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ALLELE FREQUENCIES OF INTRAGENIC, AND 5′ AND 3′ MARKERS OF THE DYSTROPHIN GENE IN JAPANESE FAMILIES AFFLICTED WITH DUCHENNE OR BECKER MUSCULAR DYSTROPHY

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Summary Using the polymerase chain reaction method (PCR), we examined the allele frequencies and heterozygosities of 7 polymorphic sites (pERT87, and CA polymorphisms in the 5′ and 3′ regions) of the dystrophin gene in 20 Japanese Duchenne muscular dystrophy and Becker muscular dystrophy (DMD or BMD) families consisting of 36 males, including 23 cases of DMD and BMD, and 28 females. The allele frequencies of three primer and enzyme sets in the pERT87 locus were well comparable to those in the previously reported Japanese female cases but different from in other countries. The frequencies of 5′ markers of the dystrophin gene in Japanese were different from the reported Caucasian frequencies. As for 5′DYS-I and 5′DYS-II, the numbers of alleles in our cases were less than in Caucasians, and the heterozygosities of all three markers (5′DYS-I, II and III) were lower than in Caucasians. However, the 3′CA polymorphisms showed almost the same frequencies and heterozygosities as in Caucasians. All of our females showed a heterozygous pattern for at least one locus, with the combination of the seven markers. The usefulness of linkage analysis involving PCR methods with these intragenic, and 5′ and 3′ markers of the dystrophin gene in the carrier and prenatal diagnosis of DMD and BMD was confirmed by the successful prenatal diagnoses in 15 fetuses, the exception being one case considered to have a new mutation.

Key Words dystrophin gene, polymerase chain reaction, pERT87, CA repeat, allele frequency

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INTRODUCTION

The Duchenne and Becker muscular dystrophies (DMD and BMD) are allelic, X-linked disorders resulting in progressive muscular degeneration. DMD occurs with an incidence of one in 3,500 male births, making it the most common lethal X-linked disorder in man (Emery, 1988). After isolation of the complete cDNA of dystrophin, about 60% of DMD mutations have been detected by Southern blot analysis (Koenig *et al.*, 1989; Lindlof *et al.*, 1989), and the polymerase chain reaction method (PCR) (Chamberlain *et al.*, 1990; Beggs *et al.*, 1990). The remaining 40% of DMD cases do not show any deletions. In such DMD families, linkage analysis involving DNA markers of flanking and intragenic dystrophin gene is necessary for carrier and prenatal diagnosis (Darras *et al.*, 1987; Lindlof *et al.*, 1986; Beggs and Kunkel, 1990). Several reports of carrier and prenatal diagnoses using polymorphic markers have been appeared (Roberts *et al.*, 1989; Feener *et al.*, 1991; Oudet *et al.*, 1990; Clemens *et al.*, 1991; Gokgoz *et al.*, 1993; Kruyer *et al.*, 1994). In this paper, we report the allele frequencies of 7 polymorphic sites (pERT87 and CA polymorphisms in the 5′ and 3′ regions) in Japanese DMD or BMD families, and the usefulness for carrier and prenatal diagnoses in 20 DMD or BMD families.

MATERIALS AND METHODS

Total DNA was extracted by the phenol-chloroform method from peripheral blood samples from 36 males and 28 females from 20 families, including 23 cases of DMD or BMD. In 4 families, DNA was extracted from cultured amniotic fluid cells of fetuses at risk for DMD or BMD. The diagnosis of DMD or BMD was made in all cases on the basis of the clinical manifestations, family history, serum creatine kinase values, and DNA analysis and muscle biopsy findings, including immunohistochemical dystrophin staining in some cases. The materials, including restriction enzymes, *Taq* polymerase and biochemical materials, were purchased from commercial suppliers.

Linkage analysis using the pERT87 locus. Three sets of oligonucleotide primers within the dystrophin gene (pERT87-15/*Bam*HI, pERT87-8/*Taq*I, and pERT87-15/*Xmn*I) were prepared (Roberts *et al.*, 1989). Amplification was performed through 30 cycles of denaturation (94°C, 30 sec), annealing (55°C, 30 sec) and extension (72°C, 3 min), using an automated thermocycler (Astec Co., Japan). After extraction with phenol and chloroform, and precipitation with ethanol and 3 M sodium acetate, the PCR products were digested with the respective restriction enzymes for 3 hr according to the manufacturers' protocols, and the digested samples were analyzed by electrophoresis on 2.5% agarose gels containing ethidium bromide.

