exons (Fig. 1e), suggesting that higher regulatory mechanisms as well as the known *cis*-control elements regulated splicing. We were unable to develop a strict set of rules to explain the observed exon skipping events.

The induction of the ribosomal read-through of nonsense mutations by gentamicin and other drugs has recently been reported as a novel method to drive dystrophin expression in DMD patients carrying nonsense mutations in the associated gene (Mankin & Liebman, 1999). Our results show that alternative rescue transcripts arising from secondary splicing alterations may be present in patients with nonsense mutations. As these transcripts may have semi-functional expression, it is important to carry out pre-analysis of dystrophin mRNA before treatment with drugs that induce ribosomal read-throughs in order to obtain an accurate assessment of the effects of the treatment.

The transformation of out-of-frame mRNA transcripts into in-frame messages through exon skipping leads to the production of semi-functional internally deleted dystrophin protein. Such a technique is a promising approach to the treatment of DMD. Individual differences in splicing despite identical mutations (Table 2) suggest that this type of treatment may have unpredictable outcomes. The therapy, however, could be effective nonetheless. In a previous report, we induced the skipping of exon 19 in a DMD patient carrying a deletion in exon 20 using antisense oligonucleotides against an splicing enhancer sequence in exon 19; this treatment lead to the production of an in-frame dystrophin mRNA and detection of dystrophin-positive skeletal muscle cells (Takeshima et al., 2006). The optimal target exon sequences for this type of therapy, however, remain unknown. Our current results suggest that mutation sites that induce exon skipping may be candidate target sites for antisense oligonucleotide treatment of patients with DMD (Surono et al., 2004).

Acknowledgments

We thank Miss T. Inagawa for her help preparing the manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Health and Labor Science Research Grant (Research on Psychiatric and Neurological Diseases and Mental Health), and a Research Grant (17A-10) for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare.

References

Barbieri, A. M., Soriani, N., Ferlini, A., Michelato, A., Ferrari, M. & Carrera, P. (1996) Seven novel additional small mutations and a new alternative splicing in the human dystrophin gene detected by heteroduplex analysis and restricted RT-PCR heteroduplex analysis of illegitimate transcripts. *Eur J Hum Genet* **4**, 183–187.

Cartegni, L., Chew, S. L. & Krainer, A. R. (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* **3**, 285–298.

Cartegni, L., Wang, J., Zhu, Z., Zhang, M. Q. & Krainer, A. R. (2003) ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res* **31**, 3568–3571.

Deburgrave, N., Daoud, F., Llense, S., Barbot, J. C., Recan, D., Peccate, C., Burghes, A. H., Beroud, C., Garcia, L., Kaplan, J. C., Chelly, J. & Leturcq, F. (2007) Protein- and mRNA-based phenotype-genotype correlations in DMD/BMD with point mutations and molecular basis for BMD with nonsense and frameshift mutations in the DMD gene. *Hum Mutat* **28**, 183–195.

Disset, A., Bourgeois, C. F., Benmalek, N., Claustres, M., Stevenin, J. & Tuffery-Giraud, S. (2006) An exon skipping-associated nonsense mutation in the dystrophin gene uncovers a complex interplay between multiple antagonistic splicing elements. *Hum Mol Genet* **15**, 999–1013.

Fairbrother, W. G., Yeo, G. W., Yeh, R., Goldstein, P., Mawson, M., Sharp, P. A. & Burge, C. B. (2004) RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucleic Acids Res* **32**, W187–190.

Fajkusova, L., Lukas, Z., Tvrdikova, M., Kuhrova, V. V., Hajek, J. & Fajkus, J. (2001) Novel dystrophin mutations revealed by analysis of dystrophin mRNA: alternative splicing suppresses the phenotypic effect of a nonsense mutation. *Neuromuscul Disord* **11**, 133–138.

Feener, C. A., Koenig, M. & Kunkel, L. M. (1989) Alternative splicing of human dystrophin mRNA generates isoforms at the carboxy terminus. *Nature* **338**, 509–511.

Ginjaar, I. B., Kneppers, A. L., VD Meulen, J. D., Anderson, L. V., Bremmer-Bout, M., Van Deutekom, J. C., Weegenaar, J., Den Dunnen, J. T. & Bakker, E. (2000) Dystrophin nonsense mutation induces different levels of exon 29 skipping and leads to variable phenotypes within one BMD family. *Eur J Hum Genet* **8**, 793–796.

Howard, M. T., Anderson, C. B., Fass, U., Khatri, S., Gesteland, R. F., Atkins, J. F. & Flanigan, K. M. (2004) Readthrough of dystrophin stop codon mutations induced by aminoglycosides. *Ann Neurol* **55**, 422–426.

Ito, T., Takeshima, Y., Yagi, M., Kamei, S., Wada, H. & Matsuo, M. (2003) Analysis of dystrophin mRNA from skeletal muscle but not from lymphocytes led to identification of a novel nonsense mutation in a carrier of Duchenne muscular dystrophy. *J Neurol* **250**, 581–587.

Li, B., Wachtel, C., Miriami, E., Yahalom, G., Friedlander, G., Sharon, G., Sperling, R. & Sperling, J. (2002) Stop codons affect 5′ splice site selection by surveillance of splicing. *Proc Natl Acad Sci USA* **99**, 5277–5282.

Mankin, A. S. & Liebman, S. W. (1999) Baby, don't stop! *Nat Genet* **23**, 8–10.

Maquat, L. E. (2004) Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat Rev Mol Cell Biol* **5**, 89–99.

Matsuo, M., Nishio, H., Kitoh, Y., Francke, U. & Nakamura, H. (1992) Partial deletion of a dystrophin gene leads to exon skipping and to loss of an intra-exon hairpin structure from the predicted mRNA precursor. *Biochem Biophys Res Commun* **182**, 495–500.

Melis, M. A., Muntoni, F., Cau, M., Loi, D., Puddu, A., Boccone, L., Mateddu, A., Cianchetti, C. & Cao, A. (1998) Novel nonsense mutation (C–>A nt 10512) in exon 72 of dystrophin gene leading to exon skipping in a patient with a mild dystrophinopathy. *Hum Mutat* **Suppl 1**, S137–S138.

Politano, L., Nigro, G., Nigro, V., Piluso, G., Papparella, S., Paciello, O. & Comi, L. I. (2003) Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results. *Acta Myol* **22**, 15–21.

Roberts, R. G., Barby, T. F., Manners, E., Bobrow, M. & Bentley, D. R. (1991) Direct detection of dystrophin gene rearrangements by analysis of dystrophin mRNA in peripheral blood lymphocytes. *Am J Hum Genet* **49**, 298–310.

Shiga, N., Takeshima, Y., Sakamoto, H., Inoue, K., Yokota, Y., Yokoyama, M. & Matsuo, M. (1997) Disruption of the splicing enhancer sequence within exon 27 of the dystrophin gene by a nonsense mutation induces partial skipping of the exon and is responsible for Becker muscular dystrophy. *J Clin Invest* **100**, 2204–2210.

Surono, A., Takeshima, Y., Wibawa, T., Pramono, Z. A. & Matsuo, M. (1997) Six novel transcripts that remove a huge intron ranging from 250 to 800 kb are produced by alternative splicing of the 5′ region of the dystrophin gene in human skeletal muscle. *Biochem Biophys Res Commun* **239**, 895–899.

Surono, A., Tran, V. K., Takshima, Y., Wada, H., Yagi, M., Takagi, M., Koizumi, M. & Matsuo, M. (2004) Chimeric RNA/ethylene bridged nucleic acids promote dystrophin expression in myocytes of Duchenne muscular dystrophy by inducing skipping of the nonsense-mutation-encoding exon. *Hum Gene Ther* **15**, 749–757.

Takeshima, Y., Nishio, H., Sakamoto, H., Nakamura, H. & Matsuo, M. (1995) Modulation of in vitro splicing of the upstream intron by modifying an intra-exon sequence which is deleted from the dystrophin gene in dystrophin Kobe. *J Clin Invest* **95**, 515–520.

Takeshima, Y., Yagi, M., Wada, H., Ishibashi, K., Nishiyama, A., Kakumoto, M., Sakaeda, T., Saura, R., Okumura, K. & Matsuo, M. (2006) Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. *Pediatr Res* **59**, 690–694.

Tay, S. K., Khng, H. H., Low, P. S. & Lai, P. S. (2006) Diagnostic strategy for the detection of dystrophin gene mutations in Asian pateints and carriers using immortalized cell lines. *J Child Neurol* **21**, 150–155.

