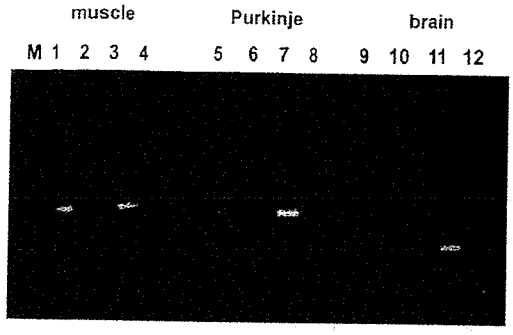

Fig. 2. Dystrophin isoform expression in myotubes. Dystrophin isoform expression was checked in myotubes by RT-PCR. The three set of primers were located at muscle type exon1 (lanes 1–4), Purkinje type (lanes 5–8), and brain type (lanes 9–12). PCR using genomic DNA as template for the positive control showed that the productions were detected in all types (lanes 3, 7, 11). PCR without adding genomic DNA for the negative control showed that the bands were not detected in all lanes (lanes 4, 8, 12). RT-PCR showed the products were detected in muscle and Purkinje type dystrophin (lanes 1 and 5), but not in brain type (lane 9). In contrast, RT-PCR without reverse transcriptase to check for the contamination of genomic DNA, showed that the bands were not detected in all lanes (lanes 2, 6, 10). Therefore, muscle and Purkinje type dystrophin was expressed in myotubes that were differentiated from fibroblasts in vitro. M: 1 kb DNA ladder (Gibco BRL, Grand Island, NY, USA).

Each promoter and first exon of muscle, brain, and Purkinje type dystrophin is independent [15], but their protein size is almost identical. PCR using genomic DNA for a positive control showed that the production of protein was detected in all types (Fig. 2, lanes 3, 7, and 11). PCR without genomic DNA as the template showed that no bands were detected in any of the negative control lanes (Fig. 2, lanes 4, 8, and 12). RT-PCR showed that the products were detected in muscle and Purkinje type dystrophin, but were not detected in brain type dystrophin (Fig. 2, lanes 1, 5, and 9). In contrast, no bands were detected in lanes of the same products using RT-PCR without reverse transcriptase, assuring that there was no contamination of genomic DNA (Fig. 2, lanes 2, 6, and 10). Therefore, muscle and Purkinje type dystrophin was expressed in myotubes that were differentiated from fibroblasts in vitro.

3.2. In vitro dystrophin staining

Fibroblasts that were cultured with 700 and 900 μg/ml of gentamicin did not survive at 2 weeks post-transfection while only some of the cells survived when cultured with a concentration of 500 μg/ml. Therefore, the concentration of 300 μg/ml of gentamicin was chosen for our experiments to allow cell survival and differentiation. At 14 days post-transfection, fibroblasts began to fuse together to form myotubes.

The in vitro immunofluorescence staining for dystrophin showed that dystrophin expression was not detected in the myotubes of patient 1 (deletion of dystrophin gene) cultured with and without gentamicin (Fig. 3C and D). In contrast, dystrophin expression was detected in myotubes of patients

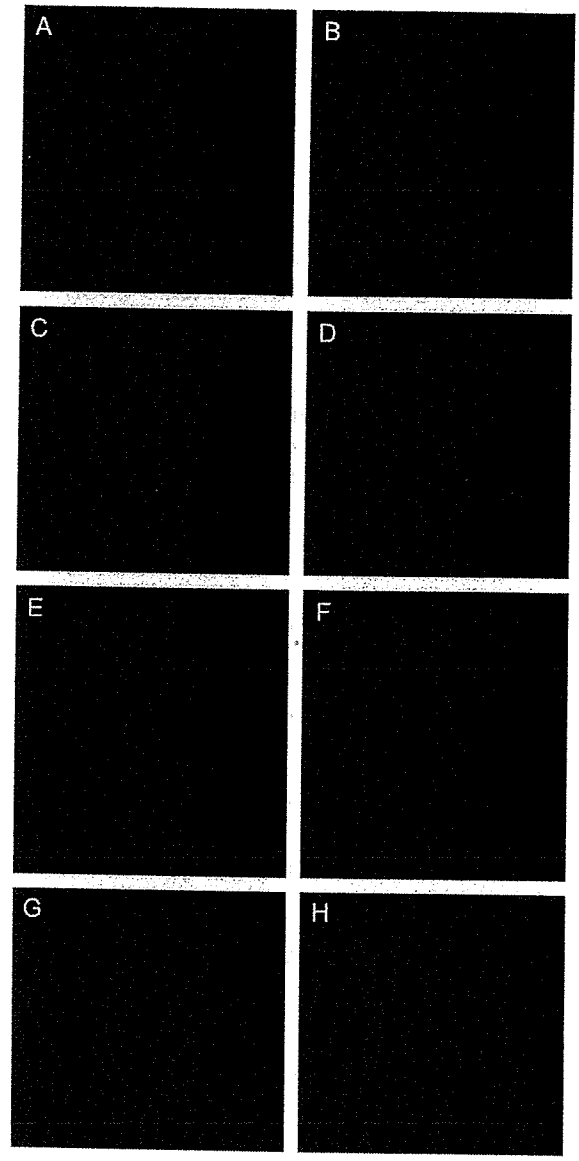

Fig. 3. Immunofluorescent analysis of myotubes differentiated from fibroblasts and cultured both in the presence (A, C, E, and G) and absence (B, D, F, and H) of gentamicin. The immunohistochemical staining results of the non-DMD patient (A and B), patients 1 (C and D), 3 (E and F), and 5 (G and H) are shown in this figure. Dystrophin expression is observed in myotubes from patient 3 after treatment of gentamicin (E), but not from patients 1 and 5, despite the treatment of gentamicin (C and G). Dystrophin expression was not observed in the myotubes of DMD patients not treated with gentamicin (D, F, and H). The dystrophin positive myotubes of the non-DMD patient were observed in both treated and non-treated (A and B) samples. Patients 2 and 4 also demonstrated the same pattern as patient 3, while patient 6 was similar to patient 5.

2, 3 and 4 (having the stop codon mutation, TGA) cultured with 300 μg/ml of gentamicin (Fig. 3E), but not detected in myotubes cultured without genatamicin (Fig. 3F). Interestingly, dystrophin expression was not observed in myotubes from patients 5 and 6 with the stop codon mutation TAA, in spite of being cultured with 300 μg/ml of gentamicin

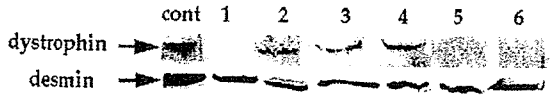

Fig. 4. Western blotting analysis of dystrophin. The cells cultured with 300 μg/ml of gentamicin were loaded in the corresponding lanes (control: non-DMD patient and lanes 1–6 for patients 1–6). Dystrophin expression was detected in the non-DMD patient (control), patients 2, 3, and 4, but not in patients 5 and 6. Desmin expression was detected in all patients.

(Fig. 3G and H). Dystrophin expression was detected in myotubes from fibroblasts of a non-DMD patient after culturing them with and without gentamicin (Fig. 3A and B).

3.3. Western blotting analysis of dystrophin

Western blotting analysis confirmed the results of the in vitro dystrophin staining (Fig. 4). The dystrophin expression was detected in the myotubes of patients 2, 3, 4 and the control (non-DMD patient) when cultured in 300 μg/ml of gentamicin, but not in the myotubes of patients 1, 5 and 6 even when they were cultured in gentamicin. The bands of dystrophin were not observed in gentamicin unexposed myotubes of DMD patients (data not shown). Desmin expression, used as an internal control, was detected in all lanes.

3.4. The quantity of dystrophin which is expressed by read-through

Desmin is expressed in early stage myoblasts as well as myotubes [16–18], whereas MHC is only expressed in myotubes [19,20]. There are differences among the efficiency of differentiation of fibroblasts from each patient into myotubes using AdMyoD. Therefore, MHC was deemed to be suitable as an internal control for myotubes.

In order to estimate the quantity of dystrophin in differentiated myotubes of DMD patients as compared with those derived from a non-DMD patient, we performed advanced experiments for western blotting using an alternative method. The density of band of MHC intensity was used as an internal control. Dystrophin expression was detected in patient 7, but not in patients 8, 9, and 10. However, the density of band of the control cells (non-DMD patient) was 2.1 times higher than those of patient 7 for MHC and 6.3 times higher for dystrophin. Therefore, the quantity of dystrophin in cells from patient 7 was approximately 36% of control. In contrast to our findings, dystrophin expression was not detected in the patient 8 despite having the TGA stop codon (Fig. 5).

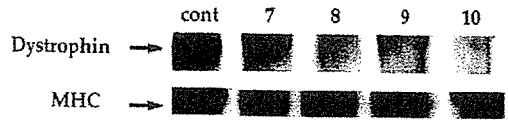

Fig. 5. The quantity of dystrophin which is expressed by read-through after gentamicin treatment. Dystrophin expression was detected in patient 7, but not in patients 8, 9, and 10. Based on measuring density of the band of Myosin Heavy Chain and dystrophin, the quantity of dystrophin expression in patient 7 is approximately one third of non-DMD patients.

4. Discussion

We have developed a system to identify DMD patients eligible for gentamicin treatment. By monitoring dystrophin expression of myotubes differentiated from fibroblasts transfected with AdMyoD and cultured in gentamicin, we are able to determine which patients will benefit from such treatments. In addition, we report for the first time that dystrophin expression was detected in myotubes of DMD patients (differentiated from fibroblasts) using gentamicin.

The dystrophin gene has at least five various isoforms and the independent promoters specify the transcription of the respective alternative first exons. Three of the five isoforms, brain, muscle, and Purkinje, are almost of the same size protein. RT-PCR shows that dystrophin expression in myotubes that were differentiated from fibroblasts was of muscle and Purkinje type. Holder et al. [21] reported that dystrophin mRNA transcripts from the Purkinje promoter were expressed in skeletal muscle, heart, and brain. Therefore, it is possible that the myotubes that were differentiated from fibroblasts can be used for monitoring the dystrophin expression in skeletal muscle.

The advantage of our system is that it is a faster and easier way to identify applicable DMD patients for aminoglycoside therapy for the following reasons. (1) It is not necessary to isolate myoblasts from DMD patients, which is much more difficult than isolating fibroblasts from skin biopsies. (2) The laborious task of analyzing the sequence of dystrophin cDNA is not necessary and can be avoided. (3) We can easily recognize the gentamicin-induced dystrophin expression by in vitro dystrophin staining and western blotting analysis.

Our results showed that the gentamicin induced read-through of the nonsense mutation TGA in dystrophin gene was detected by western blotting analysis; the nonsense mutations TAA and TAG were not. Howard et al. [10] reported that significant differences in the efficiency of aminoglycoside-induced read-through were observed, with UGA showing greater translational read-through than UAG or UAA, which coincides with our findings. Therefore, we cannot expect the same results of gentamicin treatment in all DMD patients who have stop codon mutations. It is speculated that this treatment is more effective for DMD patients with the TGA stop codon than for those with the TAA and TAG stop codon. However, dystrophin expression was not detected in patient 8, in spite of having a TGA stop codon. Further experimentation is required to gain a better understanding of these results. It should be noted that experiments by Howard et al. used fragments of the dystrophin gene to analyze the efficiency of read-through

of each nonsense mutation while we used original full-length dystrophin from the genome. Considering that the mechanism of read-through by gentamicin is complicated, we speculate that the differences in our results may be attributed to the different methods. Therefore, our monitoring system is important to identify DMD patients for gentamicin therapy.

