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SOUTHERN BLOT AND PCR ANALYSES OF DYSTROPHIN GENE DELETIONS IN JAPANESE PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY

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INDEXING WORDS

Duchenne muscular dystrophy; dystrophin gene; gene deletion; polymerase chain reaction; Southern blot analysis.

SYNOPSIS

We have analyzed 34 Japanese patients of 31 families with Duchenne muscular dystrophy (DMD) by Southern blot and PCR (polymerase chain reaction) to detect large deletions in the genomic dystrophin gene on the X chromosome. Fifteen families (48%) had various deletions in the dystrophin gene by Southern blot analysis using the dystrophin cDNA probes 1-2a, 2b-3 and 8 which effectively covers the high-risk regions of deletion. Lengths and positions of the gene deletions detected were variable. Although there were no common deletions of exons in the gene of the DMD patients examined with the above cDNA probes, the deletion frequency of each exon was almost the same as the result previously reported in Caucasian DMD patients. The PCR method was also applied to confirm gene deletions in 9 cases from the above 15 families by amplifying 6 different regions of the dystrophin gene. Eight out of 9 DMD cases had gene deletions at the same genomic regions as found by Southern blot analysis. In a single DMD case, the Southern blot analysis indicated the exon 12 deletion, but PCR successfully amplified a 331 bp DNA fragment containing exon 12 and flanking introns. This difference may arise by a large deletion except for the above small 331 bp sequence in the HindIII digests of dystrophin gene.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked, frequent (1 per 3000 live male birth) and fatal gene defect. The dystrophin gene, that results in DMD when defective,[1,7] consists of at least 70 exons spread over more than 2.5 Mbp on the X chromosome.[9] According to the extensive studies in North America and Europe,[8,11,12] partial deletions of the DMD gene account for as much as 60% of the total gene defects responsible for the DMD pathogenesis.[9] Deletion hotspots have been known to be around two specific regions of the gene that correspond to the 5'-end and central region of the dystrophin cDNA.[9] Analysis of deletions in the dystrophin gene can be used effectively for prenatal diagnosis and genetic counseling.[6]

In 1987, the dystrophin gene has been successfully cloned by Kunkel and co-workers[9] and then Southern blot analysis using dystrophin cDNA as probes has been the most popular and useful method to analyze deletions in the genomic DMD gene.[8,9] However, Southern blot analysis may have some errors arising by restriction fragment length polymorphism (RFLP). To avoid RFLP effects, PCR (polymerase chain reaction) is an advantageous method, since it amplifies a rather short specific sequence of DNA using heat-stable Taq DNA polymerase. PCR has been applied to detect gene abnormalities in many hereditary disorders.[17] Thus, employment of both Southern blot and PCR together improves the gene analysis.

In this communication, we studied dystrophin gene deletions in 34 DMD cases using Southern blot and PCR analyses.

PATIENTS and METHODS

Patients

Thirty-four Japanese DMD patients from 31 families, who were admitted in Hyogo Chuo National Hospital, were enrolled into this study. All cases were previously diagnosed as being affected with DMD by clinical course, high serum creatine kinase and histopathological findings of biopsied muscle.[16]

Method for Southern blot analysis

Genomic DNA was isolated from peripheral white blood cells of 10 to 20 ml of heparinized blood with the standard procedure.[5] Isolated DNA (10-15 μ g) was digested to completion with HindIII (Takara shuzo Co.,Ltd., Kyoto, Japan)

under the manufacturer's recommended conditions. Samples were electrophoresed in 0.8% agarose gels and transferred to nitrocellulose filters (BA-85, Schleicher & Schuell, Germany).

cDNA probes were ^{32}P-labeled to a specific activity of about 10^8 cpm/μg DNA using Multiprime™ DNA labeling system (Amersham, U.K.). The dystrophin cDNA subclones used were 1-2a, 2b-3 and 8[8,9] (purchased from the American Type Culture Collection), which had been reported to cover hotspots of gene deletions.