Tran, V. K., Takeshima, Y., Zhang, Z., Habara, Y., Haginoya, K., Nishiyama, A., Yagi, M. & Matsuo, M. (2007) A nonsense mutation-created intraexonic splice site is active in the lymphocytes, but not in the skeletal muscle of a DMD patient. *Hum Genet* **120**, 737–742.

Tran, V. K., Takeshima, Y., Zhang, Z., Yagi, M., Nishiyama, A., Habara, Y. & Matsuo, M. (2006) Splicing analysis disclosed a determinant single nucleotide for exon skipping caused by a novel intra-exonic four-nucleotide deletion in the dystrophin gene. *J Med Genet* **43**, 924–930.

Tuffery, S., Bareil, C., Demaille, J. & Claustres, M. (1996) Four novel dystrophin point mutations: detection by protein truncation test and transcript analysis in lymphocytes from Duchenne muscular dystrophy patients. *Eur J Hum Genet* **4**, 143–152.

Vuoristo, M. M., Pappas, J. G., Jansen, V. & Ala-Kokko, L. (2004) A stop codon mutation in COL11A2 induces exon skipping and leads to non-ocular Stickler syndrome. *Am J Med Genet A* **130**, 160–164.

Wang, J., Hamilton, J. I., Carter, M. S., Li, S. & Wilkinson, M. F. (2002) Alternatively spliced TCR mRNA induced by disruption of reading frame. *Science* **297**, 108–110.

Wang, Z., Rolish, M. E., Yeo, G., Tung, V., Mawson, M. & Burge, C. B. (2004) Systematic identification and analysis of exonic splicing silencers. *Cell* **119**, 831–845.

Zatkova, A., Messiaen, L., Vandenbroucke, I., Wieser, R., Fonatsch, C., Krainer, A. R. & Wimmer, K. (2004) Disruption of exonic splicing enhancer elements is the principal cause of exon skipping associated with seven nonsense or missense alleles of NF1. *Hum Mutat* **24**, 491–501.

Zhang, X., Lee, J. & Chasin, L. A. (2003) The effect of nonsense codons on splicing: a genomic analysis. *RNA* **9**, 637–639.

Zhang, X. H., Kangsamaksin, T., Chao, M. S., Banerjee, J. K. & Chasin, L. A. (2005) Exon inclusion is dependent on predictable exonic splicing enhancers. *Mol Cell Biol* **25**, 7323–7332.

Received: 26 September 2007
Accepted: 13 June 2008

ELSEVIER

High Incidence of Electrocardiogram Abnormalities in Young Patients With Duchenne Muscular Dystrophy

Yuichi Takami, MD, Yasuhiro Takeshima, MD, PhD, Hiroyuki Awano, MD, Yoh Okizuka, MD, Mariko Yagi, MD, PhD, and Masafumi Matsuo, MD, PhD

Electrocardiogram abnormalities are reported to be complicated in Duchenne muscular dystrophy. Although Duchenne muscular dystrophy can be genetically diagnosed in young patients, extensive electrocardiogram studies have not been reported. Here, electrocardiogram abnormalities were examined in Duchenne muscular dystrophy cases with *dystrophin* gene mutations. Sixty-nine patients, aged ≤18 years, received 136 electrocardiogram examinations. Sixty-four patients (91.3%) displayed one or more abnormalities. Furthermore, patients adolescent <10 years (84.8% of patients) displayed electrocardiogram abnormalities, and the most common abnormality was deep Q-waves. Remarkably, the abnormality incidence of both deep Q-waves and low RV5 + SV1 (R-wave V5 + S-wave V1) were significantly high in adolescent patients. Although the patterns or positions of *dystrophin* gene mutations were compared with electrocardiogram abnormalities, no predisposing mutation was disclosed. These results indicate that electrocardiogram abnormalities in Duchenne muscular dystrophy are a result of dystrophin deficiency, regardless of types of gene mutations. The disease can be divided into two types: age-dependent and age-independent. Deep Q-waves and low RV5 + SV1 are proposed as markers of age-dependent cardiac complications.

Takami Y, Takeshima Y, Awano H, Okizuka Y, Yagi M, Matsuo M. High incidence of electrocardiogram abnormalities in young patients with Duchenne muscular dystrophy. Pediatr Neurol 2008;39:399-403.

Introduction

Duchenne muscular dystrophy, the most common inherited myopathy, affects approximately one in 3,500 males, and is characterized by muscle dystrophin deficiency, a result of translational reading frame shifts or nonsense mutations in the *dystrophin* gene on the X-chromosome [1]. Currently, a diagnosis of Duchenne muscular dystrophy is based on identification of mutations in the *dystrophin* gene. Duchenne muscular dystrophy is characterized by frequent, progressive cardiac involvement, ultimately developing into dilated cardiomyopathy. In addition, mutations in limited regions of the *dystrophin* gene were demonstrated to cause X-linked dilated cardiomyopathy [2], indicating a direct relationship between genotype and cardiac problems. Electrocardiographic studies of Duchenne muscular dystrophy help characterize Duchenne muscular dystrophy. Many patterns of electrocardiogram abnormalities, such as sinus tachycardia, tall right precordial R-waves with increased R-wave/S-wave (R/S) ratios, deep and narrow Q-waves in I, augmented vector of left arm (aVL), and V_{5-6}, or in II, III, and augmented vector of left foot (aVF), a short PR interval, and high-frequency notches on the QRS complexes were described [3-13]. These abnormalities were identified in 77.3-95.3% of Duchenne muscular dystrophy patients [5,6,9,10,12], but were unrelated to age, duration of the disease, and its severity [8,12]. In these studies, however, a diagnosis of Duchenne muscular dystrophy was generally based on clinical features. Although X-linked dilated cardiomyopathy suggests a strong correlation between *dystrophin* gene mutations and electrocardiogram abnormalities, to

From the Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan.

Communications should be addressed to:
Dr. Takami; Department of Pediatrics, Kobe University School of Medicine; 7-5-1 Kusunoki-cho; Chuo-ku, Kobe 650-0017, Japan.
E-mail: takami@hrc-hp.com
Received March 26, 2008; accepted August 5, 2008.

doi:10.1016/j.pediatrneurol.2008.08.006 • 0887-8994/08/$—see front matter

date, no studies have compared these abnormalities with *dystrophin* gene mutations.

Recently, several Duchenne muscular dystrophy treatments were proposed, some of which were applied to Duchenne muscular dystrophy patients [14,15]. Methods of expressing dystrophin in skeletal muscle were not demonstrated to ameliorate cardiac abnormalities in Duchenne muscular dystrophy. Considering that the main cause of death in Duchenne muscular dystrophy is cardiomyopathy, the assessment of effective cardiac treatment is essential. Nevertheless, echocardiography examinations rarely disclose cardiac complications in young Duchenne muscular dystrophy patients. Therefore, it is of the utmost importance to assess cardiac involvement properly in young Duchenne muscular dystrophy patients.

In the present study, electrocardiogram findings from Duchenne muscular dystrophy patients were retrospectively assessed and compared with *dystrophin* gene mutations. Our findings suggest no correlation between *dystrophin* gene mutation patterns and electrocardiogram abnormalities; however, electrocardiogram abnormalities were more common in young patients.

Materials and Methods

Subjects

Duchenne muscular dystrophy patients who were genetically diagnosed at Kobe University Hospital were enrolled in the present study. Clinical diagnosis of Duchenne muscular dystrophy was based on onset of progressive weakness before age 5 years and other signs, elevated serum creatine kinase levels, and either loss of unassisted ambulation before age 13 years or muscle biopsy findings of dystrophin deficiency. Patients who received electrocardiogram examination at ≤18 years old were recruited for the present study. However, patients who had been treated with a β-blocker, angiotensin-converting enzyme inhibitor, angiotensin II receptor blocker, diuretic drugs, or prednisolone before electrocardiogram examination were excluded from the study. In total, 69 patients with a genetic diagnosis of Duchenne muscular dystrophy were enrolled, and 136 electrocardiogram examinations were performed (1-6 examinations/patient; mean, 2 per patient). All patients were Japanese boys, aged 4-18 years (mean age, 11.0 years) at time of electrocardiogram examination.

Genetic Diagnosis

The analysis of genomic DNA or complementary DNA confirmed the genetic diagnosis of Duchenne muscular dystrophy, as previously described [16,17]. The *dystrophin* gene mutations that resulted in premature stop codons in dystrophin messenger RNA were revealed in 69 patients: 44 patients exhibited mutations that induced a translational reading frame shift because of exon deletions or duplications (37 cases with deletions, and 7 cases with duplications); 18 patients exhibited nonsense mutations; 5 patients displayed mutations of one or a few nucleotides deletion; and 2 patients manifested intron mutations that induced a splicing error (data not shown).