Wagner et al. [9] showed that four Duchenne/Becker muscular dystrophies with various stop codons including TGA were treated once daily with intravenous gentamicin at 7.5 mg/kg/day for 2 weeks. The peak serum concentration of gentamicin was 40 μg/ml. However, full-length dystrophin was not detected in pre- and post-treatment muscle biopsies. They concluded that the lack of detection was caused by the lower doses and short duration of gentamicin therapy.

The quantity of dystrophin was calculated by density of band of MHC and dystrophin. The results show that the quantity of dystrophin in differentiated myotubes derived from patient 7 was approximately 36% of non-DMD patients. The results suggested that muscle weakness may be improved by this therapy by the increase in dystrophin.

Fuji et al. (submitted for publication, I. Fuji et al., Adenoviral mediated MyoD gene transfer to human fibroblasts and application to genetic diagnosis of myogenic diseases) showed that dystrophin expression was detected in normal myotubes that differentiated from human fibroblasts 2 weeks after transfection with AdMyoD. The efficiency of differentiation of fibroblasts to myotubes using AdMyoD is approximately 80%, which is much higher than other transfection methods. Although we only obtained an efficiency of 30–40%, this method is still convenient and easy for inducing fibroblasts to differentiate into myotubes in vitro. We therefore, conclude that our system is useful for finding DMD patients eligible for aminoglycoside treatment.

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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

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Abstract

The splicing pattern of pre-mRNA is unpredictable in genes harboring a single-nucleotide change within the consensus sequence of a splice-donor site. In the dystrophin gene, a transition from G to A at the fifth position of intron-32 (4518 + 5G > A) has been reported as a polymorphism within the consensus sequence or a mutation identified in Duchenne muscular dystrophy (DMD). Here, we report both in vivo and in vitro evidence that shows inactivation of the splice-donor site caused by this mutation. In one Japanese DMD case, two novel dystrophin mRNAs were identified in the patient's lymphocytes, one with a 98 bp deletion of the 3′ end of exon-32 (dys32 – 98) and the other with a 28 bp intron retained between exons 32 and 33 (dys32 + 28). Genomic sequencing disclosed a single-nucleotide change from G to A at the fifth position of intron-32 (4518 + 5G > A). To demonstrate in vitro the inactivation of this splice-donor site by this nucleotide change, mini-dystrophin genes comprising three exons harboring either normal or mutant intron-32 sequences were expressed in HeLa cells, and the splicing products were analyzed by reverse-transcription PCR amplification. A normal transcript consisting of three exons was obtained from the normal construct. From the mutant, we obtained one product containing a 98 bp deletion at the 3′ end of exon-32, indicating complete inactivation of the native splice-donor site. Thus, both in vivo and in vitro experiments demonstrate that 4518 + 5G > A causes a splicing error leading to transcript termination; it did not behave like a silent polymorphism. Our results indicate that the in vitro splicing system is a powerful tool for determining the underlying mechanism of a disease-causing mutation in a splicing consensus sequence.

Keywords: Dystrophin; DMD/BMD; Extra-exon; Splicing; Mutation

Introduction

Splicing is the process by which introns are removed from pre-mRNA. Consensus sequences located at intron/exon boundaries have been extensively characterized; they act as *cis*-elements that define the splice site. Mutations at consensus sequences have been reported to result in exon skipping, activation of cryptic splice sites, or both, producing aberrant mRNAs in all cases [1]. At the splice-donor site at the 5′ end of an intron, the sequence AG/GTAAG (/ denotes the exon/intron border) is the consensus sequence; the five bases in the intronic portion of the splice-donor site base-pair with U1 RNA as a prerequisite step for splicing [2,3]. The GT dinucleotide of the consensus sequence is strictly

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conserved, and changes in this sequence uniformly result in splicing errors. In contrast, only 20% of mutations at the +5 position cause aberrant splicing, even though the G at +5 position is very well conserved [1].

Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease, affecting approximately one in 3500 males, and is caused by mutations of the dystrophin gene, the largest human gene, which covers 3000 kb on the X chromosome. The dystrophin gene comprises 79 exons encoding 14 kb of cDNA, such that more than 99% of the sequence are intronic. More than 1000 single-nucleotide changes have been identified as disease-causing mutations (http://www.dmd.nl/, viewed on September 2004). Although most of them are nonsense mutations, nearly 15% are located in splicing consensus sequences, leading to either exon skipping or activation of cryptic splice sites [4–7].

Previous extensive sequence analysis of genomic DNA disclosed two instances of a single-nucleotide change from G to A at the fifth position of intron-32 of the dystrophin gene (4518 + 5G > A); one was a polymorphism (http://www.genomes.utah.edu/DMD/dystrophysnps, viewed on September 2004), whereas the second led to a case of DMD (http://www.dmd.nl/, viewed on September 2004) [8]. Although the latter suggests that 4518 + 5G > A is a disease-causing mutation, no evidence of the mutation affecting the coding sequence was shown. To conclude the 4518 + 5G > A is indeed a disease-causing mutation, evidence of the splicing error caused by 4518 + 5G > A must be provided.

Here, 4518 + 5G > A was first identified in a Japanese DMD patient. Subsequently, both in vivo and in vitro experiments disclosed inactivation of the native splice-donor site by 4518 + 5G > A.

Case and methods

Case

A 12-year-old boy was referred to Kobe University Hospital (KUHG329GA) for a genetic diagnosis of his muscle weakness. At age 1, his serum creatine kinase (CK) level was found to be markedly elevated, and Gower's sign was positive at age 3. At age 4, he was diagnosed with Duchenne muscular dystrophy (DMD) upon demonstration of a lack of dystrophin staining in a muscle biopsy. His muscle weakness progressed, and he was wheelchair-bound at the age of 10. There was no family history of neuromuscular disease. Laboratory examination disclosed an elevated level of serum CK (1646 IU/L, control: <169 IU/L). No abnormalities were detected by either X-ray or ECG examination. The Ethical Committee of Kobe University Graduate School of Medicine approved this study and consent for this study was obtained from his parents.

Methods

Mutation analysis

For mutation analysis of the dystrophin gene, DNA was isolated from peripheral blood of the index case and his family members by standard phenol–chloroform extraction methods. Southern-blot analysis was performed using *Hin*dIII restriction enzyme-digested DNA as a template as described previously [9]. This technique allows for the full extent of any deletions or duplications to be recognized.

To find a mutation not detectable by Southern-blot analysis, the dystrophin mRNA expressed in lymphocytes was analyzed using reverse-transcription nested PCR (RT-nested PCR) as described previously [10]. Full-length dystrophin cDNA was amplified as 10 separate, partially overlapping fragments [11]. A fragment showing an ambiguous sequencing result was further examined by narrowing the amplified region. A region encompassing exons 31–33 was amplified using a forward primer corresponding to a segment of exon-31 (3C = 5′-GCC CAA AGA GTC CTG TCT CA-3′) and a reverse primer complementary to a segment of exon-33 (2B = 5′-CTG CTT TTT CTG TAC AAT CTG ACG-3′).

To characterize the genomic nucleotide change, a region encompassing exon-32 (174 bp of exons 32 and 68, and 145 bp of neighboring introns 31 and 32, respectively) was PCR-amplified from the genomic DNA samples using the following pair of primers: a forward primer on intron-31: g32F = 5′-CAG AAA TAA AGG CAG ATC TAT CAA-3′ and a reverse primer complementary to intron-32: g32R = 5′-CAC AGA ATA GGC CAC AAT AC-3′.

All PCR amplifications were conducted essentially under conditions described previously [10]. PCR-amplified products were purified and subjected to sequencing either directly or after subcloning into T7 blue T vector [12]. Sequence analysis was done using a Dye terminator cycle sequencing kit (Amersham Bioscience, Piscataway, NJ) with an automatic DNA sequencer (model ABI Prism 310, Perkin Elmer Applied Biosystems, Foster City, CA).

In vitro splicing

To study splicing in vitro, three dystrophin exons (18, 19, and 20) and the intervening two introns [13] were inserted into a pcDNA3.0 mammalian expression vector (Invitrogen, Carlsbad, CA) that employs a cytomegalovirus (CMV) enhancer–promoter for high-level expression and contains a polyadenylation signal for complete synthesis of mRNA. To analyze any secondary effects of 4518 + 5G > A on splicing, the sequence of the central exon-19-encompassing region was replaced with that of

exon-32 and its flanking introns. An exon-32-encompassing fragment was amplified from DNA samples of a normal individual or the index case using primers containing restriction-enzyme recognition sites (g32F-*Xma*I = 5′-CgA CCC ggg TCT gTA TAT CTT CAg AAA TAA Agg-3′ and g32R-*Bam*HI = 5′-gCT ggA TCC AgT ATA ATT ATT ATg gTT ATC TgA-3′). After digestion of the amplified product with both *Xma*I and *Bam*HI restriction enzymes, the digested product was inserted into the dystrophin mini-gene that was predigested with the same two restriction enzymes. The resulting mini-dystrophin gene construct was then transfected into HeLa cells.

HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium) dissolved in distilled water (NANO pure DIAMOND VV/UF Water, Barnstead, Dubuque, IA) containing 10% fetal bovine serum (Trace Biosciences, Castle Hill, Australia) at 37 °C under 5% CO_2. The cells were plated on six-well tissue culture plates. One microgram of the plasmid was transfected into semi-confluent HeLa cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA) [13], and the cells were incubated for 24 h. Total RNA was extracted from HeLa cells and 3 μg RNA was subjected to reverse transcription using random hexamer primers as described previously [12]. To amplify the exogenous dystrophin mini-gene transcript, the following primers recognizing the vector/mini-gene junction were used: c18F*Kpn*I = 5′-CGA GGT ACC ACA GCT GGA TTA CTC GCT CA-3′ and c20R*Xho*I = 5′-GCT CTC GAG CAG CCA GTT AAG TCT CTC ACT-3′. PCR products were separated by 3% agarose gel electrophoresis and stained using ethidium bromide. To confirm the nucleotide sequences, each amplified product was excised from the gel and its sequence was determined as described above.

Results

Mutational analysis of the dystrophin gene was performed on genomic DNA of the index case using Southern blot. No gross gene rearrangement was disclosed. Dystrophin mRNA in lymphocytes was amplified as 10 separate fragments by RT-nested PCR amplification. Direct sequencing of these fragments revealed a completely normal sequence, except in the fragment encompassing exons 27–35; therefore, mutational analysis was focused on this region. Unexpectedly, amplification of a fragment from exons 31 to 33 disclosed two amplified products (Fig. 1A′). Subcloning and subsequent sequencing of both products disclosed two novel dystrophin mRNAs: the larger product showed a 28 bp insertion between exons 32 and 33 (dys32 + 28) (Fig. 1B′), whilst the smaller product had a 98 bp deletion of the 3′ end of exon-32 (dys32 − 98) (Fig. 1B′). Both novel dystrophin mRNAs were shown to result in a premature stop codon; this nonsense mutation accords with the lack of dystrophin staining in his muscle. However, the mechanism by which these two mRNAs were produced was not clear.

