DETECTION OF PARTIAL DELETION AND PARTIAL DUPLICATION OF DYSTROPHIN GENE IN JAPANESE PATIENTS WITH DUCHENNE OR BECKER MUSCULAR DYSTROPHY

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Summary The dystrophin gene was analyzed in 59 Japanese patients with Duchenne muscular dystrophy (DMD) from 48 unrelated families, including 11 pairs of siblings, and three patients with Becker muscular dystrophy (BMD) from two unrelated families, including one pair of siblings. The relationship between the type of gene abnormality and clinical symptoms was examined. Twenty-seven of 50 (54.0%) unrelated DMD or BMD patients were found to have partial deletions, and five (10%) appeared to have partial duplications in the dystrophin gene. Nine DMD patients, including three pairs of siblings, showed mental retardation, the existence of which was coincident in each pair of siblings, but deletion of an identical exon was not always related to mental retardation in unrelated patients.

Key Words Duchenne muscular dystrophy, deletion, duplication, mental retardation, sibling

INTRODUCTION

Duchenne muscular dystrophy (DMD) and its milder allelic variant of Becker muscular dystrophy (BMD) are the result of abnormalities in the X-linked dystrophin gene. Partial deletions or partial duplications of the dystrophin gene have been found in more than 60% of DMD/BMD patients (Koenig *et al.*, 1989; Den Dunnen *et al.*, 1989; Hu *et al.*, 1990), using a series of fragments covering a whole cDNA of 14 kb of the dystrophin gene cloned by Koenig *et al.* (1987) as probes. A molecular mechanism to explain the clinical difference in severity between DMD and BMD patients with partial deletions of the same gene locus was proposed by Monaco

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et al. (1988). They observed that the severe DMD phenotype was produced by deletions causing a disruption of the reading frame in the gene, precluding any production of functional dystrophin, whereas the milder BMD phenotype was produced by deletions that do not alter the reading frame, resulting in the production of a truncated dystrophin. Deletions in the former and the latter situations are called "out-of-frame" and "in-frame" deletions, respectively, and the proposed mechanism is called "reading-frame theory" (Liechti-Gallati *et al.*, 1989) or "reading-frame rule" (Chelly *et al.*, 1990).

In the present study, Southern blot analysis of DNA samples from DMD/BMD patients was carried out using nine probes, and partial deletions and partial duplications were identified. With respect to the "reading-frame theory," the relationship between DNA abnormalities and clinical symptoms, such as severity as well as mental retardation, was studied.

CASES

A total of 59 DMD patients from 48 unrelated families, including 11 pairs (22 cases) of siblings, and three BMD patients from two unrelated families, including one pair (two cases) of siblings, were examined for dystrophin gene abnormalities. Most were hospitalized or examined at the Hara Byoin National Sanatorium. Two patients were referred to us for analysis from a hospital in Kyushu. Patients who were unable to walk by age 16 years were diagnosed as DMD; those who were able to walk after age 16 years as BMD (Worton and Thompson, 1988). Patients who could not comprehend daily conversations and had an IQ less than 40 were diagnosed as mentally retarded. The mental retardation was observed in nine DMD patients from six unrelated families, including three pairs of siblings.

METHODS

From six clones covering the 14-kb cDNA of the dystrophin gene obtained from the American Type Culture Collection (ATCC), nine fragments (1-2a, 2b-3, 4-5a, 5b-6, 7, 8, 9-10, 11, 12-14) were produced according to the procedure of Liechti-Gallati *et al.* (1989) and used as probes.

Mononuclear cells were isolated from peripheral blood of the patients. Lymphoblastoid cell lines were established from some of the patients by Epstein-Barr virus transformation. DNA was extracted from intact mononuclear cells or the cell lines using proteinase K and sodium dodecyl sulfate (Maniatis *et al.*, 1982). DNA samples (10 μ g) were digested with a restriction enzyme, *Hind*III, electrophoresed on 0.8% or 1.0% agarose gel, and then blotted onto nitrocellulose filters.