Prehybridization (5 x SSC, 3 x Denhardt, 0.05 % SDS, 45 % formamide and 0.1 mg yeast tRNA/ml) and hybridization (the same solution with labeled probes) were performed at 42°C overnight and at 42°C for two days, respectively. Filters were washed successively, twice for 5 min. at room temperature in 2 x SSC, once for 30 min. at 65°C in 2 x SSC/0.1 % SDS, and finally once at 55°C in 0.5 x SSC/0.1 % SDS. Films were exposed with an intensifying screen for 7 days at -70°C . Deletions were diagnosed when one or more of the restriction fragments were absent in the autoradiograms.

Method for PCR

Twelve primers of 24 to 28 oligonucleotides were selected according to Chamberlain et al.[3,4] to cover 6 regions having frequently deleted exons (exon 4, 8, 12, 17, 19 and 48) of the dystrophin gene. The primers were synthesized and purified as described previously.[13,15] The sequences of 12 primers and length of each amplified fragment were summarized in Table 1. The locations of 6 amplified fragments in the genomic dystrophin gene are shown in Fig. 1. Enzymatic amplification of DNA was carried out as described previously[15] in the cases presenting gene deletions in at least one of 6 exons by Southern blot analysis. A part of genomic DNA prepared for Southern blot analysis and a newly prepared genomic DNA by mini-scale rapid method[15] from nine cases expected to have gene deletion by Southern blot analysis, were used for templates. Amplified DNA fragments were separated by electrophoresis through 3% NuSieve agarose gels and photographed after ethidium bromide staining. A deletion was diagnosed when an expected fragment was not detected. The negative amplification result was confirmed by repeating the amplification and electrophoresis steps.

Table 1. Sequences of 12 promers and lengths of each amplified fragment.

Fragment	Exon	Primer Sequence	Amplified
I	4	F-TTGTCGGTCTCCTGCTGGTCAGTG R-CAAAGCCCTCACTCAAACATGAAGC	196bp
A	8	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCTTCTCATGTTCTAATTAG	360bp
G	12	F-GATAGTGGGCTTTACTTACATCCTTC R-GAAAGCACGCAACATAAGATACACCT	331bp
B	17	F-GACTTTCGATGTTGAGATTACTTTCCC R-AAGCTTGAGATGCTCTCACCTTTCC	416bp
C	19	F-TTCTACCACATCCCATTTTCTTCCA R-GATGGCAAAAGTGTTGAGAAAAAGTC	459bp
F	48	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAGTCTTCCTTACCACAC	506bp

Six sets of primers were used to amplify 6 regions in the dystrophin gene. Each of 6 amplified regions encompases one of following exons: 4, 8, 12, 17, 19 and 48.

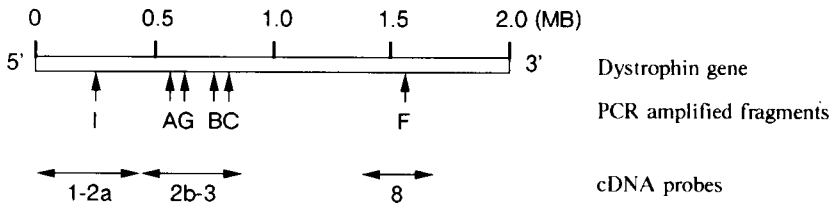

Fig. 1 Positions of 6 amplified fragments on human dystrophin gene.

RESULTS

Southern blot analysis

In normal subjects, Southern blot analysis detected the 8 fragments (10.5, 8.5, 8.0, 7.5, 4.6, 4.2, 3.2 and 3.1 kbp) using the dystrophin cDNA 1-2a probe (Fig. 2, left), the 9 fragments (12.0, 10.5, 7.3, 6.6, 6.1, 4.2, 3.0, 2.7, and 1.7 kbp) using cDNA 2b-3 (Fig. 2, center), and the 6 fragments (10.0, 7.0, 3.8, 3.1, 1.6 and 1.2 kbp) using cDNA 8 (Fig. 2, right). These results were the same as those reported previously in Caucasian,[9] suggesting no large racial differences in the dystrophin gene between Japanese and Caucasian.