Electrocardiogram

Standard 12-lead electrocardiogram recordings were conducted, using the interpretive ECG-1550 (Nihon Kohden, Tokyo, Japan). Electrocardiogram abnormalities in heart rate, P-waves in II, P-R interval, QRS complex, R/S ratios in V_1, ST-T waves, corrected QT intervals, and RV5 + SV1 were compared with published, standard, age-matched, normal values from Japanese children [18]. Each electrocardiogram abnormality was defined as follows: deep Q-wave was >0.4 mV, shortened PR interval was <0.12 second, tall P-wave was >0.25 mV, axis deviation was <0° or >120°, the prolonged corrected QT interval was >0.44 second, the abnormal R/S ratio in V1, tall R-wave in V1, sinus tachycardia, and high RV5 + SV1 were >98th percentile of age-matched normal values, and low RV5 + SV1 was <2nd percentile of age-matched normal values.

Statistical Analysis

Fisher's exact test was used for data comparisons.

Results

Among 69 patients with Duchenne muscular dystrophy, 136 electrocardiogram recordings were performed. Abnormal electrocardiogram findings were disclosed in 63 patients (91.3%; Fig 1). Although a variety of electrocardiogram abnormalities were evident, the most common abnormality was deep Q-waves (55.1%). Deep Q-waves in anterolateral leads (I, aVL, or V_{4-6}) were evident in 53.6% of patients and in 15.9% in inferior leads (II, III, and aVF). Abnormal R/S ratios in V_1 (37.7%), tall R-waves in V_1 (36.2%), shortened PR intervals (33.3%), high-frequency notches on QRS (33.3%), low RV5 + SV1 (24.6%), and sinus tachycardia (17.4%) were frequently detected.

Electrocardiogram results were divided into 3 age groups (4-9, 10-13, and 14-18 years) to evaluate age differences (Table 1, Fig 1). Remarkably, 84.8% of patients <10 years of age exhibited one or more abnormalities. All patients >13 years of age displayed at least one abnormality. There was no significant difference between electrocardiogram abnormalities among age groups, except for deep Q-waves and low RV5 + SV1. Abnormality rates of deep Q-waves and low RV5 + SV1 were significantly greater in the older, rather than the younger, group (Table 1, Fig 2).

The relationship between patterns or types of mutations (genotypes) and electrocardiogram abnormalities (phenotypes) was subsequently analyzed. However, with all parameter combinations between genotype and phenotype, no significant correlation was disclosed. For example, patterns or types of mutations and deep Q-waves were

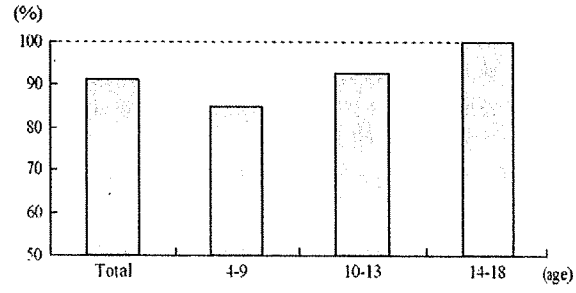

Figure 1. Rate of electrocardiogram abnormalities in age groups: 91.3% of patients displayed electrocardiogram abnormalities. Electrocardiogram abnormalities were evident in 84.8% of patients <10 years of age. There was no significant difference among age groups.

Table 1. Electrocardiogram abnormalities in patients with Duchenne muscular dystrophy

Age	Total (N = 69)		4-9 years (N = 33)		10-13 years (N = 39)		14-18 years (N = 14)	
	n	%	n	%	n	%	n	%
Deep Q waves	38	55.1	18	54.5*†	18	46.2*‡	12	85.7*†‡
in anterolateral leads (I, aVL, V4-6)	37	53.6	18	54.5	18	46.2*	11	78.6*
in inferior leads (II, III, aVF)	11	15.9	6	18.2	5	12.8	4	28.6
Abnormal R/S ratio in V1	26	37.7	9	27.3	13	33.3	7	50.0
Tall R wave in V1	25	36.2	14	42.4	14	35.9	4	28.6
Shortened PR interval	23	33.3	9	27.3	11	28.2	6	42.9
High frequency notches on QRS	23	33.3	10	30.3	17	43.6	5	35.7
Low RV5+SV1	17	24.6	1	3.0*¶‡	9	23.1*¶	7	50.0*‡
Sinus tachycardia	12	17.4	3	9.1	6	15.4	4	28.6
High RV5+SV1	2	2.9	2	6.1	1	2.6	0	0.0
Tall P wave in II	2	2.9	0	0.0	1	2.6	1	7.1
Right axis deviation	2	2.9	0	0.0	1	2.6	1	7.1
Left axis deviation	2	2.9	1	3.0	2	5.1	0	0.0
PSVT	2	2.9	1	3.0	1	2.6	0	0.0
Prolonged QTc interval	1	1.4	1	3.0	0	0.0	0	0.0

* $P < 0.05$.
** $P < 0.01$.
† Difference between 4-9 years and 14-18 years.
‡ Difference between 10-13 years and 14-18 years.
¶ Difference between 4-9 years and 10-13 years.

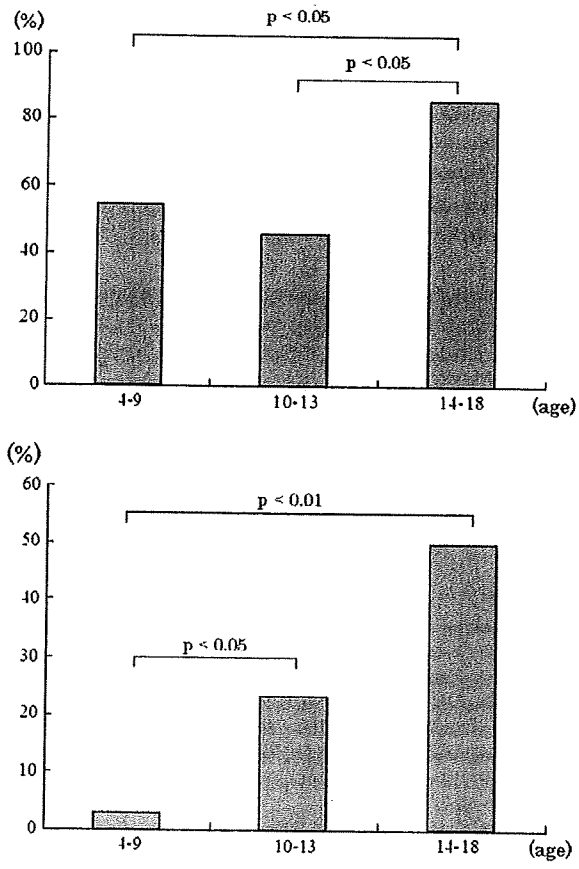

Figure 2. Rate of abnormal electrocardiograms. (a) Deep Q-waves. (b) Low RV5 + SV1. Deep Q-waves and low RV5 + SV1 were significantly greater in the older group.

compared (Fig 3). However, no clear association between the two parameters was detected. Because a higher incidence of low RV5 + SV1 was evident in older patients, genotype characteristics of low RV5 + SV1 were examined; however, no particular findings were disclosed.

