PCR primers and probes (5-FAM and 3-MGB) were designed with Primer Express software (Applied Biosystems Inc.), and the sequences of the primers and probes are shown in Supplementary Table S1.

Western blotting with the patient's myotubes. The patient's myotube cells were rinsed twice with PBS and then collected using 1× Cell Lysis Buffer (Cell Signaling Technology). Total protein (60 μg) was loaded on a 3–10% gradient polyacrylamide gel (PAGEL, ATTO). The fractionated proteins were transferred to HYBOND-P (GE Healthcare). Western blotting was performed using the ECL advance Western Blotting Detection kit (GE Healthcare) according to the manufacturer's instructions. The membrane was incubated with an antibody against the C terminus (NCL-DYS2, Leica) and exon 31 (MANDYS8, a gift from Dr Glenn E. Morris) of dystrophin at a dilution of 1:10 and 1:100, respectively. The dystrophin–antidystrophin immune complexes were detected with anti-mouse IgG (GE Healthcare). Western blotting for desmin was performed using the same protocol as described above. The Desmin antibody (H-76, Santa Cruz) was used at a dilution of 1:50. The desmine–antidesmine immune complexes were detected with anti-rabbit IgG (GE Healthcare).

Preparation of recombinant proteins. All proteins were overexpressed in BL21 (DE3) CodonPlus-RIPL cells (Stratagene). GST-His, GST-hnRNP A1-His and GST-SRp30c-His were induced at 20 °C overnight, purified according to the manufacturer's instructions (Pharmacia) and dialysed against PBS. Dialysed proteins were further purified on nickel resin (Qiagen) as recommended by the manufacturer and dialysed against buffer E (20-mM HEPES-KOH pH 7.9, 100-mM KCl, 0.2-mM EDTA, 10% glycerol and 1-mM DTT). Purified proteins were quickly frozen by liquid nitrogen and stored at −80 °C.

***In vitro* transcription and splicing assay.** As templates, pCDNA3–dys Ex31 and pCDNA3–dys Ex31m were linearized with *Xho*I, and pCDC–dys Ex31w and pCDC–dys Ex31m were linearized with *Sma*I. *In vitro* transcription and purification of the transcribed RNAs were performed as described previously[37]. HeLa cell nuclear extracts were obtained from Cilbiotech. *In vitro* splicing assay was carried out in a 10-μl scale as described previously[37]. The RNAs were analysed by 6% denaturing polyacrylamide gel electrophoresis and autoradiography.

Gel mobility shift assay. Gel mobility shift assays were essentially carried out as described previously[54]. The binding buffer that was used contained 16-mM HEPES-KOH (pH 7.9), 80-mM KCl, 0.16-mM EDTA, 0.8-mM DTT, 8% glycerol, 100 ng μl^{-1} of BSA, 50 ng μl^{-1} of *E. coli* tRNA (Sigma Chemical Co.), 5×10^4 c.p.m. of RNA (dystrophin exon 31 wild type or mutant RNA) and 1 U μl^{-1} of RNasin (Promega). Eight percent native polyacrylamide gels were used to analyse the complexes.

Statistical analyses. Statistical analyses were performed with Prism5 statistical software (GraphPad) using a paired *t*-test or a one-way analysis of variance, followed by Tukey's multiple comparison test.