Various gene deletions in the dystrophin gene were revealed in 16 Japanese DMD cases from 15 families. The patterns of gene deletion in DMD cases by Southern blot analysis were shown in Fig. 3a, 3b and 3c.

Using cDNA subclone 1-2a, case B32 had only one fragment of 3.2 kbp, but no other seven fragments (Fig. 3a). This deletion of dystrophin gene corresponded to the whole cDNA subclone 1-2a, except exons 1 and 2 contained in a 3.2 kbp HindIII fragment. In case B24, both 10.5 and 7.5 kbp fragments corresponding to exon 8 to 11 were deleted (data not shown). The remaining cases of 29 families were demonstrated to have no deletions using the cDNA 1-2a probe.

Using cDNA 2b-3 probe, a deletion of 4.2 kbp fragment corresponding to exon 12 and flanking introns was demonstrated in cases B2 and B4 (Fig. 3b). Case B24 and B32 failed to show any fragments hybridized with the cDNA 2b-3 probe (data not shown). These results suggested a gene deletion, at least long enough to span all exons (exon 10 to 20) detectable by this probe.

Using the cDNA 8 probe, four fragments, i.e., 10.0, 3.8, 1.6 and 1.2 kbp, out of 6 fragments were deleted in case B33 and a 3.1 kbp fragment in case B30. In B34 and B35 siblings, two successive 7.0 and 3.1 kbp fragments, which contained exon 52 and 51, respectively, were not detected (Fig. 3c).

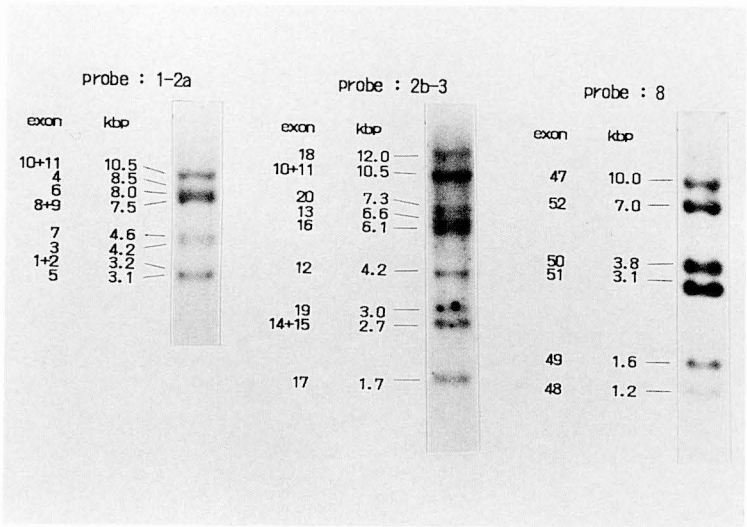

Fig. 2. Hybridization patterns of normal subject.
Hybridization patterns of DNA from normal subject with dystrophin cDNA. Probes used were, from left to right, cDNA 1-2a, 2b-3, and 8 respectively. Numbers on the left, and far left to the lanes correspond to lengths of fragments (kbp) and exon numbers, respectively.

