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Analysis of Muscle Dystrophin mRNA in Duchenne/Becker Type Muscular Dystrophy Patients with a Partial Genomic DNA Deletions

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In order to elucidate the effects of dystrophin gene deletions on the mRNA levels in the Duchenne and Becker muscular dystrophy, we analyzed human muscle dystrophin mRNA from eleven Duchenne muscular dystrophy (DMD) and six Becker muscular dystrophy (BMD) patients, with internal deletions of the dystrophin gene, using RT-PCR. We also quantified the resultant transcripts by means of semi-nested PCR to estimate the dystrophin mRNA in four BMD and three DMD cases. We found that the quantities of muscle dystrophin mRNA were drastically decreased in DMD and BMD patients as compared with in control subjects ($p<0.05$), but that there was no significant difference between DMD and BMD. In each case, three regions of the DMD gene (the deletion-containing region, and the upstream and downstream regions) were amplified, and we focused on the deletion boundary regions by sequencing the PCR products. Truncated transcripts were found in 16 cases, while in the remaining case (DMD), no RT-PCR product was amplified for either muscle or lymphocytes, and thus transcription was assumed to be disturbed. We detected a difference between the muscle mRNA and lymphocyte DNA deletions in a patient who died of a rhabdomyosarcoma. In this case, there was an apparent deletion of exon 48 in the lymphocyte DNA, while only 15 bp of the 3′ region of exon 48 was deleted from the muscle mRNA. This raises the possibility that DNA diagnosis can miss a deletion. Another DMD case showed two distinct deletion patterns in muscle versus lymphocyte mRNA due to somatic mosaicism. Thus, it is important to analyze mRNA to clarify the molecular mechanisms of DMD/BMD.

Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked recessive hereditary disorders, with allelic mutations in the dystrophin gene[1]. The dystrophin gene has been localized to the short arm of the X chromosome (Xp21), and determined to encompass at least 2.5 Mb of DNA[2)3)]. A 14 kb mRNA encodes dystrophin, which has a molecular weight of 427 kD and is composed of 3,685 amino acids arranged in four distinct domains. Dystrophin is one of the cytoskeletal components of muscle cells, being embedded in the sarcolemma (plasma membrane) as a dystrophin-associated glycoprotein complex[4)~7)]. The molecular basis for DMD/BMD has been increasingly clarified over the past decade.

In about 60% of DMD/BMD cases, the mutations comprise deletions that vary in extent and location, and two hot spot regions for mutation

have been identified; one at the 5′ terminus, and the other around exons 44-53 in the distal half of the central rod domain[8] in DMD/BMD. Based on the results of analysis of DNA from DMD/BMD patients, Monaco et al[9] and Koenig et al[10] developed the reading frame theory, i.e. DNA deletions in relatively mild BMD maintain the translational reading frame while in more severe DMD the translational reading frame is disrupted. Hoffman et al[11] showed that the clinical phenotype is correlated with the status of muscle dystrophin, which is absent or drastically reduced (<3%) in DMD patients, while a modest reduction in its amount (>10%) and/or size is seen in BMD patients.

However, on clinical screening of DMD/BMD, we found cases whose disease cannot be explained on the basis of dystrophin immunostaining. In such cases, we are not able to predict the phenotype from the DNA deletion, and even one showing the same pattern of DNA deletion may have different phenotypes. As mRNA is transcribed and spliced from genomic DNA, and then translated into dystrophin, it is important to analyze the actual mRNA, which might be better correlated with the clinical severity than the hypothetical mRNA structure determined from genomic DNA data[12].

We studied the muscle dystrophin mRNA in 17 DMD/BMD cases as well as lymphocyte dystrophin mRNA in two DMD case with partial genomic DNA deletions by RT-PCR, and examined the boundary sequences of the deletions. We found that in 14 cases the muscle transcripts were consistent with the lymphocyte DNA deletion. On the other hand, we found one case with no transcript for either muscle or lymphocytes, one with a muscle mRNA deletion inconsistent with the lymphocyte DNA data, and another with somatic mosaicism.

Subjects and Methods

Subjects

We studied muscle dystrophin mRNA in 17 DMD/BMD cases as well as lymphocyte dystrophin mRNA in two DMD case with partial genomic DNA deletions by RT-PCR, and examined the boundary sequences of the deletions. The eleven DMD patients ranged in age from 2 years (y) 2 months (m) to 15y7m and the six BMD patients from 2y7m to 14y (Table 1). All had been diagnosed based on the clinical features, serum creatine kinase (CK) level, and muscle dystrophin staining results with monoclonal DYS1, DYS2 and DYS3 antibodies (Novocastra L. Ltd. Bretton, UK), which corresponded to nucleotides (N) 3543-4164, N 11007-11055 and N 963-1494, respectively. The onset age was 0-4 years in the eleven DMD patients, and 2y11m to 14y8m in the six BMD patients. Patient No. 6 showed a delay of initial walking, mental retardation and an autistic tendency, in spite of being diagnosed as having BMD on muscle dystrophin staining. The motor function status of the six BMD patients was stationary or very slowly progressive. All were able to walk at the time this molecular genetic analysis was conducted, and their condition was relatively good, while the eleven DMD patients showed rapid disease progression (Fig. 1). Three of the eleven DMD patients and two of the six BMD patients showed mental retardation, and one DMD patient (No.10) showed a high intelligence and excellent school achievement. Among the six DMD patients who were followed until they began losing the ability of ambulation, the wheelchair-bound age was between 7y7m to 10y8m, the average being 9y4 m. All 17 patients showed high serum CK activity (Table 1), and immunohistochemical examination of biopsied muscle specimens with dystrophin monoclonal antibodies showed a discontinuous faint staining pattern in the six BMD patients, while staining was negative in nine of the DMD patients. However, in DMD patient Nos. 9 and 10, 1-2% of the fibers were revertant. Clinical evaluation of disease progression was based on the stage I-VIII classification devised with the 1980 Annual Report of the Research Group on the Immunologically Compromised Neurological Disease sponsored