Linkage analysis using CA repeat polymorphism. Eight reported oligonu-

cleotide primers (5′DYS-I, 5′DYS-II, 5′DYS-III, 3′CA) (Feener *et al.*, 1991; Oudet *et al.*, 1990) were synthesized. Each antisense primer was labelled at its 5′ end with ^{32}P-dATP (6,000 Ci/nmol, NEN, Japan) using T4 polynucleotide kinase according to the instructions (TOYOBO, Japan). PCR was carried out in a volume of 5 μl comprising 20 ng DNA, 2 pmol unlabelled primer, 0.2 pmol end-labelled primer, 200 μM each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, and 0.1 U *Taq* polymerase (Promega, USA). Two different sets of amplification conditions were used. For the primer sets of 5′DYS-I and 3′CA, denaturation was carried out at 94°C for 30 sec, and annealing and extension at 65°C for 4 min (Beggs *et al.*, 1990; Feener *et al.*, 1991). The amplification conditions for primer sets 5′DYS-II and 5′DYS-III were denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 63°C for 4 min (Chamberlain *et al.*, 1988; Feener *et al.*, 1991). The reaction products were each mixed with 3 μl sequence loading buffer and boiled for 3 min. A 1.5 μl of each mixtures was loaded onto each lane of 8% polyacrylamide and 40% urea sequencing gels with a known sequence DNA as a size marker. Electrophoresis was performed at 35 W constant power for 2.5 hr. The gels were fixed in 10% acetic acid and 10% methanol, dried, and then autoradiographed on films at −80°C for 1–24 hr.

RESULTS

pERT87 analysis

The allele frequencies in the 64 members (28 females) in 20 DMD families are shown in Table 1. The heterozygosities in the females were 46%, 36% and 50% for pERT87-15/*Bam*HI, pERT87-8/*Taq*I and pERT87-15/*Xmn*I, respectively, with polymorphism information content (PIC) values in the range of 0.455–0.500. Sixty-eight percent of the females showed a heterozygous pattern for at least one

Table 1. Allele and heterozygote frequencies of the pERT87 locus.

Allele	Our cases				Reported cases	
	Frequency		Heterozygous (%)	PIC	Frequency	PIC
	Total	Female				
	64	28				
A: Allele (+)	0.652	0.625			0.62*	
(−)	0.348	0.375	46	0.455	0.38*	0.47
B: Allele (+)	0.348	0.357			0.74**	
(−)	0.652	0.643	36	0.455	0.26**	0.38
C: Allele (+)	0.510	0.536			0.68***	
(−)	0.490	0.464	50	0.500	0.32***	0.44

A: pERT87-15/*Bam*HI: (+), *Bam*HI site (+); (−), *Bam*HI site (−). B: pERT87-8/*Taq*I: (+), *Taq*I site (+); (−), *Taq*I site (−). C: pERT87-15/*Xmn*I: (+), *Xmn*I site (+); (−), *Xmn*I site (−).

*, *** Kunkel *et al.* (1986) Nature **322**: 73–77. ** Kunkel *et al.* (1985) Proc Natl Acad Sci USA **82**: 4778–4782.

of the three combinations (Tables 1 and 4).

CA repeat analysis

The frequencies of observed alleles and heterozygous percentages in the females for each 5′ end marker (5′DYS-I, 5′DYS-II and 5′DYS-III) and the 3′ end marker (3′CA) are summarized in Tables 2 and 3. In our Japanese cases, only two

Table 2. Allele and heterozygote frequencies of 5′ dystrophin markers.

Marker and allele	Our cases				Reported cases*		
	Frequency		Heterozygous (%)	PIC	Frequency	Heterozygous (%)	PIC
	Total 63	Female 27				Female 42	
5′DYS-I			44.4	0.384		78.6	0.608
A1 (185 bp)	0	0			0.048		
A2 (183 bp)	0	0			0.071		
A3 (181 bp)	0.311	0.259			0.310		
A4 (179 bp)	0.689	0.741			0.536		
A5 (177 bp)	0	0			0.036		
5′DYS-II			51.9	0.626		82.0	0.768
A1 (228 bp)	0.167	0.130			0.090		
A2 (226 bp)	0.144	0.148			0.077		
A3 (224 bp)	0	0			0.103		
A4 (222 bp)	0.056	0.074			0.026		
A5 (220 bp)	0	0			0.103		
A6 (218 bp)	0.022	0			0.192		
A7 (216 bp)	0.078	0.074			0.013		
A8 (214 bp)	0.533	0.574			0.397		
5′DYS-III			70.4	0.707		51.0	0.586
A1 (225 bp)	0.122	0.130			0.057		
A2 (223 bp)	0.356	0.333			0.529		
A3 (221 bp)	0.133	0.167			0.057		
A4 (219 bp)	0.389	0.370			0.357		

* Feener *et al.* (1991) Am J Hum Genet **48**: 621–627.

Table 3. Allele and heterozygote frequencies of 3′ dystrophin markers.