The nine probes mentioned above were labeled with [α - 32 P]dCTP by the random primer method, and each of them was hybridized with *Hind*III fragments on Southern filters. Thereafter, the filters were washed three times with $0.1 \times$ standard saline-

citrate ($1 \times = 0.15 \text{ M NaCl}$, 15 mM sodium citrate)/ 0.1% sodium dodecyl sulfate at 65°C for 20 min. Autoradiography was performed at -80°C with intensifying screens for one to five days. For cases with possible gene duplications, analyses of band intensities on the autoradiographic film were carried out with a densitometer (ATTO densitograph, ATTO, Tokyo).

RESULTS

Some of bands derived from the dystrophin gene were absent on the Southern filters in 26 (54.2%) out of 48 unrelated DMD patients and one (50%) out of two unrelated BMD patients, suggesting the existence of partial deletions. In five DMD patients, some of bands showed about a 2-fold increase in intensity, suggesting the existence of partial duplications. Each pair of siblings showed the same pattern in Southern blotting, which demonstrated hereditary transmission. All deletions and duplications identified are shown in Fig. 1. Exon numbers were assigned based on the report of Koenig *et al.* (1989), and the numbers and the sizes of *Hind*III fragments including exons were referred to the report of Den Dunnen *et al.* (1989).

Results of Southern blotting conducted on Cases 45, 22, and a normal female (N) using cDNA9-10 as a probe are shown in Fig. 2. In Case 45, the intensity of the *Hind*III fragments of 8.8, 8.3, 7.8, 6.0, and 2.3 kb, seems relatively strong than that of the *Hind*III fragments of 6.6, 3.5, 2.8, 2.55, 2.4, and 1.0 kb, as compared with the patterns of a normal female (N) and Case 22, where neither deletion nor duplication is observable. Results of the densitometric analyses of the three cases are shown in Fig. 3. The ratio of the peak-area values for Peak No. 7 for Cases 45 and 22 was 0.7:1. With the pattern of Case 22 used as a standard, expected values of individual peaks for Case 45 were calculated by multiplying the peak-area values of individual peaks for Case 22 by the ratio mentioned above (0.7:1). When the observed peak-area values of Peaks 1-5 and 9-12 for Case 45 were divided by their expected values, 1.9, 1.6, 1.5, 0.8, 1.7, 0.8, 1.0, 1.1, and 2.0 were obtained (shown in column a in the table of Fig. 3). Similarly, when the pattern of a normal female (N) was used as a standard, the ratio of the peak-area values for Peak No. 7 for Case 45 and the normal female was 0.5:1, and the observed value/expected value ratios of the Peaks No. 1-5 and 9-12 for Case 45 were 1.9, 1.9, 1.8, 0.9, 2.1, 1.2, 1.5, 1.2, and 2.7 (shown in column b in the table of Fig. 3). Judging from these results, the values of Peaks No. 1, 2, 3, 5, and 12 (shown with asterisks in the table in Fig. 3) for Case 45 were about twofold greater than the expected values. This suggests that the five *Hind*III fragments with strong intensity are duplicated, that is, in Case 45 the region between exon 53 and exon 59 (*Hind*III fragments 42-48) is considered to be duplicated.

Based on the report of Koenig *et al.* (1989) about the exon-specific border type, in which they classified exon-intron and intron-exon borders in three types accord-

Fig. 1. Deletions and duplications in dystrophin cDNA in patients with Duchenne or Becker muscular dystrophy. All 32 abnormalities identified are shown except for those detected in siblings. B, BMD case; I, in-frame deletion; J, Junction fragment with one or both of the breakpoints within; M, with mental retardation; dashed line, not determined.

Fig. 2. Southern blot analysis of a partial duplication case (Case 45) and a partial deletion case (Case 44) identified with the cDNA 9-10 probe. Case 22, a Duchenne muscular dystrophy case, neither deletion nor duplication was detected; N, normal female.

ing to their locations in a coding triplet, we determined whether the deletions or the duplications detected in our study would disrupt the reading frame (out-of-frame) or would not (in-frame, "I" in Fig. 1). Among them, one DMD case as well as one pair of BMD siblings seemed to maintain the reading frame, whereas all of the remaining DMD patients including duplication cases were considered to disrupt the reading frame.