The 28 bp insertion in dys32 + 28 was found to be homologous to the 5′-end sequence of wild-type intron-32, except for the presence of a G-to-A transition at the fifth position of that intron. To confirm this transition in the patient's genomic DNA, we determined the genomic nucleotide sequence of intron-32 and found the same mutation to be present (4518 + 5G > A) (Fig. 1C′). The complete match of the inserted 28 bp sequence to the genomic sequence led us to conclude that the dys32 + 28 mutation constituted a retention of the 5′ end of intron-32.

These results led us to believe that the two novel dystrophin mRNAs were aberrant splicing products resulting from inactivation of the wild-type splice-donor site by 4518 + 5G > A. To validate this hypothesis, Shapiro's splicing probability score for the splice-donor site [14] was examined for the observed aberrant splice-donor sites (Fig. 2). These scores were found to be 0.68 and 0.82 at the sites 28 bp downstream and 98 bp upstream from the original splice-donor site, respectively; these scores are both higher than that of the mutated original splice-acceptor site (0.66; normal: 0.81), suggesting that the two aberrant dystrophin mRNAs (dys32 + 28 and dys32 − 98) are indeed splicing products that result from the use of cryptic splice-donor sites. Therefore, the in vivo results show that the 4518 + 5G > A mutation causes DMD by activating cryptic splice-donor sites.

Although our results indicated that 4518 + 5G > A is a disease-causing mutation, there has been a report describing 4518 + 5G > A as a polymorphism (http://www.genomes.utah.edu/DMD/dystrophysnps). To confirm the inactivation of the splice-donor site by 4518 + 5G > A, an in vitro splicing system was employed in which the dystrophin mini-gene was subjected to splicing in HeLa cells. Mini-gene constructs consisting of three dystrophin exons (18, 32, and 20) and the intervening intronic sequences containing either wild-type or mutant intron-32 sequences were prepared (Fig. 3) and transfected into HeLa cells. The mini-gene transcript was analyzed by RT-PCR using primers recognizing vector and exon junctions. When the construct containing the normal intron-32 sequence was transfected, one splicing product consisting of exons 18, 32, and 20 was obtained, indicating that splicing from the mini-gene transcript had occurred normally. On the other hand, a single smaller-sized amplified product was obtained from the mini-gene transcript containing the mutant intron-32 sequence (Fig. 3). Sequence analysis of the product disclosed that 98 bp of the 3′ end of exon-32 were deleted in this tran-

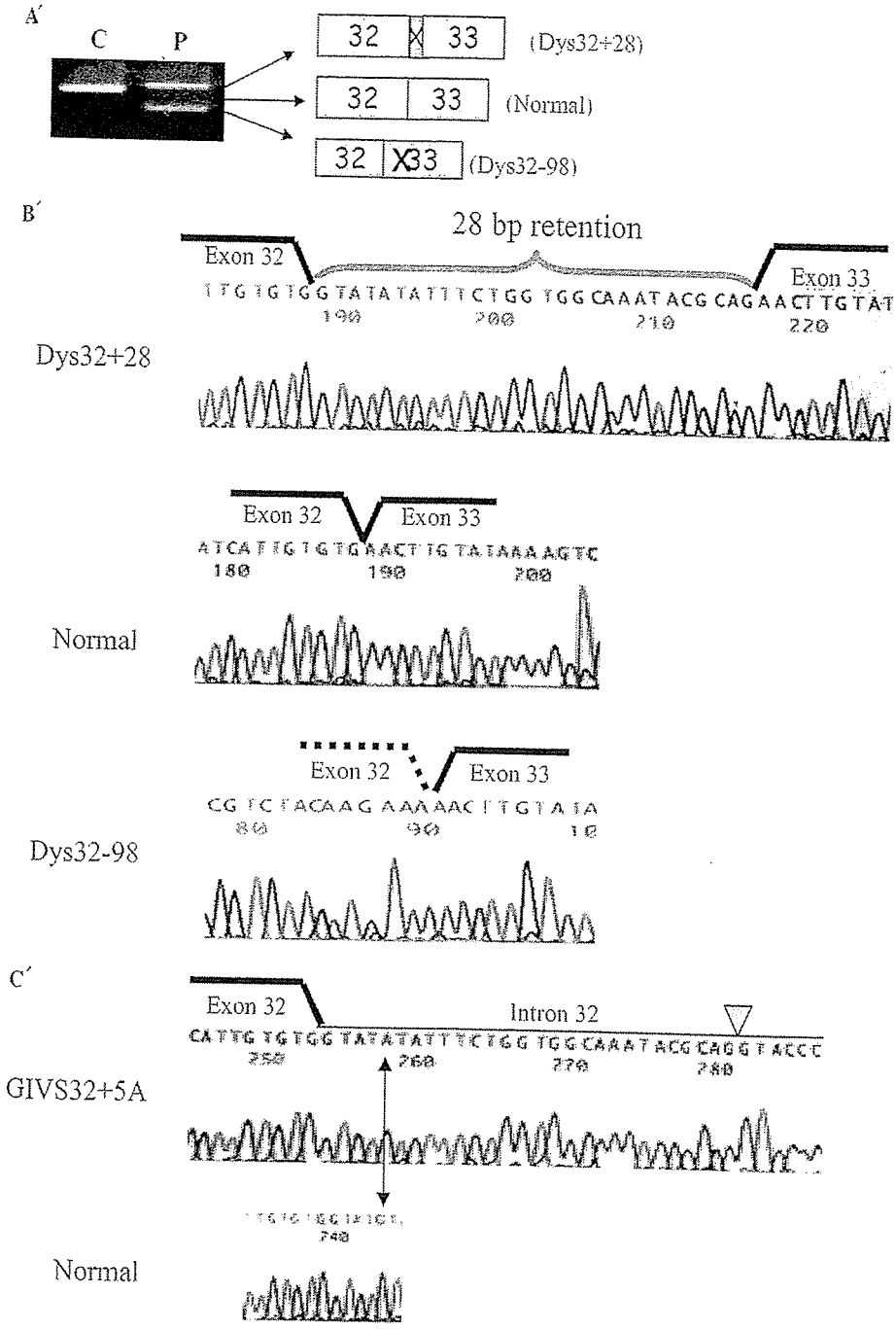

Fig. 1. Mutation analysis of the dystrophin gene. (A′) RT-PCR products. A fragment encompassing exons 31–33 was amplified from the patient's cDNA. One clear band was visualized from the normal (C), whereas two bands were visualized in the index case (P). The upper band contains an extra 28 bp between exons 32 and 33 (dys32 + 28), creating a stop codon in its sequence (X). In contrast, the lower band has a 98 bp deletion of the 3′ end of exon-32 (dys32 – 98), creating a stop codon in exon-33 (X). The structure of the product is shown schematically on the right side. Boxes and the numbers in boxes indicate the exon and exon number, respectively. The box inserted between exons 32 and 33 is the 28 bp of inserted sequence. (B′) cDNA sequences. Each sequence showed completely normal exons 31 and 33. The sequence of the 3′ end of exon-32 (5′-GTGTG-3′) is joined to two different sequences: GTATA (top) and AACTT (middle). At top, the 28 bp insertion was followed by a completely normal exon-33. In the bottom panel, 98 bp of the 3′ end of exon-32 were deleted, and the truncated exon-32 joined directly to exon-33. (C′) Intron-32 sequences. The PCR product amplified from genomic DNA was directly sequenced. The fifth nucleotide from the 5′ end of intron-32 was A in the index case (AIVS32 + 5) (upper) and G in the control (GIVS32 + 5) (lower). The inverted triangle indicates the activated cryptic splice-donor site.

script; all other sequences were normal. The sequence of the 98 bp deletion matched perfectly to that identified in the patient's lymphocytes (dys32 – 98). Neither the normal nor the dys32 + 28 transcript could be identified. These results showed that 4518 + 5G > A inactivates the original splice-donor site completely.

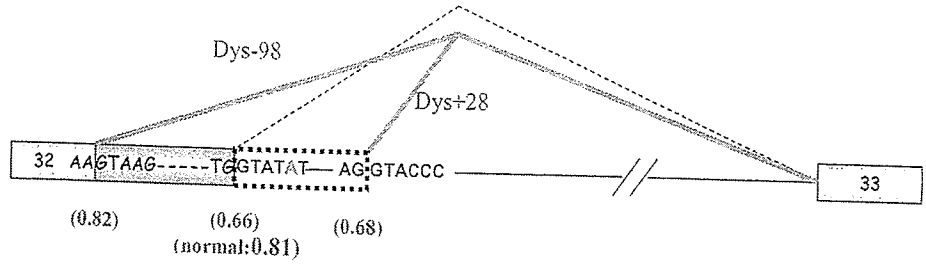

Fig. 2. Splicing patterns of intron-32 and cryptic splice-donor sites near the original splice-donor site. Two novel dystrophin mRNAs are produced by activating two cryptic splice-donor sites in 4518 + 5G > A. Splicing patterns identified in the index case are represented schematically (two solid diagonal lines; Dys + 28 and Dys – 98). One dotted line indicates a normal splicing pattern. Boxes and horizontal lines indicate exons and introns, respectively. The numbers in parentheses indicate the Shapiro's probability scores. The figure is not drawn to scale.

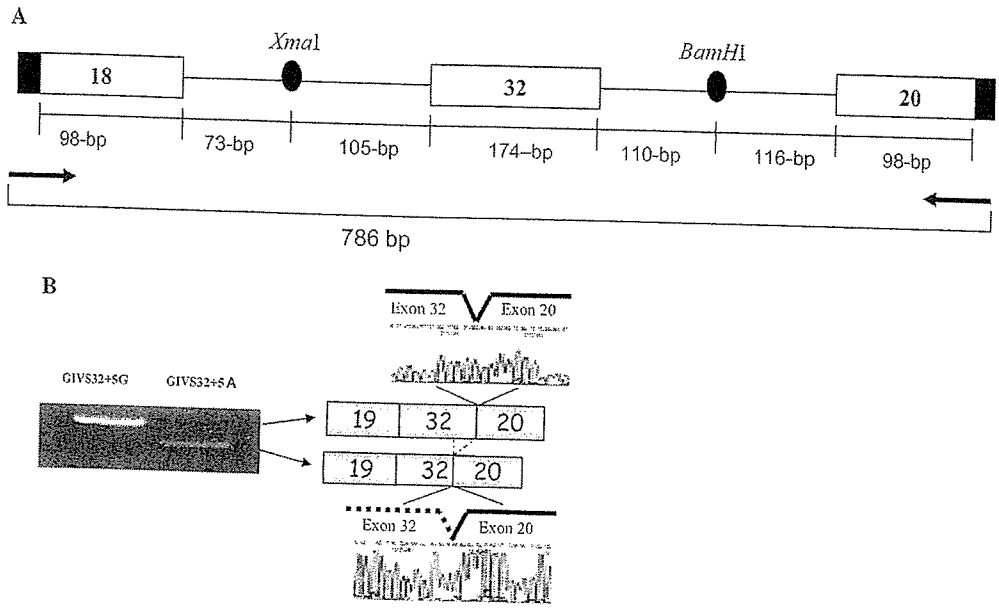

Fig. 3. In vitro splicing. (A) Structure of the mini-dystrophin gene. The mini-dystrophin gene contains sequences corresponding to exons 18 (98 bp), 32 (174 bp), and 20 (98 bp), and portions of introns 18 (73 bp) and 32 (105 bp) between exons 18 and 32, and introns 32 (110 bp) and 20 (116 bp) between exons 32 and 20, respectively. *Xma*I and *Bam*HI represent *Xma*I and *Bam*HI restriction-enzyme recognition site, respectively. Horizontal arrows indicate the positions and the directions of the primers. The bracket indicates the amplified product (786 bp). (B) RT-PCR products. The mini-gene transcript was analyzed by RT-PCR amplification of a fragment encompassing two vector–cDNA junctions. One amplified product was obtained each from the normal (GIVS32 + 5) and mutant (AIVS32 + 5), but the size differed clearly (left). Sequencing of the normal product disclosed the presence of exons 18, 32, and 19. From the mutant, however, 98 bp at the 3′ end of exon-32 were found to disappear, and the resulting truncated exon-32 was inserted between exons 19 and 20 (left).

