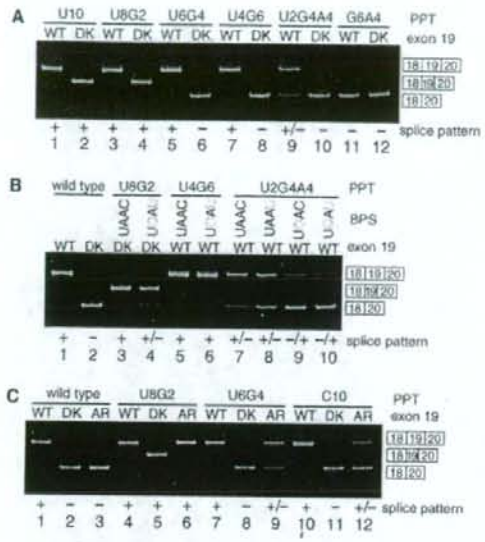

Fig. 3. **Splice pattern analysis using HeLa cells.** (A) Representative results of PPT exchange experiments. Samples were prepared in the same way as those shown in Fig. 1B. A 267 bp fragment (for example, lane 1) and a 179 bp fragment (for example, lane 6) show that WT exon 19-included and skipped splice pattern shown as Fig. 1B, respectively. A 220 bp fragment (for example, lane 2) shows that DK exon 19 is included in the mature mRNA. A schematic description of these RT–PCR products is shown on the right. The names of the tested PPTs (Fig. 2) and exon 19 types (Fig. 1A) are shown on the top. Splice patterns for exon 19 inclusion (+) and exon 19 skipping (−) are shown on the bottom. If the sample showed both included and skipped bands, when included bands were dominant they are shown as +/−, and when skipped bands were dominant they are shown as −/+. (B) Representative results of BPS exchange experiments. Samples were prepared in the same way as above. The names of the tested PPTs (Fig. 2), the last 4 nt of the BPS and the type of exon 19 are shown on the top. PPT name 'wild type' represents the same sequence as the genomic dystrophin gene, the same as the upper sequence shown in Fig. 1C. The first 3 nt (UAC) of the BPS are not shown as they are the same for all tested pre-mRNAs; the last 4 nt are shown in solid letters where they are the same nucleotides as consensus (UAAC) and outlined letters represent mutated nucleotides. A schematic description of the RT–PCR products and splice pattern for exon 19 is shown as in (A). (C) Representative results of exon length exchange experiments. Samples were prepared in the same way as above. The names of the tested PPTs, type of exon 19, schematic description of RT–PCR products and splice pattern for exon 19 are shown as in (A).

with purines or all 10 uridines were replaced with cytosines (Fig. 2). The results showed that dys-ESE19 is not necessary for proper splicing if the PPT contains eight or more uridines; however, if the PPT contains six or fewer uridines, dys-ESE19 is necessary (Fig. 3A, lanes 4 and 6). On the other hand, when the exon contains dys-ESE19, as few as four uridines are sufficient for proper exon 19 usage (Fig. 3A, lane 7), and even two uridines are enough for proper splicing of ~90% of the transcript (Fig. 3A, lane 9; Fig. 4). These results suggest that

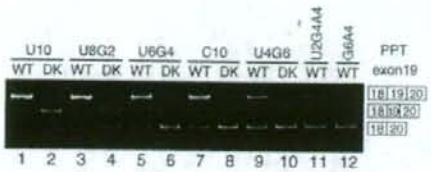

Fig. 4. ***In vitro* splicing analysis using HeLa nuclear extract.** Representative results of an *in vitro* splicing experiment are shown. After the *in vitro* splicing reaction, RNA was purified from the reaction mixture. RT–PCR and acrylamide gel electrophoresis were then performed. The names of the tested PPTs, type of exon 19 and schematic description of RT–PCR products are the same as those shown in Fig. 3.

if exon 19 does not contain the ESE, its proper splicing requires a strong PPT, and that if exon 19 contains the ESE, the upstream PPT is not necessary for proper splicing. It also suggests that the original PPT, which looks like a strong PPT, is not strong enough to promote the inclusion of exon 19 without the ESE.

Effects of BPS Mutation and Exon Length Alteration on Exon 19 Incorporation—Among the three acceptor site elements, the BPS and PPT are known for their variety. The minigene reporter used in the PPT exchange analysis illustrated in Fig. 1C contained the best BPS, UACUAAC (*12*, *15*). On the other hand, the genomic sequence of the intron 18 BPS is UACUCAU, 2 nt of which differ from the best BPS (underlined) (Fig. 1C). We analysed the effect of the alteration of these 2 nt on the splice pattern of exon 19. The fifth and seventh position of the BPS (underlined above) were mutated in minigene reporters, which were cloned into an expression vector and the resultant plasmids were introduced into HeLa cells for splicing pattern analysis using the same methods as above. For the reporter containing the PPT U8G2 and the DK exon 19, the change in the BPS slightly reduced exon 19 usage (Fig. 3B, lanes 3 and 4). For the reporter containing the U2G4A4 PPT and the WT exon 19, of the change in the BPS showed that both the fifth and seventh positions of the BPS are important for proper splicing and that when these are mutated, exon 19 usage was gradually reduced (Fig. 3B, lanes 7–10). For other cases of PPT mutation, no difference was observed due to the change in the BPS (Fig. 2; Fig. 3B, lanes 5 and 6) Considering all of these results together (Fig. 2), these2 nt changes in the BPS has an effect on the splicing efficiency of exon 19 both with and without the dys-ESE19 sequence; however, this change does not have as drastic an effect as a uridine to purine change in the PPT.

It is known that short exons are sometimes difficult to splice correctly. To confirm that the DK exon 19 (41 nt) is not too short for proper splicing compared to the WT exon 19 (88 nt), we constructed minigene reporters in which dys-ESE19 was replaced with other gene fragments that lack the ESE. For this purpose, we used a 46 nt fragment of an ampicillin resistance (AR) gene, which should not have any ESE activity because it is a bacterial gene (Fig. 1A). The fragment was selected by eye, so as not to contain too many purine residues

name, sequences \ exon19		WT	DK	AR
U10	UUUUUUUUU	+	+/−	
U8G2	UUGUUUGUU	+	+/−	+/−
U6G4	UUGUUGUGUG	+/−	−	−
C10	CCCCCCCCCC	+/−	−	−
U4G6	GGUGGUGUGU	+/−	−	
U2G4A4	GAUGGAAUGA	−/+	−	
G6A4	GGAGGAGAGA	−		
A6G4	AAGAAGAGAG	−		

Fig. 5. **All results of *in vitro* splicing assay using HNE.** PPTs, exon 19 types and splice pattern categorization are the same as in Fig. 2. For BPS, only UACUAAC was tested for *in vitro* splicing and so is not shown in this figure.

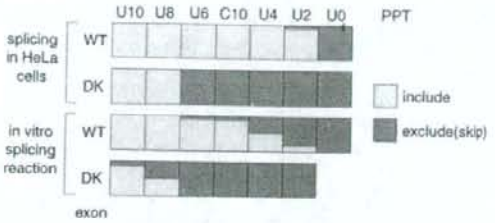

Fig. 6. **Results summary and quantification analysis of the splicing assay in HeLa cells and the *in vitro* splicing reaction with different PPTs.** Each box represents a splicing assay result and the light and dark shadowed areas in the boxes represent the molecular ratio of exon 19 inclusion/skipping. For the *in vitro* splicing assay, samples that showed both exon 19-included and exon 19-skipped splicing patterns (in Fig. 4, lanes 2, 4, 5, 7, 9 and 11) were subjected to capillary electrophoresis in an Agilent2100 and each band was quantified using peak height. For the splicing assay in HeLa cells, only the U2G4A4 sample (Fig. 3A, lane 9), showed both included and skipped splice patterns; this was amplified by a reduced number of PCR cycles (15 cycles) and analysed by same method as above. The molecular ratio of included/skipped was calculated and shown by the extent of light and dark in the shadowed area. The names of the PPTs (purine residues are omitted) are shown on the top; exon types and assay methods are shown on the left.

to avoid cloning a purine-rich ESE by accident (*16*); this fragment showed no ESE activity in an *in vitro* splicing reaction (*6*). When the exon length was expanded, AR exon 19 (93 nt) was incorporated into mRNA with a slightly higher efficiency than the DK exon 19 with a U6G4 PPT or a C10 PPT (Fig. 3C, lanes 9 and 12). Without the presence of the dys-ESE19 sequence, the U8G2 and U6G4 PPT are on the border of exon 19 inclusion/skipping determination (Fig. 3C, lanes 5 and 8). This result suggests that an exon length expansion leads to slightly more effective exon 19 recognition. The 41 nt DK exon 19 is probably slightly shorter than standard exon length required for proper exon recognition. In addition to that, AR exon 19 with a wild-type acceptor site (completely the same as the genomic dystrophin gene acceptor site) did not show exon inclusion (Fig. 3C, lane 3), confirming that dys-ESE19 is essential for the proper splicing of exon 19.

These BPS mutations and exon length alterations suggest that both the BPS and exon length have an effect on exon 19 usage in the splicing reaction; however, the effects were not drastic compared to those with/without ESE or PPT alteration.