Discussion

It was reported that distinctive electrocardiograms in Duchenne muscular dystrophy patients are a result of sinus tachycardia, tall right precordial R-waves, increased R/S

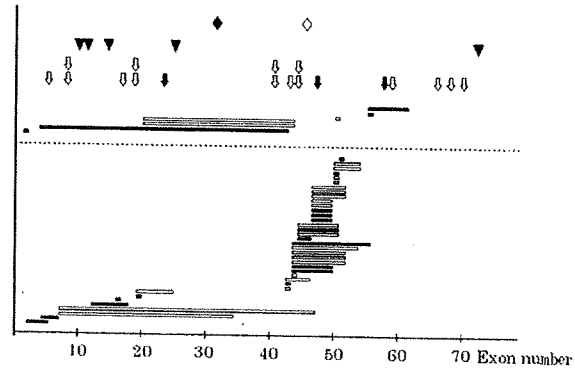

Figure 3. Distribution of dystrophin *gene mutations in Duchenne muscular dystrophy patients who exhibited deep Q-waves. Mutations were broadly distributed, and did not localize. Diamond = splice-site mutation; triangle = deletion of one or a few nucleotides; arrow = nonsense mutation; bar (upper row) = exon duplication; bar (lower row) = exon deletion; open symbols = mutations of patients with electrocardiogram abnormalities.*

ratios, deep and narrow Q-waves in I, aVL, and V_{5-6} or in II, III, and aVF, short PR intervals, and high-frequency notches on QRS complexes [3-13]. In the present study, the same electrocardiogram abnormalities were also evident in genetically confirmed Duchenne muscular dystrophy patients. Deep Q-waves were the most common abnormality, demonstrating a significantly higher incidence in older patients. It was suggested that fibrosis replacement of normal cardiac tissue, with lateral extension of scarring, might explain the presence of deep Q-waves in patients with Duchenne muscular dystrophy [19,20]. The high incidence of deep Q-waves in older patients is compatible with this supposition, indicating the progression of disease process. Considering that dystrophin is present at the membrane surface of the Purkinje fiber, Bies et al. [21] speculated that dystrophin deficiency leads to cardiac conduction disturbances. Sanyal et al. suggested that dystrophic changes in the conduction system might cause left-axis deviations and short PR intervals in Duchenne muscular dystrophy patients [11]. Ishikawa suggested that the Purkinje block might cause high-frequency notches on QRS waves [7]. These abnormalities are supposedly attributable to dystrophin deficiency, and are thought to increase incidence in older patients.

The present study targeted Duchenne muscular dystrophy patients whose diagnosis was based on genetics, as well as on clinical features and muscle biopsies. In addition, this study aimed to determine whether electrocardiogram abnormalities in Duchenne muscular dystrophy patients correlated with genetic diagnosis. To our knowledge, this is the first report of electrocardiogram abnormalities in Duchenne muscular dystrophy patients to be confirmed by *dystrophin* gene mutations.

In the present study, deep Q-waves, abnormal R/S ratio in V_1, tall R-wave in V_1, shortened PR intervals, high-frequency notches on QRS, low RV5 + SV1, and sinus tachycardia were frequently evident. These abnormalities were similar to features described in previous reports. However, low RV5 + SV1 was not previously described. Low RV5 + SV1 might be caused by a QRS anterior shift, resulting in tall, right, precordial R-waves. The rate of low RV5 + SV1 increased in the older group of patients, although the rate of tall R-waves in V1, as the result of a QRS anterior shift, decreased. Low RV5 + SV1 could indicate QRS-complex low voltage caused by myocardial dystrophy rather than a QRS anterior shift.

Abnormal electrocardiogram findings in Duchenne muscular dystrophy, with no cardiac signs, were reported during early childhood. Nigro et al. reported that 55 of 105 patients (52.4%) <10 years of age exhibited electrocardiogram changes [22]. In the present study, 84.8% of patients <10 years of age displayed electrocardiogram abnormalities. Furthermore, electrocardiogram abnormality rates were similar among the three age groups, with the exception of deep Q-waves and low RV5 + SV1. Consequently, it might be impossible to conclude that the above-described electrocardiogram abnormalities indicate cardiomyopathy progression. The rates of deep Q-waves and low RV5 + SV1 increased in the older group, which may be an indicator of cardiomyopathy. Further investigation is necessary to assess whether low RV5 + SV1 can predict the progression of early cardiomyopathy.

It was reported that specific *dystrophin* gene mutations bring about mental retardation or X-linked dilated cardiomyopathy [2]. Developmental delays and mental retardation are preferentially associated with deletions or duplications affecting the 3′ end of the *dystrophin* gene [23]. The X-linked dilated cardiomyopathy is caused by mutations in the *dystrophin* gene. It was assumed that certain regions of the *dystrophin* gene are responsible for cardiomyopathy. According to our hypothesis, there might be a hotspot region in the *dystrophin* gene that causes electrocardiogram abnormalities and X-linked dilated cardiomyopathy. Therefore, the features and patterns of *dystrophin* gene mutations in Duchenne muscular dystrophy patients who displayed a distinctive electrocardiogram were analyzed. Nevertheless, no hotspot region was detected. The distribution of mutations was scattered, although patients <10 years of age displayed an abnormal electrocardiogram. Gulati et al. examined deletions in exons 48 and 49, which were reported to be more frequent in Duchenne muscular dystrophy and Becker's muscular dystrophy with severe cardiac complications [6]. They reported that exons 48 and 49 were deleted in 6 (30%) of 30 patients with Duchenne muscular dystrophy. However, no correlation was observed with disease severity or cardiac dysfunction.

Branco et al. reported that mdx^{5cv} mice exhibited several typical electrocardiogram features observed in Duchenne muscular dystrophy patients, such as tall R-waves, short PR intervals, and mild arrhythmia vulnerability [24]. However, histopathology demonstrated no increased myocardial fibrosis.

In conclusion, electrocardiogram abnormalities of Duchenne muscular dystrophy patients <10 years of age were frequently observed. Abnormalities of deep Q-waves and low RV5 + SV1 were of significantly higher incidence in older patients. However, correlations with *dystrophin* gene mutations and distinctive abnormal electrocardiograms were not revealed. Electrocardiogram abnormalities in Duchenne muscular dystrophy may be based on dystrophin deficits, regardless of the type of gene mutations.

References

[1] **Monaco** AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988;2:90-5.

[2] **Cohen** N, Muntoni F. Multiple pathogenetic mechanisms in X linked dilated cardiomyopathy. Heart 2004;90:835-41.

[3] **Bhattacharyya** KB, Basu N, Ray TN, Maity B. Profile of electrocardiographic changes in Duchenne muscular dystrophy. J Indian Med Assoc 1997;95:40-7.

[4] **D'Orsogna** L, O'Shea JP, Miller G. Cardiomyopathy of Duchenne muscular dystrophy. Pediatr Cardiol 1988;9:205-13.

[5] **Fitch** CW, Ainger LE. The Frank vectorcardiogram and the electrocardiogram in Duchenne progressive muscular dystrophy. Circulation 1967;35:1124-40.

[6] **Gulati** S, Saxena A, Kumar V, Kalra V. Duchenne muscular dystrophy: Prevalence and patterns of cardiac involvement. Indian J Pediatr 2005;72:389-93.

[7] **Ishikawa** K. Cardiac involvement in progressive muscular dystrophy of the Duchenne type. Jpn Heart J 1997;38:163-80.

[8] **Lukasik** E, Liszewska-Pfejfer D, Rubach K, Szwed H. Long-term electrocardiographic studies in patients with Duchenne progressive muscular dystrophy. Acta Med Pol 1980;21:181-91.

[9] **Perloff** JK, de Leon AC Jr, O'Doherty D. The cardiomyopathy of progressive muscular dystrophy. Circulation 1966;33:625-48.

[10] **Sanyal** SK, Johnson WW. Cardiac conduction abnormalities in children with Duchenne's progressive muscular dystrophy: Electrocardiographic features and morphologic correlates. Circulation 1982;66: 853-63.

[11] **Sanyal** SK, Johnson WW, Thapar MK, Pitner SE. An ultrastructural basis for electrocardiographic alterations associated with Duchenne's progressive muscular dystrophy. Circulation 1978;57:1122-9.

[12] **Slucka** C. The electrocardiogram in Duchenne progressive muscular dystrophy. Circulation 1968;38:933-40.

[13] **Wahi** PL. Cardiac changes in myopathy. Am Heart J 1963;66: 748-54.

[14] **Hamed** SA. Drug evaluation: PTC-124—A potential treatment of cystic fibrosis and Duchenne muscular dystrophy. Idrugs 2006;9: 783-9.

[15] **Takeshima** Y, Yagi M, Wada H, et al. Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. Pediatr Res 2006;59: 690-4.

[16] **Matsuo** M, Masumura T, Nakajima T, et al. A very small frame-shifting deletion within exon 19 of the Duchenne muscular dystrophy gene. Biochem Biophys Res Commun 1990;170:963-7.

[17] **Matsuo** M, Masumura T, Nishio H, et al. Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy Kobe. J Clin Invest 1991;87:2127-31.

[18] **Okuni** M. Shoni sindenzu no seijochi [in Japanese]. Tokyo: Igaku Shoin, 1985. 7-131.

[19] **Perloff** JK, Henze E, Schelbert HR. Alterations in regional myocardial metabolism, perfusion, and wall motion in Duchenne muscular dystrophy studied by radionuclide imaging. Circulation 1984;69:33-42.

[20] **Perloff** JK, Roberts WC, de Leon AC Jr, O'Doherty D. The distinctive electrocardiogram of Duchenne's progressive muscular dystrophy. An electrocardiographic-pathologic correlative study. Am J Med 1967;42: 179-88.

