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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ORIGINAL ARTICLE

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

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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.[3] 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.[3–10]

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.[13–16]

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 (KUHPCG414) was referred to the Kobe University Hospital, Japan, for a genetic diagnosis of his

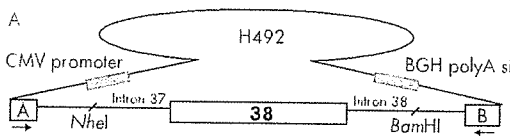
Abbreviations: BMD, Becker's muscular dystrophy; DMD, Duchenne's muscular dystrophy; PCR, polymerase chain reaction; RT, reverse transcription; TVK-Δ, mutant hybrid minigene; TVK-WT, wild-type hybrid minigene

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 1074 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.[11] 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).



B

	3' end of exon 38 ↓
1 TVK-WT	GAGGAAGAC**TTCA**ATAAAGATATG
2 TVK-Δ	GAGGAA[GAC]ATAAAGATATG
3 TVK-WT5434T→A	GAGGAAGAC[A]**TCA**ATAAAGATATG
4 TVK-WT5435T→A	GAGGAAGAC**T**[A]**CA**ATAAAGATATG
5 TVK-WT5436C→G	GAGGAAGAC**TT**[G]**A**ATAAAGATATG
6 TVK-WT5437A→T	GAGGAAGAC**TTC**[T]ATAAAGATATG
7 TVK-WT5436C→A	GAGGAAGAC**TT**[A]**A**ATAAAGATATG
8 TVK-WT5436C→T	GAGGAAGAC**TT**[T]**A**ATAAAGATATG
9 TVK-WT5436Cdel	GAGGAAGAC**TT**[ ]**A**ATAAAGATATG

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

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).[18] 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.

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.[11] PCR was carried out using a forward primer

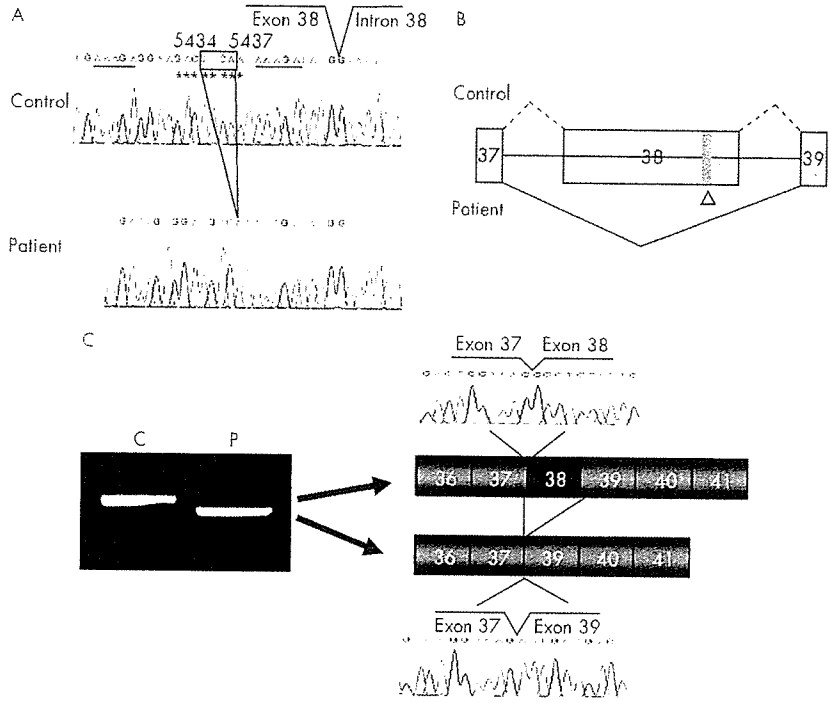


Figure 2 Analysis of the dystrophin gene and mRNA. (A) Genomic DNA analysis. Sequences of the 3′ end of exon 38 and the 5′ end of intron 38 of the control (upper panel) and the patient (lower panel) are shown. Four nucleotides (TTCA; boxed) located 11 bp upstream from the 3′ end of exon 38 were missing 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 GCA 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.[20] 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.[21]

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 9804A→G in exon 67), these changes were variants leading to an amino acid change.[22] 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 5434–5437del TTCA; 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