The mental retardation was observed in seven (from four unrelated families including three pairs of siblings) out of 34 DMD cases (from 26 unrelated families including eight pairs of siblings) with deletions and in two (unrelated) out of 18 DMD cases (from 16 unrelated families including two pairs of siblings) with neither deletion nor duplication detected. No specific characteristics were observed due to the presence, location, or length of deletions. No mental retardation was observed in six (one pair of siblings included) duplication cases nor in three BMD cases (one pair of siblings included).

Peak No.	HindIII fragment Size (kb)	Exon No.	Exon No.	Observed Peak Area Values			Observed/Expected ratio	
				Case 45	Case 22	N	a	b
1	8.8	47	57	786	581	877	1.9*	1.9*
2	8.3	44	54	1013	890	1139	1.6*	1.9*
3	7.8	42	53	810	778	949	1.5*	1.8*
4	6.6	52	-	301	515	719	0.8	0.9
5	6.0	48	58, 59	2461	2135	2433	1.7*	2.1*
7	3.5	49	60	514	738	1079	1.0	1.0
9	2.8	53	-	239	412	431	0.8	1.2
10	2.55	54	-	843	1197	1177	1.0	1.5
11	2.4	50	-	107	146	184	1.1	1.2
12	2.3	45	55	986	718	781	2.0*	2.7*

Fig. 3. Densitometric analysis of the Southern blotting film exhibited in Fig. 2. Relative ratios (observed peak-area value)/(expected value) for peaks of Case 45 were calculated using Case 22 and a normal female (N), respectively, as standards as described in the text, shown in columns a and b in the table.

DISCUSSION

Duplication of the dystrophin gene was first reported by Bertelson *et al.* in 1986. In this report, 25 DMD families were studied, and one affected male was diagnosed as having a duplication of the pERT87 region by Southern blot analysis. Thereafter, deletions and duplications identified by means of densitometry and pulsed-field gel electrophoresis were reported (Den Dunnen *et al.*, 1989; Hu *et al.*, 1990). Recently, new techniques using the polymerase chain reaction (PCR) have been devised to detect abnormalities in the dystrophin gene (Chamberlain *et al.*, 1988, 1992; Roberts *et al.*, 1990). However, duplications can be easily overlooked unless data are analyzed with that possibility in mind.

In this study, most of deletions were detected in regions of cDNA 1-2a, 7, and 8, in which hot spots for DMD mutations are considered to exist (Koenig *et al.*, 1987). However, since a single case each was detected in the region of cDNA 9-10 and cDNA 11, all the regions of the dystrophin cDNA should be examined for screening purpose.

There was one DMD patient with a deletion that seemed to produce no frame shift (in-frame deletion) in apparent disagreement with the "reading-frame theory."

Asano *et al.* (1990) also reported DMD cases having in-frame deletions and BMD cases having out-of-frame deletions among Japanese muscular dystrophy patients. Recently, Chelly *et al.* (1990) reported DMD cases in which dystrophin protein could hardly be detected, despite the in-frame deletions, due to abnormality in translation after mRNA transcription and/or instability of the protein, and BMD cases that showed truncated proteins despite having the out-of-frame deletions, due to the use of new splice sites that excluded the out-of-frame deletion sequences. By increasing the number of cases for study, common characteristics in the sequence may be found in those deletions that contradict the frame-shift theory.

Presence or absence of mental retardation was coincident with all of the 12 pairs of siblings. However, there were unrelated cases with or without mental retardation, despite having deletion of an identical exon 52 (*Hind*III fragment 41), which is expected to disrupt the reading frame. In addition, mental retardation was not observed in a case having duplication of the identical exon 52. The mechanism of development of mental retardation in some DMD patients has not yet been clarified, but the abnormality of dystrophin in the brain may possibly be involved. The base sequence at the 5' end of DMD mRNA has been reported to be different in the brain and muscle due to the possible difference of promoter and splice sites, depending on the tissue (Nudel *et al.*, 1989; Feener *et al.*, 1989). Therefore, mental retardation might be related to regions other than the exons previously reported (Koenig *et al.*, 1987).

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