demonstrate that there is a racial difference in the spectra of these polymorphisms.

It is remarkable that four of the 102 patients were heterozygous for p164E>K, which corresponds an allele frequency of 2.0% in the Japanese patients (Table 1). The p164E>K allele was identified in only 2 out of 189 individuals in the USA, and was not observed in 120 Italians and 57 Belgians [15-17]. In one of the Japanese DMD patients carrying p.164E>K, a second p.156L>I mutation was found in the same allele (Fig. 3). Considering that p.164E>K was identified in both the Americans and Japanese populations, p.164E>K may be an old polymorphism that originated in a common ancestor of the two populations or the polymorphism is located at hot spot for nucleotide changes.

Conclusion

The present study, although limited to DMD cases, showed the rare occurrence of mutations in the myostatin gene in Japanese subjects. Our results indicate that heterozygous missense polymorphisms including two novel mutations did not produce an apparent increase in muscle volume or strength in Japanese DMD cases, even in a patient carrying two amino acid substitutions.

List of abbreviations

DMD: Duchenne muscular dystrophy

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AN carried out the molecular genetic studies and drafted the manuscript. YT carried out the clinical and molecular genetic studies. KS and AN participated molecular genetic study. YO and MY participated in clinical examinations. MM conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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SHORT REPORT

A nonsense mutation-created intraexonic splice site is active in the lymphocytes, but not in the skeletal muscle of a DMD patient

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Abstract Production of semi-functional dystrophin mRNA from the dystrophin gene encoding a premature stop codon has been shown to modify the severe phenotype of Duchenne muscular dystrophy (DMD). In this study, we report the tissue-specific production of semi-functional dystrophin mRNA via activation of a nonsense mutation-created intraexonic splice acceptor site. In a DMD patient a novel nonsense mutation was identified in exon 42. In his lymphocytes semi-functional dystrophin mRNA with a 63-nucleotide deletion in exon 42 (dys-63) was found to be produced. In vitro splicing assay using hybrid minigenes disclosed that the mutation-created intraexonic splice acceptor site was activated. In his skeletal muscle cells, however, only the authentically spliced dystrophin mRNA was found. This finding identifies the modulation of the splicing of muscle dystrophin mRNA in cases of DMD as a potential target for therapeutic strategies to generate a milder phenotype for this disease.

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Introduction

The severe Duchenne muscular dystrophy (DMD, MIM 310200) and the more benign Becker muscular dystrophy (BMD, MIM 300376) are caused by mutation of the dystrophin gene. More than 100 nonsense mutations located at various positions along the 14-kb dystrophin mRNA have been reported (http://www.dmd.nl). Despite the wide variation in the coding potentials of the mutated mRNAs (0–98.6% of the full-length protein), these truncating mutations are surprisingly associated with a uniformly severe DMD phenotype. A limited number of nonsense mutations, however, have been reported to result in a mild phenotype. In some of these cases, the production of semi-functional in-frame mRNA due to skipping of the exon containing the mutation has been shown as the cause of the phenotype modification (Barbieri et al. 1996; Disset et al. 2006; Shiga et al. 1997).

The production of semi-functional mRNA via activation of a mutation created-splice site has never been reported for nonsense mutations in the dystrophin gene. In this report, semi-functional mRNA was shown to be produced in the lymphocytes of a DMD patient via activation of a mutation created-splice site; this patient was found to have a novel nonsense mutation in the dystrophin gene. Only authentic splicing products containing a nonsense codon, however, were obtained from the patient's skeletal muscle, which explained his severe DMD phenotype. Because in vitro splicing analysis of a hybrid minigene carrying the nonsense mutation disclosed the production of in-frame mRNA, modulation of the splicing of dystrophin mRNA in muscle cells is a potential target for therapeutic strategies that aim to make the DMD phenotype milder.

Case and methods

Case

The proband (KUCG 593) was a 5-year-old boy. He was born at 36 weeks of gestation and was admitted to a neonatal care unit. During a routine blood examination, his serum creatine kinase (CK) level was found to be 4,050 IU/l (normal < 169 IU/l). When the subject was 3 months old, his CK level was elevated to 25,550 IU/l. He started to walk independently at 1 year of age and his motor development was normal. During the following period his serum CK remained elevated. A muscle biopsy when the boy was 3 years old disclosed no dystrophin staining, confirming a DMD diagnosis. At 5 years old, he was referred to Kobe University Hospital to examine his dystrophin gene for a mutation. These studies were approved by our ethics committee.

Methods

Mutation analysis

DNA was isolated from blood samples by standard phenol-chloroform extraction methods. The region encompassing exon 42 was amplified by the PCR with g42F (5′-CAATTGTCAGCTGTAGAATGAGACC-3′) as the forward primer and g42R (5′-TGAAGCCAACCAC ACTATCAAGTA-3′) as the reverse primer.

Total RNA was isolated from peripheral lymphocytes that were collected from whole blood using Ficoll–Paque density gradients (Amersham Biosciences AB, Uppsala, Sweden) or from thin-sliced (6 μm) muscle sections of frozen muscle samples. Reverse-transcription PCR (RT-PCR) and RT-nested PCR were employed to analyze the dystrophin mRNA expressed in skeletal muscle and lymphocytes, respectively. For dystrophin mRNA from lymphocytes, a region encompassing exons 36 to 45 was first amplified using a forward primer corresponding to a segment of exon 36 (3E: 5′-CCCAGCAA AAAGAAGACGTG3–3′) and a reverse primer complementary to a segment of exon 45 (3B: 5′-ACTGGC ATCTGTTTTTGAGGAT-3′). The PCR product was then used as a template for a second PCR amplification using a nested set of primers (forward-c40F: 5′-CAGC CTACCTGAGCCCAGAGATG-3′ and reverse-3F: 5′-CTTCCCCAGTTGCATTCAAT-3′). For dystrophin mRNA from skeletal muscle, a region encompassing exons 40 to 45 was amplified using the inner set of primers.

The amplified products were purified and sequenced either directly or after subcloning into the pT7 Blue-T vector (Novagen, Madison, WI). The DNA sequences were determined using an automated DNA sequencer (model 310; Applied Biosystems, Foster City, CA).

In vitro splicing assay

Using a minigene (H492) constructed from the pcDNA 3.0 mammalian expression vector (Invitrogen, Carlsbad, CA) (Thi Tran et al. 2005; Tran et al. 2006), hybrid minigenes were created by inserting a test sequence consisting of exon 42 and its flanking introns into the multicloning site (Fig. 3a). The region encompassing exon 42 was amplified from both the control sample and the patient's genomic DNA by the PCR with primers that correspond to introns 41 and 42 and included *Nhe*I and *Bam*HI restriction enzyme recognition sites, respectively (In42F-Nhe: 5′-GCCGCTA GCAGCCCAGTTTGTAGATTC-3′ and In42R-Bam: 5′-CGGGATCCGTCAAAATGCCATCATGATG C-3′). Amplified products were digested with *Nhe*I and *Bam*HI (New England Biochem, UK), and inserted into the minigene that had been digested with the same restriction enzymes. In this way, we constructed both wild-type (TVK-42) and mutant (TVK-42 m) hybrid minigenes that carried wild-type exon 42 and exon 42 with the nonsense mutation, respectively. After checking their sequences, these hybrid minigenes were transfected into HeLa cells for splicing assays as described before (Thi Tran et al. 2005; Tran et al. 2006). Cells were harvested 24 h after the transfection and total RNA was extracted as using an Isogen Kit (Nippon Gene Co., Toyama, Japan).

Five microgram of total RNA was subjected to reverse transcription using random hexamer primers and the PCR was performed using a forward primer corresponding to a segment of upstream exon A and a reverse primer complementary to a segment of the downstream exon B as previously described (Thi Tran et al. 2005) (Fig. 3a). PCR products were analyzed by electrophoresis on an 8% polyacrylamide gel.

Results

In the index case, a novel disease-causing mutation was identified in exon 42; a transition mutation from a thymine to a guanine was located at the 63rd nucleotide of exon 42, corresponding to the 5,985th nucleotide of the dystrophin mRNA (c.5,985T > G) (Fig. 1). The nucleotide change converted a TAT codon, which encodes a tyrosine residue at the 1,995th amino acid position of the dystrophin protein, to a TAG stop codon (p.1,995Y > X) (Fig. 1). The predictive truncation of

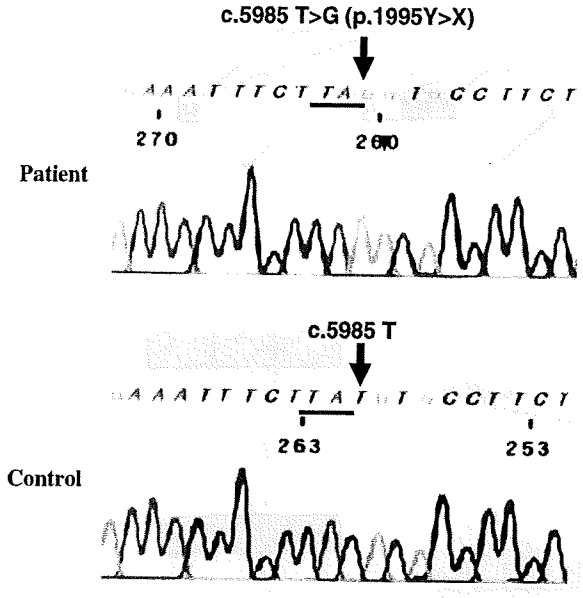

Fig. 1 Sequencing results from exon 42 are shown. The PCR products amplified from genomic DNA were directly sequenced. The 63rd nucleotide of exon 42 was a G in the index case (patient), whereas it was a T in the control sample (control). The nucleotide position corresponds to the 5,985th nucleotide of the dystrophin mRNA (c.5,985T > G). The mutation changed a tyrosine codon (TAT) to a stop codon (TAG) at the 1,995th amino acid residue of dystrophin (p.1,995Y > X)

dystrophin was consistent with the clinical diagnosis of DMD in this patient.