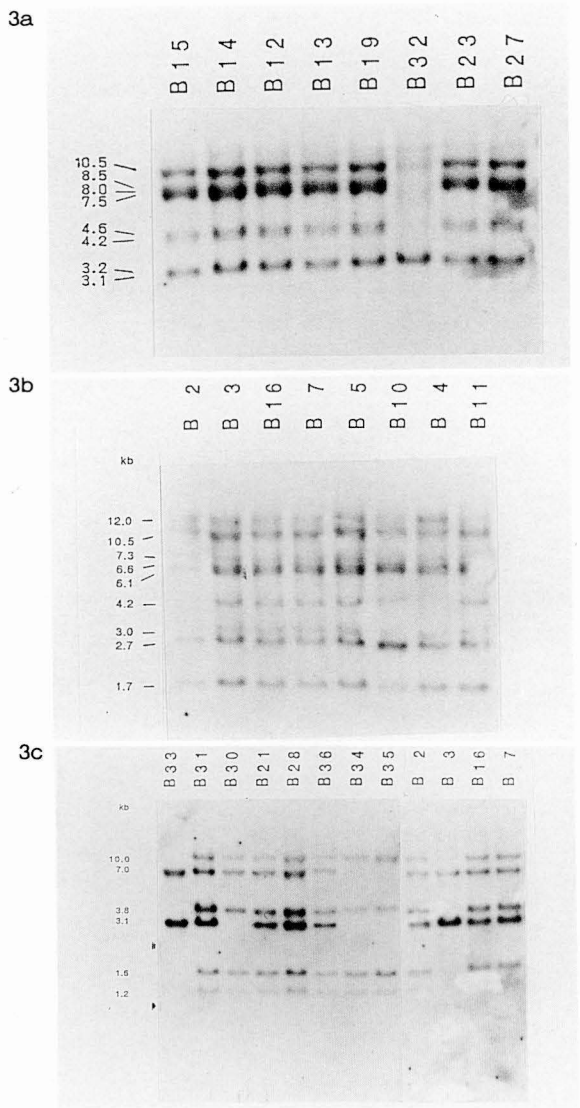

Fig. 3. Hybridization patterns of DMD patients.
Hybridization patterns of HindIII digested DNA from DMD patients with dystrophin cDNA are presented. The numbers above and left to lanes correspond to the case numbers and lengths of the fragments (kbp), respectively. Probes used are cDNA 1-2a, 2b-3 and 8 in Fig. 3a, 3b and 3c, respectively.

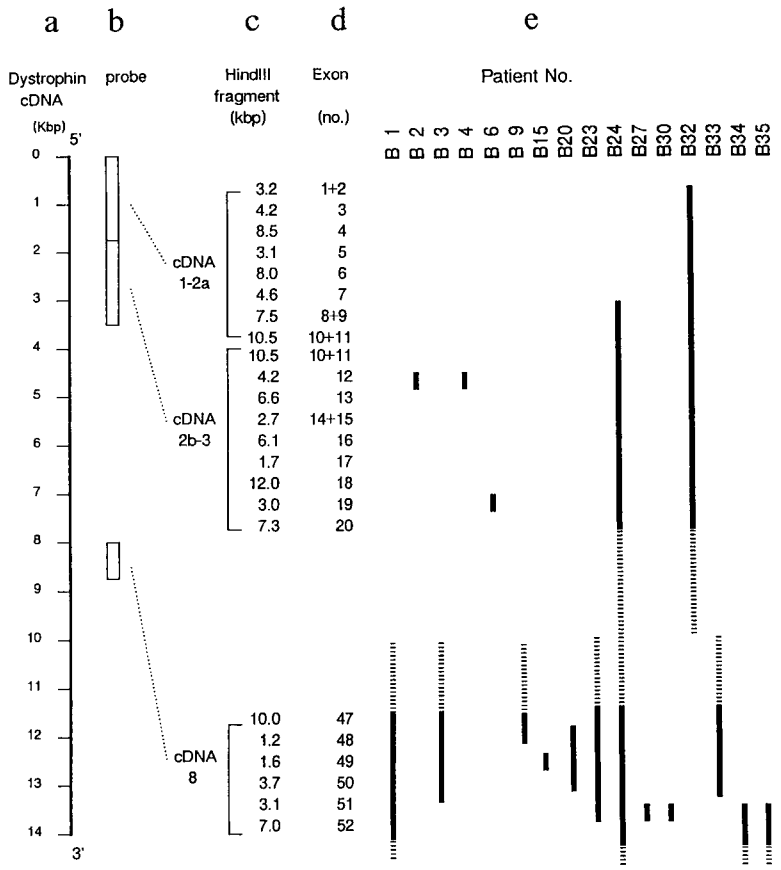

Fig. 4. Gene deletion patterns of the dystrophin gene in DMD patients.

a. Dystrophin cDNA. (sizes in kbp).
b. Position of cDNA probes used. From top to bottom, cDNA 1-2a, 2b-3, 8 are in line.
c. Lengths of the fragments detected from HindIII digested DNA of normal subjects by cDNA probes.
d. Exon numbers contained in the HindIII fragments written left.
e. Deletion patterns of the dystrophin gene in Japanese DMD patients are presented. Numbers on the top of the figure correspond to the case numbers. Solid lines correspond to deletion portion. Dotted lines mean that limits of the deletion portion were not yet clarified.