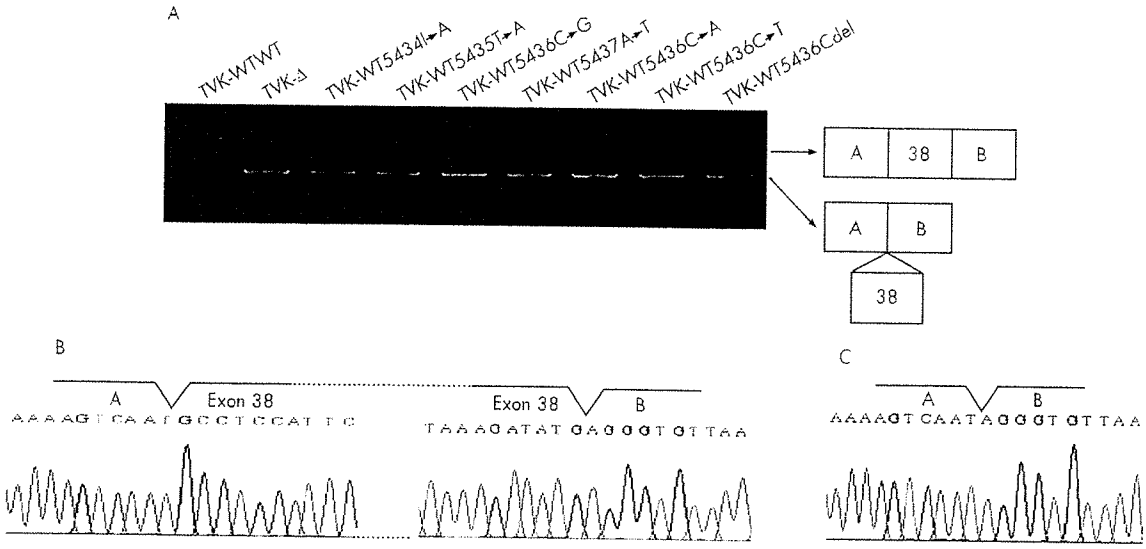


Figure 3 Hybrid minigene splicing. Hybrid minigenes containing the indicated variants were tested in an in vivo splicing assay and the resulting mRNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). (A) Products electrophoresed on a polyacrylamide gel is shown. A 319-bp full-length 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,[3] 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.[24] 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 (14 of 20 nucleotides are purines). Purine-rich sequences are known to serve as recognition motifs for SR proteins, which are required to define exons

