DELETION DETECTION FOR DIAGNOSIS OF DUCHENNE MUSCULAR DYSTROPHY IN THE JAPANESE POPULATION—COMPARISON BETWEEN THE POLYMERASE CHAIN REACTION AND THE SOUTHERN BLOT ANALYSIS—

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Summary We compared the efficacy of the multiplex PCR with that of the cDNA analysis for detection of deletions of the DMD gene in the Japanese patients. Thirty males with DMD from 27 Japanese families were studied by the multiplex PCR, and 24 of them were also investigated by Southern blot analysis. We used five dystrophin cDNA probes for deletion analysis. A total of 19 regions were amplified by the PCR to detect deletions, 9 regions by the method of Chamberlain *et al.* and another 10 regions by the method of Beggs *et al.* Deletions were detected in 14 (52%) out of 27 DMD families by the PCR. Southern blot analysis detected deletions in 14 (64%) out of 22 families. Thirteen (93%) of the 14 DMD families with deletions detected by Southern blotting were also confirmed by the multiplex PCR. Provided care is taken in cases where the deletion is limited to a single exon, the multiplex PCR appears to be an efficient and useful alternative to conventional Southern blot analysis for detecting deletions during the prenatal and postnatal diagnosis of DMD.

Key Words Duchenne muscular dystrophy, deletion detection, cDNA probe, multiplex PCR

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most devastating human X-linked recessive disease with an incidence of about one in 4,000 male live births (Moser, 1984). The number of patients with DMD is estimated to be about 7,000, and every year 150 new patients are born in Japan. A boy affected with DMD generally

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develops symptoms before the age of 6 years, is confined to a wheelchair by age 12, and dies by age 20. Since DMD is a fatal disease with no known cure, determining whether or not an at-risk fetus is affected is very important for women considering childbirth who have DMD in their families. The diagnosis of carrier status and the prenatal diagnosis of DMD are both significant issues with respect to genetic counseling.

Rapid progress in the field of DNA diagnosis of DMD has been made recently. Restriction fragment length polymorphism (RFLP) analysis with genomic DNA probes (Monaco AP *et al.*, 1985) and deletion detection analysis with cDNA probes (Koenig *et al.*, 1987) have been developed utilizing the Southern blotting method. In addition, the multiplex polymerase chain reaction (PCR) has become available, which is a technique for amplifying multiple exons within a gene to detect deletions (Chamberlain *et al.*, 1988), while PCR-RFLP analysis can be used to look for DNA polymorphisms within a gene by digesting the PCR products with appropriate restriction enzymes (Roberts *et al.*, 1989). PCR analysis usually enables results to be obtained within 1–2 days by a relatively simple procedure and is now emerging as an alternative to Southern blot analysis, which is a laborious procedure, requires the handling of radioactive materials, and usually takes 1–2 weeks to provide a results. Deletion detection with PCR analysis is especially useful in diagnosing DMD and in antenatal diagnosis. Several separate studies on the efficacy of Southern blot analysis with cDNA probes and PCR analysis are available for detecting deletions in the Japanese population (Sugino *et al.*, 1989; Asano *et al.*, 1991; Kitoh *et al.*, 1992).

This study compared the efficacy of multiplex PCR with that of the Southern blot analysis with cDNA probes for the detection of DMD deletions because these two analytical methods have not been compared enough previously in the Japanese population.

MATERIALS AND METHODS

Thirty males with DMD from 27 Japanese families were studied by the multiplex PCR to detect deletions. Twenty-two of these DMD families were also investigated by Southern blot analysis and the detection of deletions was compared. Fourteen of the families had only one sporadic male with DMD in their pedigree, while the other 13 families had at least two males affected with DMD. DNA was extracted from peripheral white blood cells by the standard method.