Combining both the in vivo and in vitro results, we conclude that 4518 + 5G > A is a disease-causing mutation responsible for DMD.

Discussion

In this report, a G-to-A transition at the fifth nucleotide of intron-32 of the dystrophin gene (4518 + 5G > A) is shown to be a disease-causing mutation. Both in vivo and in vitro analyses disclosed complete inactivation of the original splicing donor site (Figs. 1 and 3). Though skipping of an upstream exon is a common outcome of a mutation of the consensus sequence of the splice-donor site [1], exon-32 skipping was not demonstrated in our patient. Instead, cryptic splice-donor activation was disclosed, and the resulting two dystrophin mRNAs were found to encode a premature stop codon (Fig. 1). Therefore, 4518 + 5G > A was concluded to be a disease-causing mutation.

Our conclusion was corroborated by in vitro splicing experiments showing inactivation of the original splice site (Fig. 3). Although two cryptic splice-donor sites were shown to be activated in vivo, only one was found in vitro. The limited activation of one cryptic splice site in vitro may be due to environmental differences in the splicing machinery, *including tissue difference and differences in pre-mRNA secondary structure or in the size of intron-32*. As demonstrated here, our current mini-gene is a powerful tool for confirming disease-causing mutations in a splicing consensus sequence.

Because some of the introns in the dystrophin gene are extraordinarily large (more than 100 kb), splicing of dystrophin pre-mRNA must be regulated by a very sophisticated mechanism. Nucleotide changes at the fifth position within introns of the dystrophin gene have been identified in 10 cases. Their resulting splicing patterns are described in several ways: five result in upstream exon skipping, one gives activation of nearby cryptic splice-donor sites, one results in both exon skipping and cryptic splice-site activation, one is a silent polymorphism, and the others were identified in DMD cases without examination of the mRNA transcripts. Our results add another example of cryptic splice-donor site activation. Although most previously described splice-site mutations induce exon skipping, 4518 + 5G > A induced cryptic splice-site activation only. It remains unclear what factors determine the splicing pattern when the fifth position within an intron is mutated.

At this moment, there is no effective way to treat DMD. Exon skipping of nonsense mutations present in exons 27, 29, or 72 is known to convert severe DMD to mild BMD [15–17]. Therefore, artificial induction of exon skipping is one candidate for alternative gene therapy for DMD, and antisense oligonucleotides targeting either splicing enhancer sequences or splicing consensus sequences have been reported to induce exon skipping successfully [18–23]. Our results, which disclose activation of nearby cryptic sites due to inactivation of the wild-type splice-donor site, suggest that antisense oligonucleotides against splicing consensus sequence induce not only exon skipping but also cryptic splice-site activation. Therefore, this possibility should be considered when a strategy for DMD treatment targeting splicing consensus sequences with antisense oligonucleotides is employed.

Acknowledgments

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ORIGINAL ARTICLE

Splicing analysis disclosed a determinant single nucleotide for exon skipping caused by a novel intraexonic four-nucleotide deletion in the dystrophin gene

Van Khanh Tran, Yasuhiro Takeshima, Zhujun Zhang, Mariko Yagi, Atsushi Nishiyama, Yasuaki Habara, Masafumi Matsuo

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Background: Mutations in exonic splicing enhancer sequences are known to cause splicing errors. Although exonic splicing enhancers have been identified as a stretch of purine-rich sequences, it has been difficult to precisely pinpoint the determinant nucleotides in these sequences. This article reports that a 4-bp deletion in exon 38 of the dystrophin gene induced complete exon 38 skipping in vivo. Moreover, the third nucleotide of the deletion was shown to be determinant for the exonic splicing enhancer activity in in vivo splicing analysis of hybrid minigenes encoding mutant exons.

Method: Genomic DNA analysis of a 2-year-old boy with a raised level of serum creatine kinase yielded a 4-bp deletion 11 bp upstream of the 3′ end of exon 38 of the dystrophin gene (c. 5434–5437del TTCA), disrupting a predicted SC35-binding site.

Result: Interestingly, his dystrophin mRNA was shown to completely lack exon 38 (exon 38− transcript). As the exon 38− transcript coded for a truncated dystrophin protein, this exon skipping was determined to be a modifying factor of his phenotype. In an in vivo splicing assay, a hybrid minigene encoding exon 38 with the 4-bp deletion was shown to induce complete exon 38 skipping, confirming the deleted region as a splicing enhancer sequence. Site-directed mutagenesis of the deleted sequence showed that the complete exon 38 skipping was caused by mutation of the third nucleotide position of the deletion (C5436), whereas mutations at the other three nucleotide positions induced partial exon skipping.

Conclusion: Our results underline the potential of understanding the regulation of exonic splicing enhancer sequences and exon skipping therapy for treatment of Duchenne's muscular dystrophy.

For eukaryotic splicing of pre-mRNA transcripts, a process that removes introns from pre-mRNA, splice sites are defined by splicing consensus sequences located near intron–exon boundaries. Mutation of splicing consensus sequences can result in exon skipping or the activation of cryptic splice sites, thereby producing aberrant mRNAs. Recently, exonic splicing enhancers were shown to be required for proper splicing.[1] Mutations in these sequences were shown to result in aberrantly spliced transcripts, and are currently receiving a great deal of attention as modifiers of genetic mutations.[2]

Dystrophinopathy, which is caused by mutations in the dystrophin gene, is the most common inherited myopathy, affecting approximately one in 3500 men. This genetic condition shows varying degrees of severity, ranging from the severe Duchenne's muscular dystrophy (DMD) to the milder Becker's muscular dystrophy (BMD). DMD is a rapidly progressive disease that is first recognised during childhood; children with DMD commonly lose their ability to walk before they turn 12 years old. BMD has a slower rate of progression; those with BMD remain ambulatory beyond the age of 16 years and may lead near-normal lives. In as many as 60% of patients with dystrophinopathy, a partial deletion or duplication of the dystrophin gene can be detected. According to the reading-frame rule, deletions or duplications that create a shift in the reading frame of dystrophin mRNA lead to the more severe DMD phenotype, whereas the milder BMD phenotype occurs if the open reading frame is preserved after the deletion or duplication. Exceptions to the rule however, have been reported, and the production of aberrant transcripts is considered to be a key modifier of the clinical phenotype of dystrophinopathy.[3–7]

The complex structure of the dystrophin gene, which is characterised by a large number of exons (79), extremely large introns (up to 200 kb) and tissue-specific alternative splicing, suggests an indispensable role for exonic splicing enhancer sequences in normal dystrophin pre-mRNA splicing. Analyses of splicing errors in cases of dystrophinopathy with exonic mutations in the dystrophin gene first identified splicing enhancer sequences in exon 19.[11 12] So far, splicing enhancer sequences, however, have been found only in a limited number of dystrophin gene exons.[13 14]

In this study, we report that a novel 4-bp deletion in exon 38 of the dystrophin gene leads to complete exon 38 skipping. Results of an in vivo splicing assay disclosed that this 4-bp deletion disrupted an exonic splicing enhancer, and that a single nucleotide in the deletion is determinant for proper exon 38 splicing, whereas the other three nucleotides are less essential.

CASE AND METHODS

Case

A 2-year-old boy (KUHPCG111) was referred to the Kobe University Hospital, Japan, for a genetic diagnosis of his

Abbreviations: BMD, Becker's muscular dystrophy; DMD, Duchenne's muscular dystrophy; PCR, polymerase chain reaction; RT, reverse transcription; TVK-Δ, mutant hybrid minigene; TVK-WT, wild-type hybrid minigene

raised serum creatine kinase level. There was no family history of neuromuscular disease. As an infant, he started to crawl at age 6 months and began to walk at age 1.5 years. By chance, his serum creatine kinase level was found to be 3805 IU/l (normal ≤169 IU/l), although he did not show any muscle weakness. No abnormalities were detected on either radiographs or electrocardiograms. Laboratory investigation of blood yielded a raised level of serum creatine kinase (8642 IU/l). Subsequent analyses showed a wide variation in the serum creatine kinase level, which ranged from 1071 to 10 136 IU/l, leading to a tentative diagnosis of BMD. Consent for this study was obtained from the patient's parents. The ethics committee of the Kobe University Graduate School of Medicine, Kobe, Japan, approved the study.

Mutational analysis

For mutational analysis of the dystrophin gene, genomic DNA was isolated from the lymphocytes of this index case and a control patient using a Wizard genomic DNA extraction kit (Promega Corporation, Madison, Wisconsin, USA). Nineteen deletion-prone exons were amplified by polymerase chain reaction (PCR) to look for a deletion mutation.[15 16] Southern blot analysis was carried out using *Hind*III restriction-enzyme-digested DNA as a template and a dystrophin cDNA fragment as a probe. This technique allows for the full extent of any deletions or duplications to be determined. To look for small mutations, all 79 exons and the flanking introns were amplified[17] and each amplified product was directly sequenced using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) with an automatic DNA sequencer (ABI PRISM 310, Applied Biosystems).

The dystrophin mRNA expressed in lymphocytes was examined by nested PCR. Total RNA was extracted from peripheral lymphocytes and cDNA was synthesised as described previously.[18] A fragment extending from exon 36 to exon 41 was amplified using two sets of primers: an inner set with a forward primer corresponding to a segment of exon 36 (3E, 5′-TTT GAC CAG AAT GTG GAC CA-3′) and a reverse primer complementary to a segment of exon 41 (c41r, 5′-TGC GGC CCC ATC CTC AGA CAA-3′), and an outer set with a forward primer (3A, 5′-GCT TGA AGA GAG CAT CCA GTC-3′) and a reverse primer (3B, 5′-ACT GGC ATC TGT TTT TGA GGA T-3′). The PCR-amplified product was electrophoresed on an agarose gel. The purified PCR product was sequenced either directly or after it was subcloned into vector pT7 Blue (Novagen, Madison, Wisconsin, USA).

In vivo splicing assay

Hybrid minigenes

To study the splicing pattern, a minigene vector (H492) that contained two exons (exons A and B) and one intervening intron encoding a multicloning site was constructed from the pcDNA 3.0 mammalian expression vector (Invitrogen, Carlsbad, California, USA).[19] A hybrid minigene was created by inserting a test sequence consisting of exon 38 and its flanking introns into the multicloning site (fig 1). The fragment encompassing exon 38 was amplified from both the control sample and the patient's genomic DNA by the PCR with primers that corresponded to introns 37 and 38, and included recognition sites for restriction enzymes *Nhe*I and *Bam*HI (In37F-Nhe, 5′-GCC GCT AGC GAT TAG TTT AGC AAC AGG AGG-3′ and In38R-Bam, 5′-CGG GAT CCG TGC TCT GAA AAT TCA GTT GGA G-3′). Amplified products were digested with *Nhe*I and *Bam*HI (New England Biolabs, Hertfordshire, UK), and inserted into the minigene that had been digested with the same restriction enzymes. In this way, both wild-type (TVK-WT) and mutant (TVK-Δ) hybrid minigenes, which carried wild-type exon 38 and exon 38 with the 4-bp deletion, respectively, were constructed. Site-specific mutagenesis by overlap extension was carried out to introduce mutations by PCR-based mutagenesis into the TVK-WT minigene.[19] The four nucleotides at positions 5434, 5435, 5436 and 5437, which were missing in the deletion construct, were each replaced with their complementary nucleotides to make the TVK-WT5434T→A, TVK-WT5435T→A, TVK-WT5436C→G, and TVK-WT5437A→T minigenes. Furthermore, additional mutations were introduced into position 5436 to yield the TVK-WT5436C→A, TVK-WT5436C→T and TVK-WT5436Cdel minigenes (fig 1). After checking their sequences, these hybrid minigenes were transfected into HeLa cells for a splicing assay.