In Vitro Splicing Assay with Same Reporter Minigene—Most previous reports that have analysed PPT strength (*13*, *14*) or the relationship between the ESE and the PPT (*17–19*) have used a biochemical method called '*in vitro* splicing' to analyse splicing patterns. We also tried the same *in vitro* splicing system using HNE. The synthesized pre-mRNAs, which had the same exon–intron structures used above, were incubated with HNE at 37°C for 2 h. After the *in vitro* splicing reaction, RNA was extracted and RT–PCR was used to analyse splicing patterns. Interestingly, the results using HeLa cells and HNE are slightly different. In HeLa cells, dys-ESE19 is not necessary for DK exon 19 inclusion when the PPT is U10 or U8G2, so there was no exon 19 skipping in these two reporters (Fig. 3A, lanes 2 and 4); on the other hand, a fraction of these two reporters showed skipping of exon 19 in *in vitro* splicing (Fig. 4, lanes 2 and 4). A similar phenomenon was observed for dys-ESE19-dependent splicing. In HeLa cells, there was no WT exon 19 skipping when the PPT was U6G4, C10 or U4G6 (Fig. 2; Fig. 3A lanes 5 and 7); however, a fraction of the mRNAs showed a WT exon 19-skipped splice pattern following *in vitro* splicing of these reporters (Fig. 4, lanes 5, 7 and 9; Fig. 5). As many samples showed both exon-inclusive and exon-skipped splice patterns, PCR products were quantified by capillary electrophoresis and the molecular ratio of exon inclusion/skipping was calculated (Fig. 6). It seems that on/off (or include/skip) regulation of exons is more strictly controlled in living cells than it is *in vitro* (Fig. 6).

Sequence Analysis of All 78 Acceptor Sites in the Dystrophin Gene—According to the experimental results, eight or more uridine residues in a 10 nt stretch has sufficient activity to be spliced correctly without an ESE. To try to predict how many exons could be spliced in an ESE-independent manner, we scanned all 78 dystrophin acceptor sites to determine whether they have a good PPT or not. If the acceptor site has eight or more uridines in a 10 nt stretch, it was categorized as a good PPT (Supplementary Data, Table S1). When we scanned sequence positions −4 to −20 of the acceptor sites (the 5′-end of the exon is at +1), 25 acceptor sites had a good PPT, and if the sequence position of the upstream end was extended to −35, 6 more sites were added to this category. Thus, approximately one-third of dystrophin exons have a good PPT and can be supposed to be spliced in an ESE-independent manner; meanwhile, the remaining two-thirds of dystrophin exons do not have a good PPT and can be supposed to contain ESEs.

We also scanned the BPSs because these also affected correct splicing efficiency (Fig. 3C). As the BPS is highly degenerate in humans (*12*), only five acceptor sites have a typical YNCTRAC (Y = C or T, R = A or G, N = any nucleotide) sequence in the −60 to −10 position in the acceptor sites (Supplementary Data, Table S1). Using a BPS finding program on an SSF website (Splicing Sequences Finder; http://www.umd.be/SSF), 56 of the 78 acceptor sites in the dystrophin gene have at least one BPS candidate sequence in the −60 to −10 position.

In other words, 22 of the 78 acceptor sites in the dystrophin gene do not have a BPS candidate sequence.

DISCUSSION

Our results show that dys-ESE19 is necessary for the correct splicing of exon 19 to compensate for the poor splicing activity of the PPT in intron 18. Meanwhile, a degenerate PPT, more than half of which contains purine residues, causes no problem as long as exon 19 has a fully active dys-ESE19. It was surprising that a PPT containing only two uridines (minigene with the U2G4A4 PPT) could be properly spliced in the majority of transcripts when exon 19 contained a dys-ESE19. We also showed that a biochemical method called '*in vitro* splicing', which is commonly used for splicing analysis, does not fully recapitulate the splicing pattern in living cells.

The first report to describe the relationship between exon sequence and PPT came out in 1991, using the *doublesex* (*dsx*) gene in *Drosophila melanogaster* (*17*). It was known that exon 4 of the *dsx* gene has a suboptimal PPT (containing 12 pyrimidine residues and seven purine residues) upstream of it and that this exon is skipped in males, but is used in females. Their report suggested that two female-specific proteins (Tra and Tra2) bind to exon 4 to encourage its inclusion in the mature mRNA in females. This report provided a prototype of the hypothesis that ESE-binding proteins bind to exons with suboptimal PPTs and stimulate exon inclusion. Several reports (*7–19*) followed this hypothesis, assuming that SR proteins (including Tra2) bind to ESE and stimulate binding of U2AF to the PPT for several contexts of alternatively spliced exons, in several species. Our approach to analyse the relationship between an ESE and splicing *cis*-signals, mainly PPTs, is different from previous reports in two regards. First, we analysed a human constitutively spliced exon, whereas most previous reports have analysed alternatively spliced exons among several species. It is understandable that an alternatively spliced exon would have a suboptimal PPT, because that exon should be skipped in certain conditions. However, our results showed that the constitutively spliced dystrophin exon 19 also has a suboptimal PPT, and that its splicing depends on an ESE. In addition to this, the fact that over half of the dystrophin exons could contain suboptimal PPTs suggests that a number of constitutively spliced exons, possibly over half, contain suboptimal PPTs, the proper splicing of which is dependent on an ESE. Second, we mainly used cultured cells for the splicing reporter assay, whereas a number of previous reports have used *in vitro* splicing. As far as we know, the U2G4A4 PPT that we used here is the weakest PPT, yet it showed an almost normal splicing pattern in the reporter assay that was dependent on an ESE. Why would a predominantly 'purine-tract' work well in our assay and not in others? We think this is because most previously reported splicing analysis experiments used an *in vitro* splicing system with HNE, whereas we used living cells. The on/off control of each exon is probably more strictly controlled in living cells than in nuclear extract; furthermore, ESE activity in living cells is stronger than in nuclear extract. These differences between living cells and *in vitro* splicing also suggest the possibility that when other ESE experiments show the same tendency, ESE activities could be underestimated in most splicing pattern analysis, because the experiments were performed using nuclear extract.

It is said that 'weak splice sites need ESE' (*4*); however, there is not yet a good standard method to evaluate acceptor site strength. As opposed to a splicing donor site in which the consensus sequence is relatively simple (AG|GURAGU), a splicing acceptor site consensus sequence is complicated since it contains three different elements: a BPS, a PPT and a 3′end consensus sequence AG (*11*, *12*). In addition to this, the length of the PPT and the pyrimidine/purine nucleotide ratio are various in each acceptor site, as a previous report suggested that when a PPT has a long uridine stretch, the location of the uridine stretch between the BPS and the 3′-splice site AG is variable, and that there might also be a purine stretch between these elements (*14*). YNCURAC is the consensus BPS; however, this sequence is highly degenerate and it is usually difficult to predict an actual BPS. For instance, only 5 introns have a typical YNCTRAC sequence among all 78 acceptor sites of the dystrophin gene (Supplementary Material, Table S1). Because of this flexibility, there is no good scoring method for splice acceptor sites. CV is a good indicator of splice site strength; however, it accounts for only 15 nt (−14 to +1) around the 3′ splice site (*8*) and is not sufficient to evaluate splice site strength accurately. Fairbrother *et al.* (*20*) used longer sequences (−22 to +2) than Shapiro's CV to search for ESEs in 'weak exons' and so this scoring method should be more informative than the CV; however, their calculation method also did not account for the BPS. During the actual splicing reaction in living cells, splice sites should be chosen on the basis of the balance between the strengths of each *cis*-acting signal. There are a number of reports about the role of *cis*-acting signals and how to evaluate their strength. However, the relationship between them remains unclear. There are not yet enough experiment-based conclusions to predict the contribution to splicing of each *cis*-acting signal (*21*).

The reason why we want to understand the relationship between these *cis*-acting signals and their relative contributions is for the accurate diagnosis and therapy of genetic disorders. In the case of the dystrophin gene, when a mutation (in most cases, deletion of a single exon or several exons from the genome) in the gene results in the absence of protein due to disruption of the translational reading frame of the mRNA, that patient will show a severe Duchenne muscular dystrophy (DMD) phenotype. On the other hand, if several exons are deleted but the translational reading frame is maintained correctly, the patient usually expresses a truncated protein, which is partly active, and shows a milder Becker muscular dystrophy (BMD) phenotype. Thus, for the therapy of DMD, AO-induced exon skipping, which could correct the translational reading frame from out-of-frame to in-frame in DMD patients, could possibly change those patients' phenotype from severe DMD to the milder

BMD (*1*, *22*, *23*). The mechanism of this exon skipping is thought to be through the hybridization of the AO to an ESE, thereby abolishing the exon recognition (*24*). Our results suggested that two-thirds of dystrophin exons are likely to be good therapeutic targets for AO, because two-thirds of the acceptor sites in the dystrophin gene do not contain a strong PPT and so are likely to have an ESE in the downstream exon. Indeed, exon 19, which contains an ESE and does not have a strong PPT upstream of it, is a good therapeutic target exon for AO treatment. This exon was skipped by AO in *in vitro* splicing experiments (*6*) and in primary cultures of cells from a DMD patient with an exon 20 deletion in the dystrophin gene (*5*). The expression of newly synthesized dystrophin protein induced by the AO was also confirmed in cultured cells (*5*). This AO treatment was finally applied to the patient and in-frame mRNA and dystrophin protein expression was confirmed (*25*). These results suggest that two-thirds of dystrophin exons are good candidate therapeutic targets; on the other hand, it is likely not a good idea to try exon skipping for the remaining one-third of exons.