Southern blot analysis was done as follows. DNA (7 μ g) was digested for 12 hr with 5 units/ μ g DNA of the appropriate restriction endonuclease according to the manufacturer's recommendations. The digests were then subjected to electrophoresis overnight on 1% agarose gels, and the DNA was transferred from the gel to nylon filters (Hybond N+; Amersham, U.K.) using a vacuum transfer system (Pharmacia LKB, Sweden) and 0.4 N NaOH transfer buffer. The filters were then

hybridized using cDNA probes labeled by nick translation and with [α - 32 P]dCTP (Amersham, U.K.). They were washed in $2 \times$ SSC and $1 \times$ Denhardt's solution at room temperature for 45 min, then in $0.1 \times$ SSC and 0.1% sodium dodecyl sulfate (SDS) gentle shaking at 52°C for 90 min, and finally four times in $0.1 \times$ SSC. Filters were then exposed to Kodak X-OMAT AR films between intensifying screens at -80°C overnight and developed. We used five dystrophin cDNA probes

Table 1. Oligonucleotide sequences of the primers used in the multiplex PCR analysis.

Chamberlain's primers				Sequence	
Exon	Size(bp)				
4	196	F		TTG TCG GTC TCC TGC TGG TCA GTG	
		R		CAA AGC CCT CAC TCA AAC ATG AAG C	
8	360	F		GTC CTT TAC ACA CTT TAC CTG TTG AG	
		R		GGC CTC ATT CTC ATG TTC TAA TTA G	
12	331	F		GAT AGT GGG CTT TAC TTA CAT CCT TC	
		R		GAA AGC AGG CAA CAT AAG ATA CAC CT	
17	416	F		GAC TTT CGA TGT TGA GAT TAC TTT CCC	
		R		AAG CTT GAG ATG CTC TCA CCT TTT CC	
19	459	F		TTC TAC CAC ATC CCA TTT TCT TCC A	
		R		GAT GGC AAA AGT GTT GAG AAA AAG TC	
44	268	F		CTT GAT CCA TAT GCT TTT ACC TGC A	
		R		TCC ATC ACC CTT CAG AAC CTG ATC T	
45	547	F		AAA CAT GGA ACA TCC TTG TGG GGA C	
		R		CAT TCC TAT TAG ATC TGT CGC CCT AC	
48	506	F		TTG AAT ACA TTG GTT AAA TCC CAA CAT G	
		R		CCT GAA TAA AGT CTT CCT TAC CAC AC	
51	388	F		GAA ATT GGC TCT TTA GCT TGT GTT TC	
		R		GGA GAG TAA AGT GAT TGG TGG AAA ATC	
Beggs' primers				Sequence	
Exon	Size(bp)				
Pm	528	F		TAG ACA GTG GAT ACA TAA CAA ATG CAT G	
		R		TTC TCC GAA GGT AAT TGC CTC CCA GAT CTG AGT CC	
3	410	F		TCA TCC ATC ATC TTC GGC AGA TTA A	
		R		CAG GCG GTA GAG TAT GCC AAA TGA AAA TCA	
6	202	F		CCA CAT GTA GGT CAA AAA TGT AAT GAA	
		R		GTC TCA GTA ATC TTC TTA CCT ATG ACT ATG G	
13	238	F		AAT AGG AGT ACC TGA GAT GTA GCA GAA AT	
		R		CTG ACC TTA AGT TGT TCT TCC AAA GCA G	
43	357	F		GAA CAT GTC AAA GTC ACT GGA CTT CAT CG	
		R		ATA TAT GTG TTA CCT ACC CTT GTC GGT CC	
47	181	F		CGT TGT TGC ATT TGT CTG TTT CAG TTA C	
		R		GTC TAA CCT TTA TCC ACT GGA GAT TTG	
49	439	F		GTG CCC TTA TGT ACC AGG CAG AAA TTG	
		R		GCA ATG ACT CGT TAA TAG CCT TAA GAT C	
50	271	F		CAC CAA ATG GAT TAA GAT GTT CAT GAA T	
		R		TCT CTC TCA CCC AGT CAT CAC TTC ATA G	
52	113	F		AAT GCA GGA TTT GGA ACA GAG GCG TCC	
		R		TTC GAT CCG TAA TGA TTG TTC TAG CCT C	
60	139	F		AGG AGA AAT TGC GCC TCT GAA AGA GAA CG	
		R		CTG CAG AAG CTT CCA TCT GGT GTT CAG G	