In order to confirm the molecular diagnosis, the gene product was examined at the mRNA level. When dystrophin mRNA extending from exons 40 to 45 was analyzed in the patient's lymphocytes by RT-nested PCR amplification, three separate products were obtained (Fig. 2a). The largest product consisted of the sequence of exons 40 to 45, and included the same nonsense mutation in exon 42 as observed in the genomic DNA. In the second largest product, 63 bp of the 5′ end of exon 42 was missing, whereas the sequences of the other exons were completely normal (dys-63). Interestingly, in the smallest product, the 3′ end of exon 41 was directly joined to the 5′ end of exon 43, which removed all 195 bp of exon 42 (dys-exon 42) (Fig. 2b). The latter two transcripts were considered natural products, because the exon boundaries were conserved and no other nucleotide changes were present in the sequenced exons. Dys-exon 42 was assumed to be a result of exon 42 skipping caused by the single nucleotide change. The exon 42 skipping observed in the lymphocytes of in the index case may have been due to the creation of a splicing silencer.

Examination of sequences near the mutation site disclosed that a novel AG dinucleotide, which is a conserved splice acceptor sequence, was introduced into the exon sequence by c.5,985T > G (Fig. 1). Therefore, the creation of the novel splice acceptor site was likely to cause the aberrant splicing that led to the production of dys-63. In order to confirm the activity of the nonsense mutation-created AG dinucleotide, experimental splicing analysis was conducted (Fig. 3). Either the wild-type or mutant exon 42 together with the flanking intron sequences were inserted into the preconstructed minigene to make hybrid minigenes and transcripts from the hybrid minigenes were analyzed by RT-PCR amplification. One PCR product containing the entire exon 42 sequence between the cassette exons A and B was obtained from the minigene encoding the wild-type exon 42 (Fig. 3b). On the other hand, two amplified products were obtained from the hybrid minigene containing exon 42 with the mutation: a major product corresponding to the normally spliced product and a minor, smaller product containing exon 42 without 63 bp of its 5′ end between exons A and B (Fig. 3c); this was the same as one of the aberrant splicing products (dys-63) identified in lymphocytes. The result indicated that the mutation-created splicing acceptor site was actually active in this hybrid minigene in HeLa cell. Therefore, dys-63 was confirmed to be a real splicing product that was transcribed from the mutated gene.

Dystrophin mRNAs obtained from lymphocytes were examined for their protein coding abilities. The authentically spliced product containing a premature stop codon in exon 42 was nonfunctional. On the other hand, dys-63 and dys-exon 42 maintained the translational reading frame and did not carry premature stop codons, and were therefore expected to produce truncated variants of dystrophin that lacked 21 and 65 amino acid residues in the rod domain, respectively. The index case, however, was diagnosed with DMD based on the lack of dystrophin in his skeletal muscle.

Considering that the dystrophin mRNA produced in muscle cells more accurately reflects the clinical phenotype than that produced in lymphocytes, muscle dystrophin mRNA from the patient was examined by RT-PCR amplification. Remarkably, the amplification of the region encompassing exons 40 to 45 produced a single PCR product (Fig. 2a). Sequencing of the product disclosed sequences of exons 40 to 45, including the nonsense mutation. It was concluded that authentic splicing was completely maintained in the skeletal muscle and no in-frame aberrant mRNA was produced in this tissue. This is compatible with the dystrophin deficiency in his muscle cells and the clinical phenotype of DMD.

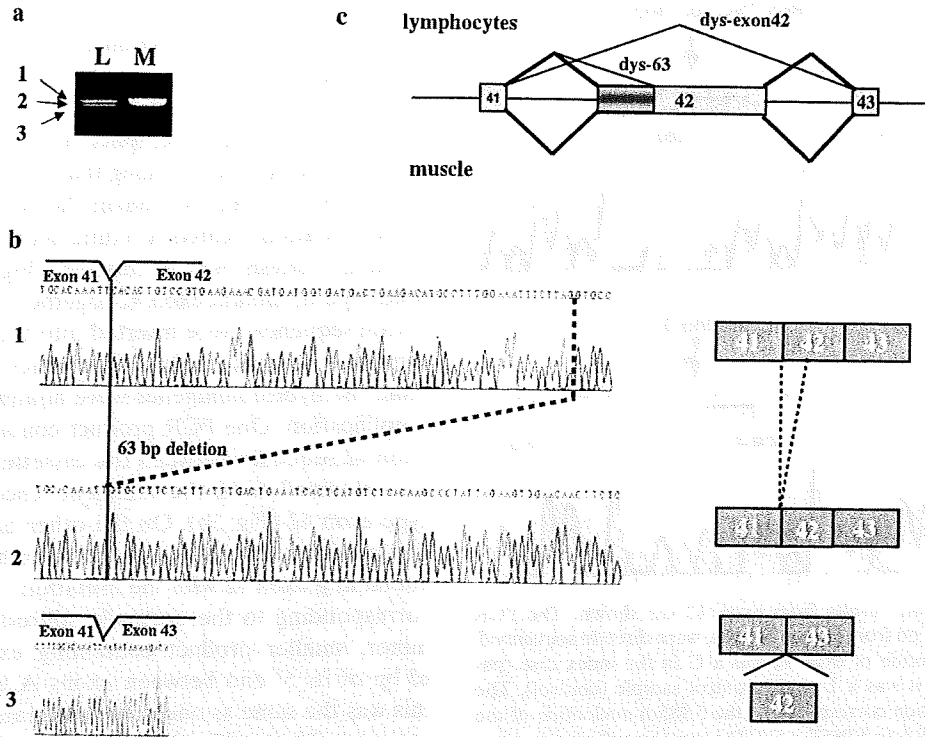

Fig. 2 Analysis of dystrophin mRNA. **a** The amplified products encompassing exons 40 to 45 are shown. Fragments encompassing exons 40 to 45 were amplified from cDNA prepared from the patient's lymphocytes and skeletal muscle. Three bands were visualized from the lymphocyte cDNA (L), whereas one clear band was visualized from the skeletal muscle (M). Numbers on the left side of the panel correspond to the numbers in panel **b** (*lower panel*). **b** The sequences of three different clones are shown. Each sequence has completely normal exons 40, 41, 43, 44, and 45. The sequence of the 3′ end of exon 41 (5′-AAATT-3′) is joined to the three different sequences in the three clones: CACAC (1), GTGCC (2), and AATAT (3). In the *top panel* (1), the normal exon structure from exon 40 to 45 is maintained, but the mutation is present. In the *middle panel* (2), 132 bp of the truncated exon 42 was followed by a completely normal exon 43. In the *bottom panel* (3), exon 41 joins directly to exon 43. The exon structure of each product is shown schematically on the right side. **c** The splicing patterns identified in the index case are represented schematically. The *diagonal lines* above and below the boxes indicate the splicing events that were observed in lymphocytes and skeletal muscle, respectively. The dys-63 and dys-exon 42 transcripts are aberrantly spliced gene products. *Boxes* and *horizontal lines* indicate exons and introns, respectively. The figure is not drawn to scale

Discussion

A novel single nucleotide change of c.5,985T > G in exon 42 of the dystrophin gene that changed a tyrosine codon to a stop codon (p.1,995Y > X) was identified in a Japanese boy diagnosed with DMD. Further molecular analysis revealed the mutation had a number of effects. In the patient's lymphocytes, the mutation caused three molecular events: (1) a premature stop codon was introduced into the authentically spliced mRNA product, (2) a mutation-created AG dinucleotide acted as a splice acceptor site, producing the aberrantly spliced dys-63 transcript, and (3) exon 42 skipping, producing the dys-exon 42 transcript (Fig. 2c). In skeletal muscle, however, only the authentically spliced product was observed. Although the patient's phenotype was expected to be mild due to the detection of in-frame dys-63 and dys-exon 42 in his lymphocytes, the patient had a typical DMD phenotype because all the dystrophin transcripts in his skeletal muscle carried the nonsense mutation.

In previous reports, the detection of aberrant splicing products in lymphocytes, which can be easily obtained, successfully led to the identification of the same transcripts in skeletal muscle (Barbieri et al. 1996; Shiga et al. 1997), thereby facilitating the molecular understanding of dystrophinopathy. Similar to previous reports (Adachi et al. 2003), however, our results showed different dystrophin mRNA splicing patterns in skeletal muscle cells and lymphocytes (Fig. 2a). This suggests that the regulators of splicing are not exactly the same in these tissues.