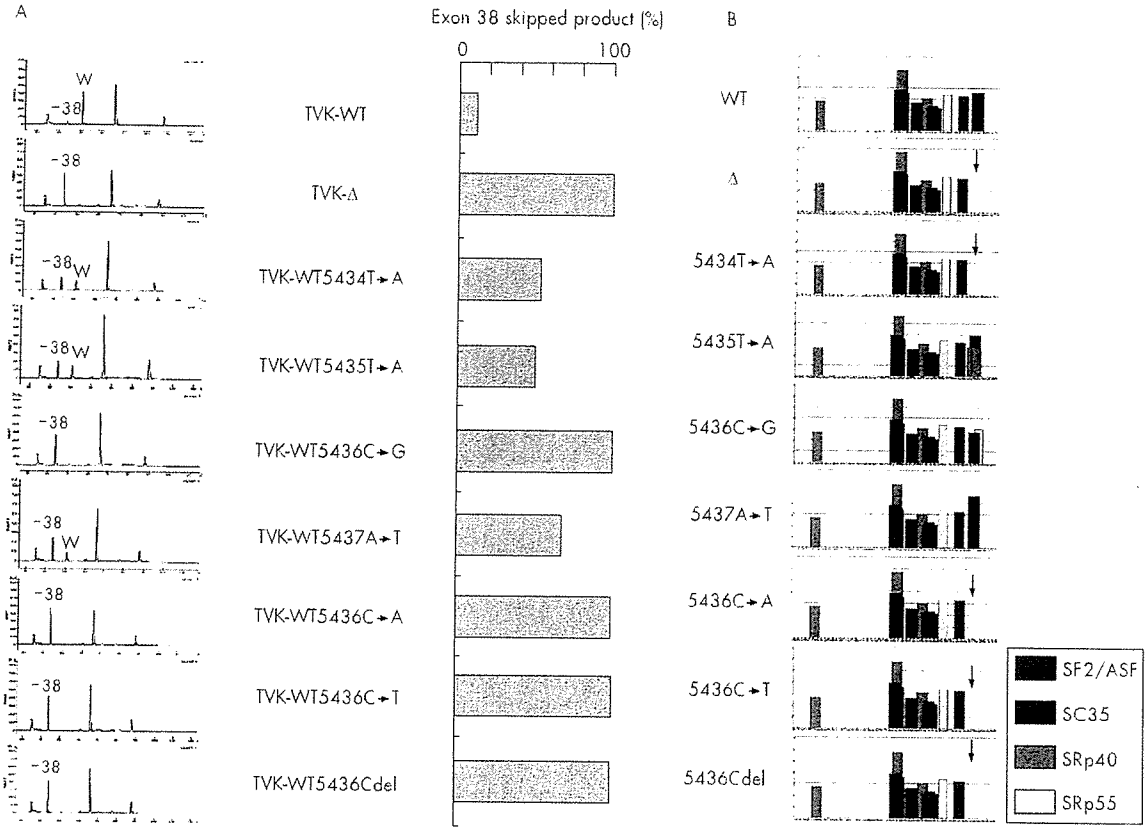


Figure 4 Splicing enhancer. (A) Semiquantitative measurement of in vivo splicing products. Capillary electrophoretic patterns from semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of minigene transcripts are shown (left). A fragment encompassing exon A to exon B was 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.[26] When exon 38 was analysed for candidate splicing enhancer motifs using the ESE-finder program,[27] 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,[26] 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.[27] The sequence AAAGA, however, was identified both 7 bp upstream and 2 bp downstream from the deletion (fig 2). Although the slipped-mispairing model[28] 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.[31–33]

A growing number of disease-causing mutations have been shown to inactivate exonic splicing enhancers, and thereby cause exon skipping.[28 31] For the dystrophin gene, the disruption of exonic splicing enhancers has been reported in exons 19 and 27.[6 11 12] 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.[32 34] Among the 79 dystrophin exons, exon 38 is classified as a normal exon according to the exon definition model[35]; 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.[13 14]

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.[18] 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.[25] 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,[7] 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.[13 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′) 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).

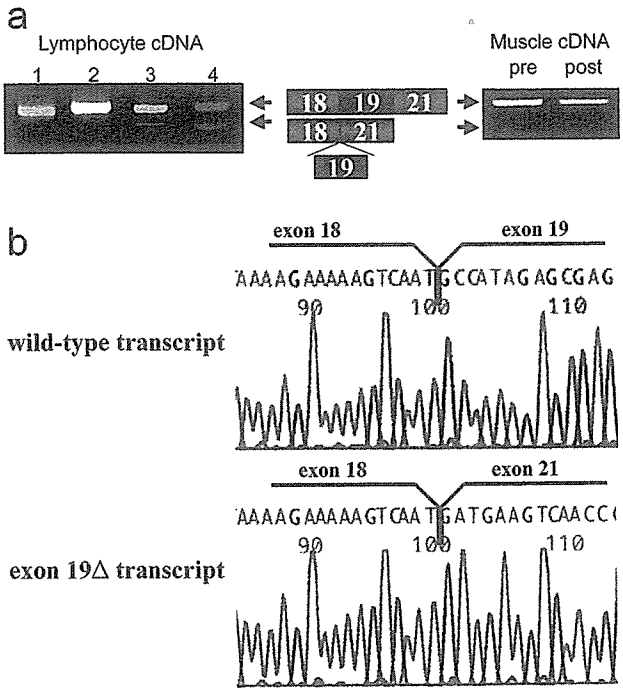

Figure 1. Dystrophin mRNA analysis. *Panel A*: Amplified products of dystrophin mRNA from exons 18 to 21. A fragment spanning exons 18 to 21 of dystrophin mRNA in lymphocytes (left) and muscle (right) was amplified using RT-nested or RT-PCR, respectively. One product was visualized in lymphocytes after 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).