In summary, we show that either a strong PPT or a strong ESE is sufficient for proper exon recognition during the splicing reaction. It was surprising that a predominantly 'purine tract' does not disturb splicing when the downstream exon has an ESE, suggesting that when a downstream exon contains a strong ESE, the PPT is almost dispensable. This high activity of an ESE is seen only in living cells, not in '*in vitro* splicing' reactions, suggesting the possibility that previous experiments may have underestimated ESE activity. Analysis of all of the dystrophin acceptor sites suggests that two-thirds of the dystrophin exons are potentially spliced in an ESE-dependent manner and the remaining one-third are possibly ESE-independent exons. Here we elucidated the relationship between the dys-ESE19 and the upstream PPT, only one relationship of many between two *cis*-acting signals that regulate splicing patterns. Splicing events are the result of a fine 'balance of power' between a number of regulatory elements (*21*); thus, clarifying each relation could be a way to achieve an understanding of the splicing machinery and it enables the development of therapies for genetic disorders by splicing-associated methods.

Supplementary Data are available at JB Online.

We thank Dr K. Inoue and Dr K. Saiki for helpful advice and discussion. This work was supported by grant from the Ministry of Education, Culture, Sports, Science and Technology. Y.H. was supported by a grant from the Japan Foundation of Neuroscience and Mental Health.

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J Hum Genet (2008) 53:215–219
DOI 10.1007/s10038-007-0235-1

ORIGINAL ARTICLE

Tandem duplications of two separate fragments of the dystrophin gene in a patient with Duchenne muscular dystrophy

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Received: 7 November 2007 / Accepted: 3 December 2007 / Published online: 27 December 2007

Abstract Mutations in the dystrophin gene result in the most common inherited muscle disease, Duchenne muscular dystrophy (DMD). Duplications spanning one or more exons have been found to be the second most common disease-causing mutation in the dystrophin gene. Although the duplicated exons are commonly thought to be arranged in tandem, rare noncontiguous exon duplications have been disclosed without clarifying their location or orientation. Here we present the first report that details the exact locations and orientations of noncontiguous duplications in the dystrophin gene. Multiplex ligation-dependent probe amplification analysis of the dystrophin gene of a Japanese boy with DMD revealed that his genomic DNA contained duplications of exons from two separate fragments of the gene: one from exon 45 to exon 48 and the other from exon 55 to exon 63. To clarify the locations and orientations of the duplicated exons, reverse transcription-nested PCR analysis of dystrophin mRNA was conducted. Interestingly, the extra copies of exons 45–48 and exons 55–63 were found to be properly oriented between exons 48 and 49 and exons 63 and 64, respectively. These results indicated that two tandem duplication events occurred in the dystrophin gene of this patient and should contribute to the understanding of the duplication mechanisms that contribute to the development of DMD.

Keywords Dystrophin · Mutation · Duplication

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Introduction

Duchenne muscular dystrophy (DMD) is the most common inherited muscle disease, affecting 1 in every 3,500 male births. This disease is caused by mutations in the dystrophin gene located on Xp21. Deletions involving one or more exons are the most common type of mutation associated with DMD, accounting for nearly two-thirds of all cases. Duplications are the second most common type of mutation in this gene, occurring in approximately 5–10% of DMD patients (Hu et al. 1990; White et al. 2002, 2006).

Because the dystrophin gene consists of 79 exons spanning more than 2,500 kb in the human genome, it has been difficult to examine every exon for deletions or duplications. Instead, PCR amplification of deletion-prone exons has been used to genetically diagnosis patients suspected of having DMD (Chamberlain et al. 1988; Beggs et al. 1990). Recently, multiplex ligation-dependent probe amplification (MLPA) analysis, which is used to examine every exon for deletion and/or duplications, has been developed, resulting in a marked improvement in the mutation detection rate (Janssen et al. 2005).

Interestingly, MLPA analysis produced ambiguous results in eight cases in whom twice as much genomic DNA was detected for two separate fragments of the dystrophin gene (Janssen et al. 2005; White et al. 2006; Zeng et al. 2007). Although these cases are thought to carry tandem duplications of two separate dystrophin gene fragments, details of the duplicated exon fragments have not been reported, thereby making it possible that the extra genomic fragments were located outside of the dystrophin gene. In this article, we describe a patient with novel noncontiguous duplications in his dystrophin gene; detailed mRNA analysis revealed tandem duplication of two separate fragments from this gene.

Case and methods

Case

More than 400 DMD patients at the Kobe University Hospital were subjected to mutation analysis of their dystrophin genes. Among these patients, 27 cases were shown to carry duplications of contiguous exons. Recently, one case was found to have noncontiguous duplications. The proband (KUCG759) was a 5-year-old boy. At 1 year old, his serum creatine kinase (CK) level was found to be markedly elevated (12,320–17,712 IU/l), and a muscle biopsy disclosed no dystrophin-specific staining, confirming a diagnosis of DMD. At 4 years old, the patient's Gowers' sign was positive, and he was referred to our hospital for an examination of his dystrophin gene. The Ethics Committee of the Kobe University Graduate School of Medicine approved this study, and consent was obtained from his parents.

MLPA analysis

DNA was isolated from lymphocytes obtained from the patient and normal individuals using standard phenol–chloroform extraction methods. MLPA was performed with the P034 and P035 kits from MRC-Holland (Amsterdam, The Netherlands) as described (Lalic et al. 2005). This technique allowed the full dystrophin gene to be examined for any deletions or duplications.

Polymorphism analysis

To characterize the duplicated fragments, genomic regions encompassing each set of duplicated exons were amplified using primers specific for the flanking sequences (Table 1), resulting in the amplification of dinucleotide repeat markers from intron 45 (STR-45) and intron 62 (DI623) (Clemens et al. 1991). Amplified products were directly sequenced using an automated DNA sequencer (model 310; Applied Biosystems, Foster City, CA).

Analysis of dystrophin mRNA

The dystrophin mRNA expressed in lymphocytes was examined by reverse transcription (RT)-nested PCR

Table 1 Primer sequence

Target region	Forward primer	Reverse primer
gDNA		
Exon 45	5′ TGCCAGTACAACTGCATGTGGTAG 3′	5′ GCTTATAATCTCTCATGAAATATTC 3
Exon 46	5′ GTTTGTGTCCCAGTTTGCATTAAC 3′	5′ GGCAGAAAACCAATGATTGAATTA 3′
Exon 47	5′ GGGGTGAGTGTTTCAGTCAATC 3′	5′ CATATAGCCAAAGCAAACGGTC 3′
Exon 48	5′ TAAACATTTTGGCTTATGCCTTGA 3′	5′ TGGTGCCTGTGCCTATTGTGTTAT 3′
Exon 55	5′ CCATCTTTCTCTTTTTATGGAGTT 3′	5′ TTGTCCCTGGCTTGTCAGTT 3′
Exon 56	5′ TACGCCAAGAAAAGGGATTTGAGA 3′	5′ CCAGTTACTTGTGCTAAGACAATGAGG 3′
Exon 57	5′ ACACTTCTAGATATTCTGACATGG 3′	5′ GTCACTGGATTACTATGTGCTTAAC 3′
Exon 58	5′ GCACCCAGGATTAATTTTGAGAAGA 3′	5′ CCAGACCCTGGCAGCAAGAAT 3′
Exon 59	5′ CAGTAGGTTACCCTCTTGTTCAAC 3′	5′ GGGAAGATAACACTGCACTCAAGT 3′
Exon 60	5′ CCCTAAAGAGAATAAGCCCAGGTA 3′	5′ TCCTATCCTCACAAATATTACCATGAA 3′
Exon 61	5′ GTTGCTTTAGTGTTCTCAGTCTTGGA 3′	5′ GGATGATTTATGCTTCTACTGCTACTG 3′
Exon 62	5′ CCTGTTTGCGATGAATTTGACCTC 3′	5′ ACAGGTTAGTCACAATAAATGCTCTT 3′
Exon 63	5′ GCAAAAATCATGTTGTTGTTATTG 3′	5′ CAAGTAACTTTCACACTGCAAACT 3′
Intron 45	5′ GAGGCTATAATTCTTTAACTTTGGC 3′	5′ CTCTTTCCCTCTTTATTCATGTTAC 3′
Intron 62	5′ ACCTGCCTAGTCAAGGTA 3′	5′ CACTGCCATGGTGAATGATC 3′
mRNA		
Exon 44	4A: 5′ TGGCGGCGTTTTCATTAT 3′	
Exon 46		c46r: 5′ CTTGACTTGCTCAAGCTTTTC 3′
Exon 47	c47f: 5′ TTACTGGTGGAAGAGTTG 3′	
Exon 52		4D: 5′ CGATCCGTAATGATTGTTCTAGC 3′
Exon 59		4B: 5′ CGGAGTGCAGGTTCAATTTT 3′
		4F: 5′ CCCACTCAGTATTGACCTCCTC 3′
Exon 60	c60f: 5′ TCAGCACTCTGGAAGACCTG 3′	
Exon 61	c61f: 5′ GCCGTCGAGGACCGAGTCAGGCAGCT 3′	

analysis as described previously (Matsuo et al. 1991). Briefly, total RNA was isolated from peripheral lymphocytes, and cDNA was synthesized. A fragment encompassing the duplicated region of exons 45–48 was amplified using an outer set of primers with a forward primer corresponding to a segment of exon 44 and a reverse primer complementary to a segment of exon 52. The PCR product was then used as a template for a second PCR using an inner set of primers with a forward primer specific for exon 47 and a reverse primer complementary to exon 46 (Table 1). A fragment encompassing the duplicated region of exons 55–63 was also amplified using two sets of primers: an outer set with a forward primer specific for exon 60 and a reverse primer complementary to exon 59 and an inner set with a forward primer specific for exon 61 and a reverse primer complementary to exon 59 (Table 1). The amplified products were purified and directly sequenced.