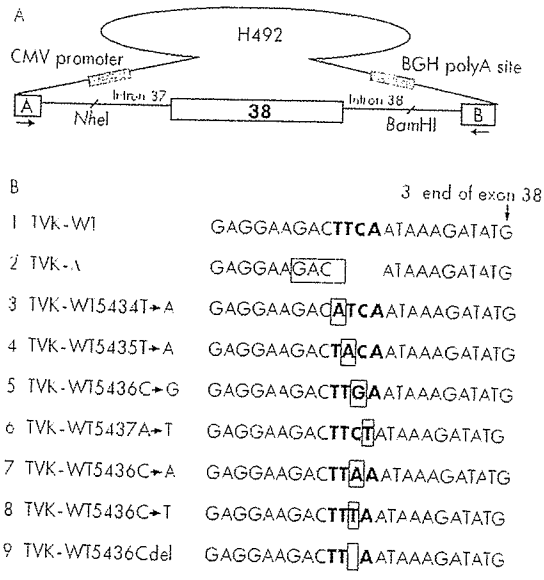

Figure 1 In vivo splicing analysis. (A) Schematic representation of the hybrid minigene. A minigene vector (H492) was constructed to encode two cassette exons (A and B) and an intervening sequence containing a multicloning site. The polymerase chain reaction (PCR)-amplified regions encompassing exon 38 were inserted into the multicloning site after digestion with *Nhe*I and *Bam*HI. The minigene vector contained a cytomegalovirus enhancer–promoter and a polyadenylation signal (BGH; dark shaded boxes) for complete synthesis of mRNA. Box and lines indicate exon and its flanking introns, respectively. The primers used in the reverse transcriptase (RT)-PCR assay are represented by arrows. (B) Sequences of the 3′ ends of exon 38 in the hybrid minigenes. TVK-WT and TVK-Δ denote the sequences of wild-type exon 38 and exon 38 with the 4-bp deletion, respectively. Single nucleotide changes were introduced into TVK-WT by mutagenesis to produce seven other hybrid minigenes. Boxes highlight the mutated nucleotides and open boxes indicate deletions. Bold letters correspond to the deleted nucleotides identified in the index case.

Transfection

HeLa cells were grown in six-well plates to approximately 70% confluency in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum (Trace Biosciences, Castle Hill, Australia) at 37°C under 5% carbon dioxide. Hybrid minigenes (1.5 μg each) were transfected into the cells using Plus Reagent and Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells were harvested 24 h after transfection and total RNA was extracted using ISOGENE (Nippon Gene, Toyama, Japan).

Analysis of splicing products

Five μg of total RNA was subjected to reverse transcription (RT) using random hexamer primers as described previously.[18] PCR was carried out using a forward primer

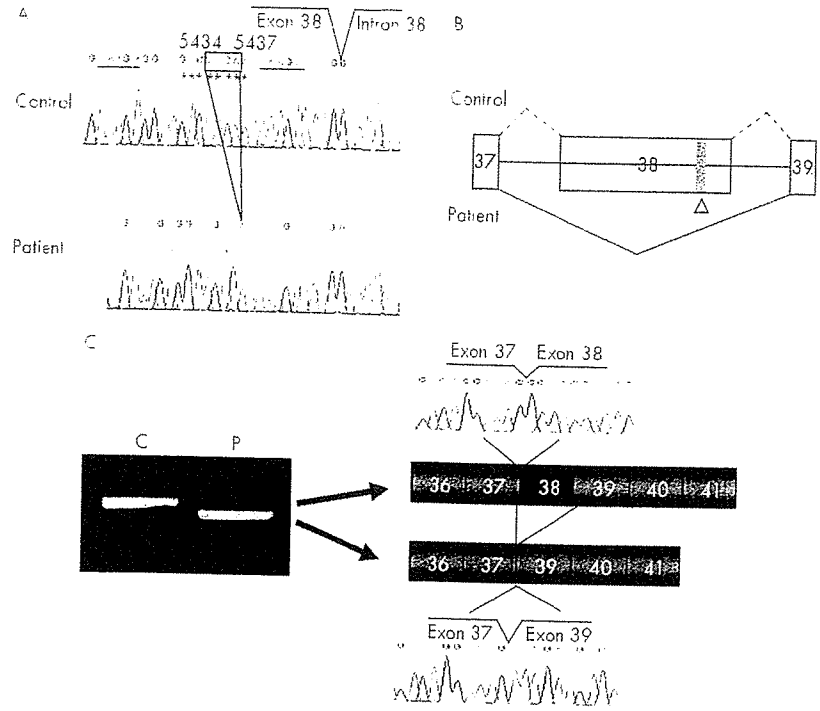

Figure 2 Analysis of the dystrophin gene and mRNA. (A) Genomic DNA analysis. Sequences of the 3' end of exon 38 and the 5' end of intron 38 of the control (upper panel) and the patient (lower panel) are shown. Four nucleotides (TTCA; boxed) located 11 bp upstream from the 3' end of exon 38 were missing from the patient. The nucleotide numbers correspond to the position in the wild-type dystrophin cDNA. The 4-bp deletion is predicted to introduce a stop codon in the sixth codon after the deletion. The deletion does not change Shapiro's probability score for splicing. The sequence AAAGA is found on both sides of the deletion (underlined). A predicted SC35-binding site (GACTTCAA) is marked by asterisks. (B) Schematic representation of the dystrophin exon 38 and the flanking exons. Splicing events in the control (dotted lines) and the patient (solid lines) are indicated. The arrowhead indicates the position of the four-nucleotide deletion. The figure is not drawn to scale. (C) The dystrophin mRNA. Dystrophin cDNA from exon 36 to 41 was amplified by polymerase chain reaction (PCR). Although the control (C) and the patient (P) both produced one PCR product, the size of the products clearly differed. The patient produced a smaller PCR product, and sequencing of this product showed that exon 38 was missing. On the right, the exon structure of each band is schematically presented. Boxes and the numbers in the boxes indicate the exon and exon number, respectively. The sequence at the junction of the exons is shown.

corresponding to a segment of the upstream exon A (YH303, 5'-GGT ACC ACA GCT GGA TTA CTC GCT C-3') and a reverse primer complementary to a segment of the downstream exon B (YH304, 5'-CTC GAG CAG CCA GTT AAG TCT CTC AC-3', fig 1). Amplification was carried out in a total volume of 20 µl containing 4 µl cDNA, 2 µl 10× Ex Taq buffer, 2 µl 2.5 mM deoxyribonucleotide triphosphates, 10 pmol of each primer and 1 U Ex Taq Polymerase (Takara Bio, Kyoto, Japan). The PCR cycling conditions were as follows: an initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. PCR products were analysed by electrophoresis on an 8% polyacrylamide gel.

To identify the differences between the splicing products, semiquantitative PCR was used. PCR was carried out as described earlier, except that the number of PCR cycles was reduced from 30 to 18. To quantify the amplified products, 1 µl of each reaction mixture mixed with 5 µl of the loading buffer solution containing size markers (15 and 1500 bp) was analysed by capillary electrophoresis (Agilent 2100 Bioanalyzer with DNA 1000 LabChip; Agilent Technologies, Palo Alto, California, USA). The amount of each product was quantified by measuring fluorescence of the peak areas (Agilent 2100 Bioanalyzer).

Analysis of the exonic splicing enhancer

Two programs designed to find exonic splicing enhancers were used to examine exon 38: (1) ESE finder and (2) RESCUE-ESE. ESEfinder (http://rulai.cshl.edu/ESE/) is a web-based resource that facilitates rapid analysis of exon sequences to identify binding motifs for four serine/arginine-rich (SR) proteins: SF2/ASF, SC35, SRp40 and SRp55.[26] The RESCUE-ESE program (http://genes.mit.edu/burgelab/rescue-ese/) uses a computational method that identifies exonic splicing enhancers in human genomic sequences by searching for specific hexanucleotides.[27]

RESULTS

The dystrophin gene of an index patient who had been clinically diagnosed with dystrophinopathy was analysed for mutations. Conventional mutational analysis of deletion-prone exons by PCR amplification and Southern blotting did not disclose any mutations. Next, all 79 exons of the dystrophin gene were examined by direct sequencing of PCR-amplified products. Although two single-nucleotide changes were found (ca 7096C→A in exon 48 and ca 9801A→G in exon 67), these changes were variants leading to an amino acid change.[28] In the amplified region encompassing exon 38, four nucleotides (TTCA) 11 bp upstream and 108 bp downstream from the 3' and 5' ends of exon 38, respectively, were found to be absent (ca 5434–5437delTTCA; fig 2A). We did not find this deletion in more than 100 other patients with DMD or those with BMD, and this deletion has not been previously reported in the literature. This novel deletion shifted the translational reading frame of the dystrophin mRNA, which resulted in a premature stop codon after the fifth amino acid residue of the wild-type protein. A

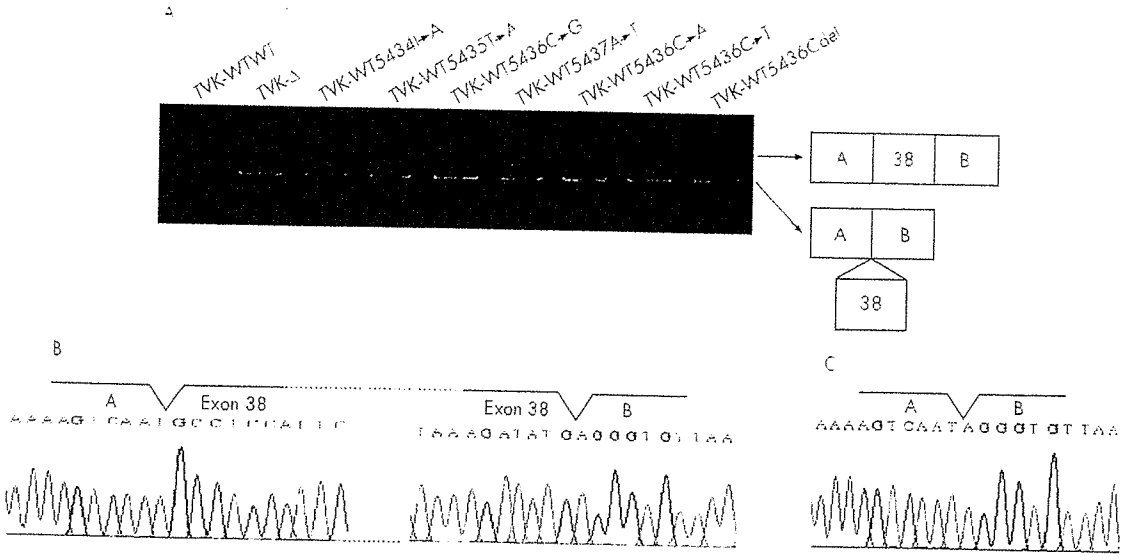

Figure 3 Hybrid minigene splicing. Hybrid minigenes containing the indicated variants were tested in an in vivo splicing assay and the resulting mRNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). (A) Products electrophoresed on a polyacrylamide gel is shown. A 319-bp full-length transcript was generated from a minigene carrying the wildtype sequence of exon 38 (TVK-WT), whereas a 196-bp transcript was generated from a minigene carrying the mutant exon 38 (TVK-Δ). TVK-WT and TVK-Δ contain the wild-type exon 38 and the patient's genomic sequence with the 4-bp deletion, respectively. Each of the four nucleotides of the deleted region in the TVK-WT minigene was replaced with respective complementary nucleotide to produce the TVK-WT5434T→A, TVK-WT5435T→A, TVK-WT5436C→G and TVK-WT5437A→T minigenes. To analyse the position effect, 5436C was deleted (TVK-WT5436Cdel), or replaced with A (TVK-WT5436G→A) or T (TVK-WT5436G→T). A schematic description of the RT-PCR products is shown on the right. The wild-type product (top) consists of exons A, 38 and B, whereas the smaller product did not contain exon 38, with exon A joining directly to exon B (bottom). (B,C) Nucleotide sequences at the junctions between exons are shown.

deletion leading to a translational frame shift suggested a severe DMD phenotype.