[21] **Bies** RD, Friedman D, Roberts R, Perryman MB, Caskey CT. Expression and localization of dystrophin in human cardiac Purkinje fibers. Circulation 1992;86:147-53.

[22] **Nigro** G, Comi LI, Politano L, Bain RJ. The incidence and evolution of cardiomyopathy in Duchenne muscular dystrophy. Int J Cardiol 1990;26:271-7.

[23] **Mehler** MF. Brain dystrophin, neurogenetics and mental retardation. Brain Res Brain Res Rev 2000;32:277-307.

[24] **Branco** DM, Wolf CM, Sherwood M, Hammer PE, Kang PB, Berul CI. Cardiac electrophysiological characteristics of the mdx (5cv) mouse model of Duchenne muscular dystrophy. J Interv Card Electrophysiol 2007;20:1-7.

ELSEVIER

Brain & Development 31 (2009) 169–172

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

www.elsevier.com/locate/braindev

Case report

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

Shigemi Kimura [a,*], Kaori Ito [b], Hiroe Ueno [a], Makoto Ikezawa [a], Yasuhiro Takeshima [c], Kowashi Yoshioka [a], Shiro Ozasa [a], Kyoko Nakamura [a], Keiko Nomura [a], Makoto Matsukura [b], Koichi Mitsui [a], Masafumi Matsuo [c], Teruhisa Miike [a]

[a] *Department of Child Development, Kumamoto University Graduate School, 1-1-1 Honjou, Kumamoto 860-0811, Japan*
[b] *Laboratory of Clinical Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan*
[c] *Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Japan*

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

* Corresponding author. Tel.: +81 96 3735197; fax: +81 96 3735200.
E-mail address: kimusige@kumamoto-u.ac.jp (S. Kimura).

0387-7604/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
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-

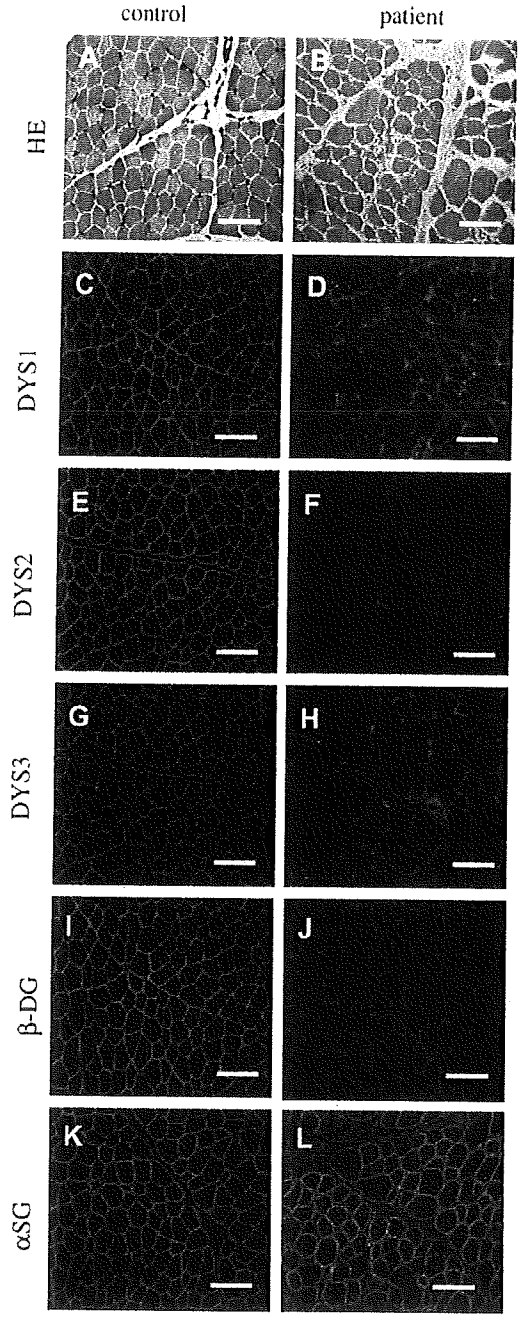

Fig. 1. HE staining and immunohistochemical study. Cryostat sections were prepared from non-dystrophinopathy controls (A, C, E, G, I, K) and patients (B, D, F, H, J, L). HE staining from the patient (B) showed slight variations in myofiber size, as well as scattered regenerating fibers and necrotic fibers. 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

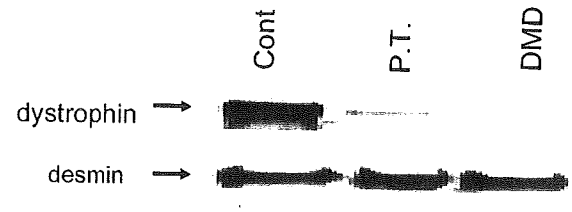

Fig. 2. Western blotting analysis for dystrophin of skeletal muscle. The size of dystrophin from the non-dystrophinopathy control (Cont) and the patient (P.T.) was almost the same; but, the quantity of the dystrophin expression in the patient was lower than that of the control. DMD, Duchenne muscular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

References

[1] Hoffman EP, Brown Jr RH, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919–28.

[2] Koenig M, Beggs AH, Moyer M, Scherpf S, Heindrich K, Bettecken T, et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. Am J Hum Genet 1989;45:498–506.

[3] Becker PE, Kiener F. New X-chromosomal muscular dystrophy. Arch Psychiatr Nervenkr 1955;193:427–48.

[4] Arahata K, Ishiura S, Ishiguro T, Tsukahara T, Suhara Y, Eguchi C, et al. Immunostaining of skeletal and cardiac muscle surface membrane with antibody against Duchenne muscular dystrophy peptide. Nature 1988;333:861–3.

[5] Tinsley JM, Blake DJ, Zuellig RA, Davies KE. Increasing complexity of the dystrophin-associated protein complex. Proc Natl Acad Sci USA 1994;91:8307–13.

[6] Suzuki A, Yoshida M, Ozawa E. Mammalian alpha 1- and beta 1-syntrophin bind to the alternative splice-prone region of the dystrophin COOH terminus. J Cell Biol 1995;128:373–81.

[7] Sadoulet-Puccio HM, Rajala M, Kunkel LM. Dystrobrevin and dystrophin: an interaction through coiled-coil motifs. Proc Natl Acad Sci USA 1997;94:12413–8.

[8] Surono A, Takeshima Y, Wibawa T, Ikezawa M, Nonaka I, Matsuo M. Circular dystrophin RNAs consisting of exons that were skipped by alternative splicing. Hum Mol Genet 1999;8:493–500.

[9] Crawford GE, Faulkner JA, Crosbie RH, Campbell KP, Froehner SC, Chamberlain JS. Assembly of the Dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain. J Cell Biol 2000;150:1399–410.

[10] Suminaga R, Takeshima Y, Wada H, Yagi M, Matsuo M. C-terminal truncated dystrophin identified in skeletal muscle of an asymptomatic boy with a novel nonsense mutation of the dystrophin gene. Pediatr Res 2004;56:739–43.

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

Zhujun Zhang · Yasuhiro Takeshima · Hiroyuki Awano · Atsushi Nishiyama · Yo Okizuka · Mariko Yagi · Masafumi Matsuo

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

Z. Zhang · Y. Takeshima · H. Awano · A. Nishiyama · Y. Okizuka · M. Yagi · M. Matsuo (✉)
Department of Pediatrics, Graduate School of Medicine Kobe University, 7-5-1 Kusunokicho, Chuo, Kobe 650-0017, Japan
e-mail: matsuo@kobe-u.ac.jp

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

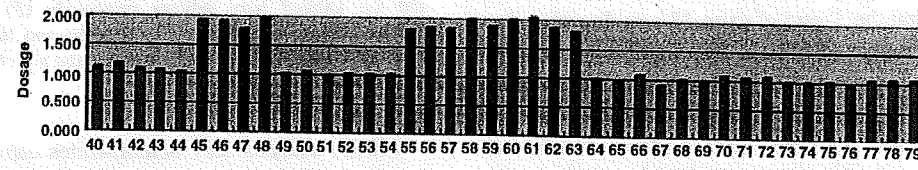

Fig. 1 MLPA analysis of the dystrophin gene. *Bars* represent the amount of amplified product expressed as a ratio to the results obtained with a control sample. *Numbers below the bars* indicate the exon numbers. The *heights of the bars* represent the relative signals of the corresponding exons compared 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