Results

MLPA analysis of the dystrophin gene in the index patient disclosed that all of the exons were present. For a total of 13 exons, however, the signals resulting from MLPA analysis were twice those observed in samples from a normal male control subject, indicating that these exons were duplicated (Fig. 1). Surprisingly, these 13 exons were

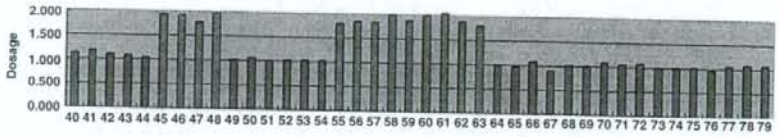

Fig. 1 MLPA analysis of the dystrophin gene. *Bars* represent the amount of amplified product expressed as a ratio to the results obtained with a control sample. *Numbers below the bars* indicate the exon numbers. The *heights of the bars* represent the relative signals of the corresponding exons compared to those obtained from a male control sample. Exons 45–48 and exons 55–63 produced signals that were twice those obtained for the other exons

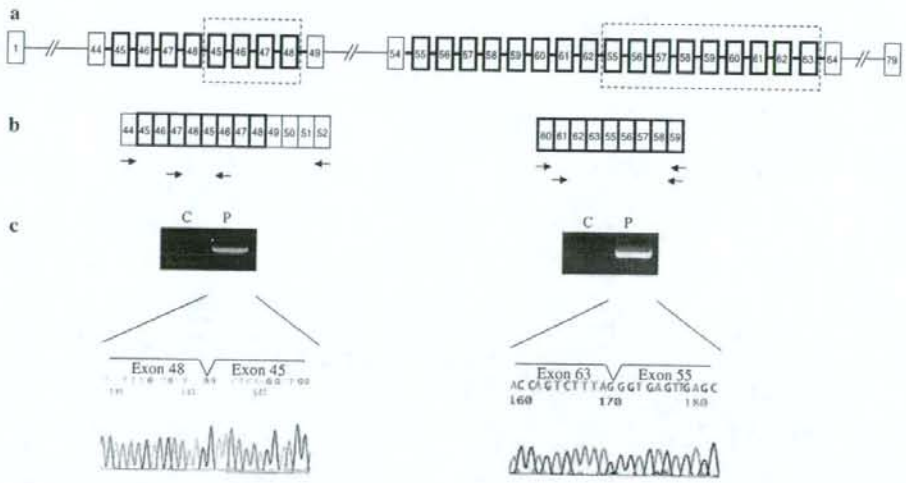

Fig. 2 RT-PCR amplification of the duplication borders of exons 45–48 and exons 55–63. **a** The predicted genomic structure of the duplicated region of the dystrophin gene. The *boxes* represent exons and the *numbers in the boxes* indicate the exon numbers. *Horizontal lines* between the *boxes* indicate introns. Duplicated fragments have been denoted with *dotted lines*. **b** The predicted fragments of the dystrophin transcripts covering the two duplicated regions. *Boxes* represent exons, and *horizontal arrows* show the locations and orientations of the outer and inner primers that were used for RT-PCR analysis. **c** RT-PCR products for the index patient (*P*) and a control subject (*C*) encompassing the duplication breakpoints. PCR products were visualized on a gel (*top panel*). No amplified product was obtained from the control sample, whereas the index case produced a clear band. A partial sequence of the amplified product is shown (*bottom panel*); the 3′ terminal sequence of exon 48 is joined to the 5′ end of exon 45, and the 3′ end of exon 63 is joined to the 5′ end of exon 55

clustered in two separated regions of the dystrophin gene: one extending from exon 45 to exon 48 and the other spanning from exon 55 to exon 63. Normal signal levels were observed for exons 49–54 located between the two duplicated regions, indicating that these exons were present as single copies. Although duplications of exons in the dystrophin gene are commonly thought to occur in tandem, the locations and orientations of the two duplicated fragments were unclear.

To determine the exact location as well as the orientations of the duplicated exons, dystrophin mRNA from lymphocytes was analyzed using RT-nested PCR amplifications. When we amplified a fragment extending from exon 47 to exon 46, a product was obtained from the index case, but not from the control subject. Direct sequencing of the product disclosed the sequences of exons 47, 48, 45, and 46, revealing that the 3′ end of exon 48 was directly joined to the 5′ end of exon 45 (Fig. 2). Similarly, a fragment extending from exon 61 to exon 59 was only amplified from the index case, but not from the control subject. Sequencing of the product revealed that the 3′ end of exon 63 was directly joined to the 5′ end of exon 55 (Fig. 2). Sequences of the other portions of the dystrophin mRNA from the index case, including from exon 55 to exon 63, were the same as was observed in the control sample (data not shown). According to the reading frame rule, the more 3′ duplication of exons 55–63 disrupted the reading frame of the patient's dystrophin mRNA, resulting in a severe DMD phenotype. Our mRNA analysis disclosed that each exon duplicated in his dystrophin gene was incorporated into the fully spliced mRNA and that both of the duplicated fragments were present in tandem to their respective fragment.

To identify the source of the two duplicated fragments, all of the duplicated exons were sequenced, and polymorphic markers in introns 45 and 62 were also analyzed. No heterozygosities, however, were disclosed in the exon sequences (data not shown). Moreover, no differences were observed for markers in introns 45 and 62, at which heterozygosities have been reported. This suggests that both duplicated fragments were derived from the same chromosome.

Discussion

We have previously identified 27 subjects with duplication mutations in their dystrophin genes, all of which were contiguous. In this study, we report a novel case carrying noncontiguous duplications in the dystrophin gene; two separate fragments from exon 45 to exon 48 and from exon 55 to exon 63 were duplicated. The index case is the first reported Japanese case with noncontiguous duplications in the dystrophin gene.

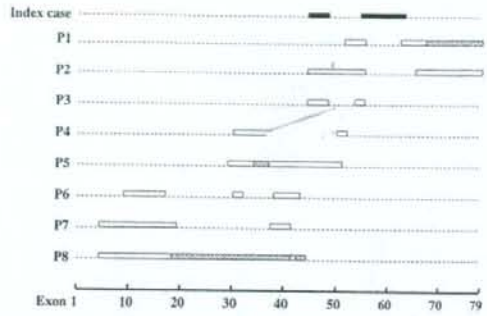

Fig. 3 A schematic representation of the distribution of identified noncontiguous duplications in the dystrophin gene. *Horizontal bars* indicate duplicated regions that have been identified in patients with DMD. The *black bars* indicate duplications identified in this study, and the *unfilled bars* indicate previously (*P*) reported cases of noncontiguous duplications. The *shaded* and *dotted bars* denoted previously reported partial triplications and a partial quadruplication, respectively

MLPA analysis only demonstrates changes in the copy number of individual exons, and it is difficult to know the exact organization of the duplicated exons. Duplications can result in transpositions, which would have no effect on the reading frame if the duplicated regions are inserted outside of the dystrophin gene (White et al. 2006). Several examples of such transposition events have been described for the gene encoding proteolipid protein 1 (*PLP1*), in which an additional copy of *PLP1* gene was found integrated at noncontiguous sites on the X chromosome (Xp22 and Xq26) (Woodward et al. 2005). Though eight noncontiguous duplications, three of which involved partial triplications or a quadruplication, have been previously identified in the dystrophin gene (Janssen et al. 2005; White et al. 2006; Zeng et al. 2007), there are no descriptions of the locations and orientations of the duplications. Our results indicated that both of the duplications in the index case were inserted in tandem and maintained the same orientation as that of the dystrophin gene. This provides the first evidence for the tandem organization of duplications in the dystrophin gene.

Our case is the ninth reported patient with noncontiguous duplications in the dystrophin gene. Although the majority of the reported duplications have clustered toward the 5′ end of the dystrophin gene (White et al. 2006), the distribution of noncontiguous duplications is shifted slightly toward the 3′ end of the gene: four, two, and three of the duplications were located in the 3′ portion, in the middle, and in the 5′ portion of the dystrophin gene, respectively (Fig. 3). Eight of the nine duplications were located in major (exons 45–52) and minor (exons 3–19) deletion hot spots. In particular, four noncontiguous duplications had breakpoints in intron 44, whereas three

had breakpoints in intron 55. The extraordinary size of intron 44 (250 kb) and intron 55 (120 kb) are likely to make these two introns prone to rearrangement and recombination events that result in duplications.

What was the source of the duplicated exons? Because the duplicated alleles showed no nucleotide differences (data not shown), they likely arose as a result of an interchromosomal event. It has been reported that unequal crossing over between sister chromatids, rather than between nonsister chromatids from two X chromosomes, is the predominant cause of duplications in the dystrophin gene (Hu et al. 1991). Another possible mechanism that may generate a duplication is synthesis-dependent nonhomologous end joining (Helleday 2003). This process results in a tandem duplication at the site of a double-stranded break, and unequal crossing over does not take place. This mechanism has recently been proposed for a duplication of exon 2 in the dystrophin gene and *PLP1* (White et al. 2006). Whether this mechanism is involved in the formation of noncontiguous duplications, however, is unclear. Further refinement of the duplication breakpoint will enable us to understand the mechanism underlying the rearrangement.

In conclusion, we identified novel noncontiguous duplications in the dystrophin gene of a patient with DMD. Detailed RNA analysis confirmed that the RNA in fact did contain two duplications. In addition, we were able to show that both of the noncontiguous duplications were organized in tandem, which provides insight into the mutational mechanisms that cause noncontiguous duplications.

Acknowledgments We would like to thank Ms. Inagawa for her secretarial help. This work was supported by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science; Health and Labor Sciences Research Grants for Research on Psychiatric and Neurological Diseases and Mental Health; a research grant for nervous and mental disorders from the Ministry of Health, Labor, and Welfare; and the Mitsubishi Foundation.