References

1. Koenig, M., Monaco, A. P. & Kunkel, L. M. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* **53,** 219–228 (1988).
2. Monaco, A. P., Bertelson, C. J., Liechti-Gallati, S., Moser, H. & Kunkel, L. M. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* **2,** 90–95 (1988).
3. Nishiyama, A. *et al.* Dystrophin nonsense mutations can generate alternative rescue transcripts in lymphocytes. *Ann. Hum. Genet.* **72,** 717–724 (2008).
4. Shiga, N. *et al.* 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.* **100,** 2204–2210 (1997).
5. Disset, A. *et al.* An exon skipping-associated nonsense mutation in the dystrophin gene uncovers a complex interplay between multiple antagonistic splicing elements. *Hum. Mol. Genet.* **15,** 999–1013 (2006).
6. Fajkusova, L. *et al.* Novel dystrophin mutations revealed by analysis of dystrophin mRNA: alternative splicing suppresses the phenotypic effect of a nonsense mutation. *Neuromuscul. Disord.* **11,** 133–138 (2001).
7. Deburgrave, N. *et al.* Protein- and mRNA-based phenotype-genotype correlations in DMD/BMD with point mutations and molecular basis for BMD with nonsense and frameshift mutations in the DMD gene. *Hum. Mut.* **28,** 183–195 (2007).
8. Matsuo, M. Duchenne/Becker muscular dystrophy: from molecular diagnosis to gene therapy. *Brain Dev.* **18,** 167–172 (1996).
9. Aartsma-Rus, A. & van Ommen, G. J. Less is more: therapeutic exon skipping for Duchenne muscular dystrophy. *Lancet Neurol.* **8,** 873–875 (2009).
10. Le Roy, F., Charton, K., Lorson, C. L. & Richard, I. RNA-targeting approaches for neuromuscular diseases. *Trends Mol. Med.* **15,** 580–591 (2009).
11. Wood, M. J., Gait, M. J. & Yin, H. RNA-targeted splice-correction therapy for neuromuscular disease. *Brain* **133,** 957–972 (2010).
12. Pramono, Z. A. *et al.* 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.* **226,** 445–449 (1996).
13. 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.* **95,** 515–520 (1995).
14. Takeshima, Y. *et al.* Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. *Pediatr. Res.* **59,** 690–694 (2006).
15. van Deutekom, J. C. *et al.* Local dystrophin restoration with antisense oligonucleotide PRO051. *N. Engl. J. Med.* **357,** 2677–2686 (2007).
16. Kinali, M. *et al.* Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurol.* **8,** 918–928 (2009).
17. Hirawat, S. *et al.* Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers. *J. Clin. Pharmacol.* **47,** 430–444 (2007).
18. Welch, E. M. *et al.* PTC124 targets genetic disorders caused by nonsense mutations. *Nature* **447,** 87–91 (2007).
19. Long, J. C. & Caceres, J. F. The SR protein family of splicing factors: master regulators of gene expression. *Biochem. J.* **417,** 15–27 (2009).
20. Shepard, P. J. & Hertel, K. J. The SR protein family. *Genome Biol.* **10,** 242 (2009).
21. Fukuhara, T. *et al.* Utilization of host SR protein kinases and RNA-splicing machinery during viral replication. *Proc. Natl Acad. Sci. USA* **103,** 11329–11333 (2006).
22. Nowak, D. G. *et al.* Regulation of vascular endothelial growth factor (VEGF) splicing from pro-angiogenic to anti-angiogenic isoforms: a novel therapeutic strategy for angiogenesis. *J. Biol. Chem.* **285,** 5532–5540 (2010).
23. Muraki, M. *et al.* Manipulation of alternative splicing by a newly developed inhibitor of Clks. *J. Biol. Chem.* **279,** 24246–24254 (2004).
24. Yomoda, J. *et al.* Combination of Clk family kinase and SRp75 modulates alternative splicing of adenovirus E1A. *Genes Cells* **13,** 233–244 (2008).
25. Kaida, D. *et al.* Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA. *Nat. Chem. Biol.* **3,** 576–583 (2007).
26. Kotake, Y. *et al.* Splicing factor SF3b as a target of the antitumor natural product pladienolide. *Nat. Chem. Biol.* **3,** 570–575 (2007).
27. Thi Tran, H. T. *et al.* A G-to-A transition at the fifth position of intron-32 of the dystrophin gene inactivates a splice-donor site both *in vivo* and *in vitro*. *Mol. Genet. Metab.* **85,** 213–219 (2005).
28. Tran, V. K. *et al.* Splicing analysis disclosed a determinant single nucleotide for exon skipping caused by a novel intraexonic four-nucleotide deletion in the dystrophin gene. *J. Med. Genet.* **43,** 924–930 (2006).
29. Tran, V. K. *et al.* A nonsense mutation-created intraexonic splice site is active in the lymphocytes, but not in the skeletal muscle of a DMD patient. *Hum. Genet.* **120,** 737–742 (2007).
30. Piva, F., Giulietti, M., Nocchi, L. & Principato, G. SpliceAid: a database of experimental RNA target motifs bound by splicing proteins in humans. *Bioinformatics* **25,** 1211–1213 (2009).
31. Paradis, C. *et al.* hnRNP I/PTB can antagonize the splicing repressor activity of SRp30c. *RNA (New York, NY)* **13,** 1287–1300 (2007).
32. Burd, C. G. & Dreyfuss, G. RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. *EMBO J.* **13,** 1197–1204 (1994).
33. Siomi, M. C. *et al.* Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. *J. Cell. Biol.* **138,** 1181–1192 (1997).
34. Kiledjian, M., Burd, C., Görlach, M., Portman, D. & Dreyfuss, G. *Structure and Function of hnRNP Proteins* (Oxford University Press, 1994).
35. Hanamura, A., Caceres, J. F., Mayeda, A., Franza, B. R. Jr. & Krainer, A. R. Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA (New York, NY)* **4,** 430–444 (1998).
36. Monier, K., Armas, J. C., Etteldorf, S., Ghazal, P. & Sullivan, K. F. Annexation of the interchromosomal space during viral infection. *Nat. Cell Biol.* **2,** 661–665 (2000).
37. Kataoka, N. *et al.* Pre-mRNA splicing imprints mRNA in the nucleus with a novel RNA-binding protein that persists in the cytoplasm. *Mol. Cell* **6,** 673–682 (2000).
38. Zahler, A. M., Neugebauer, K. M., Stolk, J. A. & Roth, M. B. Human SR proteins and isolation of a cDNA encoding SRp75. *Mol. Cell. Biol.* **13,** 4023–4028 (1993).
39. Cartegni, L., Hastings, M. L., Calarco, J. A., de Stanchina, E. & Krainer, A. R. Determinants of exon 7 splicing in the spinal muscular atrophy genes, SMN1 and SMN2. *Am. J. Hum. Genet.* **78,** 63–77 (2006).
40. Cartegni, L. & Krainer, A. R. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. *Nat. Genet.* **30,** 377–384 (2002).
41. Kashima, T. & Manley, J. L. A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nat. Genet.* **34,** 460–463 (2003).
42. Kashima, T., Rao, N., David, C. J. & Manley, J. L. hnRNP A1 functions with specificity in repression of SMN2 exon 7 splicing. *Hum. Mol. Genet.* **16,** 3149–3159 (2007).
43. Zhu, J., Mayeda, A. & Krainer, A. R. Exon identity established through differential antagonism between exonic splicing silencer-bound hnRNP A1 and enhancer-bound SR proteins. *Mol. Cell* **8,** 1351–1361 (2001).