Despite the genetic diagnosis of DMD for the patient, his serum creatine kinase level was not constantly raised (the lowest measured value was 1074 IU/l), a phenotype that did not completely match with that of DMD. To examine this discrepancy, the peripheral lymphocytes of the patient were examined for the production of aberrant dystrophin mRNA transcripts. The region spanning from exon 36 to 41 was amplified by a nested PCR. Interestingly, the amplified product from the index case was found to be smaller than the expected product (fig 2C), suggesting that an aberrant splicing product was being amplified. In fact, sequencing the product disclosed that the dystrophin mRNA no longer contained the 123-bp exon 38 (an exon 38− transcript). Subcloning and sequencing of the PCR-amplified product did not produce any clone that retained exon 38. We concluded that the ca 5434–5437delTTCA genomic deletion induced complete exon 38 skipping in vivo. The exon 38− transcript was expected to produce a truncated but semifunctional dystrophin protein, thereby resulting in the observed mild phenotype.

As in other dystrophinopathies,[9] the exon 38− transcript was expected to be produced by disruption of one of the strictly conserved splicing consensus sequences. No mutations, however, were identified in the flanking introns. Moreover, the ca 5434–5437delTTCA deletion did not influence Shapiro's probability score for the splicing donor site.[10] It was therefore likely that the deletion interrupted an exonic splicing enhancer. To examine this, the mutant exon was subjected to in vivo splicing analysis. A hybrid minigene containing exon 38 and its flanking introns between two cassette exons was constructed (fig 1) and transfected into HeLa cells. The resulting splicing products were amplified by RT-PCR amplified. In the control sample (TVK-WT), a mature hybrid mRNA consisting of exons A, 38 and B was produced (fig 3). When a mutant exon 38 with the 4-bp deletion replaced the wild-type exon 38 (TVK-Δ), a smaller PCR product was exclusively obtained (fig 3). Sequencing of the product disclosed the absence of exon 38, indicating that this exon was skipped. Therefore, the four nucleotides were concluded to be part of an exonic splicing enhancer that was required for normal splicing.

To explore the role of each of the four nucleotides in the regulation of exon 38 splicing, site-directed mutagenesis was used. Four hybrid minigenes in which each of the four nucleotides was replaced with its complementary nucleotide were prepared (TVK-WT5434T→A, TVK-WT5435T→A, TVK-WT5436C→G and TVK-WT5437A→T) and transfected into HeLa cells. RT-PCR analysis of hybrid mRNA from cells expressing TVK-WT5436C→G produced a single band corresponding to a transcript lacking exon 38, indicating complete exclusion of exon 38; the other three constructs resulted in two amplified bands that corresponded to hybrid mRNAs with and without exon 38 skipping, indicating partial exon 38 skipping (fig 3). Quantification of the splicing products showed that TVK-Δ and TVK-WT5436C→G induced complete exon 38 skipping, whereas the production of mRNA without exon 38 was not absolute in cells expressing TVK-WT5434T→A, TVK-WT5435T→A and TVK-WT5437A→T (fig 4).

As the 5436C→G mutation produced the same result as the complete disruption of the splicing enhancer sequence, the role of nucleotide 5436 in the splicing reaction was further examined by deleting this nucleotide or substituting the nucleotide with A or T. Three hybrid minigenes (TVK-WT5436C→A, TVK-WT5436C→T and TVK-WT5436Cdel) were constructed and their transcripts were analysed. As all three mutants showed the complete exon 38-skipping phenotype (fig 4A), we believe that nucleotide 5436C is determinant for proper exon 38 splicing.

The deletion identified in the index case is located in a purine-rich sequence (11 of 20 nucleotides are purines). Purine-rich sequences are known to serve as recognition motifs for SR proteins which are required to define exons

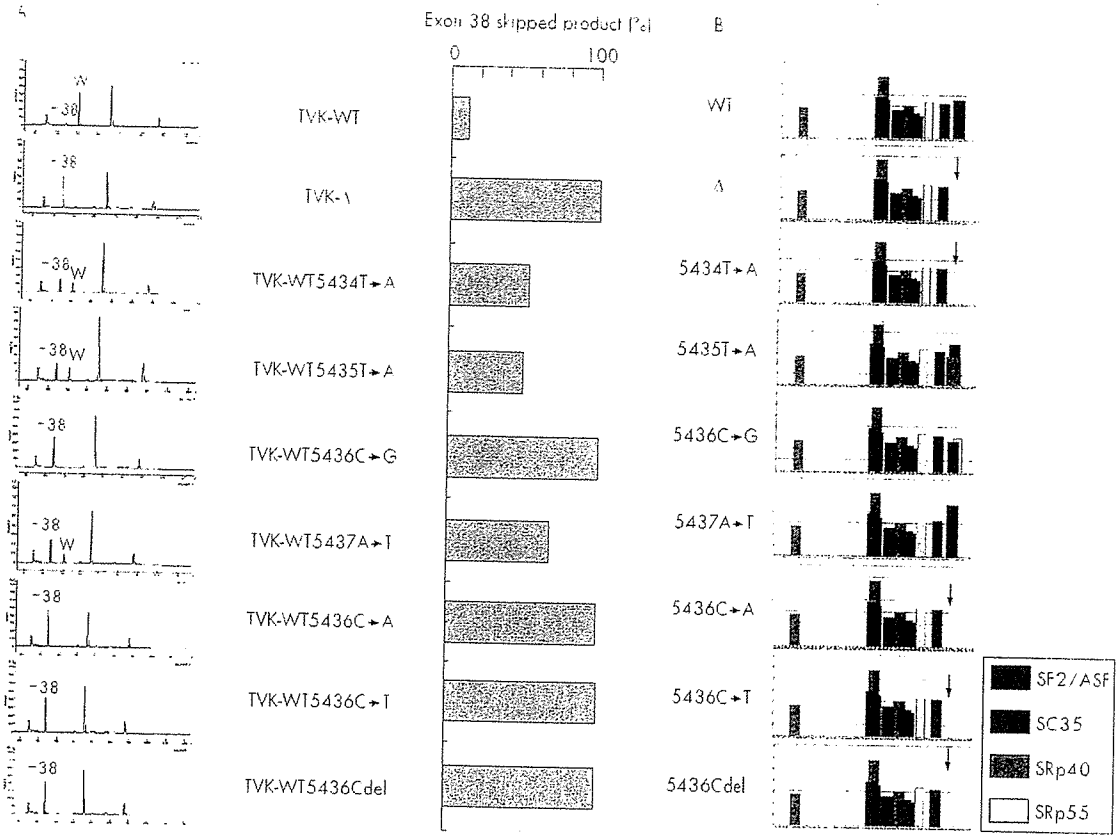

Figure 4 Splicing enhancer. (A) Semiquantitative measurement of in vivo splicing products. Capillary electrophoretic patterns from semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of minigene transcripts are shown (left). A fragment encompassing exon A to exon B was amplified. From their size, two peaks were identified: the 319-bp wild-type (WT) and the 196-bp exon 38-skipped product (−38). The area under the peak of each amplified product was quantified and the percentage of the total product, which was the exon 38-skipped product, was calculated and is graphically shown (right panel). Five minigene products (TVK-Δ, TVK-WT5436C→G, TVK-WT5436G→A, TVK-WTG5436→T and TVK-WT5436Gdel) completely lacked exon 38. (B) ESEfinder predictions of exonic splicing enhancers. ESEfinder predictions of exonic splicing enhancers in the wild-type exon 38 (WT) and a variety of exon 38 mutants including the 4-bp deletion (Δ) are shown. The arrows indicate the SC35 motif that disappeared as a result of the 4-bp deletion and some of the single nucleotide mutations. Exonic splicing enhancer motifs that had scores above the threshold for each SR protein (SF2/ASF, SC35, SRp40 and SRp55) are indicated with differently shaded bars. The heights of the bar reflect the scores of the motifs.

and consequently for splicing.[17] When exon 38 was analysed for candidate splicing enhancer motifs using the ESE-finder program,[18] many SR protein-binding motifs were identified (fig 4B). In particular, an SC35-binding site was found to be located at the deletion site (GACTTCAA, the underlined sequence was deleted in the index case). These findings suggested that disruption of a predicted SC35-binding site caused the skipping of exon 38 in the index case. Similarly, introduction of the C5436A, C5436T and C5436del mutations into exon 38 disrupted the predicted SC35-binding site (fig 4). This agrees with our experimental results (fig 4). Four results, however, indicated that the identified motifs were not absolutely responsible for the in vivo splicing reaction (fig 4). Firstly, C5436G induced complete exon skipping but maintained a predicted SC35-binding site and introduced an additional SRp55-binding site. Secondly, T5434A induced partial enhancement of exon skipping in vivo and disrupted a predicted SC35-binding site. Thirdly, T5435A improved exon skipping in vivo, but produced two additional SR-protein-binding sites while maintaining a predicted SC35-binding site. Finally, A5437T induced partial exon skipping but maintained the same binding site as the wild-type sequence.

The RESCUE-ESE program identified exonic splicing enhancers in several regions of exon 38. Two predicted enhancers, AAGACT and ACTTCA (the underlined sequences were deleted in the index case), were affected by the deleted region (data not shown). This again indicated that the disruption of an exonic splicing enhancer by the deletion caused the exon skipping observed in the index case.

DISCUSSION

Genomic DNA analyses led to the identification of a novel molecular basis for dystrophinopathy: a 4-bp deletion (ca 5434–5437delTTCA) in exon 38 of the dystrophin gene. The 4-bp deletion mutation created a premature stop codon and was expected to cause DMD. The patient's moderately raised serum creatine kinase level, however, was more indicative of BMD. Interestingly, the exon 38− transcript was found to be the sole product of the mutant dystrophin gene (fig 2C). The exon 38− transcript contained an uninterrupted translational reading frame, leading to the production of an internally deleted, semifunctional dystrophin protein. Careful follow-up with the index case will be necessary to make a conclusion about his phenotype.