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Brain & Development xxx (2008) xxx–xxx

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

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Case report

A 2-bp deletion in exon 74 of the dystrophin gene does not clearly induce muscle weakness

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Received 7 November 2007; received in revised form 10 March 2008; accepted 12 March 2008

Abstract

Duchenne muscular dystrophy (DMD) is caused by mutation of the dystrophin gene. Cases of dystrophinopathy with a 2-bp deletion in the dystrophin gene commonly result in DMD. We report here a case of dystrophinopathy in a 9-years-old boy with a 2-bp deletion in exon 74 of the dystrophin gene; however, the boy had no clear clinical signs of muscle weakness. Immunohistochemical studies with N-terminal (DYS3) and rod-domain anti-dystrophin (DYS1) antibodies revealed that the dystrophin signals were weaker than in the control sample (non-dystrophinopathy) at the sarcolemma of myofibers, and the studies with C-terminus anti-dystrophin antibody (DYS2) were negative. Our patient's mutation is located between the binding sites of α-syntrophin and α-dystrobrevin. These results suggest that this mutation does not clearly induce muscle weakness at least through the age of 9 years.

Keywords: Dystrophin; Exon 74; Duchenne muscular dystrophy; Out of frame; Non-muscle weakness

1. Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are caused by defective expression of the dystrophin gene, resulting in the absence of the dystrophin protein in muscle fibers [1]. DMD results from an out-of-frame deletion(s) in the dystrophin gene resulting in the lack of dystrophin expression in myofibers [2]. The muscle weakness caused by the disease is progressive. The symptoms initially appear as muscle weakness at 2–3 years of age; patients typically lose the ability to walk by themselves before the age of 12. In contrast, BMD, which results from an in-frame deletion(s) in the dystrophin gene, causes a milder muscle weakness [3]. In skeletal muscle specimens from DMD and BMD patients, necrosis and regenerating fibers are often observed. An immunohistochemical study of DMD with anti-dystrophin antibodies revealed no staining of the surface membrane of the muscle fibers, but a corresponding study of BMD showed weak and patchy staining [4].

Dystrophin binds both to cytoskeletal actin and to the cytoplasmic tail of the transmembrane dystrophin–glycoprotein complex [5], important members of which are α-, β-dystroglycan, α-, β-, γ-, δ-sarcoglycan, laminin α2, and integrin α7. In addition, dystrophin also binds to syntrophin and dystrobrevin as a peripheral cytoplasmic subcomplex at exons 73–74 and 74–75, respectively [6,7], which is thought to function like a signaling protein. The members of this subcomplex include neuronal

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doi:10.1016/j.braindev.2008.03.004

nitric oxide synthase (nNOS) and aquaporin-4. Therefore, exon 74 of the dystrophin gene is the key exon. We report here the case of a 9-years-old boy with a 2-bp deletion in exon 74 of the dystrophin gene who shows no clear clinical signs of muscle weakness.

2. Methods and case report

2.1. Case report

The patient is a 9-years-old boy with no familial history of muscle disease. The patient began to walk independently when he was one-year old. He reported muscle pain in his foot and showed drop foot when he was 2-years old. His family doctor suspected cerebral palsy and referred the boy to our hospital staff to confirm the diagnosis when the patient was 8-years-old. The boy had slight pseudohypertrophy of his calf muscles and mild contracture of his ankle joints, but he did not show Gowers' sign. A manual muscle test did not show muscle weakness. Although the patient could not run fast during the tests, he did exhibit the ability to hop. His Achilles and patellar tendon reflexes were hyperactive, and he had an increased level of serum creatine kinase (1596 U/mL; normal range, 57–284). The results from magnetic resonance imagery of the brain, a chest X-ray, and electrocardiography were normal. The work that the boy has done in school combined with a conversion ability in line with that of normal 9-years-old boys suggests that he does not have mental retardation.

2.2 Histological studies and Western blotting analysis for dystrophin

Muscle biopsy specimens were obtained from the biceps brachii according to a standard protocol. The biopsy specimens were studied with hematoxylin and eosin (HE). In addition, immunohistochemical stainings were performed on cryostat sections with monoclonal anti-dystrophin, anti-β-dystroglycan, and anti-α-, β-, γ-sarcoglycan antibodies (DYS1, DYS2, DYS3, dystroglycan and sarcoglycan: Novocastra Laboratories Ltd., UK). Western blotting was performed with the antibody against the rod domain of dystrophin (DYS1, Novocastra Laboratories Ltd.).

2.3. Mutation analysis of muscle dystrophin gene

Mutational analysis of the dystrophin gene was performed according to the protocol of Surono et al. [8]. The genomic region encompassing exon 74 was amplified by PCR with g74f (5′-CAAATACACTCCTGAGTCCCTAACC-3′) as the forward primer and g74r (5′-AGATTCCTGGCACTTTTCTATGTGT-3′) as the reverse primer. The amplified product was purified and subjected to sequencing either directly or after subcloning into a pT7 blue T vector (Novagen, Madison, WI).

3. Results

3.1. Histological studies

The HE staining of the muscle biopsy (Fig. 1A and B) from the biceps brachii showed slight variations in myofiber size, as well as scattered regenerating fibers and necrotic fibers (Fig. 1B). An immunohistochemical study with anti-N-terminus (DYS3) and anti-rod-domain dystrophin (DYS1) antibodies showed that the immunoreactivity of the patient was weaker (i.e., less staining) than that of the control at the surface membrane of the muscle fibers; but, patchy staining was not observed. Immunoreactivity with antibody for the C-terminus (DYS2) was not observed (Fig. 1C–H). Immunohistochemical studies with anti-β-dystroglycan (DG) and anti-α-, β-, γ-sarcoglycan (SG) revealed weaker staining signals for the patient than the control at the sarcolemma of myofibers in skeletal muscle (anti-β-, γ-sarcoglycan: data not shown) (Fig. 1I–L).

The Western blotting analysis with DYS1 for dystrophin of skeletal muscle showed that the band size of dystrophin in the patient was almost the same as that of the control and that the quantity of expression in the patient was lower than that of the control (Fig. 2). Again, immunoreactivity with DYS2 was not detected (data not shown).

3.2. Mutation analysis of the dystrophin gene

The dystrophin gene of the index case was analyzed for mutations. PCR amplification of all 79 exons did not reveal any deletion mutations. Next, all exons of the dystrophin gene were examined by direct sequencing of PCR-amplified products. In the amplified region encompassing exon 74, two nucleotides (AG) 104 bp downstream or 55 bp upstream from the 5′ and 3′ ends of exon 74, respectively, were absent (c. 10498–10499delAG), resulting in a frame-shift mutation. This pattern of mutation usually leads to DMD. The mutation is located before the epitope of C-terminus (DYS2), which recognizes amino acids 3669–3685 (nucleic acids 11,004–11,054) in exons 78–79 of dystrophin. These data coincide with immunohistochemical studies.

4. Discussion

We report here a case of dystrophinopathy with a 2-bp deletion in exon 74 of the dystrophin gene. The patient's diagnosis should be DMD, as the mutation typically leads to a frame shift in the dystrophin gene. However, the patient does not show any clear clinical signs of muscle weakness at 9 years of age. Western blot-

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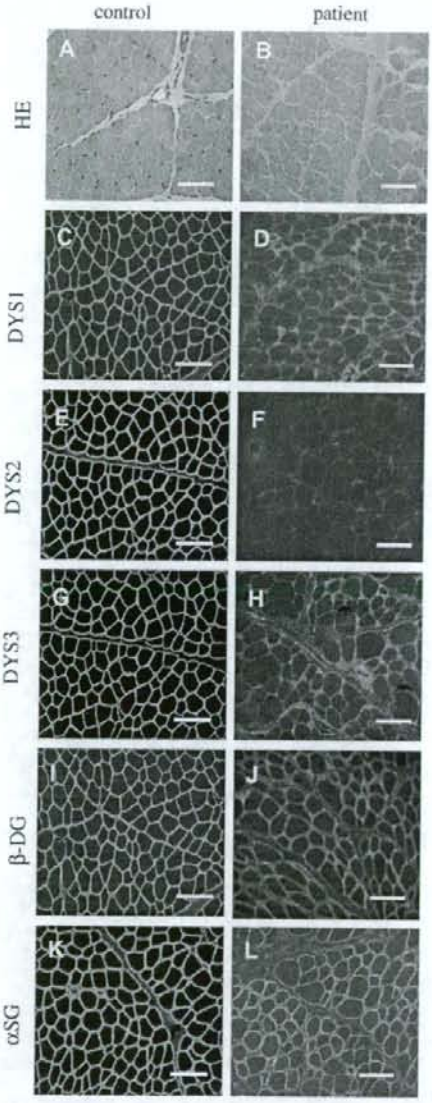

Fig. 1. HE staining and immunohistochemical study. Cryostat sections were prepared from non-dystrophinopathy controls (A, C, E, G, I, K) and patients (B, D, F, H, J, L). HE staining from the patient (B) showed slight variations in myofiber size, as well as scattered regenerating fibers and necrotic fibers. An immunohistochemical study with anti-N-terminus (DYS3), anti-rod-domain dystrophin (DYS1), anti-β-dystroglycan (β-DG), and α-sarcoglycan (α-SG) antibodies (D, H, J, L) showed that the immunoreactivity of the patient was weaker than that of the control (C, G, I, K) at the surface membrane of the muscle fibers. However, immunoreactivity with antibody for the C-terminus, DYS2 (F), was not detected in the patient. Bar scale, 100 mm.

ting analysis of the boy's skeletal muscle tissue demonstrated some expression of dystrophin, albeit low. This

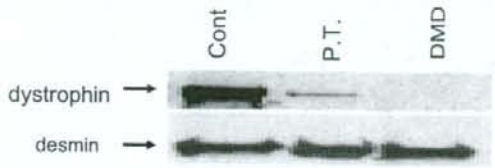

Fig. 2. Western blotting analysis for dystrophin of skeletal muscle. The size of dystrophin from the non-dystrophinopathy control (Cont) and the patient (P.T.) was almost the same; but, the quantity of the dystrophin expression in the patient was lower than that of the control. DMD, Duchenne muscular dystrophy; desmin, internal control.

data suggests that the phenotype more closely resembles BMD than DMD.