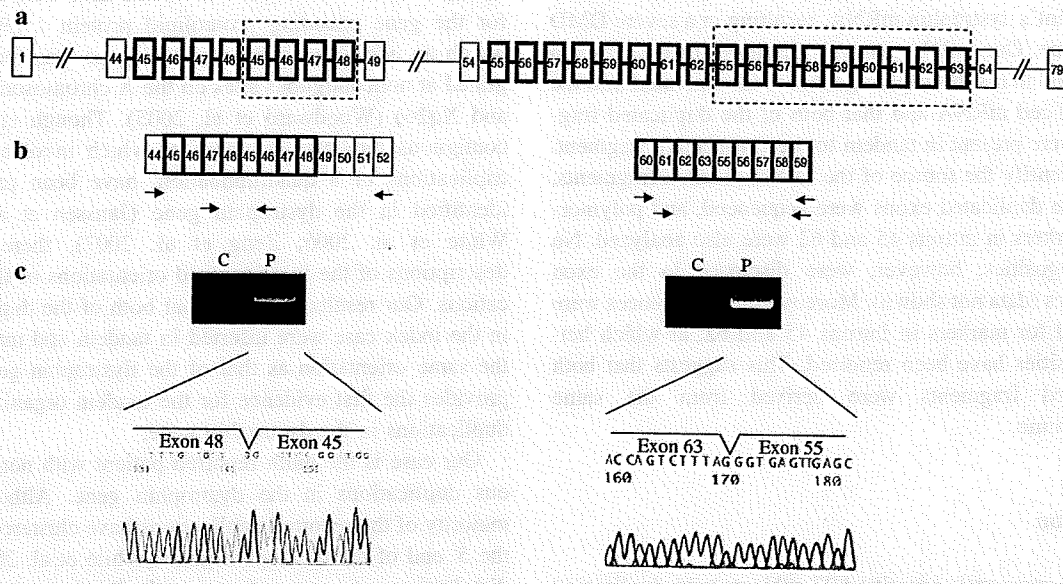

Fig. 2 RT-PCR amplification of the duplication borders of exons 45–48 and exons 55–63. **a** The predicted genomic structure of the duplicated region of the dystrophin gene. The *boxes* represent exons and the *numbers in the boxes* indicate the exon numbers. *Horizontal lines* between the *boxes* indicate 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.

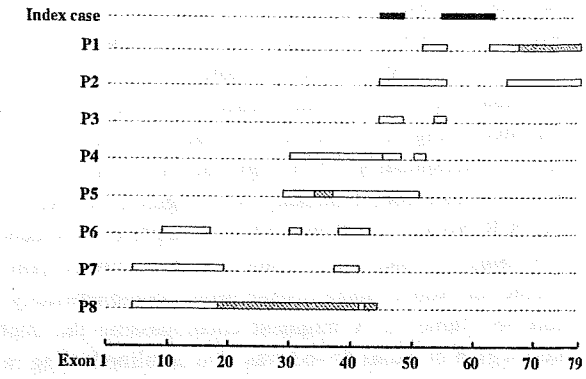

Fig. 3 A schematic representation of the distribution of identified noncontiguous duplications in the dystrophin gene. *Horizontal bars* indicate duplicated regions that have been identified in patients with DMD. The *black bars* indicate duplications identified in this study, and the *unfilled bars* 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.

References

Beggs AH, Koenig M, Boyce FM, Kunkel LM (1990) Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. Hum Genet 86:45–48

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16:11141–11156

Clemens P, Fenwick R, Chamberlain J, Gibbs R, de Andrade M, Chakraborty R, Caskey C (1991) Carrier detection and prenatal diagnosis in Duchenne and Becker muscular dystrophy families, using dinucleotide repeat polymorphisms. Am J Med Genet 49:951–960

Helleday T (2003) Pathways for mitotic homologous recombination in mammalian cells. Mutat Res 532:103–115

Hu XY, Ray PN, Murphy EG, Thompson MW, Worton RG (1990) Duplicational mutation at the Duchenne muscular dystrophy locus: its frequency, distribution, origin, and phenotypegenotype correlation. Am J Hum Genet 46:682–695

Hu XY, Ray PN, Worton RG (1991) Mechanisms of tandem duplication in the Duchenne muscular dystrophy gene include both homologous and nonhomologous intrachromosomal recombination. Embo J 10:2471–2477

Janssen B, Hartmann C, Scholz V, Jauch A, Zschocke J (2005) MLPA analysis for the detection of deletions, duplications and complex rearrangements in the dystrophin gene: potential and pitfalls. Neurogenetics 6:29–35

Lalic T, Vossen R, Coffa J, Schouten J, Guc-Scekic M, Radivojevic D, Djurisic M, Breuning M, White SdDJ (2005) Deletion and duplication screening in the DMD gene using MLPA. Eur J Hum Genet 13:1231–1234

Matsuo M, Masumura T, Nishio H, Nakajima T, Kitoh Y, Takumi T, Koga J, Nakamura H (1991) Exon skipping during splicing of dystrophin mRNA precursor due to an intraexon deletion in the dystrophin gene of Duchenne muscular dystrophy Kobe. J Clin Invest 87:2127–2131

White S, Kalf M, Liu Q, Villerius M, Engelsma D, Kriek M, Vollebregt E, Bakker B, van Ommen GJ, Breuning MH, den Dunnen JT (2002) Comprehensive detection of genomic duplications and deletions in the DMD gene, by use of multiplex amplifiable probe hybridization. Am J Hum Genet 71:365–374

White SJ, Aartsma-Rus A, Flanigan KM, Weiss RB, Kneppers AL, Lalic T, Janson AA, Ginjaar HB, Breuning MH, den Dunnen JT (2006) Duplications in the DMD gene. Hum Mutat 27:938–945

Woodward KJ, Cundall M, Sperle K, Sistermens EA, Ross M, Howell G, Gribble SM, Burford DC, Carter NP, Hobson DL, Garbem JY, Kamholz J, Heng H, Hodes ME, Malcolm S, Hobson GM (2005) Heterogeneous duplications in patients with Pelizaeus–Merzbacher disease suggest a mechanism of coupled homologous and nonhomologous recombination. Am J Hum Genet 77:966–987

Zeng F, Ren ZR, Huang SZ, Kalf M, Mommersteeg M, Smit M, White S, Jin CL, Xu M, Zhou DW, Yan JB, Chen MJ, van Beuningen R, Huang SZ, den Dunnen J, Zeng YT, Wu Y (2007) Array-MLPA: comprehensive detection of deletions and duplications and its application to DMD patients. Hum Mutat 13 Sep (Epub ahead of print)

J. Biochem. 143, 303–310 (2008)
doi:10.1093/jb/mvm227

A Strong Exonic Splicing Enhancer in Dystrophin Exon 19 Achieve Proper Splicing Without an Upstream Polypyrimidine Tract

Yasuaki Habara[1], Mari Doshita[2], Sadako Hirozawa[2], Yuka Yokono[2], Mariko Yagi[1,*], Yasuhiro Takeshima[1] and Masafumi Matsuo[1]

[1]*Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe 650-0017; and* [2]*Kobe Pharmaceutical University, Kobe 658-8558, Japan*

Received October 17, 2007; accepted November 3, 2007; published online November 26, 2007

Proper splicing is known to proceed under the control of conserved *cis*-elements located at exon–intron boundaries. Recently, it was shown that additional elements, such as exonic splicing enhancers (ESEs), are essential for the proper splicing of certain exons, in addition to the splice donor and acceptor site sequences; however, the relationship between these *cis*-elements is still unclear. In this report, we utilize dystrophin exon 19 to analyse the relationship between the ESE and its upstream acceptor site sequences. Dystrophin exon 19, which maintains adequate splicing donor and acceptor consensus sequences, encodes exonic splicing enhancer (dys-ESE19) sequences. Splice pattern analysis, using a minigene reporter expressed in HeLa cells, showed that either a strong polypyrimidine tract (PPT) or a fully active dys-ESE19 is sufficient for proper splicing. Each of these two *cis*-elements has enough activity for proper exon 19 splicing suggesting that the PPT, which is believed to be an essential *cis*-element for splicing, is dispensable when the downstream exon contains a strong ESE. This compensation was only seen in living cells but not in '*in vitro* splicing'. This suggests the possibility that the previous splicing experiments using an *in vitro* splicing system could underestimate the activity of ESEs.

Key words: acceptor site sequences, dystrophin, exonic splicing enhancer, polypyrimidine tract, splicing.