Although 4-bp deletions have been found in some exons of the dystrophin gene, none has been reported in exon 38. The genomic region surrounding the deletion was searched for sequences that could have predisposed the area to a deletion

Intrastrand complementarity, which is known to cause small intraexonic deletions,[32] was ruled out because the exon 38 sequence is unlikely to form the required hairpin structure. Additionally, deletion motifs were not found in the genomic sequences.[33] The sequence AAAGA, however, was identified both 7 bp upstream and 2 bp downstream from the deletion (fig 2). Although the slipped-mispairing model[34] suggests that this repeat may have had a role in the generation of the 4-bp deletion, the exact mechanism that led to the TTCA deletion remains unclear.

Three models have been proposed to explain exon skipping caused by an exonic mutation. The first model is a nuclear scanning model in which a translation-like machinery in the nucleus preserves the integrity of the reading frame by surveying the sequence and inducing the skipping of exons containing premature termination codons during pre-mRNA splicing.[29] This model, however, does not explain our results, because the transcripts produced in the in vivo splicing assay did not contain a continuous open-reading frame. The second model is a secondary structure model in which a change in the pre-mRNA secondary structure leads to exon skipping.[30] The results from the in vivo splicing assay using minigenes that did not contain the full wild-type introns make it unlikely that this model underlies the skipping of exon 38. The third model, which is supported by our results, is a *cis*-element model in which disruption or creation of exonic splicing enhancers or silencers causes mutation-associated exon skipping.[31 35]

A growing number of disease-causing mutations have been shown to inactivate exonic splicing enhancers, and thereby cause exon skipping.[29 31] For the dystrophin gene, the disruption of exonic splicing enhancers has been reported in exons 19 and 27.[6 11 15] Our results showed that exon 38 contains an exonic splicing enhancer sequence. It has been reported that the strength of the splice sites and the position of the exonic splicing enhancer along the exon have major roles in the activity of the splicing enhancer.[31 36] Among the 79 dystrophin exons, exon 38 is classified as a normal exon according to the exon definition model[36], weak exons of the dystrophin gene are reported to need an exonic splicing enhancer sequence for proper recognition of the exon. In fact, exonic splicing enhancers have been identified in several dystrophin exons in experiments that searched for antisense oligonucleotides inducing exon skipping.[11 15]

The hybrid minigene proved to be a good model for the exon skipping induced by the deletion mutation (fig 3). This hybrid minigene system will facilitate the molecular understanding of mutations that are not classified as disease-causing mutations.[15] Although tissue-specific splicing patterns are well documented, our results suggest that lymphocytes and HeLa cells contain the same splicing machinery for dystrophin pre-mRNA splicing. As the deleted region was shown to be essential for proper splicing in two different cell types, the deleted sequence probably contributes to the constitutive splicing of exon 38 instead of alternative or tissue-specific splicing.[36]

Purine-rich exonic splicing enhancers activate splicing by binding SR proteins, which recruit the splicing machinery to adjacent splice sites.[37] Although individual SR proteins are known to exhibit distinct substrate specificities, the degeneracy of the RNA recognition sequences for the different SR proteins makes the prediction of SR-protein-dependent enhancers uncertain. The ESE-finder program showed that the deletion removed a predicted SC35-binding motif (GACTTCAA) from exon 38. Moreover, our in vivo splicing study (fig 4) suggests that a single nucleotide (C5436) is essential for proper splicing of exon 38. Although splicing error due to abnormal SC35 binding has been shown to cause genetic diseases,[38] a single nucleotide in a predicted SC35-binding site has never been shown to be determinant for proper splicing. Our finding will facilitate the understanding of splicing regulation by SR proteins.

Presently, there is no effective way to treat DMD. Recent treatments for DMD have focused on converting the DMD phenotype to a BMD phenotype by changing dystrophin mRNAs from out of frame to in frame. We have shown that induction of exon 19 skipping in a patient with DMD carrying a deletion in exon 20 led to the production of in-frame dystrophin mRNA and dystrophin-positive skeletal muscle cells.[18] Therefore, artificial induction of exon skipping using antisense oligonucleotides is now being extensively studied.[15 39–42] Our findings indicated that the 4-bp sequence TTCA is a good target for treatment with antisense oligonucleotides to induce exon 38 skipping. Seven nonsense mutations have been identified in exon 38 of patients with DMD. If these patients are treated with antisense oligonucleotides that are able to induce exon 38 skipping, the resulting dystrophin mRNA is expected to produce a truncated and semifunctional dystrophin protein. This would shed new light on designing antisense oligonucleotides used for treatment of DMD.

Our results provide important insights for the understanding of the molecular basis of splicing regulation and the clinical application of exon skipping treatment for patients with DMD.

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Competing interests: None declared

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Intravenous Infusion of an Antisense Oligonucleotide Results in Exon Skipping in Muscle Dystrophin mRNA of Duchenne Muscular Dystrophy

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ABSTRACT: Duchenne muscular dystrophy (DMD) is a fatal muscle wasting disease that is characterized by muscle dystrophin deficiency. We report that intravenous (IV) infusion of an antisense oligonucleotide created an in-frame dystrophin mRNA from an out-of-frame DMD mutation (*via* exon skipping) which led to muscle dystrophin expression. A 10-year-old DMD patient possessing an out-of-frame, exon 20 deletion of the dystrophin gene received a 0.5 mg/kg IV infusion of an antisense 31-mer phosphorothioate oligonucleotide against the splicing enhancer sequence of exon 19. This antisense construct was administered at one-week intervals for 4 wk. No side effects attributable to infusion were observed. Exon 19 skipping appeared in a portion of the dystrophin mRNA in peripheral lymphocytes after the infusion. In a muscle biopsy one week after the final infusion, the novel in-frame mRNA lacking both exons 19 and 20 was identified and found to represent approximately 6% of the total reverse transcription PCR product. Dystrophin was identified histochemically in the sarcolemma of muscle cells after oligonucleotide treatment. These findings demonstrate that phosphorothioate oligonucleotides may be administered safely to children with DMD, and that a simple IV infusion is an effective delivery mechanism for oligonucleotides that lead to exon skipping in DMD skeletal muscles. (*Pediatr Res* **59: 690–694, 2006**)

Duchenne muscular dystrophy (DMD) is the most common inherited muscular disease, affecting one in 3,500 male births. DMD is characterized by rapid, progressive muscle wasting that typically kills patients in their twenties. Complete muscle dystrophin deficiency is a common mechanism for DMD. Deletion mutations of the dystrophin gene that result in production of out-of-frame dystrophin mRNA have been identified in two-thirds of DMD cases. Although substantial progress has been made in the study of gene-replacement therapy for DMD, clinically significant results remain a distant goal (1–3). Therefore, alternative strategies for DMD treatment that use the endogenous, although defective, dystrophin gene are now attracting attention. Transformation of an out-of-frame mRNA into an in-frame dystrophin message—by inducing exon skipping and thereby enabling production of semi-functional internally deleted dystrophin—is considered one of the approaches most likely to lead to success (1,4,5).

We have reported that transfection of an antisense oligodeoxynucleotide that binds to a splicing enhancer sequence of exon 19 (AO19) induced exon-19 skipping in cultured lymphocytes (6). Transfection of AO19 into cultured myocytes isolated from a DMD patient with a deletion of exon 20 was shown to result in dystrophin expression through induction of exon 19 skipping (7). Along with our studies, induction of exon skipping through the use of antisense oligonucleotides has also been shown to stimulate dystrophin expression in cultured DMD myocytes harbouring dystrophin gene mutations (8–11). Antisense oligonucleotides are considered to have great potential for DMD treatment.

Although *in vitro* evidence for successful dystrophin expression with antisense oligonucleotides is accumulating, a clinically useful delivery method to transport these constructs into the skeletal muscles of DMD patients has not been established. Several *in vivo* studies have succeeded in producing muscle dystrophin expression in *MDX* mice, an animal model of DMD (1,12–15), but these studies used a carrier for delivery of the antisense oligonucleotides, which hampers its clinical application.

In several non-muscular diseases, phosphorothioate oligonucleotides have been injected IV (intravenous) without significant side effects (16,17). Recently, intraperitoneal injection of AO19, without a carrier, was shown to induce exon 19 skipping in both skeletal and cardiac muscles of *MDX* mice (18). Based on these findings, IV infusion of antisense oligonucleotide in the absence of a carrier was proposed as a potential mechanism for systemic delivery. However, no stud-

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Abbreviations: **AO19,** antisense oligodeoxynucleotide of exon 19; **BMD,** Becker muscular dystrophy; **CK,** creatine kinase; **DMD,** Duchenne muscular dystrophy; **nt,** nucleotides

ies have addressed infusion of antisense oligonucleotides in children. Infusion during childhood would be indispensable for an oligonucleotide treatment of DMD because the disease is fatal for patients in their early twenties.

This paper provides the first report of antisense oligonucleotide treatment of DMD. The purpose of the study was to examine both the safety of IV injection of antisense oligonucleotides in young patients and to determine whether the antisense construct induced exon skipping of dystrophin mRNA in diseased DMD muscles. These findings may pave the way for novel clinical applications of antisense oligonucleotide for the treatment of DMD.

MATERIALS AND METHODS

Case. A 10-year-old wheelchair-bound DMD patient with the deletion of exon 20 (242 nt) of the dystrophin gene was enrolled in this study. In our previous study, transfection of the AO19 (31-mer phosphorothioate oligodeoxynucleotides, 5′-GCCTGAGCTGATCTGCTGGCATCTTGCAGTT-3′)

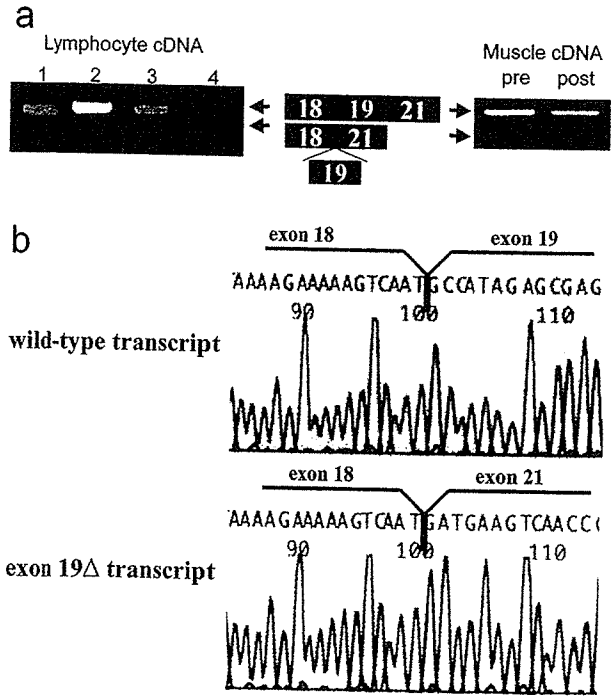

Figure 1. Dystrophin mRNA analysis. *Panel A*: Amplified products of dystrophin mRNA from exons 18 to 21. A fragment spanning exons 18 to 21 of dystrophin mRNA in lymphocytes (left) and muscle (right) was amplified using RT-nested or RT-PCR, respectively. One product was visualized in lymphocytes after the first and second infusions (lanes 1 and 2). An additional small band became visible on the third and fourth infusions (lanes 3 and 4), and the intensity of this band increased from the third to the fourth infusion. Sequencing of the two bands after sub-cloning indicated that the larger product corresponded to the wild-type product and consisted of exons 18, 19, and 20 (*panel B*). The lower-molecular-weight product contained the sequence of the 3′ end of exon 18 joining directly to the 5′ end of exon 21, lacking sequences from exon 19 completely (the exon 19Δ transcript) (*panel B*). RT-PCR amplification of dystrophin mRNA from a sample of the patient's right biceps biopsied one week after the last infusion showed that the exon 19Δ transcript was present in addition to the wild-type transcript (right). Densitometric analysis of the products showed that nearly 6% of the total amplification products were exon 19Δ transcript (post). Before treatment, no exon 19Δ transcript was visible (pre). *Panel B*: Sequencing results. Sequences at the junction of exon 18 and its downstream exons are shown for the wild-type transcript (upper) and exon 19Δ transcript (lower).

was shown to express dystrophin in the patient's cultured myocytes *via* production of an in-frame dystrophin by induction of exon 19 (88 nt) skipping (7).