The immunohistochemical study of the patient showed that a low level of the dystrophin–sarcoglycan complex at the sarcolemma still remained, because weaker immunoreactivity of β-dystroglycan and α-, β-, γ-sarcoglycan was observed. Our patient's mutation (c. 10498–10499delAG in exon 74) is located between the binding sites of α-syntrophin [amino acids 3444–3494 (nucleic acids 10,330–10,482) in exons 73–74] and α-dystrobrevin [amino acids 3501–3541 (nucleic acids 10,501–10,623) in exons 74–75 of the dystrophin gene] [6,7]. Transgenic mice that express dystrophins with deletions of exons 71–78 (amino acids 3402–3675) had normal muscle function and normal localization of syntrophin and dystrobrevin [9]. These results support the report by Crawford et al. that the dystrophin–sarcoglycan complex may directly bind dystrobrevin, which binds to syntrophin [9]. Our Western blot data also showed that nNOS, which binds to α-syntrophin, was expressed in the patient's muscle (data not shown). Suminaga et al. reported that a boy with a point mutation (c. C11081T) that produces an aberrant stop codon in exon 76 of the dystrophin gene also does not show muscle weakness [10]. The mild phenotype of our patient may be due to exon skipping excluding exon 74, which results in an in-frame shift of the dystrophin gene (as in the above case). Further experiments are needed to examine this possibility.

We conclude that a deficiency of the cytoplasmic subcomplex of dystrophin that binds to syntrophin and dystrobrevin does not clearly induce muscle weakness, at least through 9 years of age.

Acknowledgments

The authors thank Dr. Ryan Pruchnic (Cook Myo-Site, Inc., USA) for his assistance. This study was supported by research Grants for nervous and mental disorders and for brain science from the Ministry of Health, Labour and Welfare; and a Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Please cite this article in press as: Kimura S et al., A 2-bp deletion in exon 74 of the dystrophin gene ..., Brain Dev (2008), doi:10.1016/j.braindev.2008.03.004

ANNALS OF human genetics

doi: 10.1111/j.1469-1809.2008.00468.x

Dystrophin Nonsense Mutations Can Generate Alternative Rescue Transcripts in Lymphocytes

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Summary

Secondary alterations in splicing have been reported to produce semi-functional mRNA from several nonsense mutations in the dystrophin gene. Disruptions of exonic splicing enhancers by single nucleotide changes are thought to underlie such alterations. The precise frequencies of such nonsense mutation-dependent splicing alterations, however, remain unknown. Here we analyzed the splicing patterns of dystrophin mRNA in lymphocytes from 38 patients with dystrophinopathies due to nonsense mutations in the dystrophin gene. In seven of the cases (18%), we observed partial skipping of the nonsense-encoding exon. Two of the seven cases, however, exhibited complex activation of a nonsense mutation-created splice site, which resulted in the generation of novel transcripts. Examination of *cis*-regulatory splicing elements through calculation of splicing probability scores and identification of potential splicing enhancer or silencer sequences failed to disclose a single cause for exon skipping. Remarkably, individual differences in splicing patterns were observed for cells from patients with identical nonsense mutations (C.5899C>T). Although five cases produced semi-functional dystrophin mRNAs, only one of these exhibited a mild clinical course. These results provide important insights about targets for exon skipping induced by candidate antisense oligonucleotides and for ribosomal read-through of nonsense mutations.

Keywords: dystrophin, Duchenne muscular dystrophy, nonsense mutation, exon skipping, splicing

Introduction

The splicing machinery that removes introns from pre-mRNA is strictly regulated to avoid aberrant splicing. The locations of splice sites are determined by conserved *cis*-elements or splicing conserved sequences located at intron/exon boundaries and branch points. Splicing enhancer or silencer sequences within exons also regulate the recognition of splice sites (Zhang et al., 2005; Wang et al., 2004). In addition, the translatability of an exon influences its splicing (Zhang et al., 2003). Nonsense-associated alterations in splicing have been shown to occur in minigenes and T-cell receptor transcripts (Li et al., 2002; Wang et al., 2002).

Mutations in the dystrophin gene cause either the severe Duchenne muscular dystrophy (DMD; MIM 310200) or the more benign Becker muscular dystrophy (BMD; MIM 300376). The gene has a complex structure, including a large number of exons, lengthy introns, and several alternative promoters. Therefore, dystrophin pre-mRNA splicing requires strict regulation. Single nucleotide changes creating nonsense mutations in the dystrophin gene, however, can cause secondary splicing alterations, including exon skipping and nonsense mutation-associated splice site activation (Barbieri et al., 1996; Shiga et al., 1997; Melis et al., 1998; Ginjaar et al., 2000; Fajkusova et al., 2001; Disset et al., 2006; Deburgrave et al., 2007; Tran et al., 2007). Exon skipping results in in-frame open reading frames that encode semi-functional dystrophin proteins; these proteins ameliorate the severity of the DMD phenotype (Barbieri et al., 1996; Shiga et al., 1997; Melis et al., 1998; Ginjaar et al., 2000; Fajkusova et al., 2001; Disset et al., 2006; Deburgrave et al., 2007). Nonsense mutation-associated splice site activation, following nonsense mutation of exon 42 of the dystrophin gene, produces a novel mRNA transcript (Tran et al., 2007). Thus, single nucleotide changes resulting in nonsense mutations in the dystrophin gene can alter splicing in two independent ways.

Drug-induced suppression of nonsense mutations by inducing the ribosomal read-through effect is currently being examined as a treatment for DMD patients carrying nonsense mutations in dystrophin. The possibility that dystrophin protein can be produced following the creation of semi-functional mRNA transcripts by altered splicing had not been

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considered during read-through effect stimulation (Howard et al., 2004; Politano et al., 2003). Artificial induction of exon skipping using antisense oligonucleotides specific for the exon sequence has attracted attention as a promising treatment for DMD, as it transforms out-of-frame dystrophin mRNAs into in-frame transcripts, thereby producing semi-functional internally deleted dystrophin protein. Although disruption of the splicing enhancer sequence with antisense oligonucleotides induces exon skipping (Takeshima et al., 1995), methods facilitating the identification of optimal antisense oligonucleotides with the maximal ability to induce exon skipping have not been established. To assist with the identification of ideal target sites for designing antisense oligonucleotides, it is crucial to identify key nucleotides involved in splicing regulation. Nonsense mutations are a good model with which to validate whether single nucleotide changes can alter splicing regulation.

Here we analyzed 38 unique nonsense mutations in the dystrophin gene for their ability to cause alterations in the splicing of dystrophin pre-mRNA isolated from patient lymphocytes. Secondary alterations in splicing were observed in seven cases with the identification of alternative rescue transcripts in five cases.

Cases and Methods

Cases

More than 400 DMD patients from throughout Japan were referred to the specialized clinic for DMD patients at Kobe University Hospital (Kobe, Japan). For each clinically diagnosed case of DMD, extensive testing was performed to identify the causative mutation in the dystrophin gene. Fifty-six cases were determined to have nonsense mutations in the dystrophin gene. We then analyzed dystrophin mRNA expressed by lymphocytes for 38 of these patients as described below. Subjects' ages ranged from two to 32 years old (average: 10 years old). The protocols used in this study were approved by the ethics committee of Kobe University School of Medicine. Blood samples were acquired after obtaining written informed consent.

Analysis of Dystrophin mRNA

Total RNA was isolated from peripheral blood lymphocytes. Dystrophin mRNA transcripts expressed by the lymphocytes were analyzed by reverse transcription (RT)-nested PCR (Roberts et al., 1991; Matsuo et al., 1992). The first amplification utilized an outer set of primers (Table 1). An aliquot of this reaction was then subjected to a secondary amplification using an inner primer set. An aliquot of each of the PCR reactions was separated on an agarose gel (Tran et al., 2006).

After purification, amplified products were subcloned into the pT7 vector (Novagen, Madison, WI); the inserted DNA was sequenced using a Big Dye terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) on an automatic DNA sequencer (ABI PRISM model 310, Applied Biosystems) as described (Surono et al., 1997).

Analysis of Exonic Splicing Enhancers and Silencers

Two web-based algorithms identified exonic splicing enhancers. The ESE finder (http://exon.cshl.edu/ESE/) is a web-based resource that rapidly analyzes exon sequences to identify binding motifs for four serine/arginine-rich (SR) proteins, SF2/ASF, SC35, SRp40, and SRp55 (Cartegni et al., 2003). RESCUE-ESE software (http://genes.mit.edu/burgelab/rescue-ese/) employs a computational method identifying exonic splicing enhancers in human genomic sequences by searching for specific hexanucleotides (Fairbrother et al., 2004).

Exonic splicing silencers were analyzed using the ACESCAN2 web-based computational method (http://genes.mit.edu/acescan2/index.html). All putative splicing enhancer and silencer motifs were identified, allowing us to calculate the density of enhancer or silencer motifs per 100 bp.