In vitro splicing analysis using a hybrid minigene clearly showed the nonsense mutation-created splice acceptor site was used by the spliceosome (Fig. 3). Using this hybrid minigene, a small amount of an aberrant splicing product that was produced using the novel

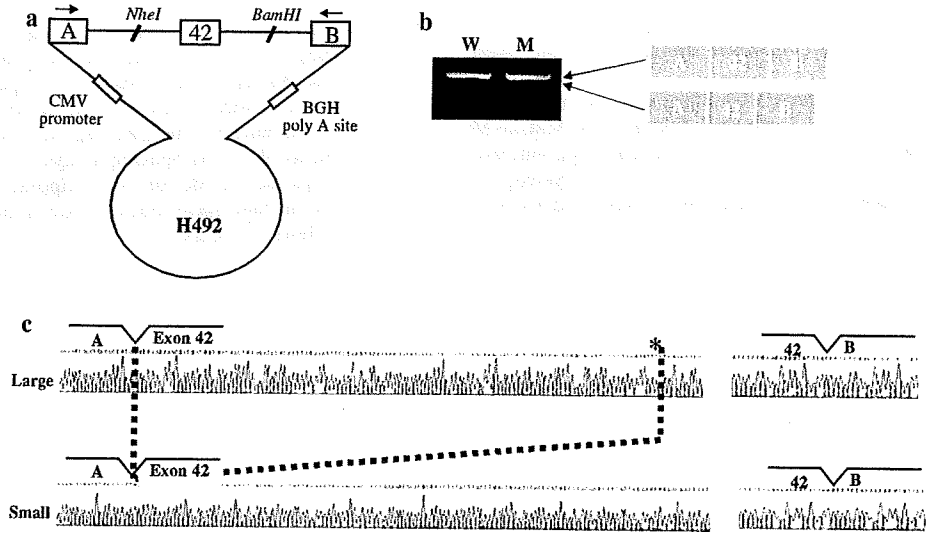

Fig. 3 Hybrid minigenes containing the indicated variants were tested in an in vitro splicing assay. **a** The hybrid minigene construct is schematically described. A minigene (H492) was constructed to encode two cassette exons (A and B) and an intervening sequence containing a multicloning site. The minigene contained a cytomegalovirus (CMV) enhancer-promoter and a bovine growth hormone gene (BGH) polyadenylation signal (*dark shaded boxes*) for complete synthesis of mRNA. The primers used in the RT-PCR assay are represented by arrows. **b** RT-PCR amplified products of hybrid minigene transcripts. A single transcript was generated from a minigene carrying the wild-type exon 42 sequence (W). From a minigene carrying the mutant exon 42, two transcripts were generated (M) and their nucleotide sequences are shown in panel **c**. A schematic description of the RT-PCR products is shown on the right. **c** Two transcripts from the mutant hybrid minigene. Nucleotide sequences at the junctions between exons are shown. The large product (*top*) consists of exon A, the complete exon 42, and exon B, whereas the small product lacked 63 bp of the 5′ end of exon 42 (*bottom*). c.5,985T > G is marked by an *asterisk*

splice acceptor site was obtained (Fig. 3). This indicates that the novel site can be recognized by the splicing machinery in HeLa cells. In contrast, the novel splice acceptor site was not used in the patient's skeletal muscle (Fig. 2). These differences in the use of the novel splice acceptor site suggest that trans-elements, such as nuclear proteins expressed in tissue-specific patterns, instead of cis-elements, such as splicing enhancer and silencer sequences, regulate the activation of the novel splice acceptor site. Future studies should clarify the trans-elements that determine whether or not the novel splice acceptor site is used.

Presently, there is no effective way to treat DMD. Recent DMD treatments have focused on converting the DMD phenotype to a BMD phenotype by changing dystrophin mRNAs from out-of-frame to in-frame. In our previous study, we showed that the induction of exon 19 skipping in a DMD patient carrying a deletion in exon 20 led to the production of in-frame dystrophin mRNA and dystrophin-positive skeletal muscle cells (Takeshima et al. 2006). Our present findings indicate that modulating the splicing of dystrophin mRNA in skeletal muscle to produce in-frame transcripts coding for truncated, semi-functional dystrophin is a potential target for treatment of this disease.

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RESEARCH ARTICLE

Multiexon Skipping Leading to an Artificial DMD Protein Lacking Amino Acids from Exons 45 Through 55 Could Rescue Up to 63% of Patients With Duchenne Muscular Dystrophy

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Approximately two-thirds of Duchenne muscular dystrophy (DMD) patients show intragenic deletions ranging from one to several exons of the *DMD* gene and leading to a premature stop codon. Other deletions that maintain the translational reading frame of the gene result in the milder Becker muscular dystrophy (BMD) form of the disease. Thus the opportunity to transform a DMD phenotype into a BMD phenotype appeared as a new treatment strategy with the development of antisense oligonucleotides technology, which is able to induce an exon skipping at the pre-mRNA level in order to restore an open reading frame. Because the DMD gene contains 79 exons, thousands of potential transcripts could be produced by exon skipping and should be investigated. The conventional approach considers skipping of a single exon. Here we report the comparison of single- and multiple-exon skipping strategies based on bioinformatic analysis. By using the Universal Mutation Database (UMD)-DMD, we predict that an optimal multiexon skipping leading to the del45-55 artificial dystrophin (c.6439_8217del) could transform the DMD phenotype into the asymptomatic or mild BMD phenotype. This multiple-exon skipping could theoretically rescue up to 63% of DMD patients with a deletion, while the optimal monoskipping of exon 51 would rescue only 16% of patients. Hum Mutat 28(2), 196–202, 2007. © 2006 Wiley-Liss, Inc.

KEY WORDS: multiple-exon skipping; DMD; BMD; dystrophin; muscular dystrophy

INTRODUCTION

DMD (MIM# 310200) and BMD (MIM# 300376) are two X-linked recessive allelic disorders characterized by mutations in the dystrophin gene (MIM# 300377; NM_004010.1; Xp21.2) [Hoffman et al., 1987; Koenig et al., 1987]. DMD affects approximately 1 out of 3,500 live male newborns, while BMD is five times less frequent. The phenotypes of these two diseases are quite different. In DMD, clinical symptoms resulting from progressive muscle fiber degeneration are observed between 2 and 3 years old (yo) and the muscle wasting will lethally affect heart and lungs in adulthood. In contrast, BMD has a slower

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disease progression and a wider spectrum of phenotypes ranging from mild DMD to almost asymptomatic forms [Morrone et al., 1997]. The dystrophin Dp427 muscular isoform (3,685 amino acids) harbors four domains [Koenig et al., 1988]: the N-terminal actin binding domain (amino acids [aa] 12–240); a large central domain with 24 spectrin motif repeats (aa 253–3112); a cysteine-rich domain (aa 3113–3299) that interacts with various proteins of the dystroglycan complex; and a C-terminal complex (aa 3300–3685). Dystrophin plays a critical role in the preservation of the structure and function of muscle fibers while interacting with proteins of the associated dystrophin-glycoprotein complex (DAG), thus establishing a bond between the extracellular matrix and the cytoskeletal actin. The absence of dystrophin in humans leads to disorganization and slow degeneration of the muscular cells. Approximately two-thirds of patients show intragenic deletions ranging from one to several exons of the *DMD* gene. The remaining cases arise from genomic duplications or micro-rearrangements (nonsense mutations, small deletions or insertions, intronic mutations, and rare missense mutations). The reading frame rule [Monaco et al., 1988] explains the two different phenotypes resulting from mutations in the same gene. Mutations that change the translational reading frame of the gene elicit formation of premature stop codons and consequent abortion of the translation process that results in dystrophin deficiency and the DMD phenotype, whereas mutations that conserve the translational reading frame of the mRNA result in the BMD phenotype. The reading frame hypothesis explains the phenotypic differences observed in approximately 92% of the DMD/BMD cases [Koenig et al., 1989]. Nevertheless, several exceptions to this rule have been described such as BMD with the out-of-frame exon 3–7 deletion. Thus, dystrophin can be detected in patients with out-of-frame mutations, theoretically leading to absence of protein while large in-frame deletions can be detected in DMD patients. In parallel, in many DMD patients as well as in animal models (X-linked muscular dystrophy [*mdx*] and Golden Retriever muscular dystrophy [GRMD] dogs), rare dystrophin-positive fibers have been reported [Crawford et al., 2001; Wilton et al., 1997]. It has been suggested that restoring the reading frame by exon skipping is the most likely cause of this natural phenomenon. In fact, it is well known that the phenotype is directly correlated with the functional importance of the deleted protein domains, the repeated central domain being more tolerant to deletions than the N- and C-terminal domains because of its modular structure [Beggs et al., 1991]. Thus the opportunity to transform a DMD phenotype into a BMD phenotype appeared as a new therapeutic strategy with the development of the antisense oligonucleotide (AO) technology, which is able to induce an exon skipping at the pre-mRNA level in order to restore an open reading frame. Genomic deletions of one or more exons being the most frequent mutations among DMD patients and mainly localized in the repeated central domain, exon skipping in this area should allow the production of a partially functional dystrophin. Such exon skipping exists among patients and can explain exceptions to Monaco's rule. This has prompted many groups to investigate the possibility of designing strategies for gene repair/modulation based on the use of compounds interfering with splicing, thus inducing exon skipping [Aartsma-Rus et al., 2004b; Goyenvalle et al., 2004; Kapsa et al., 2003]. Because the DMD gene contains 79 exons, thousands of potential transcripts can be produced by exon skipping and should be investigated. Here we report the prediction of an optimal exon skipping to transform the DMD phenotype into the asymptomatic or mild BMD phenotype. This work was performed by using the UMD-DMD mutation database.