Pm, muscle-specific promotor plus a quarter of exon 1; F, forward; R, reverse.

for the detection of deletions (1-2a, 2b-3, 4-5a, 5b-7, and 8 from the 5' end of the DMD gene—all obtained from the American Type Culture Collection).

The multiplex PCR was performed as follows. A total of 19 regions were amplified to detect deletions, 9 regions (exons 4, 8, 12, 17, 19, 44, 45, 48, and 51) by the method of Chamberlain *et al.* (1988) and the other 10 regions (muscle-specific promoter, and exons 3, 6, 13, 43, 47, 49, 50, 52, and 60) by the method of Beggs *et al.* (1990). Primers were purchased from a commercial laboratory (Sawady Technology, Tokyo). Oligonucleotide sequences of the primers used in the multiplex PCR are shown in Table 1. One hundred microliters of the total reaction mixture (250 ng of template DNA, 1.5 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 10× reaction buffer, 15 μmol of each primer, and 2.5 units of Taq polymerase) was amplified for 23 cycles after 6 min of initial denaturation at 94°C. Each cycle consisted of 30 sec of denaturation at 94°C, 30 sec of annealing at 60°C, and 4 min of elongation at 72°C. These 23 cycles were followed by a final 7 min of elongation at 65°C. Amplified products were subjected to electrophoresis on two separate 6% polyacrylamide gels (14 mA, 45 min), stained with ethidium bromide, and photographed with a Polaroid camera for analysis.

RESULTS

Deletions were detected in 14 (52%) out of 27 DMD families when a total of 19 regions were amplified by the PCR. Southern blot analysis detected deletions in 14 (64%) out of 22 DMD families using the five cDNA probes (Table 2). Thirteen families (93%) of the 14 DMD families with deletions detected by cDNA probes were also confirmed with the multiplex PCR. The presence of deletions in the remaining one DMD family (7%) whose deletions were detected by cDNA probes could not be confirmed with the multiplex PCR (Table 3).

Figure 1 shows the extent and location of the deletions detected by the five cDNA probes in relation to the 19 exons amplified in the PCR. Two brothers with DMD (patients No. 1 and 1' had deletions in exon 3 detected by cDNA probe 1-2a which could not be confirmed using the multiplex PCR. In patient No. 8, the deletion extended from exon 45 to 49 on PCR analysis while it was from exon 45 to 48 on cDNA analysis. Among the 13 families with 15 deletions confirmed by the multiplex PCR, three families (23%) were detected by Chamberlain's method

Table 2. Detection of deletions by the multiplex PCR and the Southern blot analysis using cDNA probes.

Method	Number of DMD families examined	Number of DMD families with deletions	Detection rate (%)
Multiplex PCR	27	14	52
Southern blotting	22	14	64

Table 3. Correspondence of deletion detection between the multiplex PCR and the Southern blot analysis.

Method	Number of DMD families examined	Number of DMD families with deletion	Correspondence rate (%)
Southern blotting	22	14	
Multiplex PCR	14	13	93

Fig. 1. Extent and location of the deletions detected by the five cDNA probes in relation to the 19 exons amplified in the PCR. a) Two brothers. b) Two cousins. c) Deletions detected with cDNA probes could not be confirmed by the multiplex PCR. d) The deletion extended from exon 45 to 49 on the PCR, while it extended from exon 45 to 48 on cDNA analysis. e) A junctional fragment was detected between exons 45 and 53 by cDNA probe 8, but exon 50 was amplified by the PCR.

only, five (38%) by the Begg's method only (1990), and five (38%) by both methods. Thus, Chamberlain's method (1988) detected eight (62%) of the 13 families with deletions, and the detection rate was increased considerably by adding Begg's method.