Fig. 4 summarizes schematically the locations of gene deletions detected by Southern blot analysis with the above three different cDNA subclones. The analysis in 34 cases using the cDNA subclones 1-2a, 2b-3 and 8 revealed deletions in 2, 4 and 12 cases, respectively, and thus gene deletion has been demonstrated in 15 out of 31 families (48.4%). Gene deletions in a part of the dystrophin gene corresponding to cDNA subclone 8 which contained exons 47 to 52 were the most frequent. However, lengths and positions of deletions differed from cases to cases.

The calculated deletion frequency of each exon in 31 families and that of Caucasian reported by Koenig et al.[8] are shown in Fig. 5. The deletion frequency of a 4.2 kbp HindIII fragment using cDNA subclone 2b-3 (exon 12) were slightly higher, though not significant, in Japanese than in Caucasian, and deletion frequencies in other exons were very similar between Japanese and Caucasian.

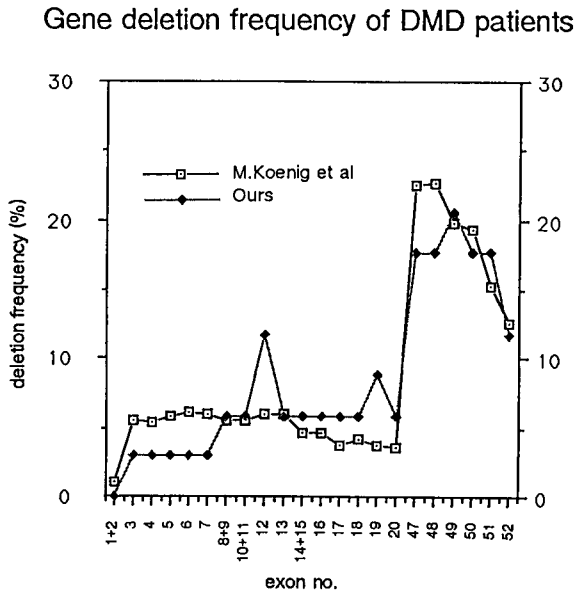

Fig. 5. Comparison of deletion frequencies of each exons between DMD patients in Japanese and Caucasian.
Deletion frequencies of each exons are plotted in % form. Solid block and open square correspond to our results (Japanese) and that of Caucasian, respectively.

Confirmation by PCR

Six DNA fragments (designated as I, A, G, B, C and F, after Chamberlain et al, [3,4] which contain exon 4,8,12,17,19 and 48 and their flanking introns, respectively), were PCR-amplified from the DNA of normal subjects. The results in Fig. 6. showed that the lengths of the above 6 amplified fragments were as expected.

Table 2 summarizes the PCR-detected deletions. In nine of 15 Japanese DMD families studied by Southern blot analysis, 6 regions were analyzed by PCR. In all of these nine cases, at least one DNA fragment failed to be amplified. Five successive fragments were not amplified in two cases of B24 and B32 (Fig. 6). Only one fragment failed to be amplified in the remaining seven cases (B1, B3, B4, B6, B20, B23 and B33) (Fig. 6 and Table 2). The exons corresponding to the deletions detected in Southern blot analysis were not amplified by PCR in eight out of 9 cases studied. A single case of B4 was different, because Southern blot analysis using cDNA 2b-3 revealed a deletion of only the 4.2 kbp HindIII fragment (exon 12 and flanking introns) but PCR detected fragment G (exon 12) of apparently normal size (Fig. 6). This result suggested that the deletion might not eliminate the 331 bp fragment G. In this case, PCR detected another deletion of the 360 bp PCR fragment A containing exon 8.