PATIENTS AND METHODS

Database of Mutations

We used the UMD® software [Beroud et al., 2000, 2005] to build the UMD-DMD database of mutations from dystrophin gene–mutated patients identified in French reference diagnostic laboratories. For each patient, data have been collected at the molecular, protein, and clinical levels. On July 1, 2006, the UMD-DMD database included 602 records from 409 DMD patients, 160 BMD patients, and eight patients with an intermediate phenotype. Phenotype for the remaining 25 patients was not assessed because of their young age. In order to evaluate the impact of various exon skipping events, specific tools have been developed. The "exon-phasing" tool gives access to a graphical presentation of all exons of the DMD gene according to their phasing. Thus the direct consequence of the deletion of one or more exons on the gene's reading frame can be easily evaluated. When deletions result in a new junctional codon (the first or the two first nucleotides of the junctional codon comes from the 5′ exon while the remaining nucleotide(s) come(s) from the 3′ exon) this could theoretically lead to an allo- or an isosemantic impact and ultimately to a stop codon. In this latter situation the apparent reading frame preservation based on the exon phasing is invalidated at the nucleotide level. We therefore created the "AA junction after exon skipping" tool, which displays for each artificial deletion generated by exon skipping its consequences both at the reading frame and at the junctional codon levels. As mentioned previously, the dystrophin protein is tolerant to internal deletions. Therefore, to restore the reading frame of an out-of-frame deletion found in a DMD patient, one can imagine additionally deleting one or more exons either on the 5′ and/or 3′ side of the deletion. More than 3,000 artificial deletions can theoretically be generated by exon skipping of one or more exons. As it is impossible to evaluate the therapeutic potential of all the possible resultant dystrophins, various groups have designed mini-, micro-, or quasidystrophins [Kapsa et al., 2003; Kobinger et al., 2003; Li et al., 2005; Liu et al., 2005; Nonaka, 2004; Takeda, 2004; Yoshimura et al., 2004]. In addition, microdystrophin has been designed as an alternative to the incorporation of a full-length dystrophin cDNA (14 kb) into an AAV vector because of the limited size of the cassette. Using a 4.9-kb rod-truncated microdystrophin CS1, Takeda [2004] has almost completely ameliorated the dystrophic phenotypes in transgenic *mdx* mice. Concomitantly, Liu et al. [2005] have used a C-terminal truncated DeltaR4-R23/DeltaC microgene (DeltaR4/DeltaC). These are promising approaches to rescue muscular dystrophy in young *mdx* skeletal muscle. Nevertheless, if the percentage of centrally nucleated myofibers was reduced to approximately 22% in microdystrophin-treated muscle [Liu et al., 2005], one can expect that a larger functional dystrophin could give better results. We designed an automatic tool that presents, for each mutation, the largest in-frame artificial dystrophin that can be generated by exon skipping. All other alternative exon skippings restoring the frame are also accessible. In addition, we created the "in-frame correction table" module, which summarizes the number of patients with a deletion that could be rescued by a skipping of one, two, or three exons either in the 5′ or 3′ side of the deletion. Direct access to data of corresponding patients is also provided. Because patients with an in-frame deletion are frequently associated with the milder form of the disease (BMD), they are natural mutants that give valuable information about the phenotypic consequence of various minidystrophins. We therefore created the "Large rearrangements rescuable by mono-exon skipping" tool. It evaluates all monoexon

skippings that can rescue out-of-frame deletions for patients described in the UMD-DMD database. For each monoexon skipping, it lists the number of patients eligible for this skipping, their deletion, the amino acid junctional impact, and the patients or individuals naturally harboring the artificial deletion. Furthermore, each deletion rescuable by monoexon skipping is associated with one of the following groups: 1) exon skipping with a junctional impact being a stop codon; 2) exon skipping with an allosemantic junctional impact (such protein has never been reported in patients); 3) exon skipping with an isosemantic junctional impact (such protein has never been reported in patients); 4) exon skipping leading to a protein found exclusively in DMD patients; 5) exon skipping leading to a protein found both in DMD and BMD patients; and 6) exon skipping leading to a protein found exclusively in BMD patients. The goal of the exon skipping strategy in the context of the DMD being to transform a DMD phenotype into a BMD phenotype, deletions belonging to groups 2, 3, or 6 above are the best target for such strategies. In parallel to this monoskipping analysis, we wished to evaluate multiexon skipping. We then designed a specific routine to display the full set of correcting events for each mutation. In addition, to evaluate the potential use of multiexon skipping to rescue the largest set of DMD patients, we developed the "Exon skipping leading to BMD" algorithm. This routine selects all in-frame deletions found in BMD patients and searches for all deletions found in DMD patients that can be transformed into in-frame deletions through various multiexon skipping. The list of all corresponding cases is available.

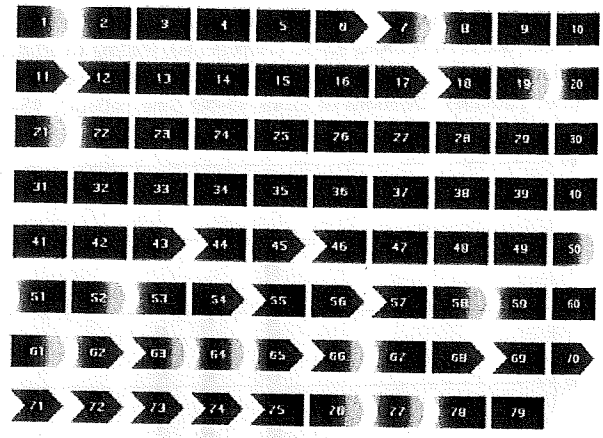

FIGURE 1. **Exon phasing of the DMD gene. Each exon is presented by a gray box. Each extremity of the box represents the specific phasing of the exon. Left end of exons: 1) dark gray vertical lane means that the exon begins by the first nucleotide of a codon; 2) light gray curve means that the exon begins by the second nucleotide of a codon; and 3) gray arrow means that the exon begins by the third nucleotide of a codon. Right end of exons: 1) dark gray vertical lane means that the exon ends by the last nucleotide of a codon; 2) light gray curve means that the exon ends by the first nucleotide of a codon; and 3) gray arrow means that the exon ends by the second nucleotide of a codon. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

Patients

Using UMD-DMD tools, we identified 11 male individuals carrying the deletion of exons 45 to 55 (abbreviated as "del45-55"; approved mutation nomenclature c.6439_8217del based on GenBank NM_004010.1 and using the A of the ATG initiation codon as +1) of the DMD gene. We also collected four individuals with this mutation from Japan. For each patient, clinical data were collected from the caring physicians. These data included age and symptoms of onset, age of ambulation loss, age of last examination, neuromuscular, cardiological, respiratory and behavioral data, creatine phosphokinase (CPK) level, and muscle biopsy results. When muscle biopsy samples were available, dystrophin analysis was performed by conventional techniques [Anderson and Davison, 1999; Bornemann and Anderson, 2000].

RESULTS

Junctional Impact of Exon Skipping

The DMD gene is composed of 79 exons that are distributed in various phases, as shown in Figure 1. When a deletion occurs, it can result into a frameshift and therefore lead to a premature stop codon. This is achieved when two exons in different phases are joined end to end (for example, exons 51 and 53). When two exons in the same phase are involved, it is usually assumed that no premature stop codon is generated as no frameshift occurs as predicted by the DMD exonic deletions/duplications reading frame checker 1.6 from the Leiden database (www.dmd.nl) [Fokkema et al., 2005]. Nevertheless, this does not take into account the impact at the junctional codon. The automatic tool developed in the UMD-DMD database allowed us to demonstrate that among the 3,081 possible deletions, seven deletions that are not predictive of a frameshift in fact lead to a junctional stop codon. These are del2-19 (c.32_2380del), del2-58 (c.32_8668del), del 2-77 (c.32_11014del), del51-58 (c.7310_8668del), del51-77 (c.7310_11014del), del65-77 (c.9362_11014del), and del67-77 (c.9650_11014del). Only del51-58 is of interest in the context of exon skipping.

Monoexon Skipping

To evaluate if mono-, bi-, or triexon skipping are equally efficient to rescue out-of-frame deletions described in patients, we used the "in-frame correction table" module. For 71 patients, the skipping of one exon was the only available approach, while for six patients their deletion could be rescued by deletion of one, two, or three exons and for 111 patients by deletion of one or three exons. Interestingly, monoskipping could theoretically restore the frame of deletions found in 215 patients, biskipping in 76 patients, and triskipping in 160 patients. If only patients exclusively rescued by one of these approaches are evaluated, monoskipping is required for 71 patients, biskipping for eight patients, and triskipping for two patients (Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). Therefore, monoskipping is the most efficient approach in this cohort. The rescue by monoexon skipping of various deletions will lead to individualized therapeutic approaches. To evaluate if some exons could rescue various types of mutations and therefore could be the best targets for clinical trials, we used the "Large rearrangements rescuable by mono-exon skipping" tool. It reveals that only 11 monoexon skipping events could potentially rescue more than 10 patients (Fig. 2). If we consider that some deletions can be rescued by monoskipping of either the 5′ or the 3′ exons, only nine monoskipping events are relevant. Indeed, the nine patients with deletion of exon 44 can be rescued alternatively by monoskipping of exons 43 or exon 45. Similarly, the 15 patients with deletion of exon 51 can be rescued either by monoskipping of exons 50 or 52.

The monoskipping of exons 51 is the best choice, as it can rescue deletions of exons 48–50 (12 patients), leading to artificial deletions found exclusively in BMD patients, i.e., exons 48–51 (four patients). Deletions of exons 43–50 (one patient), 45–50 (13 patients), 47–50 (one patient), 49–50 (eight patients), 50 (four patients), 52 (five patients), and 52–63 (one patient) that can be rescued by monoskipping of exon 51 result in artificial dystrophins that so far have not been reported in the UMD-DMD database.