Abbreviations: AO, antisense oligonucleotide; AR, ampicillin resistant gene; BMD, Becker muscular dystrophy; BPS, branch point sequence; CV, Shapiro's splice site consensus value; DK, dystrophin Kobe; DMD, Duchenne muscular dystrophy; dys-ESE19, dystrophin exonic splicing enhancer 19; ESE, exonic splicing enhancer; PPT, polypyrimidine tract; RT–PCR, reverse transcription–PCR.

The dystrophin gene, one of the largest genes in the human genome, is characterized by the extraordinary length of its introns (*1*), and its splicing regulatory mechanism is not fully understood. In dystrophin Kobe, which harbours a 52 nt deletion in exon 19 of the dystrophin gene, exon 19 was totally skipped in mature mRNA; however, this deletion does not disturb either splicing donor or acceptor site consensus sequences (*2*). This exon skipping suggested that the deleted region contains an exonic splicing enhancer (ESE), a *cis*-acting splicing signal located within an exon that stimulates the inclusion of that exon in the mature mRNA (*3*). ESEs are thought to be recognized by protein factors such as SR proteins, which comprise a family of splicing factor containing an N-terminal RNA-binding domain and a C-terminal RS (arginine/serine-rich) domain (*4*). The exon 19 skipping observed in patients was replicated when an antisense oligonucleotide (AO), which hybridized to the region deleted in dystrophin Kobe, was introduced into cultured cell (*5*). Both *in vitro* and *in vivo* studies suggest that part of the exon sequence is essential for proper splicing; this part was named dystrophin exonic splicing enhancer 19 (dys-ESE19).

*To whom correspondence should be addressed. Tel: +81-78-382-6090, Fax: +81-78-382-6099, E-mail: myagi@med.kobe-u.ac.jp

It is not clear why exon 19 requires ESE for proper splicing. Our previous study indicated that dys-ESE19 is necessary for intron 18 splicing, but not for intron 19 splicing (*6*). This suggested that the acceptor site of exon 19 is a weak splice site, as it has been suggested that ESEs are essential for the proper splicing of an exon with weak splice consensus sequences (*4*, *7*). However, the Shapiro's splice site consensus value (CV) for the acceptor site of exon 19 (*8*) is not notably low among all of the 78 acceptor sites in the dystrophin gene exons: the CV for the exon 19 acceptor site is 0.91, while the average CV for all 78 acceptor sites in dystrophin gene exons is 0.87 (*9*). Therefore, there are unknown factors or rules that determine the exon 19 splicing patterns.

In this report, we investigate the relationship between dys-ESE19 and the quality of a polypyrimidine tract (PPT) using a minigene reporter made from dystrophin exons 18, 19, 20 and the adjacent introns. Our results suggest that either a strong PPT or a fully active dys-ESE19 is sufficient for the correct splicing of dystrophin exon 19.

MATERIALS AND METHODS

Minigene Reporter Construction—To study splicing patterns, minigene expression plasmids containing three exons (exon 18, 19 and 20 of the human dystrophin gene)

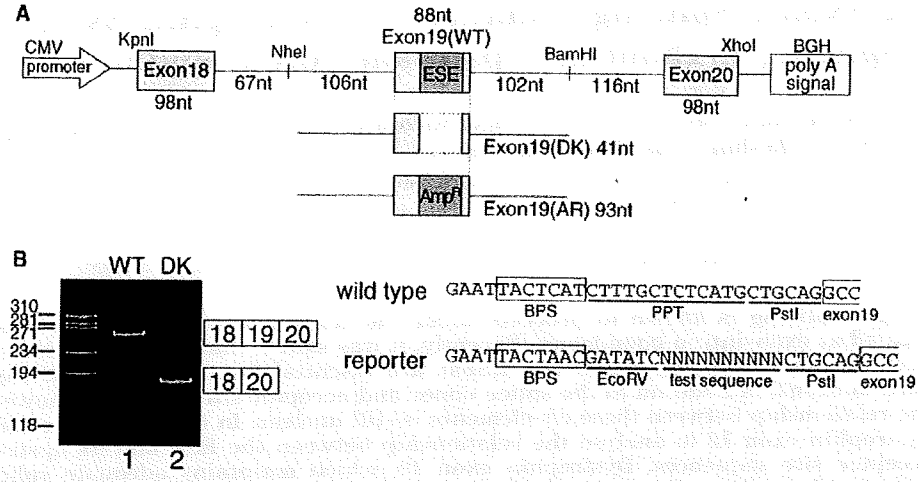

Fig. 1. **The structure of the dystrophin exon 18-19-20 minigene and examples of its splice pattern analysis.** (A) The structure of the dystrophin exon 18-19-20 minigene. Dystrophin exons 18, 19 and 20, and the adjacent introns, were amplified by PCR using primers containing a restriction enzyme cutting site, as shown in the figure. Then, three fragments (KpnI–NheI, NheI–BamHI and BamHI–XhoI) were ligated step by step using cloning vectors, and finally cloned into the pcDNA3 human expression vector with KpnI and XhoI. Two modified exon 19 constructs—DK exon 19, from which dys-ESE19 was replaced by HindIII cutting site, and AR exon 19, in which dys-ESE19 was replaced by a part of the AR gene—are shown below the WT exon 19. Open arrow and open box indicate the CMV promoter and the BGH polyadenylation signal of the pcDNA3 vector, respectively. Shaded boxes and lines indicate dystrophin exons and their flanking introns, respectively. Dark shaded boxes are dys-ESE19 (in WT exon 19) or a part of the AR gene (in AR exon 19). The sizes of the dystrophin exons and the flanking introns are also shown. The actual minigene sequences are shown in Supplementary Data Fig. S1. (B) The result of minigene reporter splice patterns. HeLa cells transfected with the minigene reporter plasmid were harvested 24 h after transfection using ISOGEN. After RNA purification and RT–PCR, samples were separated on an 8% polyacrylamide gel and stained with ethidium bromide. A 267 bp fragment (lane 1) shows that the WT exon 19 is included in the mature mRNA and a 179 bp fragment shows exon 19 skipping (lane 2). A schematic description of these RT–PCR products is shown on the right. The names of the exon 19 types are shown on the top. (C) The splice acceptor site sequence of human dystrophin exon 19 (wild type) and the sequence of the same region of the minigene reporter for PPT sequence exchange analysis (reporter). Boxes show a BPS and the first 3 nt of exon 19. Between the EcoRV and PstI sites, 11 different sequences of PPT (see Fig. 2) were inserted and tested.

and the adjacent introns were constructed (Fig. 1A) and cloned into a pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA, USA) using KpnI and XhoI sites. The actual minigene sequences are shown in Supplementary Data Fig. S1. For minigene construction and other splicing analysis, we used standard molecular cloning methods (*10*). To make PPT- and branch point sequence (BPS)-modified reporters, an altered BPS and an EcoRV recognition site were introduced into intron 18 of the minigene using PCR-based methods. Synthesized PPT oligonucleotides containing EcoRV and PstI sites were then cloned into the same restriction site (Fig. 1C). All minigene sequences amplified by PCR were confirmed by DNA sequencing using an ABI model 310 genetic analyser (Applied Biosystems, Foster City, CA, USA).

Transfection—HeLa cells were grown in 12-well plates to ~70% confluency in DMEM (Dulbecco's modified Eagle's medium) containing 5% fetal bovine serum (Trace Biosciences, Castle Hill, Australia) at 37 C under 5% CO_2. Minigene reporter expression plasmids (0.75 μg each) were transfected into cells using Plus Reagent and Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were harvested 24 h after transfection and total RNA was extracted using ISOGEN (Nippon Gene Co., Ltd., Japan).

Analysis of Splicing Products—Five micrograms of total RNA was subjected to reverse transcription (RT) using random hexamer primers or specific primers (YH353, 5′-AAGTCTCTCACTTAGC-3′) with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 μl. PCR was performed using a forward primer corresponding to a segment of exon 18 (YH307, 5′-ATTACTCGCTCAGAAGCTGTGTTGC-3′) and a reverse primer complementary to a segment of exon 20 (YH308, 5′-AAGTCTCTCACTTAGCAACTGGCAG-3′). Amplification was carried out in a total volume of 20 μl containing 4 μl of cDNA, 2 μl of 10 × Ex *Taq* Buffer, 2 μl of 2.5 mM dNTPs, 10 pmol of each primer and 1 U of Ex *Taq* Polymerase (Takara Bio, Inc., Kyoto, Japan). PCR cycling conditions were as follows: initial denaturation at 94 C for 2 min followed by 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 separated on an 8% polyacrylamide gel and stained using ethidium bromide.