44. Okunola, H. L. & Krainer, A. R. Cooperative-binding and splicing-repressive properties of hnRNP A1. *Mol. Cell. Biol.* **29,** 5620–5631 (2009).
45. Colwill, K. *et al.* The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. *EMBO J* **15,** 265–275 (1996).
46. Nayler, O., Schnorrer, F., Stamm, S. & Ullrich, A. The cellular localization of the murine serine/arginine-rich protein kinase CLK2 is regulated by serine 141 autophosphorylation. *J. Biol. Chem.* **273,** 34341–34348 (1998).
47. Johnson, K. W. & Smith, K. A. Molecular cloning of a novel human cdc2/CDC28-like protein kinase. *J. Biol. Chem.* **266,** 3402–3407 (1991).
48. Duncan, P. I., Stojdl, D. F., Marius, R. M., Scheit, K. H. & Bell, J. C. The Clk2 and Clk3 dual-specificity protein kinases regulate the intranuclear distribution of SR proteins and influence pre-mRNA splicing. *Exp. Cell Res.* **241,** 300–308 (1998).
49. Goncalves, V., Matos, P. & Jordan, P. Antagonistic SR proteins regulate alternative splicing of tumor-related Rac1b downstream of the PI3-kinase and Wnt pathways. *Hum. Mol. Genet.* **18,** 3696–3707 (2009).
50. Karlas, A. *et al.* Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. *Nature* **463,** 818–822 (2010).
51. Kataoka, N. & Dreyfuss, G. A simple whole cell lysate system for *in vitro* splicing reveals a stepwise assembly of the exon-exon junction complex. *J. Biol. Chem.* **279,** 7009–7013 (2004).
52. Nojima, T. *et al.* Herpesvirus protein ICP27 switches PML isoform by altering mRNA splicing. *Nucleic Acids Res.* **37,** 6515–6527 (2009).
53. Nakielny, S., Shaikh, S., Burke, B. & Dreyfuss, G. Nup153 is an M9-containing mobile nucleoporin with a novel Ran-binding domain. *EMBO J.* **18,** 1982–1995 (1999).
54. Kataoka, N., Ohno, M., Moda, I. & Shimura, Y. Identification of the factors that interact with NCBP, an 80 kDa nuclear cap binding protein. *Nucleic Acids Res.* **23,** 3638–3641 (1995).

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Author contributions

A.N., N.K., H.M. and M.M. designed experiments and A.N., N.K., Y.T., M.Y., H.A., M.O. and K.I. performed them. A.N., N.K., H.M. and M.M. analysed the data and wrote the manuscript.

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Brief Communication

Two closely spaced nonsense mutations in the *DMD* gene in a Malaysian family

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ABSTRACT

In Duchenne muscular dystrophy (DMD), identification of one nonsense mutation in the *DMD* gene has been considered an endpoint of genetic diagnosis. Here, we identified two closely spaced nonsense mutations in the *DMD* gene. In a Malaysian DMD patient two nonsense mutations (p.234S>X and p.249Q>X, respectively) were identified within exon 8. The proband's mother carried both mutations on one allele. Multiple mutations may explain the occasional discrepancies between genotype and phenotype in dystrophinopathy.