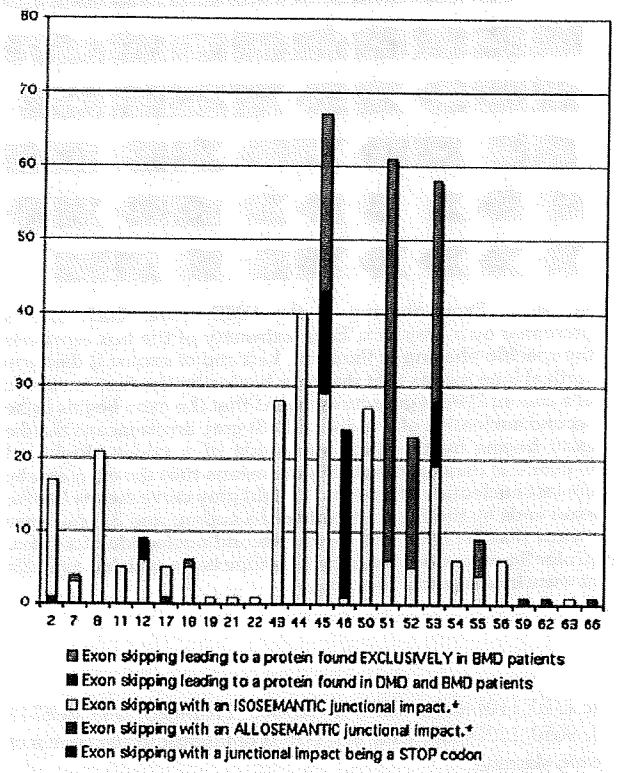

FIGURE 2. **Monoskipping of the DMD gene. For each exon is presented the number of patients from the UMD-DMD database that can be rescued by the corresponding monoskipping. Each deletion has been associated with one of the five groups. *Such protein has never been reported in patients.**

Multiexon Skipping

The work from J.S. Chamberlain's group (Department of Human Genetics, University of Michigan Medical School) led to the description of the microdystrophin and to the recognition of dispensable spectrin repeats in the dystrophin protein [Phelps et al., 1995]. Today it is recognized that repeats four to 23 (DeltaR4-R23) can be removed leading to a functional protein. Therefore, it is possible to use the exon skipping approach to generate artificial dystrophins including deletions of various sizes. This could be achieved by targeting multiple exons with various antisense oligonucleotides or modified small nuclear RNA unit #7 (U7 snRNA). In this situation, various deletions could be rescued by the same approach and therefore only one procedure will have to be developed. To evaluate which is the artificial dystrophin that can be generated by exon skipping and could rescue the largest number of patients, we developed a specific tool. As expected, such approach should invariably lead to the largest functional deletion (i.e., DeltaR4-R23). Because we have collected phenotypic data for most patients, we decided to limit this analysis to artificial dystrophin already reported in BMD individuals and therefore for which functional in vivo data were available. We developed the so-called "Exon skipping leading to BMD" algorithm. It evaluates, for each in-frame deletion reported only in BMD patients, how many deletions found in DMD patients could be rescued by multiexon skipping leading to this in-frame deletion. Figure 3 summarizes available data. This tool allowed us to identify deletion from exons 45 to 55 (c.6439_8217del) as the best solution as it could rescue deletions described in 161 DMD patients among the 254 DMD patients with a large deletion (63%) (Supplementary Table S2). This deletion could also rescue small

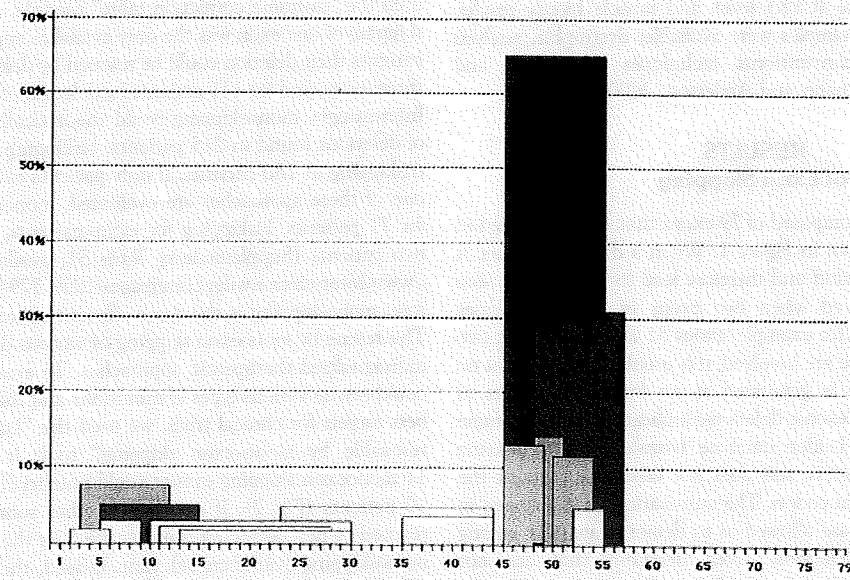

FIGURE 3. **Multiskipping of the DMD gene. X-axis: exons of the DMD gene. Y-axis: number of DMD patients rescuable by multiexon skipping. Each colored rectangle corresponds to the deletion found in BMD patients. Note the pink del45-55 deletion that can rescue 161 DMD patients with an out-of-frame deletion of the DMD gene.**

TABLE 1. Clinical Features of the 15 Documented Patients Carrying the del45-55 Dystrophin Gene Mutation

Patient	Symptoms of onset/age (years)	Age at last examination (years)	Clinical status	CPK/age (years)[a]	Ambulation (if no, then age of loss)	Cardiac involvement/ age (years)
1	Muscle pain, myoglobinuria/13	14	Mild BMD	6 times/14	Y	N
2	Chance high CK levels/8	9	AS	17 times/8	Y	N
3	Chance high CK levels/6	7	AS	6 times/7	Y	N
4	Chance high CK levels/13	18	Mild BMD	5 times/18	Y	N
5	Myalgia/12	14	Myalgia	Elevated/14	Y	—
6	Weakness/45	55	Mild BMD		Y	—
7	Weakness/4	40	Mild BMD	4 times/22	Y	—
8	Weakness/4	40	Mild BMD	4 times/22	Y	—
9	Toe walking, weakness/2	46	Mild BMD	—	Y	—
10	Weakness (very mild)/3.5	13	Exercise intolerance	19 times/3.5	Y	N
11	Chance (related to Patient 10)/57	63	AS	1.3 times/57	Y	Cardiomyopathy (LVEF 51%)/57
12	Scoliosis, weakness/19	40	Mild BMD	7 times/40	Y	N
13	Calf hypertrophy/40	54	Mild BMD	6 times/41	Y	Cardiomyopathy (LVEF 57%)/54
14	Exercise intolerance/12	19	Mild BMD	41 times/12	Y	N
15	Weakness/49	50	Mild BMD	1.5 times/50	Y	—

[a]CPK levels are expressed as number of times normal level.
–, not available; N, No; Y, Yes; BMD, Becker muscular dystrophy; AS, asymptomatic; LVEF, left ventricular ejection fraction; Chance, individual discovered by chance without any clinical sign.

rearrangements found in six DMD patients out of 129 (4.7%) and large duplications found in nine patients out of 41 (22%).

Clinical Study

According to neuromuscular features, the 15 individuals were classified as asymptomatic, exercise intolerant and/or myalgia, and mild BMD phenotypes (Table 1). Mild BMD was defined as a mild weakness with or without calf hypertrophy and autonomous ambulation. None of them had evidence for respiratory involvement or mental retardation.

Overall phenotypes of the 15 documented patients associated with del45-55 are heterogeneous ranging from asymptomatic (three individuals, ages: 7 to 63 yo), exercise intolerance and/or myalgia (two patients, ages: 13 and 14 yo) and mild BMD (10 patients, ages: 14 to 55 yo). The age of onset when available was ranging from 2 to 49 yo (mean = 19.5 ± 19.6 yo, median = 12, interquartile range = 24.5). The most frequent symptoms of onset were weakness; defined as walking, running, and stair-climbing difficulties (seven patients), exercise intolerance (three patients), and calf hypertrophy (one patient). CPK level was highly elevated (>5 times) for seven individuals (mean age = 17 ± 15 yo, median = 12, interquartile range = 19) while it was moderately elevated for others (≤5 times) (mean age = 34 ± 19 yo, median = 22, interquartile range = 28). All patients, including nine individuals at least 39 yo, were still ambulatory at last examination (ages: 7 to 63 yo). Two patients had a mild cardiac involvement identified at echocardiography without clinical manifestation.

Muscle Biopsy Results

When tissues were available (two cases with exercise intolerance and/or myalgia , one asymptomatic case, and one mild BMD), the microscopy revealed mild (two patients) or moderate (two patients) muscular dystrophy. Dystrophin immunostaining was moderately reduced with Dys-2 antibody (Novocastra Laboratories, Newcastle, United Kingdom; www.novocastra.co.uk) (aa 3668–3684) in two patients and with the C5G5 antibody (aa 1173–1728) [Fabbrizio et al., 1993] in four patients. It was negative with the Do antibody (aa 1840–2226), whose epitope overlap the deleted portion of the dystrophin, in two patients (data not shown).

The Western blot analysis was performed in three samples and a reduced amount of dystrophin was detected in all samples (data not shown). In all analyzed cases dystrophin displayed a reduced molecular weight.

Other patients with a deletion of exons 45 to 55 are reported in the Leiden database [Fokkema et al., 2005]. Only one of these patients was previously published [Sironi et al., 2001]. This patient was 60 yo and classified as a mild BMD without cardiomyopathy. The level of expression of the deleted dystrophin was estimated to 80% by Western blot analysis.

Overall, these data suggest that deletion of exons 45 to 55 is associated with mild BMD (10 patients), exercise intolerance and/or myalgia (two patients) or even no phenotype (three patients).