Results

After extensive mutation analysis, nonsense mutations in the dystrophin gene were identified in 56 Japanese patients with dystrophinopathies. We analyzed the dystrophin mRNA isolated from 38 of these patients' lymphocytes by RT-PCR analysis to identify secondary alterations in splicing patterns (Table 2). We have previously reported two examples of exon skipping due to nonsense mutations. The c.3631G>T mutation in exon 27 (p.1211E>X) induced partial skipping of exon 27, which resulted in a mild dystrophinopathy phenotype with production of semi-functional dystrophin (Shiga et al., 1997). The c.5899C>T mutation in exon 41 (p.1967R>X) induced the partial skipping of exon 41 (Surono et al., 2004). Although the c.5899C>T mutation was identified in two additional patients with DMD (cases 501 and 651), in both cases, amplification of a fragment spanning dystrophin exons 40 to 45 resulted in a single PCR product (Fig. 1a). Sequencing of this transcript revealed an authentic splicing product containing exons 40 to 45. These results demonstrated that exon 41 was not skipped in two of the three cases carrying the c.5899C>T mutation (Surono et al., 2004). This difference in the splicing patterns suggests individual differences in the splicing regulatory environment.

We also identified a novel nonsense mutation in a patient with DMD (case 496); a c.1793C>G mutation altering a serine-encoding TCA codon to a TGA stop codon (p.598S>X) within exon 15. RT-nested-PCR amplification of a fragment spanning exons 13 to 18 from this template produced two PCR products (Fig. 1b). The larger product was an authentic splicing product containing exons 13 to 18.

Table 1 Primers used to amplify dystrophin cDNA fragments

Exon	Outer primers (exon number)		Inner primers (exon number)	
	Forward	Reverse	Forward	Reverse
5	1A : TTTTTATCGCTGCCTTGATATACA (1)	1B : ACTCTGCAACACAGCTTCTGAG (18)	1C : ATGCTTTGGTGGGAAGAAGTAG (1)	c8r : GATGGACTTCTTATCTGGATA (8)
8	1A	1B	1C	1D
10	1A	1D : TCCTGTTCCAATCAGCTTACTTC (11)	c7f : GTGGTTTGCCAGCAGTCAGCCACA (7)	1D
14	1A	1B	c13f : GCTGCTTTGGAAGAACAACTT (13)	1F : CTTCTGAGCGAGTAATCCAGCT (18)
15	1A	1B	c13f	1F
17	1A	1B	c13f	1F
18	2A : ACAAGGGAACAGATCCTGGTAA (17)	2B : CTGCTTTTTCTGTACAATCTGACG (33)	2C : AGGCAGATTACTGTGGATTCTGA (17)	2D : CCCACCTTCATTGACACATGTT (25)
19	2A	2B	2C	2D
20	2A	c25r : CCCACCTTCATTGACACTGTT (25)	2C	c21r : TTGTCTGTAGCTCTTTCTCTC (21)
23	2A	2B	c21f : CAACCTCAAATTGAACGATT (21)	2D
26	2A	2B	2E : GAGCATTGTCAAAGCTAGAGGA (24)	c28r : CAATAACTCATGCCAACATGCCCA (28)
27	2A	2B	c25f : ACTCAGTGGGATCACATGTG (25)	c30r : CTCCTGGGCAGACTGGATGC (30)
28	2A	2B	2E	2F : TCCACACTCTTTGTTTCCAATG (32)
33	3A : GCTTGAACAGAGCATCCAGTC (30)	3D : TTAAACTGCTCCAATTCCTTCAA (38)	3C : GCCCAAAGAGTCCTGTCTCA (31)	3D
34	3A	3B : ACTGGCATCTGTTTTTGAGGAT (45)	3C	3D
38	3A	3B	3E	3F : CTTCCCCAGTTGCATTCAAT (45)
39	3A	3B	3E	c41r : TGCGGCCCCATCCTCAGACAA (41)
41	3A	3B	c40f : AGCCTACCTGAGCCCAGAGATG (40)	3F
42	3E : TTTGACCAGAATGTGGACCA (36)	3B	c40f	3F
43	3A	3B	3E	3F
44	3A	3B	3E	3F
47	4A : TGGCGGCGTTTTCATTAT (44)	4B : CGGAGTGCAGGTTCAATTTT (52)	4C : GCTGAACAGTTTCTCAGAAAGACACAA (44)	4B
53	c48f : CAAGGAGAAATTGAAGCTCAA (48)	c56r : GTAACAGGACTGCATCATCG (56)	4E : TGGACAGAACTTACCGACTGG (51)	c56r
59	5A : CTATTGGAGGCGACTTTCCA (57)	5B: TTATTCTGCTCCTTCTTCATCTGTC (79)	5C	5D : TTTCTGCAGCAGCCACTCT (68)
66	5C : GACAGAGCAGCCTTTGGAAG (58)	c72r : TATCATCGTGTGAAAGCTGAG (72)	c64f	5D
68	c64f : CTCCGAAGACTGCAGAAGGC (64)	5F : ATCATCTGCCATGTGGAAAAG (79)	5E	c72r
70	c64f	5F	5E	c72r
76	5E : ATTGAGCCAAGTGTCCGG (67)	5F	c70f : CAGGAGAAGATGTTCGAGC (70)	5F

Table 2 Nonsense mutations exhibiting splicing alterations

Case	Exon	Mutation	Aberrant splicing pattern	ESE	ESS	Phenotype
38	5	c.354G>A	No			DMD
571	5	c.355C>T	No	create		DMD
65	8	c.724C>T	No	disrupt	create	DMD
377	10	c.1062G>A	No	disrupt		DMD
313	10	c.1087C>T	No	disrupt		DMD
454	14	c.1684C>T	Yes*			DMD
496	15	c.1793C>G	Yes	create		DMD
582	17	c.2047G>T	Yes	disrupt	disrupt	DMD
264	17	c.2089A>T	No	disrupt	create	DMD
102	18	c.2236G>T	No	disrupt		DMD
78	19	c.2302C>T	No	disrupt		DMD
85	19	c.2365G>T	No	disrupt		DMD
203	20	c.2449C>T	No			DMD
326	23	c.3151C>T	No	disrupt		DMD
54	26	c.3562A>T	No			DMD
28	26	c.3580C>T	No	disrupt		DMD
N1	27	c.3631G>T	Yes	disrupt		BMD
522	28	c.4003G>A	No		disrupt	DMD
652	33	c.4527T>G	No	disrupt		DMD
517	33	c.4666G>T	No		disrupt	DMD
44	34	c.4729C>T	No	disrupt		DMD
52	34	c.4757G>A	No	disrupt		DMD
148	38	c.5350G>T	No	disrupt		DMD
557	39	c.5551C>T	No	disrupt		DMD
577	39	c.5551C>T	No	disrupt		BMD
225	41	c.5899C>T	Yes	disrupt	create	DMD
501	41	c.5899C>T	No	disrupt	create	DMD
651	41	c.5899C>T	No	disrupt	create	DMD
593	42	c.5985T>G	Yes*	disrupt	create	DMD
30	43	c.6283C>T	No			DMD
185	44	c.6432T>A	No	disrupt		DMD
341	47	c.6805C>T	No	disrupt		DMD
475	53	c.7855A>T	No	disrupt		DMD
110	59	c.8745G>T	No	disrupt		DMD
724	66	c.9568C>T	No	disrupt	create	DMD
445	68	c.9851G>A	No	disrupt		DMD
703	70	c.10108C>T	Yes			DMD
N2	76	c.10873C>T	No	disrupt	create	BMD

The mutations are shown in the table with the corresponding effect on the exonic splicing enhancers and silencers. Nonsense mutation-associated splice site activation was observed in two cases (asterisks; cases 454 and 593).

The smaller product, however, lacked exon 15 completely. Thus, the c.1793C>G mutation induced the skipping of exon 15, despite the fact that this nucleotide change was 89 bp downstream of the 5′ end of the exon and did not influence the splicing consensus sequences. The transcript lacking exon 15 was expected to produce an internally-deleted dystrophin protein lacking the in-frame exon 15 and the associated premature stop codon. The phenotype of this patient, however, was severe, classified as DMD (Table 2).

In one patient carrying a c.2047G>T mutation (p.683E>X) within exon 17 (case 582), we observed the partial skipping of exon 17. RT-nested PCR amplification of a fragment spanning exons 13 to 18 generated two PCR products (Fig. 1c). Sequencing of these products determined that the larger, more abundant product contained all six authentic exons, while the smaller product lacked exon 17. As a result of the absence of the 176-bp exon 17, the translational reading frame was disrupted, and no dystrophin protein was expected to be produced.

In a patient carrying a c.9851G>A mutation (p.3284W>X) within exon 68 (case 445), amplification of a fragment from exon 67 to exon 72 produced two products; the larger, more abundant product represented the full-length transcript, while the smaller lacked exon 68 (data not shown). As exon 68 has previously been shown to be alternatively spliced in normal controls (Feener et al., 1989), it was difficult to determine if exon 68 skipping was enhanced by this mutation.

We also observed the skipping of two consecutive exons in dystrophin mRNAs carrying the c.10108C>T mutation (p.3370R>X) within exon 70 (case 703). RT-nested PCR amplification of exons 67 to 72 generated two products (Fig. 1d). The major product represented the authentically spliced transcript, while the minor product lacked both exons 70 and 71. Subcloning and sequencing of the minor product failed to identify any transcripts lacking only exon 70 or exon 71, indicating that exon 70 skipping only occurred in conjunction with the previously reported alternative splicing of exon 71 (Feener et al., 1989).