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

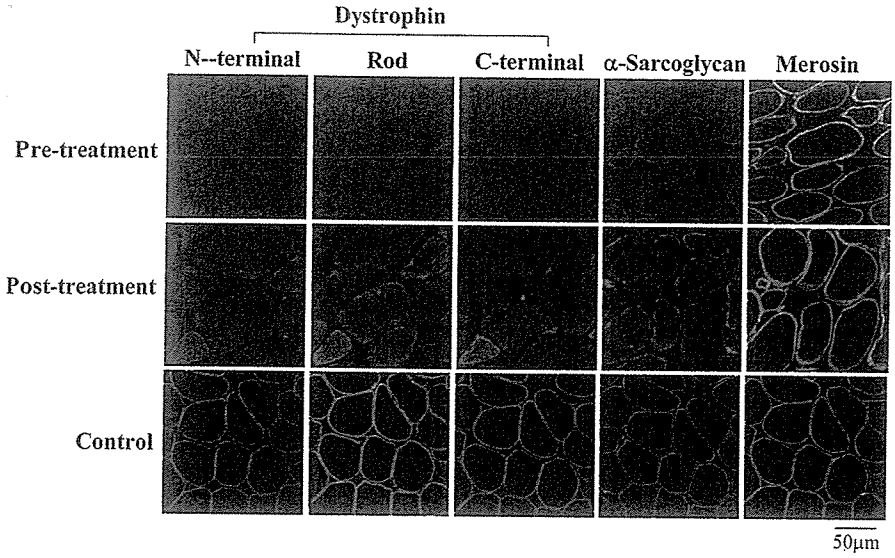

Figure 2. Immunostaining of the biopsied muscles. The biopsied muscle sample was stained for dystrophin using three MAb recognizing the N-terminal-, rod-, and C-terminal domains. No antibody-reactive material was observed in any muscle cells before the infusion (pre-treatment). Every cell stained weakly 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

post-treatment muscle tissue was examined for this possibility. Ten separate fragments were amplified. Every fragment, except the one containing exon 19, amplified exactly as expected (data not shown). This result indicated that the splicing error was limited to exon 19 skipping. This specificity was likely because our target for AO19 was a splicing enhancer sequence and not a consensus sequence for splicing.

Since our trial succeeded in producing an in-frame exon 19Δ transcript in skeletal muscle (Fig. 1), the production of internally-deleted dystrophin protein was examined. Immunohistochemical examination of the biopsied muscle obtained before treatment using three MAb that recognize different epitopes of dystrophin identified no reactive material (Fig. 2). However, the three dystrophin-reactive MAb did weakly identify protein along the sarcolemma with varied density in every muscle cell (nearly 540 muscle cells total) after treatment. These staining patterns differed from that of revertant fibers or natural dystrophin-positive fibers observed in DMD (26) as follows. Positive fibers were observed only after infusion of the antisense oligonucleotide (Fig. 2). The percentage of dystrophin-positive fibers was high (Fig. 2). This contrasts with revertant cells, which are usually observed in only a few percent of all muscle fibres (27,28). The intensity of dystrophin staining was not strong as seen with revertants. Stained cells did not cluster.

Although newly expressed dystrophin in cultured myocytes was localized in the cytosol (7), newly expressed dystrophin *in vivo* appeared to be located in the sarcolemma. This result indicates that dystrophin synthesized *in vivo* in a progressive stage of DMD maintains the ability to be localized to the sarcolemma. The intensity of α-sarcoglycan staining increased with AO19 treatment (Fig. 2), indicating a stabilization of α-sarcoglycan in the sarcolemma. This treatment appeared to restore the dystrophin-associated glycoprotein complex. Unfortunately, immunoblot analysis of the biopsied muscle sample could not be performed due to severe fatty degeneration.

The dystrophin produced from the patient's modified mRNA was expected to lack 110 amino acids of the rod-domain. It is known that a large portion of the rod domain of dystrophin can be removed without significantly affecting the patient's muscle function (29,30). The largest in-frame deletion of the dystrophin gene previously reported in a Becker muscular dystrophy (BMD) patient encompassed exons 13 to 48 (30). The truncated dystrophin produced in the patient would be expected to be semi-functional. However, in our experiment, no apparent improvement in muscle power was observed (data not shown) and the patient remained wheelchair bound after the treatment. In our case, the immunologic reaction against the newly synthesized dystrophin should be carefully monitored from this point on.

Our report is the first to show successful induction of exon skipping in skeletal muscles in a case of DMD. This study provides three important findings for the potential clinical application of antisense oligonucleotide treatment. First, IV infusion is a simple way of delivering oligonucleotides. Second, no carrier material was necessary for delivery of oligonucleotides in our experiment. Third, the amount of the infused oligonucleotides was only 0.5 mg/kg, a dose lower than that used for other diseases (31). This treatment circumvents the side effects associated with direct muscle injection, the immunogenicity of carriers, and the accumulation of degradation products of antisense oligonucleotides (1,12). This simple technique would make possible life-long treatment of DMD with antisense oligonucleotide.