DISCUSSION

We compared the efficacy between the multiplex PCR and the Southern blot analysis with cDNA probes for the detection of deletions in Japanese DMD pa-

tients. The detection rate for Southern blotting was 64% with a total of five cDNA probes. This rate was higher than that in a previous study on Japanese patients which detected 25% of deletions in DMD (5/20) and 50% of deletions in BMD (9/18) (Asano *et al.*, 1991), and was similar to the high rate found in Caucasians (Koenig *et al.*, 1987). Deletions were concentrated at two hot spots where 73% of all deletions (11/15 deletions) were detected by two cDNA probes (1-2a and 8). Therefore, these two probes are efficient to screen for deletions. However, Southern blot analysis using cDNA probes has several disadvantages, since it requires the handling of radioactive materials, is a complex procedure, and takes a long time to obtain results. The multiplex PCR detected deletions in 52% of DMD patients when 19 exons were examined. This result was slightly lower than the 65% reported by Sannomiya (1991) and somewhat higher than the 38% reported by Minami (1991) in Japanese subjects using same 19 exons.

Of the deletions detected with cDNA probes, 93% could be confirmed by the multiplex PCR. However in two brothers (patients No. 1 and 1') deletions detected with cDNA probe 1-2a could not be confirmed by the PCR. Two possible explanations for this can be considered. One is that there was a failure to detect signals on the blot due to problems with Southern hybridization. The other explanation is that it was due to contamination of the test sample by undeleted DNA. In patient No. 8, the deletion was detected between exons 45 and 47 with cDNA probes and was extended to exon 48 by the multiplex PCR. Exon 48 is shown as a combination of 3.8 and 1.25 kb fragments with the cDNA probe, and it is sometimes difficult to determine precisely on Southern blots that only the 3.8 kb fragment of exon 48 is deleted because the 3.7 kb fragment for exon 50 is located just below it. In addition, the 1.25 kb fragment is also sometimes difficult to detect. PCR analysis has the problem of falsely identifying deletions where none exist because amplification can be prevented by the template DNA sequence polymorphism at the PCR primer annealing site (Abbs *et al.*, 1991; Fujimura *et al.*, 1990). Therefore, the true reason for this discrepancy is unclear. In the patients with a junctional fragment detected by cDNA probe 8 (patients No. 13 and 13', two cousins), the deletions was between exons 45 and 53, but exon 50 was amplified in the PCR. This difference may have been due to the partial conservation of a PCR primer annealing site on the template DNA.

Comparative studies of the detection of deletions by the multiplex PCR and cDNA analysis have not accumulated enough previously in Japanese DMD patients. In Caucasians, 82% of the deletions detected with cDNA probes were confirmed by multiplex PCR amplifying a total of 9 exons (Multicenter Study Group, 1992). This rate was increased to 98% when the number of regions amplified was expanded to 19 exons (Beggs *et al.*, 1990). Fujishita *et al.* (1991) reported that 26 (92%) of 28 deletions observed with Southern analysis were detected by PCR using only seven primer sets. Our study also showed a high detection rate that was almost consistent with these results. However, the results of PCR analysis sometimes

differed from those of Southern blot analysis with cDNA probes. Although deletions covering several exons are reliably detected, reconfirmation is required by using different sets of primers for the same exons or analysis with cDNA probes in cases where deletion is limited to a single exon. This is particularly true in prenatal diagnosis.

In conclusion, provided these precautions are followed, multiplex PCR analysis appears to be an efficient, and useful alternative to conventional Southern blot analysis for detecting deletions when undertaking the prenatal or postnatal diagnosis of DMD.

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