1. Introduction

The identification of one deleterious mutation in a responsible gene has been considered an endpoint for genetic diagnosis of hereditary diseases. However, multiple mutations have been reported in some genetic diseases. In one survey, the frequency of multiple point mutations causing genetic disease was calculated as ~0.2% (the ratio of the number of multiple point mutations to the total number of reported disease-associated point mutations) [1].

The *DMD* gene is a huge gene comprising of 79 exons locating at Xp21.2. Mutations in the *DMD* gene, exon deletions being the most frequent, result in the severe Duchenne, or the mild Becker, muscular dystrophies (DMD/BMD). Identification of one disrupting mutation in the *DMD* gene is considered sufficient for genetic diagnosis. The reading frame rule explains the clinical differences between DMD and BMD: out-of-frame or nonsense mutations cause dystrophin deficiency, resulting in DMD, while in-frame mutations enable the production of internally deleted dystrophin, resulting in BMD [2]. However, there remain unanswered questions regarding this rule: 1) there are some exceptions in which in-frame mutations result in DMD and *vice versa* [3]; and 2) there can be inter-sibling differences in clinical findings [4]. The answers to these questions might lie in multiple mutations in the *DMD* gene. In the literature, there are no examples of multiple nonsense mutations in the *DMD* gene although a case has been described with a double missense mutation [5].

Here, we identified two closely spaced nonsense mutations in the *DMD* gene during a mutation analysis of Malaysian DMD patients.

2. Methods

2.1. DMD case

The proband was an 18-year-old Malaysian boy with a high serum creatine kinase level (5408 IU/l). He was born to a healthy Malaysian couple as their fifth baby of six. The parents were first-degree cousins. He was diagnosed as having DMD at the age of 4, showing an unstable gait. Genetic diagnosis was performed after obtaining informed consent.

2.2. Methods

Genomic DNA was extracted from blood as described before [6]. Mutations in the *DMD* gene were first screened using multiple ligation-dependent probe amplification (MLPA) using an MLPA DMD kit (SALSA MLPA KIT P034/P035 DMD/Becker; MRC-Holland, Amsterdam, The Netherlands) [7]. If no abnormality was identified by MLPA, four exons (exons 8, 34, 44, and 7) were examined according to Japanese recommendations [8]. The amplified fragments were sequenced directly or after subcloning as described previously [6].

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3. Results

In the proband two nucleotide changes were identified within the 182-bp exon 8: c.701C>A and c.745C>T. The two nucleotide changes were separated by only 44 bp. No nucleotide change was identified in the examined 135-bp intron 7 and 177-bp intron 8. The former would change the TCA serine codon to a TAA stop codon (p.234S>X) and the latter would change the CAG glutamine codon to a TAG stop codon (p.249Q>X). Both were nonsense mutations and were thus concluded to be the cause of DMD.

We questioned whether both mutations were carried by the proband's mother. When we sequenced the exon 8-encompassing region of the mother's genome, we found that she carried both nucleotide changes. Furthermore, subcloning sequencing of PCR-amplified products revealed that one allele was normal and the other carried both of the mutations seen in the proband. We concluded that the two nonsense mutations were inherited through the mother.

Two closely spaced nonsense mutations suggest hypermutability of the inherited allele. However, we did not find any additional nucleotide changes within exon 8, nor in 1340 bp of intron 7 and 1113 bp of intron 8 flanking the exon.

4. Discussion

Two closely spaced nonsense mutations in the *DMD* gene were disclosed in the proband. The two single nucleotide changes were located in exon 8 and were inherited from the patient's mother. To our knowledge, this is the first example of two nonsense mutations identified in the *DMD* gene. C>T changes at CpG sites are considered to arise because of spontaneous deamination of 5-methylcytosine to thymidine at methylated CpG dinucleotides [9], and accordingly, hypermutability of CGA>TGA has been revealed in the *DMD* gene [10]. However, our identified mutations (TCA to TAA and CAG to TAG) were not at CpG sites, indicating that a different mechanism is likely responsible. Moreover, because one mutation is a transition and the other a transversion, it is difficult to envisage an identical mechanism producing both.