DISCUSSION

Many teams worldwide have been engaged in monoexon skipping to rescue the dystrophic *mdx* mouse bearing a nonsense mutation in exon 23 by skipping of this exon. Recent very promising results [Goyenvalle et al., 2004; Lu et al., 2005] open the way to human DMD exon skipping. One approach aims at inducing skipping of an exon carrying a nonsense mutation (such as R1967X in exon 41) in human cultured myocytes [Surono et al., 2004]. Another approach has been to perform double skipping [Aartsma-Rus et al., 2004a]. As a broad pattern of deletions is observed in DMD patients (today 163 different deletions are recorded in the UMD-DMD database), the set of monoexon skipping events would have to be large with some being indicated for only one patient while others would correct many. In order to better define the most efficient artificial dystrophins resulting from monoexon skipping, we developed various dedicated tools. We have been able to demonstrate that only nine exons could be used to rescue deletions found in more than 10 patients (254 patients harbor a large deletion of the DMD gene). The best target is exon 51 that could rescue 45 patients (16%). We also developed tools to evaluate artificial deletions

produced by exon skipping that would lead to deletions reported in DMD patients. For example, deletion of exon 45 is associated with DMD as it results in a premature stop codon. This deletion can theoretically be rescued by monoskipping of exon 44 or 46. The resulting del44-45 has been described once in a DMD patient [Herczegfalvi et al., 1999] and the del45-46 three times, in two DMD and one BMD patients. Therefore the deletion of exon 45 could not be efficiently rescued by monoexon skipping. Furthermore, we also evaluated the consequences of the skipping on the junctional codon. We demonstrated that seven deletions apparently restoring the frame would indeed result in a premature stop codon: del2-19 (c.32_2380del), del2-58 (c.32_8668del), del2-77 (c.32_11014del), del51-58 (c.7310_8668del), del51-77 (c.7310_11014del), del65-77 (c.9362_11014del), and del67-77 (c.9650_11014del).

As suggested by Aartsma-Rus et al. [2004a], the targeting of 20 monoskipping events would theoretically be beneficial for at least 75% of patients. Nevertheless, this approach will be time consuming and many clinical trials will be required, as each monoskipping has to be addressed individually. We believe that multiexon skipping is a better solution, as we can expect that only a limited number of different multiskipping events would be required. We therefore developed a set of dedicated tools to address the multiskipping. We used the phenotypes of various individuals included in the UMD-DMD database as an in vivo control of the functional impact of artificial dystrophins generated by the multiskipping. The "Exon skipping leading to BMD" algorithm allowed us to select the del45-55 (c.6439_8217del) artificial dystrophin as the most efficient multiskipping as it would theoretically be beneficial to 63% of patients harboring a deletion. In order to assess the exact phenotypes of individuals harboring a del45-55 deletion, we used the clinical data provided by the corresponding caring physicians. After reviewing detailed clinical data, we can conclude that for all patients older than 40 yo (eight patients) the phenotype is either asymptomatic (one patient) or a mild BMD (eight patients) with cardiac involvement of variable severity (two patients). The observed clinical heterogeneity could at least partially result from genetic heterogeneity in the *DMD* gene. In fact the deletions have only been characterized in term of missing exons in the DNA but not at the intronic level (deletion breakpoints). Therefore we can expect that asymptomatic patients have a fully functional neointron 44–56 and therefore an almost normal level of del45-55 (c.6439_8217del) dystrophin, while patients with mild BMD phenotype have different breakpoints that lead to partially functional neointron 44–56. In a family with a del45-54 (c.6439_8027del) mutation, an asymptomatic female carrier with an altered dystrophin mRNA fragment generating a mRNA deletion of exon 44 has been reported [Lenk et al., 1993]. It was concluded that this fragment could be generated by an alternative splicing of exon 44. In one patient from our study, we detected a weak signal corresponding to a del44-55 (c.6291_8217del) fragment. We can therefore hypothesize that various levels of alternative splicing of exon 44 could partially explain the observed heterogeneity as del44-55 (c.6291_8217del) leads to nonsense mediated decay.

This is so far the largest series of patients harboring a large deletion of the DMD gene either asymptomatic or with mild phenotype. It reinforces the bioinformatic predictive value of the del45-55 (c.6439_8217del) deletion as being the "target of choice" for exon skipping. Furthermore, the number of individuals harboring this deletion is probably under evaluated as asymptomatic individuals exist and one of them has been identified by chance in our series. Indeed Patient 11 has been detected at 57 yo during the course of a family analysis. His grandson (Patient 10) was under investigation because the pediatrician had noticed a slight toe walking and a very mild weakness in the lower limbs. The molecular investigations revealed that he had inherited the mutated dystrophin gene from his grandfather who was asymptomatic.

To evaluate the efficiency of the exon skipping leading to the del45-55 artificial dystrophin for a specific patient, it could be necessary to use his own cells for in vitro testing prior to clinical trial. Alternatively, the precise definition of intronic breakpoints could lead to a better understanding of functional or partially functional neointrons. The identification of exonic splicing silencer (ESS) [Sironi et al., 2004; Wang et al., 2004; Zhang and Chasin, 2004] and exonic splicing enhancer (ESE) [Cartegni et al., 2003; Fairbrother et al., 2004] patterns can also give valuable information.

A major concern about this approach is the feasibility of multiexon skipping at the technical level. Various reports lead us to believe that this could be performed in the near future. So Aartsma-Rus et al. [2004a] have shown that double skipping of exons 43 and 44 or 45 and 51 can be successfully achieved. More importantly, a recent study by Fall et al. [2006] showed that a cocktail of AOs can be used to efficiently induce the skipping of exons 19 to 25 in the *mdx* mouse.

In conclusion, we demonstrated that use of dedicated algorithms from the UMD software could predict the best multiexon skipping to rescue DMD patients. We conclude that the del45-55 (c.6439_8217del) could rescue up to 63% of DMD patients harboring a deletion of one or more exons in our series, while the best monoskipping approach (monoskipping of exon 51) will only rescue 16% of these patients. This approach will also be useful for patients with small rearrangements (small insertions/deletions, splice mutations, etc.) or large duplications. Overall, the multiskipping of exons 45 to 55 could rescue 41.5% of DMD patients from our series. This "general" as opposed to "specific" approach may therefore simplify the development of clinical trials in the context of DMD.

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We want especially to thank the clinicians who gave us access to the various patients for this study. We thank all laboratory technicians and biologists who identified mutations reported in the UMD-DMD database; in particular, Sylvie Chambert, Céline Saquet, Delphine Thorel, and Philippe Khau van Kien.

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特 集　神経・筋疾患の分子標的治療

筋ジストロフィーの分子標的療法

Mutation Specific Treatments for Duchenne Muscular Dystrophy

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Masafumi Matsuo*, Yasuhiro Takeshima*

Abstract

Duchenne muscular dystrophy (DMD) is the most common form of inherited muscle disease and is characterized by progressive muscle wasting ultimately resulting in death of the patients in their twenties. DMD is characterized by a deficiency of the muscle dystrophin as a result of mutations in the dystrophin gene. Currently, no effective treatment for DMD is available. Two promising treatments strategies have been proposed specifically for correcting the mutations in the dystrophin gene.

Induction of exon skipping using antisense oligonucleotides is expected to correct the out-of-frame mutation into in-frame mutation of the translational reading frame of dystrophin mRNA. This strategy enables the production of truncated dystrophin production in DMD patients with out-of-frame exon-deletion mutations in the dystrophin gene. Our first treatment with antisense oligonucleotides against exon 19 was successful and resulted in the production of dystrophin in the skeletal muscle of a DMD patient with exon 20 deletion. It is anticipated that exon skipping will be applied extensively for the correction of deletion mutations.

Induction of the read-through effect using gentamycin or PTC124 is expected to produce dystrophin in DMD patients with nonsense mutation. The treatment with PTC124 is currently under clinical trial. In this review, these treatments strategies have been summarized.

Key words : Duchenne muscular dystrophy, dystrophin, out-of-frame, mutation, nonsense mutation

はじめに

筋ジストロフィーに関する研究は大幅に進み，肢帯型筋ジストロフィーが20近い型に細分されるなど，その多様性が明らかになってきた。なかでも，その頻度ならびに重症度からDuchenne型筋ジストロフィー(Duchenne muscular dystrophy：DMD) は筋ジストロフィーの代表的疾患である。DMDは，ジストロフィン遺伝子の異常に起因する筋肉のジストロフィン欠損を特徴とする。これまで，このジストロフィン欠損を回復させるために多くの研究がなされてきた。特に，体外から正常なジストロフィン遺伝子を導入する遺伝子治療は有効な治療法になり得るものとして大きな期待を集め，多くの研究成果が得られてきた。ところが，ジストロフィンcDNAを組み込んだベクターを筋細胞に導入する有効な方法の確立にいまだ至らず，実用化への障壁となっている。最近では，患者自身が生来持っているジストロフィン遺伝子を有効に活用しようとする分子標的療法が注目を集めている。この分子標的療法では，ジストロフィンの発現が時間的・空間的に生体本来の様式に従うためその治療効果は大きく，かつ副作用は少ないと考えられる。ここでは，DMDの分子標的治療として注目を集めているエクソンスキッピング誘導治療とリードスルー誘導治療について紹介する。