This treatment, which salvages mRNA transcribed from the endogenous dystrophin gene, would be expected to produce dystrophin in a physiologically appropriate manner, since the internally deleted dystrophin translated from the edited mRNA was expressed in the proper tissues, at the proper times, and in the proper isoforms. In fact, dystrophin expression from exon-skipped mRNA has been reported in cases of BMD with skipping of the exon encoding nonsense mutations (32–35). Antisense oligonucleotides can be synthesized chemically in large scale, reducing the cost. Therefore, more than 70% of DMD cases could be treated in this manner in the near future (10).

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ORIGINAL INVESTIGATION

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Co-occurrence of mutations in both dystrophin- and androgen-receptor genes is a novel cause of female Duchenne muscular dystrophy

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Abstract Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder. Here, we report a novel mechanism for the occurrence of DMD in females. In a Vietnamese DMD girl, conventional PCR amplification analysis disclosed a deletion of exons 12–19 of the dystrophin gene on Xp21.2, with a karyotype of 46, XY. Furthermore, a novel mutation in the androgen-receptor gene on Xq11.2-q12 was identified in this girl, which led to male pseudohermaphroditism. Co-occurrence of mutations of these two genes constitutes a novel mechanism underlying female DMD.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessively inherited muscle-wasting disorder caused by mutations in the dystrophin gene on Xp21.2. DMD is common in boys, with a worldwide incidence of about 1 in 3,500 male births; only in rare instances DMD has been diagnosed in girls. Four mechanisms are known to cause female DMD: (1) a skewed pattern of X-chromosome inactivation in carriers of the mutation of the dystrophin gene (Azofeifa et al. 1995); (2) X;autosome translocations that disrupt the dystrophin gene (Cantagrel et al. 2004); (3) monosomy X (Turner syndrome) associated with a DMD mutation on the remaining X chromosome (Chelly et al. 1986); and (4) maternal isodisomy for the X chromosome carrying a DMD mutation (Quan et al. 1997).

We report for the first time a female DMD patient with co-occurrence of mutations in both the dystrophin- and androgen-receptor genes. This constitutes the fifth reported mechanism underlying female DMD.

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Case

A 9-year-old Vietnamese girl was born to a non-consanguinous couple. At age six, she was referred to the National Children's Hospital in Hanoi, Vietnam, for muscle weakness. She showed Gowers' sign, and her serum creatine kinase (CK) level was 25,392 IU/l (normal: 57–197 IU/l). She was diagnosed clinically with female DMD. At age nine, she could not stand up by herself, but the girl could walk independently starting from a standing position. Laboratory studies demonstrated high serum CK levels, reaching 9,420 IU/l. Her 18-year-old sister did not complain of muscle weakness, and she had a serum CK level of 135 IU/l. After obtaining informed consent, genetic analysis of the girl and her family members was conducted.

Results

Conventional PCR amplification of 19 deletion-prone exons of the dystrophin gene disclosed deletions of exons 12, 13, 17 and 19 in the patient's genomic DNA (Fig. 1a). The deletion was confined to exons 12–19, as fragments of the flanking exons 11 and 20 were successfully amplified (Fig. 1a) (Adachi et al. 2003). This deletion shifted the translational reading frame and created a premature stop codon in exon 20 of the dystrophin mRNA, confirming the diagnosis of DMD. To test for monosomy X, the patient's chromosome was