Both of these nonsense mutations have been described before. c.701C>A has been identified twice in Australia and c.745C>T has also been revealed twice, in patients from France and the U.S.A. [11]. On each of these four occasions, no additional mutations were described, implying that these nonsense mutations resulted from an isolated mutation event. Multiple mutations can be the result of sequential independent single mutations or they can be generated simultaneously or quasi-simultaneously in the same cell cycle. Multiple synchronous mutations have been postulated to arise *via* transient hypermutability [1]. Multiple mutations are consistent with a model of transient hypermutability [12–14].

Closely spaced multiple mutations are divided into two classes: <100 bp and >100 bp [1]. For the former, closely separated type, which applies to our identified mutations, a so-called 'mutation shower' has been proposed [14]. In an extreme case of mutation shower, five closely separated mutations were identified [1]. The lack of additional mutations in a surrounding region of 2635 bp is slightly more supportive of sequential mutations.

Our findings suggest that unidentified second mutations may play a role in modifying the clinical phenotypes of dystrophinopathy, which has a wide range of clinical severity. Nonsense mutations have occasionally been identified in mild BMD, and exon skipping has been reported to be a modifier in these cases [15–17]. However, some cases with exon deletion do not follow the reading frame rule [8]. Our result suggests that a second mutation could explain a discrepancy between genotype and phenotype that cannot be explained by the reading frame rule. We believe that, although determination of one deleterious mutation in the *DMD* gene is sufficient for genetic diagnosis, it is also necessary to pay attention to additional mutations that may modify the phenotype.

Acknowledgments

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References

[1] J.M. Chen, C. Ferec, D.N. Cooper, Closely spaced multiple mutations as potential signatures of transient hypermutability in human genes, Hum. Mutat. 30 (2009) 1435–1448.
[2] A.P. Monaco, C.J. Bertelson, S. Liechti-Gallati, H. Moser, L.M. Kunkel, An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus, Genomics 2 (1988) 90–95.
[3] S.B. Gangopadhyay, T.G. Sherratt, J.Z. Heckmatt, V. Dubowitz, G. Miller, M. Shokeir, P.N. Ray, P.N. Strong, R.G. Worton, Dystrophin in frameshift deletion patients with Becker muscular dystrophy, Am. J. Hum. Genet. 51 (1992) 562–570.
[4] M. Sifringer, B. Uhlenberg, S. Lammel, R. Hanke, B. Neumann, A. von Moers, I. Koch, A. Speer, Identification of transcripts from a subtraction library which might be responsible for the mild phenotype in an intrafamilially variable course of Duchenne muscular dystrophy, Hum. Genet. 114 (2004) 149–156.
[5] F.A. Saad, L. Merlini, M.L. Mostacciuolo, G.A. Danieli, Double missense mutation in exon 41 of the human dystrophin gene detected by double strand conformation analysis, Am. J. Med. Genet. 80 (1998) 99–102.
[6] V.K. Tran, Y. Takeshima, Z. Zhang, M. Yagi, A. Nishiyama, Y. Habara, M. Matsuo, Splicing analysis disclosed a determinant single nucleotide for exon skipping caused by a novel intra-exonic four-nucleotide deletion in the dystrophin gene, J. Med. Genet. 43 (2006) 924–930.
[7] Y. Okizuka, Y. Takeshima, H. Awano, Z. Zhang, M. Yagi, M. Matsuo, Small mutations detected by multiplex ligation-dependant probe amplification of the dystrophin gene, Genet. Test. Mol. Biomarkers 13 (2009) 427–431.
[8] Y. Takeshima, M. Yagi, Y. Okizuka, H. Awano, Z. Zhang, Y. Yamauchi, H. Nishio, M. Matsuo, Mutation spectrum of the dystrophin gene in 442 Duchenne/Becker muscular dystrophy cases from one Japanese referral center, J. Hum. Genet. 55 (2010) 379–388.
[9] D.N. Cooper, M. Krawczak, Cytosine methylation and the fate of CpG dinucleotides in vertebrate genomes, Hum. Genet. 83 (1989) 181–188.
[10] K.M. Flanigan, D.M. Dunn, A. von Niederhausern, P. Soltanzadeh, E. Gappmaier, M.T. Howard, J.B. Sampson, J.R. Mendell, C. Wall, W.M. King, A. Pestronk, J.M. Florence, A.M. Connolly, K.D. Mathews, C.M. Stephan, K.S. Laubenthal, B.L. Wong, P.J. Morehart, A. Meyer, R.S. Finkel, C.G. Bonnemann, L. Medne, J.W. Day, J.C. Dalton, M.K. Margolis, V.J. Hinton, R.B. Weiss, Mutational spectrum of DMD mutations in dystrophinopathy patients: application of modern diagnostic techniques to a large cohort, Hum. Mutat. 30 (2009) 1657–1666.
[11] A. Aartsma-Rus, J.C. Van Deutekom, I.F. Fokkema, G.J. Van Ommen, J.T. Den Dunnen, Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule, Muscle Nerve 34 (2006) 135–144.
[12] J.W. Drake, A. Bebenek, G.E. Kissling, S. Peddada, Clusters of mutations from transient hypermutability, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 12849–12854.
[13] J.W. Drake, Too many mutants with multiple mutations, Crit. Rev. Biochem. Mol. Biol. 42 (2007) 247–258.
[14] J. Wang, K.D. Gonzalez, W.A. Scaringe, K. Tsai, N. Liu, D. Gu, W. Li, K.A. Hill, S.S. Sommer, Evidence for mutation showers, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 8403–8408.
[15] N. Shiga, Y. Takeshima, H. Sakamoto, K. Inoue, Y. Yokota, M. Yokoyama, M. Matsuo, 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. 100 (1997) 2204–2210.
[16] L. Fajkusova, Z. Lukas, M. Tvrdikova, V.V. Kuhrova, J. Hajek, J. Fajkus, Novel dystrophin mutations revealed by analysis of dystrophin mRNA: alternative splicing suppresses the phenotypic effect of a nonsense mutation, Neuromuscul. Disord. 11 (2001) 133–138.
[17] K.M. Flanigan, D.M. Dunn, A. von Niederhausern, P. Soltanzadeh, M.T. Howard, J.B. Sampson, K.J. Swoboda, M.B. Bromberg, J.R. Mendell, L. Taylor, C.B. Anderson, A. Pestronk, J. Florence, A.M. Connolly, K.D. Mathews, B. Wong, R.S. Finkel, C.G. Bonnemann, J.W. Day, C. McDonald, R.B. Weiss, Nonsense mutation-associated Becker muscular dystrophy: interplay between exon definition and splicing regulatory elements within the DMD gene, Hum. Mutat. 32 (2011) 299–308.