Marker and allele	Our cases				Reported cases*		
	Allele frequency		Heterozygous (%)	PIC	Alle frequency	Heterozygous (%)	PIC
	Total 63	Female 27				Female 27	
3′CA			40.7	0.337		37.0	0.34
A1 (137 bp)	0.144	0.167			0.22		
A2 (133 bp)	0.822	0.796			0.76		
A3 (121 bp)	0.033	0.037			0.02		
A4 (119 bp)	0	0					

* Oudet *et al.* (1990) Hum Genet **84**: 283–285.

Table 4. Heterozygosity of the pERT87 locus and the CA repeat.

Marker	% Heterozygous
1) pERT87 locus	Female 28
A: 87-15/*Bam*HI	46
B: 87-8/*Taq*I	36
C: 87-15/*Xmn*I	50
A, B, C	68
2) CA repeat	Female 27
5′DYS-I	44
5′DYS-II	52
5′DYS-II	70
3′CA	41
5′DYS-I, II, III	85
5′DYS-I, II, III, 3′CA	93
3) pERT87 (A, B, C) +5′DYS-I, II, III, 3′CA	100

alleles for 5′DYS-I and six alleles for 5′DYS-II were detected, compared to five and eight alleles detected in Caucasians (Feener *et al.*, 1991), which resulted in low heterozygosities for these alleles. However, the heterozygosity percentages for 5′DYS-III and 3′CA were higher than the values reported for Caucasians (Feener *et al.*, 1991; Oudet *et al.*, 1990). Eighty-five percent of the females showed a heterozygous pattern for at least one of the three 5′ end loci (5′DYS-I, 5′DYS-II and 5′DYS-III), and 93% heterozygosity was detected on inclusion of the 3′CA repeat (Table 4). However, only 22% of the females showed a heterozygous pattern for both the 5′ and 3′CA haplotype repeats.

All the females showed a heterozygous pattern for at least one of seven markers, three pERT87 and four CA markers (Table 4).

By PCR analysis with the seven markers, we could make successful carrier and prenatal diagnoses. On the examination of 15 fetuses at risk for DMD or BMD, only one undiagnosed male fetus was found. In this case, the haplotype of the elder brother with DMD was the same as in the healthy younger brother and in the male fetus at risk.

DISCUSSION

The allele frequencies in the pERT87 locus detected with three combinations of primer and enzyme sets were well comparable to those previously reported for Japanese and Chinese females (Akita *et al.*, 1987; Shimmoto *et al.*, 1988; Sugino *et al.*, 1989; Fujishita *et al.*, 1991; Soong *et al.*, 1991). However, they were quite different from those in the Caucasian, Spanish, Indian and black populations (Kunkel *et al.*, 1985, 1986; Lindlof *et al.*, 1987; Schwartz and Barjon, 1987; Roberts *et al.*, 1989; Gokgoz *et al.*, 1993; Al-Maghtheh *et al.*, 1993). The most characteristic difference was in the pERT87-8/*Taq*I site, which was positively

detected in only 35% of our Japanese cases, but in 74% of Caucasians (Kunkel *et al.*, 1986). The allele frequencies of 5′DYS-I, II and III were also different from those previously reported for Caucasians in the following point: only two patterns for 5′DYS-I and six patterns for 5′DYS-II were found in Japanese, five patterns and eight patterns having been found in Caucasians respectively, therefore the heterozygosity frequencies in Japanese females in the three loci were lower than those in Caucasians, the most frequent allele of 5′DYS-III being A4 (219 bp) in Japanese and A2 (223 bp) in Caucasians (Feener *et al.*, 1991). However, the heterozygosity of 3′CA was slightly higher than in Caucasians. The rare allele, A3 (121 bp), was also detected in our Japanese cases (Oudet *et al.*, 1990). These results indicated that the allele frequencies of polymorphic markers in the dystrophin gene differ among races. pERT87-15/*Xmn*I, 5′DYS-III and 3′CA are more informative markers in Japanese because each marker shows high heterozygosity and a high PIC value (Tables 1, 2 and 3).

With the three primer sets of the pERT87 locus or the four primer sets of the CA repeat, 68% and 93% of the females examined in this study showed heterozygosity in at least one of the three and four loci, respectively. With all seven primer sets, all the females showed heterozygosity. We could also make successful prenatal diagnoses in 15 fetuses at risk for DMD or BMD, there being one exception, by deletion analysis involving PCR and PCR-RFLP analysis with the seven markers. Rapid and useful PCR-RFLP analysis with these combinations of polymorphic markers in the dystrophin gene is very important and necessary for correct carrier and prenatal diagnoses in DMD or BMD families. However, about 11% of intragenic recombination has to be taken into account when these polymorphic markers are used for diagnostic application (Oudet *et al.*, 1991).

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