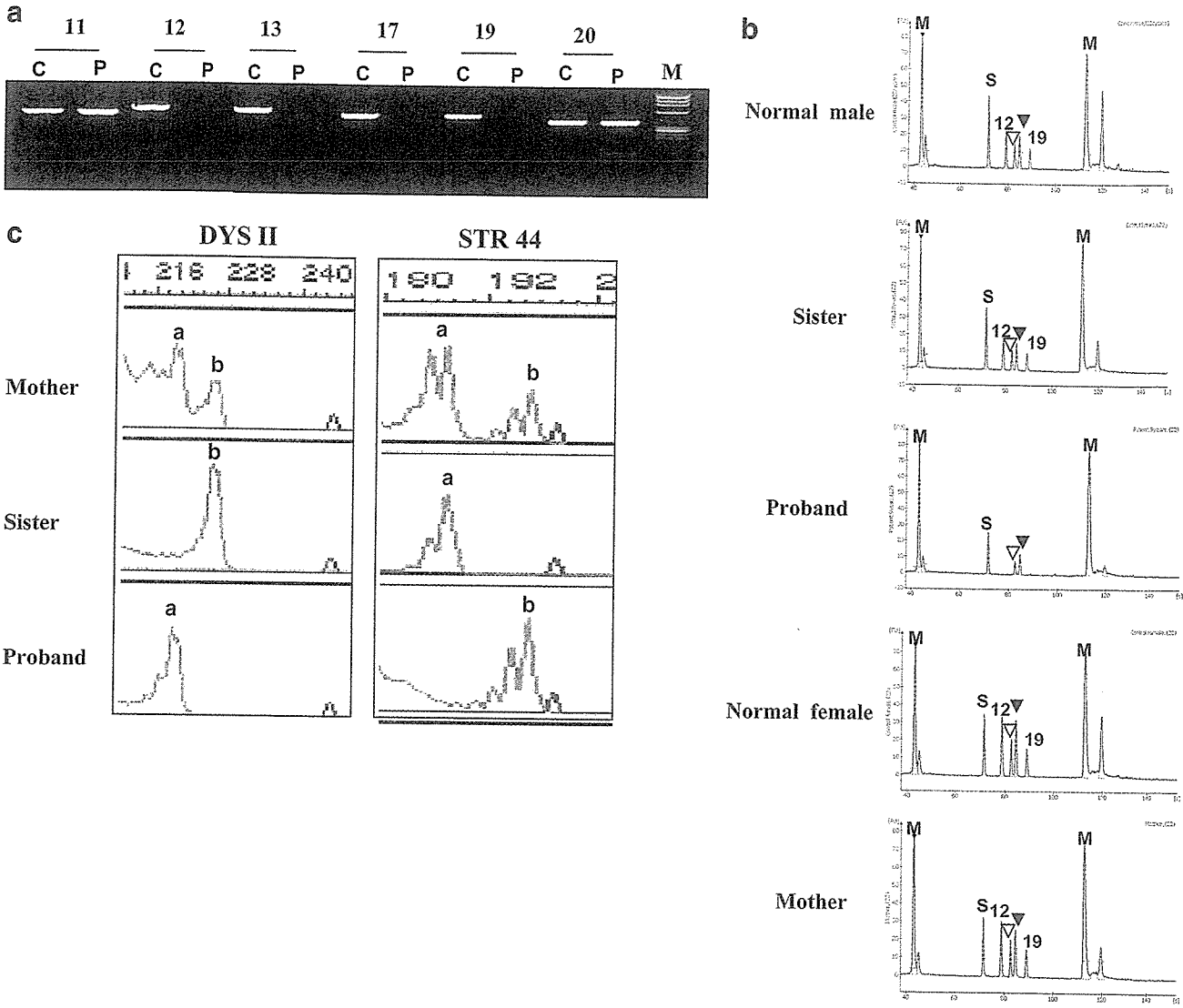

Fig. 1 Analysis of the dystrophin gene. **a** *Deletion mutation analysis of the dystrophin gene.* Results of PCR amplification of exons 11, 12, 13, 17, 19 and 20 of the dystrophin gene are shown. From the proband, no PCR products corresponding to exons 12, 13, 17 or 19 were obtained, whereas exons 11 and 20 were visualized (*P*); this pattern confirms the deletion of exons 12–19. From the control sample, every exon is shown as an amplified product (*C*). Numbers over the amplified products indicate their respective exon numbers. *M* refers to a size marker (*Hae*III-digested ΦX174 DNA, Takara Shuzo Co. Japan). **b** *Semi-quantitative PCR amplification of multiple exons.* Results of capillary electrophoresis (Agilent 2001, Bioanalyzer with DNA 1000 Lab Chips, Agilent Technologies, Palo Alto, CA) of PCR-amplified products are shown. Four exon fragments, including those corresponding to exons 11, 12, 19 and 20, and an internal control fragment (a fragment of the Ca^{2+}-sensing receptor gene on 3q13) were co-amplified in one PCR reaction. In a normal male and his sister, four peaks corresponding to four exons are observed in addition to the internal standard. Exons 12 and 19 are marked by their respective numbers, and exons 11 and 20 are marked by *open and filled arrow heads*, respectively. *S* refers to the internal standard that is an amplified product of the Ca^{2+}-sensing gene. *M* refers to size markers of 15 and 1,500 bps. The proband has no amplified product for exons 12 and 19 (*middle*). In the mother, the amount of amplified fragments of exons 12 and 19 were calculated and found to be equivalent to that of the normal female control (*bottom*). **c** *Polymorphism analysis.* Segregation analysis of the dystrophin gene was performed by analyzing $(CA)_n$ repeat markers in the promoter region (DYSII) and in intron 44 (STR44). Two peaks of amplified products at DYSII and the STR44 repeat region were obtained from the mother. At each locus, a and b allele names are assigned to the small and large products, respectively (*Top*). The proband had a and b alleles at DYSII and STR44, respectively (*bottom*), whereas her sister had b and a alleles, respectively (*middle*)