exon	PCR	Patient's ID.s								
no.	fragment	B1	B3	B4	B6	B20	B23	B24	B32	B33
4	I	+	+	+	+	+	+	+	del	+
8	A	+	+	del	+	+	+	del	del	+
12	G	+	+	+	+	+	+	del	del	+
17	B	+	+	+	+	+	+	del	del	+
19	C	+	+	+	del	+	+	del	del	+
48	F	del	del	+	+	del	del	del	+	del

Table 2. Deletion patterns in Japanese DMD patients using PCR method
+, normal; del, deletion

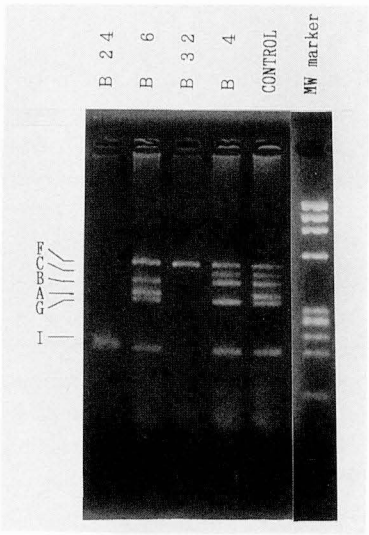

Fig. 6. PCR amplification of 6 regions of dystrophin DNA. Six fragments were amplified from DNA from normal individual (control). Case numbers are indicated on the top of the lanes. MW marker refers to HaeIII digested ϕx174 phage DNA.

DISCUSSION

In DMD, partial gene deletion was reported to be a very common genetic background for its pathogenesis.[2,8,9,12] Gene deletion analysis has become very important for genetic counseling and prenatal diagnosis.[6,11,14] Recent studies have shown the similar frequency of gene deletions in both Japanese[18] and Caucasian DMD patients[8,9,12] by Southern blot analysis using dystrophin cDNA probes.

The present study revealed the dystrophin gene deletion in 48% of Japanese DMD patients using three different deletion-prone dystrophin cDNA subclones. Lengths and positions of gene deletions in Japanese DMD patients were variable (Fig. 4). A large deletion long enough to span all of 3 cDNA subclones was detected in two cases. The frequency of deletion of each exon was almost the same between Japanese and Caucasian 8) (Fig. 5). These results suggested that gene deletion in the dystrophin gene might arise from the common genetic background for DMD and no large racial differences existed

false negative result occurs in some cases due to RFLP. PCR was first reported in 1985 by Saiki et al.[17] as a newly developed method of DNA analysis and is now widely used in DNA analysis of many hereditary disorders by its rapidity and facility.[10,17] The PCR application to DMD was first reported by Chamberlain et al.[3] in 1988 and shown to be a useful method for screening of deletions in DMD.

We applied PCR to confirm the gene deletions in the nine cases detected by Southern blot analysis. PCR analysis was carried out in six selected regions of the dystrophin gene. In eight cases the positions of gene deletion by Southern blot analysis corresponded well to those by PCR. In case B4, a 4.2 kbp HindIII fragment containing exon 12 and flanking introns was not detected by Southern blot analysis using cDNA 2b-3 probe. However, fragment G containing exon 12 was successfully PCR-amplified in the same case and fragment A containing exon 8 was never amplified. This conflict may suggest two following possibilities: one is that a large deletion except for the amplified small fragment G, which eliminates the 4.2 kbp HindIII fragment in the autoradiogram, exists in this particular case; the other is that a small deletion or point mutation may exist in the dystrophin gene of case B4 at the positions where either of the two primers for fragment A anneal. This case remains to be investigated in greater details.

Southern blot analysis is suitable for the simultaneous analysis of many exons, but sometimes makes an error due to RFLP or rearrangements. On the other hand, PCR is free from the RFLP effects, but needs numerous primers to detect gene deletions. These two methods employed together may provide more detailed and more accurate diagnosis for detecting gene deletions in DMD.

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