In Vitro Splicing Assay—To make substrate pre-mRNA, template DNA fragments for the *in vitro* transcription were synthesized by standard PCR amplification using forward [T7(*19*), 5′-TAATACGACTCACTATAGG-3′] and reverse (YH304, 5′-CTCGAGCAGCCAGTTAAGTCTCTC AC-3′) primers with plasmid templates, which were the same plasmids utilized in the splicing assays in HeLa cells. Then, fragments were purified using a MiniElute PCR Purification Kit (QIAGEN, GmbH, Germany) and purified

name, sequences \ exon19, BPS		WT UAAC	WT UAAU	WT UCAC	WT UCAU	DK UAAC	DK UAAU	DK UCAC	DK UCAU	AR UAAC
U10	UUUUUUUUUU	+	+	+	+	+	+	+	+	+
U8G2	UUGUUUUGUU	+				+	+	+	+/−	+
U8A2	UUAUUUUAUU	+				+	+	+	+/−	+
U6G4	UUGUUGUGUG	+				−			−	+/−
U6A4	UUAUUAUAUA	+				−			−	+/−
C10	CCCCCCCCCC	+				−			−	−/+
U4G6	GGUGGUGUGU	+	+	+	+	−				
U4A6	AAUAAUAUAU	+	+	+	+	−				
U2G4A4	GAUGGAAUGA	+/−	+/−	−/+	−/+	−				
G6A4	GGAGGAGAGA	−				−				
A6G4	AAGAAGAGAG	−				−				

Fig. 2. **All results of splicing assay using reporter minigenes transfected into HeLa cells.** Name and sequence columns show the names and actual sequences of the PPT in the EcoRV–PstI cassette, which is shown in Fig. 1C. Exon 19 and BPS categories show the exon 19 types (WT: wild type; DK: 52 nt deletion the same as dystrophin Kobe; AR: ampcillin resistance gene fragment inserted in DK) and the last 4 nt of the branch site sequences (the first 3 nt of the branch site are same UAC for all constructs and were not shown in the figure). The splicing patterns are categorized into four types based on the results of acrylamide gel electrophoresis. Exon 19 included: +; exon 19 skipped: −; both included and skipped bands are observed and included was dominant or the densities of the two bands were almost the same: +/−; skipped band was dominant: −/+. Blank cells mean no such plasmid was made. Each cell is coloured as described below: light shaded: + or supposed to be +; dark shaded: − or supposed to be −; shaded medium colour: +/− or −/+; white: unknown.

templates were subjected to *in vitro* transcription using a mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX, USA). After DNaseI treatment, the resultant RNAs were purified on Quick Spin Columns G-50 (Roche, IN, USA) and the concentration and size of RNAs were confirmed using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA) and an Agilent2100 bioanalyser with an RNA nano kit (Agilent Technologies, Santa Clara, CA, USA), respectively. Standard *in vitro* splicing reactions were carried out at 37 °C for 2 h in a total volume of 20 µl containing 50% (v/v) HeLa nuclear extract (HNE; CIL BIOTEC, Mons, Belgium), 1.6 mM $MgCl_2$, 0.5 mM ATP, 20 mM creatine phosphate, 25 ng pre-mRNA and 20 U of RNaseOUT (Invitrogen, Carlsbad, CA, USA). After ProteaseK treatment, RNAs were extracted by phenol–chloroform extraction and ethanol precipitation. Splice patterns were analysed using the same RT–PCR methods as described above, except that the PCR cycle number was reduced to 15. When samples showed both exon included and skipped bands, these two bands were quantified by capillary electrophoresis using an Agilent2100 bioanalyser with a DNA1000 kit. The molecular ratio of the upper and lower bands was calculated using an index of peak height number divided by nucleotide length.

RESULTS

Effects of PPT Alteration with/without ESE on the Exon 19 Splice Pattern—To investigate dys-ESE19 function during the splicing reaction, we first synthesized minigene reporters, which contain dystrophin exons 18, 19, 20 and the adjacent introns (Fig. 1A). Minigenes containing a wild-type exon 19 (WT) or a dys-ESE19 deleted exon 19, mimicking the dystrophin Kobe mutation (DK), were cloned downstream of a CMV promoter in pcDNA3 vectors and transfected into HeLa cells. One day after transfection, cells were harvested and splicing patterns were analysed by RT–PCR. The minigene with the WT exon 19 showed a normal splicing pattern (Fig. 1B, lane 1), whereas the minigene with the DK exon 19 showed an exon 19-skipped splice pattern (Fig. 1B, lane 2). This result confirmed that this minigene reporter system reproduces the *in vivo* splicing patterns seen in both healthy controls and the DK patient who has a 52 nt dys-ESE19 deletion in exon 19 (2). This splice pattern was also confirmed in other cell-lines (MRC5 and N1H3T3, data not shown). We concluded that this reporter system recapitulates the *in vivo* splice pattern as all of the cell-lines used showed the same splice patterns as healthy controls and the patient; we used HeLa cells for following experiments.

The splice acceptor site sequence contains three different elements (*11*, *12*): a BPS, a PPT and a 3′end consensus sequence AG. First, we analysed the relationship between the PPT and the dys-ESE19. For this purpose, we modified the PPT of exon 19 in reporter plasmids to make minigenes with different PPT sequences (Fig. 1C). In total, 11 different 10 nt artificial PPTs were inserted between the BPS and the 3′ splice site consensus sequence, and the splicing pattern with or without dys-ESE19 was analysed (Fig. 2). As the most efficient type of PPT sequence is a poly(U)-tract (*13*, *14*), a U10 test sequence was examined first. Reporters containing a U10 PPT and the WT exon 19 showed a splice pattern that included exon 19, as expected (Fig. 3A, lane 1). Interestingly, reporters with a U10 PPT and the DK exon 19 also showed a splice pattern that included exon 19 (Fig. 3A, lane 2; the band size is smaller than that in lane 1 because the DK exon 19 is shorter than the WT exon 19), indicating that dystrophin exon 19 does not require the ESE if the upstream PPT has enough strength. It also suggests the possibility that dystrophin exon 19 requires the ESE because the upstream PPT is too weak to define exon 19, even though the PPT contains enough pyrimidines (10 out of 13 nt) and its CV is sufficiently high (see 'Introduction' section). To elucidate the relationship between PPT sequence and ESE requirement, we tested a series of minigenes with different PPTs, in which pairs of uridines were replaced

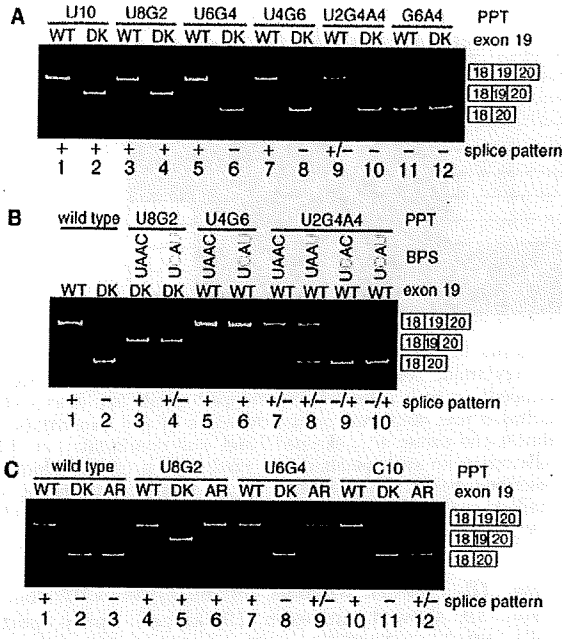

Fig. 3. **Splice pattern analysis using HeLa cells.** (A) Representative results of PPT exchange experiments. Samples were prepared in the same way as those shown in Fig. 1B. A 267 bp fragment (for example, lane 1) and a 179 bp fragment (for example, lane 6) show that WT exon 19-included and skipped 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

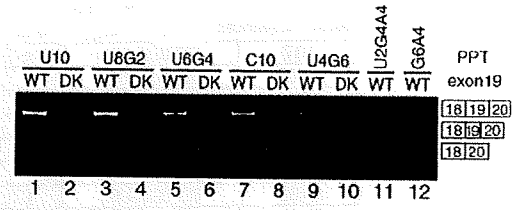

Fig. 4. ***In vitro* splicing analysis using HeLa nuclear extract.** Representative results of an *in vitro* splicing experiment are shown. After the *in vitro* splicing reaction, RNA was purified from the reaction mixture. RT–PCR and acrylamide gel electrophoresis were then performed. The names of the 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