analyzed and was found to be 46, XY (Fig. 2a). To identify the cause of the patient's male-to-female phenotypic conversion, we tested for mutation of the androgen-receptor gene. A novel single-nucleotide change from C to T at bp 82 in exon 1 (C82T) was identified (Fig. 2b). The mutation changes codon 28 from CAG for glutamine to TAG for a stop codon (Q28X). This mutation, which leads to complete androgen insensitivity, was concluded to be the cause of male pseudohermaphroditism in this case.

Remarkably, the patient's sister was also found to have a 46, XY karyotype, due to the same nonsense mutation in the androgen-receptor gene (Fig. 2b). Furthermore, their mother was found to be a carrier for the

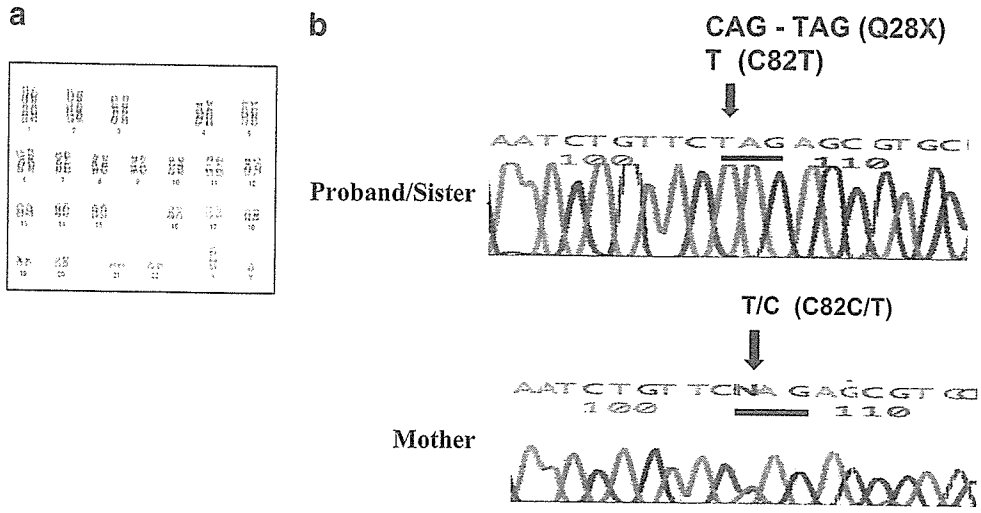

Fig. 2 Analysis of sexual state of the index case. **a** *Karyotype.* A karyotype of the proband (46, XY) is shown. **b** *Mutation of the androgen-receptor gene.* Mutation of the androgen-receptor gene was analyzed by sequencing exon-encompassing regions. The PCR amplification of exons 2–8 was carried out as described previously, and exon 1 was amplified as five separate fragments using five sets of primers. Amplified products were either directly sequenced or sequenced after subcloning into vector pT7 (Novagen Inc., Madison, WI) with an automated DNA sequencer (model 373A, Perkin-Elmer Applied Biosystems Inc., Norwalk, CT). A part of the sequence of exon 1 of the androgen-receptor gene is shown. The proband and her sister, both have a C-to-T nucleotide change at bp 82 within exon 1 (C82T) (*top*). The mother has double peaks corresponding to T and C at bp 82 (*bottom*). This nucleotide change altered the 28th codon from CAG to TAG (stop; Q28X)

nonsense mutation. It was concluded that the X chromosome harboring the nonsense mutation of the androgen receptor was inherited by both offsprings from their mother.

On the other hand, the sister's DNA had no deletions in any examined dystrophin exons, and was concluded to be free of DMD. The mother was also found to be a noncarrier for the deletion (Fig. 1b). The deletion mutation of the patient was concluded to be a de novo mutation. Analysis of polymorphisms at both the promoter region (DYSII) and intron 44 (STR44) of the dystrophin gene disclosed that the index case inherited the a and b alleles in DYSII and STR44, respectively, whereas her sister harbored the b and a alleles at those respective loci (Fig. 1c). It is reasonable to consider that complex recombination events occurred in the proband's genomic DNA.