Duchenne型筋ジストロフィーに対するエクソンスキッピング誘導治療

Exon skipping therapy for Duchenne muscular dystrophy

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◎Duchenne 型筋ジストロフィー(DMD)は，ジストロフィン遺伝子の異常によって発症する遺伝性筋疾患であり，出生男児 3,500 人に 1 人という高頻度で発症し，症状も重篤であるが，いまだ根治治療は確立していない．DMD の 60%ではジストロフィン遺伝子の 1〜数エクソンの欠失がみられ，欠失によりアミノ酸読み取り枠にずれが生じるために，機能を有するジストロフィン蛋白が産生されない．現在 DMD の治療として注目されているエクソンスキッピング誘導治療は，アンチセンスオリゴヌクレオチド(AS-oligo)を用いて mRNA 前駆体のスプライシングのレベルでエクソンのスキッピングを誘導し，アミノ酸の読み取り枠のずれを修正することでジストロフィン蛋白を産生させるものである．著者らは 2006 年に，DMD 症例に対して AS-oligo を静脈内投与しジストロフィン蛋白を発現させることが可能であることを，世界ではじめて明らかにした．著者らの報告に引き続き，イギリスやオランダでもエクソンスキッピング誘導治療の有効性が報告されている．さらに近年，著者らはエクソンスキッピングを誘導する低分子化合物を見出した．エクソンスキッピング誘導治療は近い将来，DMD の標準的な治療になるものと期待される．

Key word：Duchenne型筋ジストロフィー(DMD)，エクソンスキッピング，アンチセンスオリゴヌクレオチド，低分子化合物

Duchenne 型筋ジストロフィー(Duchenne muscular dystrophy：DMD)は小児で発症するもっとも頻度の高い遺伝性筋疾患であり，ジストロフィン遺伝子の異常によって発症する．出生男児 3,500 人に 1 人が発症する頻度の高い疾患で症状も重篤であるため，根治的な治療法の開発が望まれてきた．ジストロフィン遺伝子がクローニングされて以降，正常なジストロフィン遺伝子を導入する遺伝子治療に注目が集まったが，現在もまだ臨床での治療としての実用化には至っていない．

一方で，著者らはアンチセンスオリゴヌクレオチド(AS-oligo)により遺伝情報を修復する分子治療の検討を進めてきた．これは，AS-oligo を用いて mRNA 前駆体のスプライシングレベルでエクソンのスキッピングを誘導することでアミノ酸の読み取り枠のずれを修正し，機能を有するジストロフィン蛋白を発現させるものである．そして実際に DMD 症例に対して AS-oligo を静脈内投与し，ジストロフィン蛋白を発現させることが可能であることを 2006 年に世界ではじめて明らかにした[1]．その後，オランダ，イギリスからも DMD 症例に対する AS-oligo 投与の有効性が報告され[2-4]，AS-oligo によるエクソンスキッピング誘導治療研究が現在精力的に進められている．