Oligonucleotides. AO19 was synthesized by an automatic DNA synthesizer following good manufacturing practice (Proligo Co., Boulder, CO). The synthesized AO19 was dissolved in saline at a concentration of 10 mg/mL and filtered through a Millex-GV 0.22 μm filter unit (Milipore, Billerica, MA). The acute toxicity of AO19 was examined by injecting the solution into mice at a dose of 200 mg/kg. No harmful results attributable to infusion were observed over the 14-d observation period. Microscopic examination of mice liver and muscle obtained by necropsy reveal no significant histologic change. Adult healthy volunteers received one infusion of 0.5 mg of AO19 per kg of body weight. Clinical examinations, including liver function and coagulation tests, revealed no abnormality.

Protocol for AO19 infusion. AO19 at a dose of 0.5 mg/kg body weight was diluted in saline to make a 100-mL solution. The mixture was then infused into the peripheral vein over 2 h using an infusion pump. This infusion was repeated four times at one-week intervals.

The patient's body temperature, blood pressure, and heart rate were monitored during the infusion. Blood samples were obtained before and 2 d after each infusion. Complete blood counts, serum CK, aspartate transaminase, alanine transaminase, aldolase, lactate dehydrogenase, bilirubin, and blood coagulation activity were analyzed. The maximal voluntary isometric tongue produced by the elbow flexor muscles and knee extensor muscles was measured with a manual dynamometer (Microfet2 Digital Muscle Tester, Value Medical Supplies, Hesperia, CA) having a precision of 0.1Nm. The maximal voluntary isometric tongue was recorded in a 90° flexed position (elbow and knee) as the largest of two brief maximal extensions with a 1-min rest period between trials to prevent fatigue. One week after the final infusion, a muscle biopsy was performed on the patient's right biceps.

Ethical aspect. The protocol was approved by the ethical committee of the Graduate School of Medicine, Kobe University as a phase I/II clinical study (no. 194). Details of the protocol were fully explained to the patient's parents several times before obtaining signed informed consent.

Dystrophin mRNA analysis. Total RNA was isolated from peripheral lymphocytes that were collected from whole blood using Ficoll-Paque density gradients (Amersham Biosciences AB, Uppsala, Sweden) or thin-sliced (6 μm) muscle sections of frozen muscle samples. cDNA was synthesized using random hexanucleotides as primers and reverse transcriptase as described (19). To examine exon 19 skipping in dystrophin mRNA, a region-spanning exons 18 to 21 was amplified by nested PCR from lymphocyte cDNA or by single PCR from muscle cDNA (6,7). Amplified products were electrophoresed and the intensity of each band was measured by a densitometer (Agilent 2100, Agilent Technologies, Palo Alto, CA). Ten partially overlapping fragments spanning the entire coding region of the dystrophin mRNA were amplified to test for the possibility of a secondary splicing error (20).

The PCR-amplified products were sub-cloned into the pT7 blue T vector (Novagen, Madison, WI) and sequenced using a *Taq* dye termination-cycle sequence kit (PerkinElmer Applied Biosystems, Norwalk, CT) with an automatic DNA sequencer (model ABI Prism 310 Genetic Analyzer; PerkinElmer Applied Biosystems) as previously described (21).

Immunohistochemical analysis. The muscle biopsy sample was examined histologically. An indirect immunofluoresence analysis was performed using three dystrophin MAb that recognize the N-terminal-(NCL-Dys3), the rod-(NCL-Dys1), and the C-terminal-(NCL-Dys2) domains of dystrophin (Novocastra Laboratories, Ltd., Burlingame, CA). The sample was also stained with anti-merosin (Chemicon, Temecula, CA) and anti-α-sarcoglycan (Novocastra Laboratories, Ltd., Burlingame, CA) MAb. Normal skeletal muscle tissue was simultaneously stained with the panel of antibodies as a control. The conditions for immunostaining were previously described (22).

RESULTS AND DISCUSSION

After IV infusion of AO19, no apparent adverse reactions were observed in the patient's vital signs, complete blood counts, blood coagulation activity, or serum bilirubin levels during the treatment period. Serum CK, a marker of muscle damage in DMD, was 2402 IU/L (normal: 153 ~ 249 IU/L) before the treatment, and 4753, 2398, 3060, and 2461 IU/L

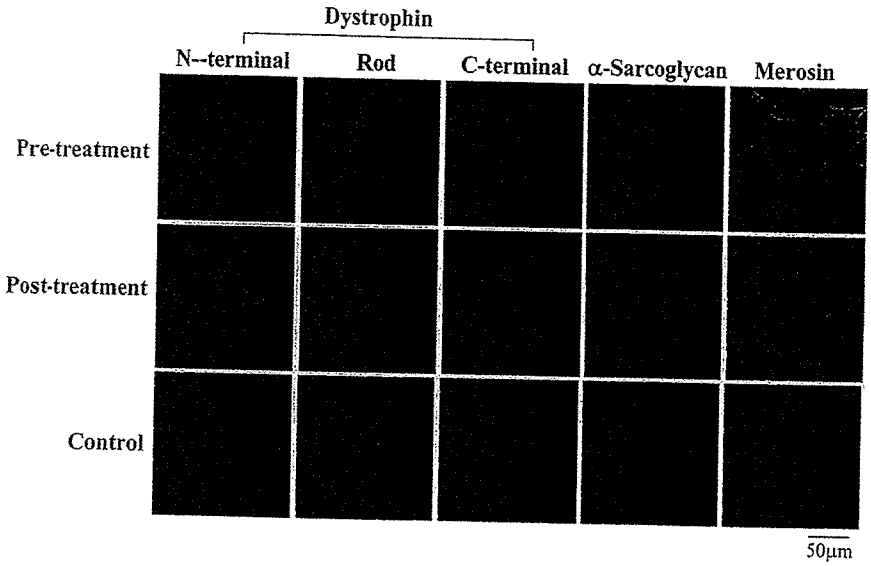

Figure 2. Immunostaining of the biopsied muscles. The biopsied muscle sample was stained for dystrophin using three MAb recognizing the N-terminal-, rod-, and C-terminal domains. No antibody-reactive material was observed in any muscle cells before the infusion (pre-treatment). Every cell stained weakly either continuously or discontinuously in the sarcolemma with the three anti-dystrophin antibodies after the infusion, indicating that the dystrophin produced as a result of exon skipping maintained the N- to C-terminal domains (post-treatment). The control was dystrophin-stained normal muscle tissue. One of the dystrophin-associated proteins was also stained (α-sarcoglycan). Merosin was stained as a reference.

after the first, second, third, and fourth infusions, respectively. The trial did not change serum CK levels. Additionally, no apparent muscle strength improvement was observed. These results demonstrate that the AO19 oligonucleotide can be administered safely to a young DMD patient.

Although IV infusion of antisense oligonucleotides has been performed for several diseases (16,17), the effect on splicing patterns in peripheral lymphocytes has never been explored. We have been analyzing illegitimate dystrophin mRNA in peripheral lymphocytes for detection of mutation of the dystrophin gene. Reverse transcription-nested PCR has been used to examine dystrophin mRNA expressed in peripheral lymphocytes, and we have succeeded in detecting splicing abnormalities in DMD patients (22). It was not known whether antisense oligonucleotides work to induce exon skipping in lymphocytes of the patient. The pattern of splicing of dystrophin pre-RNA in lymphocytes was analyzed to assess the delivery of AO19 to the peripheral lymphocyte nucleus in the index case. A fragment spanning exon 18 to 21 of dystrophin cDNA was nested-PCR amplified in the patient's peripheral lymphocytes (Fig. 1*A*). A single amplified product consisting of exons 18, 19, and 21 was obtained after the first and second infusions (Fig. 1*A*, lanes 1 and 2). This result indicated that no exon 19 skipping had occurred. Remarkably, an additional small band was observed in PCR amplification products in addition to the native fragment, after the third and fourth infusions (Fig. 1*A*, lanes 3 and 4). Sequencing of the small additional PCR product disclosed that the 3′ end of the exon 18 sequence was joined directly to the 5′ end of the exon 21 sequence, indicating a complete disappearance of the exon 19 sequence (exon 19Δ transcript, Fig. 1*B*). This result indicated that AO19 infused *via* a venous line could be delivered to the lymphocyte nucleus where it induced exon 19 skipping. In contrast with *in vitro* transfection (6), AO19 was delivered to peripheral lymphocytes without carrier.

These findings are the first to show that exon skipping can be induced in peripheral lymphocytes by infusion of antisense oligonucleotides. IV They suggest a wider application for antisense oligonucleotide treatment that targets the immunologic activity of lymphocytes. However, it should be noted that the efficient delivery of AO19 to lymphocytes may be due to a DMD-specific condition. Further study is required to determine whether lymphocytes are good therapeutic targets for antisense oligonucleotides.

The *in vivo* production of exon 19Δ transcript suggested that skipping of exon 19 may also be induced in the patient's skeletal muscles where dystrophin deficiency severely damages the membrane structure (23). The biopsied biceps muscle sample obtained after the treatment showed severe fatty degeneration and a limited number of muscle fiber clusters. Reverse transcription-PCR amplification of a fragment encompassing exons 18 to 21 revealed two products; one major product corresponding to the wild-type transcript and another weaker and smaller product corresponding to the exon19Δ transcript (Fig. 1*A*). The exon 19Δ transcript was calculated to be approximately 6% of the total PCR product. Contamination of the exon 19Δ transcript from lymphocytes present in the blood stream was ruled out because not a single PCR, but a nested-PCR amplification of dystrophin mRNA is required to produce the product from lymphocyte dystrophin cDNA (24). Before the treatment, in contrast, a single amplified product that corresponded to the wild-type transcript was obtained from muscle tissue (Fig. 1*A*). These results demonstrate that IV infused antisense oligonucleotides were delivered to the nucleus of skeletal muscles without using carrier material. This result indicates that damaged DMD muscle membranes are far more permeable to oligonucleotides than expected (23). The delivery mechanism is presumed to be diffusion from the bloodstream into the cellular cytosol, similar to the method of CK diffusion from the cytosol to the bloodstream.

Although AO19 was designed to be complementary to the exon splicing enhancer sequence (deliberately avoiding the exon-intron junction consensus sequences), additional erroneous splicing might be expected to complicate AO19 treatment. In fact, in another study, multiple splicing errors were induced by blocking a splicing consensus sequence with an antisense oligonucleotide in *MDX* mice (25). The full-length dystrophin cDNA, consisting of 79 exons, obtained from the patient's