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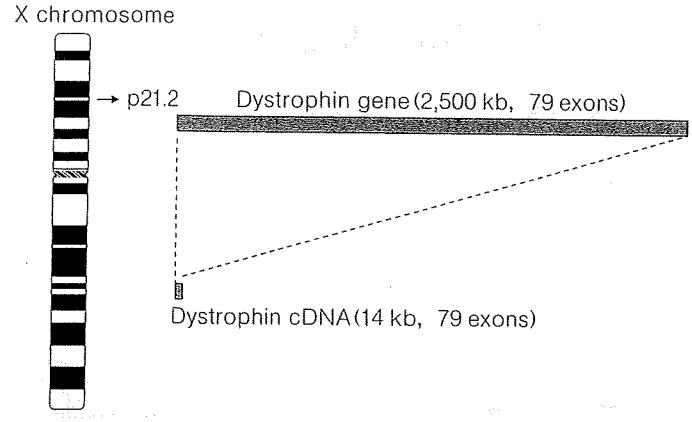

Fig. 1　ジストロフィン遺伝子
Xp21.2 上にあるジストロフィン遺伝子はエクソンが 79 からなり，サイズは 2,500 kb 以上と巨大である。しかし，転写された cDNA のサイズはわずか 14 kb である。

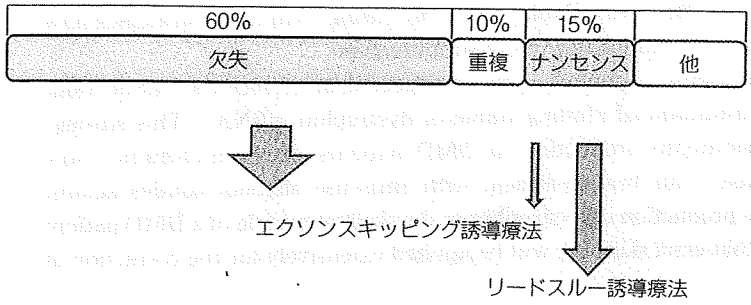

Fig. 2　ジストロフィン遺伝子の異常とその治療法
DMD で同定されたジストロフィン遺伝子異常のうち，約 6 割がエクソン単位の欠失で，1.5 割がナンセンス変異であった。エクソン欠失ではエクソンスキッピング誘導治療が，ナンセンス変異ではリードスルー誘導治療あるいはエクソンスキッピング誘導治療が治療法となる。

I．ジストロフィン遺伝子の遺伝子診断

DMD は男児 3,500 人に 1 人に発症する，最も頻度の高い遺伝性の進行性筋萎縮症である。筋萎縮は幼児期に発症し，その後も一貫して進行して，20 歳代には心不全あるいは呼吸不全により死亡する。そのため，治療法の確立が最も望まれている筋ジストロフィーである。一方，Becker 型筋ジストロフィー（BMD）は，成人期に筋力低下で発症し，筋萎縮の進行は比較的緩やかな伴性劣性遺伝性疾患である。

DMD の責任遺伝子としてクローニングされたジストロフィン遺伝子は X 染色体の短腕の Xp21.2 に座位し，その大きさは 2,500 kb 以上もあり，極めて大きな遺伝子である。ところが，ジストロフィン蛋白をコードしている領域は 79 のエクソンからなるが，そのサイズはわずか 14 kb である（Fig. 1）。遺伝子診断にとって好都合なことに，多くの DMD 例がジストロフィン遺伝子のエクソン単位の欠失を有しており，サザンブロット法で検出が可能であった。サザンブロット法により，ジストロフィン遺伝子の 2 カ所にエクソン欠失異常が生じやすいホットスポットがあることが明らかにされた。しかし，サザンブロット法は大変な時間と費用を有することから，より簡易な診断法が求められた。そこで，欠失のホットスポットにある特定のエクソンに標的を絞ってその領域に存在する多数のエクソンを同時に polymerase chain reaction（PCR）により増幅させるマルチプレックス法が，ジストロフィン遺伝子の異常を同定する最も容易な方法として頻用されてきた。しかしこれも，特定のエクソンのみを増幅するため DMD 患者の約 5 割でしか遺伝子異常が同定できず，しかも，その結果が次項で述べる「フレームシフト」則の検証に用いられないなどの欠点を有した。最近では，ジストロフィン遺伝子の 79 のエクソンすべてを対象としてその欠失・重複が解析できる multiplex ligation-dependent probe amplification analysis（MPLA）法が開発され，主流になりつつある。MLPA 法は比較的容易な方法で，しかも重複の異常も検出可能となり従来の方法に比べ遺伝子診断能力が向上している。この MLPA 法により，DMD/BMD の約 6 割の患者で遺伝子診断が可能となった。また，MLPA 法ではエクソン単位の欠失・重複の異常に加え，微細な遺伝子の異常も検出されることもあり，MLPA 法の有用性はさらに高くなっている[1]。

一方，DMD の残る 4 割の症例ではナンセンス変異のような微細な遺伝子の異常を有していると考えられる。こうした微細な遺伝子の異常の検出はゲノムを用いてジストロフィン遺伝子の各エクソンを PCR により増幅させ，その塩基配列を明らかにしなければ同定できない。

79個のエクソンを個別にシークエンスすることは時間を要するため，ジストロフィンcDNAの全長の塩基配列を決定することが行われる。こうしたことで，ほぼ全例で遺伝子診断は可能である。中には，染色体異常によるもの，あるいは巨大なイントロンの内部の変異により新たなエクソンが形成された異常もある[2]。

従来，DMDの診断は血液中のクレアチンキナーゼ（CK）を測定するなど臨床診断で十分であり，遺伝子診断の重要性が認識されていなかった。そのため，エクソン単位の欠失・重複の異常以外の遺伝子異常の解析は方法的に困難なため積極的になされていなかった。しかし，後述するようにナンセンス変異については分子標的療法での治療が可能となり，DMD患者での遺伝子異常の検出は患者の予後を左右する極めて重要なこととなった。

われわれは，ジストロフィン遺伝子異常の同定を全患者で明らかにすることを目標にDMD/BMD患者で詳細な遺伝子診断を進め，日本でのジストロフィン遺伝子異常の分布を明らかにすることに成功した（Fig. 2）。そして約7割の患者がジストロフィン遺伝子のエクソン単位の大きな欠失・重複の異常を有し，さらに，一塩基の置換がコドンをストップにかえるナンセンス変異を約15%の患者が，その他の異常を1.5割の患者が有することを明らかにしてきた（未発表データ）。

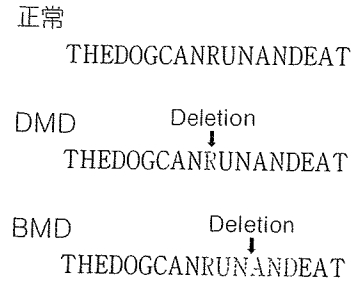

Fig. 3　フレームシフト則
遺伝子が働くときには，転写を受けてmRNA前駆体が産生され，mRNA前駆体はスプライシングによりイントロンが切り取られ，エクソン配列のみからなるmRNAとなる。そして，mRNAから蛋白へ翻訳される。この翻訳ステップでは，3塩基が1つのアミノ酸を指令する。この翻訳のルールに従えば，18文字は3文字ずつに区切ることにより，正確に翻訳される（正常）。ところが，1文字が欠失すると，区切りがずれ翻訳はできなくなる（DMD）。生体ではこうしたずれが生じるとコドンを形成する3塩基を構成する塩基の組み合わせがかわり，欠失の下流でTGA，GTA，TAAのどれかのストップコドンが出現し，翻訳を停止し，蛋白の合成が停止する。一方，6文字が欠失しても，3文字ずつの区切り方は維持されなんとか翻訳できる（BMD）。前者がDMDであり，後者がBMDである。このように，DMDとBMDの違いは欠失した塩基の総数が3の倍数か否かで決定されている。

Ⅱ．フレームシフト則

DMDとBMDでジストロフィン遺伝子の遺伝子診断を進めると，DMDもBMDも共にジストロフィン遺伝子にエクソン単位の欠失を有し，しかも重篤なDMD患者のほうが軽症のBMD患者より欠失の範囲が狭い例も同定されている。また，欠失のエクソン部位がDMDとBMDで共通している例もあり，欠失の大きさ，あるいは部位は臨床症状の決定因子でないことが判明した。現在，DMDとBMDの表現型の違いはジストロフィンmRNAのアミノ酸読み取り枠のずれの有無を確認するフレームシフト則によるものと考えられている。これによれば，DMDではジストロフィン遺伝子の欠失あるいは重複したエクソンにコードされている塩基の総数が3の倍数ではないため，ジストロフィンmRNAのアミノ酸読み取り枠にずれを生じ（アウトオブフレーム），その結果ストップコドンが出現しジストロフィンが産生されない。これに対し，症状の軽いBMDでは欠失あるいは重複したエクソンにコードされている塩基の総数が3の倍数で，ジストロフィンmRNAのアミノ酸読み取り枠は維持され（インフレーム），サイズの小さなジストロフィンが産生され症状が軽いものと考えられている。

このフレームシフト則は，英語の18文字を用いてより簡単に理解される（Fig. 3）。フレームシフト則はまた，DMDの治療の可能性があることを示唆する。すなわち，ジストロフィンmRNAのアウトオブフレームをインフレームに変換する，つまり，3の倍数でない欠失でもスプライシング時に隣接するエクソンのスキッピングを誘導して欠失範囲を広げ，欠失する塩基の総数を3の倍数にすることである。これによりストップコドンが解消され，ジストロフィンの産生が可能となる治療法が考えられる。