Discussion

We report here a novel mechanism leading to female DMD, which is caused by the co-occurrence of a deletion mutation in the dystrophin gene and a nonsense mutation in the androgen-receptor gene. This is only the fifth mechanism reported so far to cause DMD in females. Molecular genetic analysis of family members disclosed that the deletion mutation of the dystrophin gene was acquired in the index case, whereas the nonsense mutation in the androgen-receptor gene was inherited from her mother. Although the two mutations are independent, our findings suggest that the co-occurrence of mutations in both the dystrophin- and androgen-receptor genes should be examined in cases of DMD in females.

Deletion of exons 12–19 of the dystrophin gene is relatively rare among the DMD-causing deletion mutants. The segregation pattern of haplotypes of the dystrophin gene disclosed that three haplotypes within the dystrophin gene were completely different between the two sisters (Fig. 1c). However, no changes recognizable by chromosomal analysis were present in the proband's X chromosome (Fig. 2a), indicating the occurrence of subtle genomic recombinations. Given the complexity of the allelic differences, it is difficult to propose a recombination model to explain the haplotype difference.

The androgen-receptor signaling pathway plays a key role in muscle development and in sexual differentiation. Complete androgen insensitivity is usually characterized by female masculization, and therefore DMD with androgen insensitivity would be expected to manifest as an aggravated progression of muscle wasting. However, the index case, which indeed had combined DMD and complete androgen insensitivity, did not exhibit such accelerated degeneration, suggesting that muscle growth at younger ages is independent of androgen-receptor signaling. This finding may be relevant to the currently controversial issue of steroid treatment in DMD (Dubowitz 2005).

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Novel Cryptic Exons Identified in Introns 2 and 3 of the Human Dystrophin Gene with Duplication of Exons 8-11

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The dystrophin gene, which is mutated in Duchenne muscular dystrophy, is the largest known human gene and characterized by the huge size of its introns. Intron 2 has been shown to include cryptic exons termed exons 2a and 2b, while intron 3 has been shown to include a cryptic exon designated exon 3a. In the present study, we identified 2 and 1 additional cryptic exons in introns 2 and 3, respectively. A previously unknown 157-bp insertion was identified between exons 2 and 3 of a dystrophin mRNA isolated from the lymphocytes of a dystrophinopathy patient with duplication of exons 8-11. Since this sequence exhibited the typical characteristics of a genomic exon, we designated it "exon 2c-l". A more detailed examination revealed that a position 4 bp downstream from the 5' end of exon 2c-l was also used as a splice acceptor site, and this exon was designated "exon 2c-s". In the same patient, a 357-bp insertion was identified between exons 3 and 4. Since this sequence also showed the typical characteristics of an exon, and its 3' end was the same as the splice donor site of exon 3a, we designated the novel cryptic exon "exon 3a-l", and changed the name of the previously reported exon 3a to "exon 3a-s". Among these novel cryptic exons, exon 3a-l was also incorporated into the dystrophin mRNA from normal lymphocytes, whereas exons 2c-l and 2c-s were not. The physiological or pathophysiological roles of these novel cryptic exons remain to be clarified.

The human dystrophin gene, which is defective in patients with Duchenne or Becker muscular dystrophy (DMD/BMD), encodes a dystrophin protein that is located along the plasma membrane of muscle cells (3). It spans approximately 3,000 kb of the X-chromosome and encodes a 14-kb transcript consisting of 79 exons (2,21). Genomic structural analyses have revealed at least eight alternative promoters over the entire dystrophin gene, producing tissue-specific dystrophin isoforms (2,21). Consequently, more than 99% of the gene sequence is comprised of introns which have been considered to be functionless. To date, four cryptic exons (exon 1a from intron 1, exons 2a and 2b from intron 2, and exon 3a from intron 3) have been reported to be incorporated into dystrophin mRNA (7,23,30,36). Furthermore, we previously identified six novel alternative splicing patterns in the 5' region of the dystrophin gene in addition to the six already-known patterns (33), indicating that exon usage in the 5' region of the dystrophin gene is highly complicated.

Splicing is the process that removes introns from pre-mRNAs, thereby producing mature mRNAs that only consist of exons. The presence of well-defined cis elements, namely the 5' and 3' splice sites and a branch point, are necessary but not sufficient to define the intron-exon boundaries in pre-mRNAs (26). Pseudoexons that match the splice-site

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