本稿では，DMD に対するエクソンスキッピング誘導治療について概説する．

Duchenne型およびBecker型筋ジストロフィーとジストロフィン遺伝子

DMD および Becker 型筋ジストロフィー(Becker muscular dystrophy：BMD)は，伴性劣性遺伝形式を示すもっとも頻度の高い遺伝性の進行

性筋萎縮症である．DMD は 4〜5 歳ごろから筋力低下に気づかれ，その後筋力低下が進行し 12 歳までに歩行不能となり，20 歳代で心不全や呼吸不全により死に至る重篤な疾患である．一方，BMD は DMD に比べて筋力低下の程度は軽く進行も遅いため，壮年期になってはじめて症状を認める例もある．

DMD/BMD の責任遺伝子であるジストロフィン遺伝子は X 染色体短腕上に存在する 3,000 kb に及ぶ巨大な遺伝子であり，79 個のエクソンから構成される．DMD/BMD でみられるジストロフィン遺伝子の変異としては，エクソン単位の欠失や重複，ナンセンス変異，スプライシング変異などの微小変異があるが，エクソン単位の欠失がおよそ 6 割を占め，もっとも多い[5]．

ジストロフィン遺伝子にコードされているジストロフィンは筋細胞膜を裏打ちする細長い棒状の蛋白である．骨格筋の免疫染色では，DMD ではジストロフィンは筋細胞膜に染色されずジストロフィン欠損を示すが，症状の軽い BMD では筋細胞膜にジストロフィンが斑点状に染色される．

アミノ酸読み取り枠則

同じジストロフィン遺伝子の異常であるにもかかわらず，DMD と BMD では臨床像が大きく異なる．重症の DMD と軽症である BMD の違いは，アミノ酸読み取り枠則で説明される．

mRNA から蛋白が翻訳される際，3 塩基で 1 つのアミノ酸をコードしている．ジストロフィン遺伝子のエクソン単位の欠失によって失われる塩基数が 3 の倍数であれば，mRNA のアミノ酸読み取り枠は維持されるため(インフレーム)，翻訳の際に遺伝子欠失部位に相当するアミノ酸は欠失するが，機能をもったジストロフィンが産生される．そのため症状の軽い BMD となる．欠失の塩基数が 3 の倍数でない場合は mRNA の読み取り枠がずれるため(アウトオブフレーム)，ストップコドンが出現し，機能的なジストロフィン蛋白は合成されず重症型の DMD となる．

この分子病態に注目し，DMD のアウトオブフレームをインフレームに変換させることができれば，機能を有するジストロフィン蛋白を産生し，重症の DMD を軽症型へ変換することが可能となる．DMD に対するエクソンスキッピング誘導治療はまさにこの着想に基づき，患者自身のもつ遺伝子を活用してジストロフィンを発現させるものである．

神戸大学で行った エクソンスキッピング誘導治療

著者らは，エクソン 19 内に部分欠失を有する症例(ジストロフィン神戸)[6]の解析において，ゲノム DNA ではエクソン 19 内に 52 塩基の欠失が認められるのに対し，mRNA ではエクソン 19 全体が欠失しているエクソンスキッピングが生じていることを発見した．この例ではスプライシングのコンセンサス配列が維持されており，エクソンスキッピングの生じた原因が不明であった．*In vitro* スプライシング系を用いた検討により，エクソン 19 内の欠失している配列内に含まれるスプライシングを促進する配列(splicing enhancer sequence：SES，「サイドメモ」参照)が欠失したためであることを明らかにした[7]．さらに，SES に対する AS-oligo によりエクソン 19 のスキッピングを人工的に誘導しうることを世界ではじめて示した[8]．

つぎに，エクソン 19 のスキッピングを誘導する

サイドメモ

スプライシング促進配列(splicing enhancer sequence：SES)

スプライシングは，mRNA 前駆体からイントロンが取り除かれ，エクソンとエクソンが結合する反応である．スプライシング部位決定には従来よりエクソン・イントロン境界部の 5'および 3'スプライシングコンセンサス配列が重要であるとされてきた．しかし，これらのコンセンサス配列の存在のみでは十分ではなく，エクソン内の配列がスプライシング部位決定に重要な役割を果たしていることが明らかにされた．こうした配列はエクソン内スプライシング促進配列(exonic SES)とよばれ，この配列に核内のさまざまな核蛋白が結合することによりスプライシングが制御されている．AS-oligo は，これらの核蛋白の結合を阻害することにより，エクソンスキッピングを誘導すると考えられている．