Ⅲ．ジストロフィン神戸の発見とエクソンスキッピング誘導

われわれは，DMD患者でジストロフィン遺伝子の解析を進める中で，極めて特異な遺伝子異常を見出した。ジストロフィン神戸と命名したこのジストロフィン遺伝子の異常は，エクソン19内の52塩基の欠失であった（Fig. 4）[3]。ところが，ゲノムで同定されたエクソン19内の52塩基の欠失の結果からは，36塩基のエクソン19の

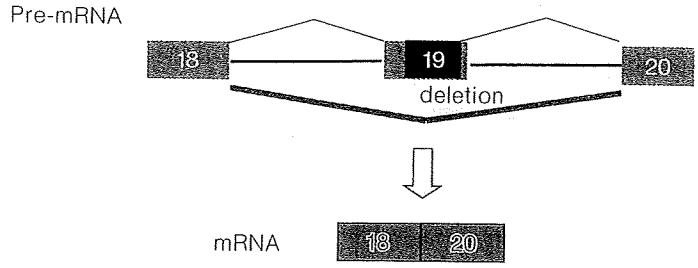

Fig. 4　ジストロフィン神戸

ジストロフィン遺伝子のエクソン 19 内に 52 塩基の欠失があり，エクソン 19 の配列はもともと 88 塩基であったのが 36 塩基に縮小している。しかし，ジストロフィン mRNA からはエクソン 19 の配列が消失したエクソンスキッピングが生じていた。

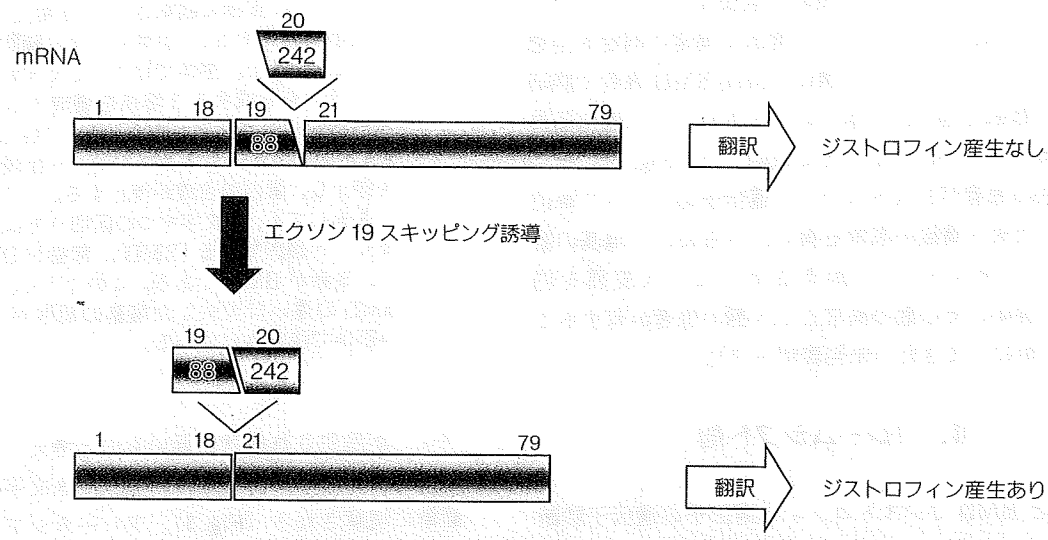

Fig. 5　DMD のエクソンス 19 のスキッピング誘導治療モデル

配列が mRNA 上で検出されると想定されたが，実際に患者のジストロフィン mRNA を解析してみると mRNA からジストロフィン 19 の配列がまったく消失してしまっていた。すなわち，ジストロフィン神戸ではゲノムの異常から 2 次的にエクソン 19 のスキッピングがスプライシング時に生じていたことが判明した。

スプライシングは，遺伝子から転写された mRNA 前駆体からイントロン配列を切り取り，エクソン配列のみからなる mRNA を産生させるステップである。スプライシングに異常が生じると，mRNA からエクソン配列が脱落するエクソンスキッピングが起こる。ジストロフィン神戸では，欠失した 52 塩基の配列がスプライシングに影響を及ぼしエクソンスキッピングを誘導したものと考えられた。欠失した配列のスプライシング反応における役割について分子生物的に検討したところ，この配列がスプライシング促進配列の機能を有しており，エクソンスキッピングが生じる原因は 52 塩基の欠失によりスプライシング促進配列が消失したことによることが明らかとなった[4]。

このスプライシング促進配列の機能を阻害することにより，人工的にエクソンのスキッピングが誘導されることが着想された。実際，このスプライシング促進配列に相補的な配列からなるアンチセンスオリゴヌクレオチド（AO）はジストロフィン遺伝子のエクソン 19 のスキッピングを誘導する能力を有することが判明した。

Ⅳ. エクソンスキッピング誘導治療

ジストロフィン神戸の分子病態の解析からエクソン AO を用いた 19 のスキッピング誘導を人為的に行うことが可能なり，その臨床応用の可能性を探った。エクソン 19 は 88 塩基からなっており，このエクソンのスキッピングによりアウトオブフレームの変異がインフレームとなるジストロフィン遺伝子の欠失異常を検討した

(Fig. 5)。ジストロフィン遺伝子のエクソン20を欠失したDMDでは242塩基が欠失し，アウトオブフレームになっている。このDMD例で，エクソン19のスキッピングを誘導すると，エクソン19の88塩基がさらにmRNAから欠けることとなり，エクソン20と19の合計330塩基がmRNAから消失する。その結果，mRNAはインフレームとなりこのDMD患者ではジストロフィンの産生が期待される。このことから，エクソン20を欠失したDMD患者がエクソン19のスキッピング誘導治療の対象となる。

この治療理論を実証するためエクソン20を欠失したDMD患者で，患児由来の培養筋細胞に先のAOを導入し，導入後の細胞でジストロフィンmRNAを解析した。予想通りにエクソンを欠失したインフレームのmRNAが産生された。さらに，AOを導入しなかった筋細胞ではまったくジストロフィンは染色されなかったが，導入した筋細胞ではエクソン19のスキッピング誘導が確認されるとともにジストロフィン染色陽性細胞の出現をみた[5]。これらの結果は本DMD患者でAOの導入によりジストロフィン発現を誘導できる治療法となることを示した。

V. 神戸大学での治療

こうした成果とマウスで得られた結果を統合して，DMD患者に経静脈的にAOを投与する治療法が考えられた。そこで神戸大学ではエクソン20を欠失したDMD患者へのAOを用いた治療を計画し，倫理委員会にて審議され承認された。AOは31塩基のものでS化されたDNAからなり，アメリカのProligo社において合成した。これを生理食塩水に溶き，0.5 mg/kg/回を2時間かけて点滴静注した。1週間に1回点滴投与し，投与後に血中リンパ球中のジストロフィンmRNAを解析した。また，4回投与した1週間後に筋生検を実施した。投与期間中のリンパ球のジストロフィンmRNAの解析でエクソンスキッピングを検出し，本治療が有効に検証していることを確認した。そして，患者骨格筋のジストロフィンmRNAをreverse-transcription-PCR（RT-PCR）解析すると，治療前には検出できなかったエクソン19のスキッピングしたmRNAを検出し，AOによるエクソンスキッピングの誘導効果を確認した。さらに，骨活筋のジストロフィンを免疫染色すると，ジストロフィンの発現をも確認することに成功した。その成果は，まったく新たなDMDの治療法として『Pediatric Research』誌の2006年5月号の表紙を飾るなど，世界から大きな注目を集めることとなった[6]。

VI. エクソンスキッピング誘導による治療の展開

ジストロフィン遺伝子のエクソン20を欠失したDMDの世界で初めての治療例を示した。しかし，エクソン20を欠失したDMDの症例数は極めて少なく，この方法の応用は限局的なものであった。ジストロフィン遺伝子には5'端と中央部にエクソン欠失の好発部位があり，この領域のエクソンのスキッピングを誘導するAOを確立することができれば，この方法による治療例は飛躍的に増加する。エクソン欠失好発部位で，スキッピングを誘導することによりアウトオブフレームからインフレームになるエクソンは合計17個である。既にvan Deutekomらは，2001年にエクソン46，Aartsma-Rusらは2003年にエクソン44，49，50，51のスキッピングがAOにより誘導され，DMD由来の筋培養細胞にこれらのAOを導入することによってジストロフィンが合成されるようになったことを報告している[7,8]。さらに，2つのエクソンを同時にスキッピング誘導する試みもなされており，1人の患者で2つのエクソンのスキッピングの誘導に成功しジストロフィンの発現を確認している[8-10]。こうした結果を踏まえ，オランダではジストロフィン遺伝子のエクソン51のスキッピングを誘導する治療が臨床試験されている[11]。

エクソンスキッピング誘導治療の問題点は，AOの安定化と効率のよい筋細胞への導入法である。神戸大学ではRNA／ENAキメラという新しい強力なAOを開発している。これは，ethylene bridged nucleic acid(ENA)という生体での安定性を増した修飾核酸とRNAをキメラにしたものである。エクソン19のスキッピング誘導を標的としたときには，RNA/ENAキメラの活性はS-オリゴと比較して40倍以上あることを確認している[12]。そして，RNA/ENAからなるエクソン45のスキッピング誘導するAOをデザインし，エクソン45のスキッピングを完全に誘導するAOのデザインに成功した。また，このAOをジストロフィン遺伝子のエクソン44を欠失したDMDあるいはエクソン46と47を欠失したDMD患者培養細胞に導入したところ，ジストロフィンの有意な発現を確認している。エクソン45のスキッピング誘導によりエクソン46の欠失例をはじめ多くの例で治療が可能となり，DMDが治療し得る疾病になることに大きく前進する。