We previously reported that a nonsense mutation in exon 42 (case 593) generated two altered splicing products: one demonstrating exon 42 skipping and the other incorporating a truncated exon 42 due to activation of a nonsense mutation-created splice acceptor site (Tran et al., 2007). In case 454, the c.1684C>T mutation identified in exon 14 altered a CAA codon to a TAA stop codon (p.562Q>X). Two bands were amplified from RT-nested-PCR of a fragment spanning exons 13 to 18 (Fig. 1e). Sequencing of the smaller product identified three consecutive missing exons (exons 14, 15, and 16). The larger band was a mixture of two products, one with the normal amplified product carrying a stop codon within exon 14 while the second PCR product lacked 22 bp from the 3′ end of exon 14. The truncation in exon 14 was likely due to aberrant splicing, as this unexpected product contained the normal sequences of exons 13 and 15. The c.1684C>T mutation, located 21 bp upstream from the 3′ end of exon 14, created a GT dinucleotide splice donor site. Therefore, the c.1684C>T mutation induced both skipping of three consecutive exons and aberrant splicing due to a nonsense mutation-generated splice donor site.

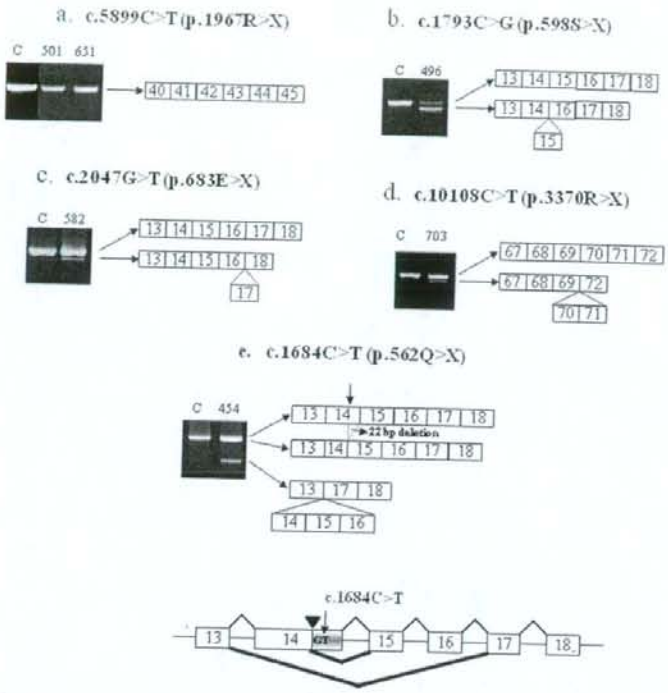

Figure 1 Analysis of dystrophin mRNAs isolated from lymphocytes. Products spanning exons indicated schematically at the right of each panel were amplified from cDNAs prepared from lymphocytes of each patient denoted by the case numbers over the amplified products. In panel e, the splicing patterns identified in the index case are represented schematically (bottom). Skipping of exons 14, 15, and 16 and activation of the splice site created by the mutation (inverted triangle) were observed. The diagonal lines above and below the boxes indicate the authentic and aberrant splicing events, respectively. The boxes and horizontal lines indicate the exons and introns, respectively. The figure is not drawn to scale.

In the remaining 30 cases, we did not detect any additional mRNA products, suggesting that the underlying nonsense mutations did not cause any aberrant splicing. Therefore, seven of the 38 nonsense mutations (18%) altered the splicing of dystrophin mRNA expressed in lymphocytes. The absence of an effect for the majority of nonsense mutations on the splicing pattern indicated that the translatability of the mRNA was not the primary cause determining splicing alterations.

Exons with low Shapiro's probability scores for the splice donor and acceptor sites are prone to exon skipping following single exonic nucleotide substitutions (Cartegni et al., 2002). We therefore calculated the splicing probability scores for the splice donor and acceptor sites of all exons that contained a nonsense mutation. No significant differences in these scores were observed between skipped exons and included exons (data not shown). When the exons were plotted based on their splicing probability scores, the skipped and non-skipped exons were distributed similarly in the plot (Fig. 2). In addition, we identified two exons (exons 17 and 41) that were either included or skipped, depending on the patient. Thus, the strength of the splice site was not a predictor of exon skipping.

Exonic splicing enhancer and silencer sequences also regulate splicing. The nonsense mutations identified in this study may have disrupted exonic splicing enhancers or created splicing silencers in skipped exons. Disruption of a splicing enhancer sequence was identified in four of the seven skipped exons (Table 2). This disruption, however, was also observed at a similar frequency (23/30) in the non-skipped exons (Table 2). Similarly, splicing silencer sequences were created in both skipped exons (two of seven cases) and non-skipped

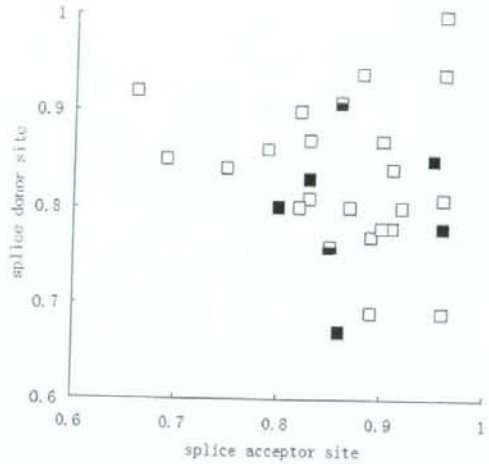

Figure 2 Quantitation of splice site strength. The Shapiro's splicing probability scores for the splice donor (vertical axis) and acceptor (horizontal axis) sites are represented graphically. Closed and open boxes represent the skipped and non-skipped exons, respectively. The half-filled boxes represent exons indicated in Table 2 that were skipped or included in a patient-dependent manner.

exons (six of 30 cases). Therefore, we concluded that splicing alterations are not primarily due to changes in the exon's splicing regulatory motifs.

As nonsense-mediated mRNA decay destabilizes mRNAs containing premature stop codons (Maquat, 2004), it was thought that detection of mRNAs containing nonsense mutations would be difficult (Ito et al., 2003). For the seven mutations producing skipped exons, five of the resulting transcripts contained an intact open reading frame, allowing them to avoid nonsense-mediated mRNA decay. In fact, the detection frequency of exon skipping was higher for in-frame exons (five of 15, 33%) than for out-of-frame exons (two of 13, 15%). This result suggested that detection of exon skipping was influenced in part by mRNA stability.

Although the observed alternative rescue transcripts would be predicted to lead to the production of semi-functional dystrophin in five cases, only one (case N1) exhibited a mild phenotype (Table 2). In the four remaining cases of DMD, dystrophin mRNA expressed in muscle was not analyzed. Rescue transcripts were demonstrated in lymphocytes. Since the splicing and processing patterns of dystrophin mRNA transcripts in myofibres may differ in lymphocytes, it is premature to conclude the effect of the presence of semi-functional dystrophin mRNAs on the DMD phenotype without specifically analyzing muscle dystrophin mRNAs. Semi-functional dystrophin mRNA transcripts may be inferred to modify the phenotype only when they are expressed in skeletal muscle.

Discussion

A few reports of nonsense-associated altered splicing have been reported in the literature (Zatkova et al., 2004; Vuoristo et al., 2004). In dystrophinopathy patients, nonsense mutations that induce exon skipping resulting in semi-functional dystrophin protein have been discovered for several exons of the dystrophin gene; such mutations appear to modify the clinical phenotype (Shiga et al., 1997; Disset et al., 2006; Deburgrave et al., 2007). The use of lymphocyte cells to identify mutations and to determine splicing patterns of transcripts have been established by both our group (Ito et al., 2003, Tran et al., 2007) and others (Tuffery et al., 1996; Barbieri et al., 1996; Roberts et al., 1991; Tay et al., 2006). Accordingly, we have previously shown that the splicing patterns of dystrophin mRNA active in lymphocytes are similar to those in skeletal muscles (Shiga et al., 1997). Similar splicing patterns of dystrophin mRNA have been observed in both lymphocyte and skeletal muscle (Shiga et al., 1997). In our current study, we observed secondary splicing alterations in nearly one-fifth (19%) of transcripts bearing nonsense mutations. The dystrophin splicing pattern in patients carrying nonsense mutations in the dystrophin gene should be analyzed in an attempt to understand those cases exhibiting a mild phenotype (Shiga et al., 1997).

An identical nonsense mutation has been reported to result in different dystrophinopathic phenotypes among siblings (Ginjaar et al., 2000). While this phenotypic difference was explained by differences in dystrophin mRNA splicing, no further examination was conducted. In this study of nonsense mutations in Japanese dystrophinopathy patients, we observed different splicing outcomes for an identical nonsense mutation (c.5899C>T) (Table 2). The c.5899C>T mutation has been identified in eight dystrophinopathy cases worldwide; these patients exhibit diverse phenotypes, ranging from severe DMD to mild BMD (http://www.dmd.nl/dmd_all.htmle. These differences in clinical phenotype may result from differences in rescue transcript production. These data suggest that there are individual differences in the regulation of splicing between patients.

We have described two types of splicing alterations that occur for dystrophin mRNA bearing nonsense mutations: exon skipping and the use of nonsense mutation-created splice sites. Exon skipping was more commonly observed than the generation of nonsense mutation-associated splice sites. Neither maintenance of the open reading frame nor the strength of the splice boundaries surrounding an exon was the primary factor determining exon skipping (Fig. 2). It was surprising that one such mutation induced the skipping of three consecutive