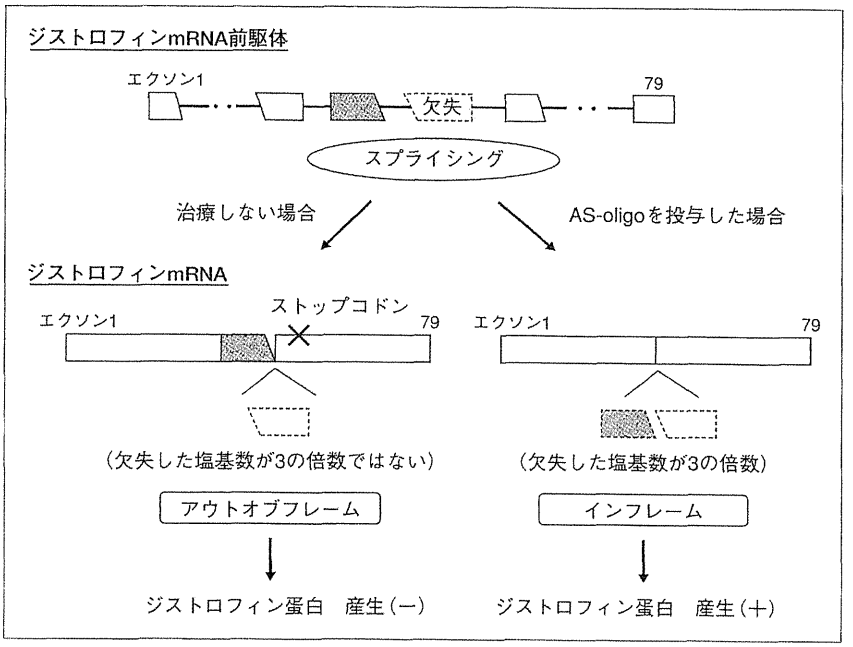

図 1　エクソンスキッピング誘導によるDMD治療モデル

欠失したエクソンに含まれる塩基数が 3 の倍数でない場合は(点線台形で示す)アウトオブフレームとなるためアミノ酸の読み取り枠にずれが生じ，ストップコドンが出現してジストロフィンは産生されない(図の左側)．アンチセンスオリゴヌクレオチド(AS-oligo)を用いて欠失したエクソンに隣接するエクソンのスキッピングを誘導し欠失する塩基数の合計を 3 の倍数とするとアミノ酸の読み取り枠をはずれないため(インフレーム)，ジストロフィンが産生される(図の右側)．

3 の倍数の塩基をコードするエクソンは四角，3 の倍数でない塩基をコードするエクソンは台形で示している．

ことによる治療を検討した(図 1)．エクソン 20 欠失症例ではエクソン 20 の 242 塩基が欠失するためアウトオブフレームとなり，エクソン 21 内にストップコドンが出現しジストロフィンは産生されない．しかし隣接する 88 塩基からなるエクソン 19 のスキッピングを誘導すればエクソン 19 と 20 の合計 330 塩基が消失するためインフレームとなり，不完全ながらも機能を有するジストロフィンの産生が期待できる．エクソン 20 を欠失した DMD 患者の培養筋細胞に AS-oligo を導入した結果，エクソン 19 のスキッピングが誘導されジストロフィンの発現がみられた[9]．

この結果を踏まえ，エクソン 20 を欠失した DMD 患者へのエクソンスキッピング誘導治療を行った．AS-oligo(0.5 mg/kg/回)を 2 時間かけて 1 週間に 1 回点滴静注し，これを 4 週間行ったところ，治療後の筋組織の解析ではジストロフィン mRNA においてエクソン 19 のスキッピングが誘導されており，免疫組織染色ではジストロフィン蛋白の発現がみられた[1]．この結果によって，AS-oligo を用いたエクソンスキッピング誘導治療の有効性を臨床の場ではじめて示すことに成功した．

世界における エクソンスキッピング誘導治療

著者らの報告に引き続き，海外からもエクソンスキッピング誘導治療の有効性が報告されている．著者らの報告を含め，これまでに AS-oligo を臨床応用した報告を表 1 に示す[1-4]．

2007 年には，オランダからエクソン 51 を標的としたスキッピング誘導治療が報告された[2]．ここでは AS-oligo として 2′-*O*-methyl phosphorothioate oligonucleotide(2-OMePS)が用いられ，対象となる DMD 患者の前脛骨筋に対して筋肉注射を行った．これによりエクソン 51 のスキッピングが