Table 1 Clinical data for the DMD/BMD patients

Patient No.	Pheno-type	Initial walking age	Onset age	Initial symptom	Mental retardation	Jum-ping	Age at initial visit	Motor function level at initial visit	Wheelchair-bound age	CK (mU/ml)	Current age	Muscle dystrophin staining
1	B	1y	2y11m	leg pain with fever	LD	+	3y7m	I-a	ambulant	10,640	12y10m	F
2	B	1y	14y	running delay	−	+	14y	I-a	ambulant	2,560	17y1m	F
3	B	1y2m	2y7m	motor development delay	−	+	2y7m	II-a	ambulant	4,340	4y6m	F
4	B	1y2m	4y	tendency to fall	−	+	7y	I-a	ambulant	5,470	12y8m	F
5	B	1y	3y	no muscle symptoms	−	+	3y	normal	ambulant	26,110	4y11m	F
6	B	1y5m	1y3m	delay of initial walking	+	−	3y1m	I-a	No	13,000	5y7m	F
7	D	1y2m	1y11m	difficulty climbing	−	+	1y11m	I-b	No	11,360	7y	−
8	D	1y2m	3y	tendency to fall	−	−	6y9m	II-c	7y7m	5,020	9y7m	−
9	D	1y5m	1y3m	tendency to fall	−	−	7y11m	II-a	No	6,910	10y11m	−(R)
10	D	1y2m	3y	leg pain with fever	−	−	14y1m	VII	9y6m	10,260	16y5m	−(R)
11	D	1y3m	3y	tendency to fall	−	−	7y8m	II-a	9y6m	5,920	9y6m	−
12	D	1y1m	3y	motor development delay	−	−	3y11m	II-c	10y2m	16,680	10y2m	−
13	D	1y6m	1y3m	delay of initial walking	+	−	2y2m	II-a	No	10,840	2y2m	−
14	D	1y1m	2y3m	lack of head control	−	−	4m	II-a	No	10,110	8y6m	−
15*	D(C)	−	after born	lack of head control	+	−	9y4m	VIII	−	11,850	14y11m	−
16	D	1y6m	1y	delay of initial walking	−	−	7y	VIII	10y1m	9,560	dead (15y7m)	−
17	D	1y3m	3y5m	motor development delay	+	−	8y9m	II-c	9y5m	8,780	9y5m	−

* : clinical features resemlbed those of congenital muscular dystrophy (CMD), D : DMD, B : BMD, C : CMD, F : faint staining, R : revertant fibers, LD : learning disability, motor function levels (I -VIII) are the same as in Fig. 1.

by the Ministry of Health and Welfare[13]. Patient Nos. 14, 15 and 16 showed the same genotype, nevertheless, the motor function status of patient No. 14 was II-a with gradual deterioration; the clinical features of patient 15 resembled those of congenital muscular dystrophy, as he was never able to walk and had severe mental retardation (DQ=21) associated with severe epilepsy[14]. Patient No. 16 had DMD complicated by a rhabdomyosarcoma. His muscle disease stage was VIII, and he was wheelchair-bound from 10 years of age, and died of tumor metastasis and respiratory insufficiency at 15y7m. Patient No. 17 was at stage II-c when his DMD was diagnosed at age 3y5m, the disease then progressed rapidly with mental retardation, and he was wheelchair-bound by 10 years of age.

In the eleven DMD and six BMD patients, and six controls who had undergone a biopsy, genomic DNA and muscle mRNA analyses were performed. The muscle transcripts from 16 patients, the exception being patient No. 17, were sequenced by with an ALF sequencer. Lymphocyte transcripts were studied in two of the DMD patients (patient Nos. 10 and 17). Quantitative analysis of dystrophin mRNA was also performed in three DMD patients, four BMD patients and six controls by means of quantitative semi-nested PCR (Table 2).

Seventeen patients and six controls in this study all gave informed consent to undergo a muscle biopsy and lymphocyte DNA/mRNA analysis of the dystrophin gene.

RNA preparation

All 17 muscle biopsy specimens, which had been stored at −80°C in a freezer, were sliced at a thickness of 7 μm, and then subjected to acid

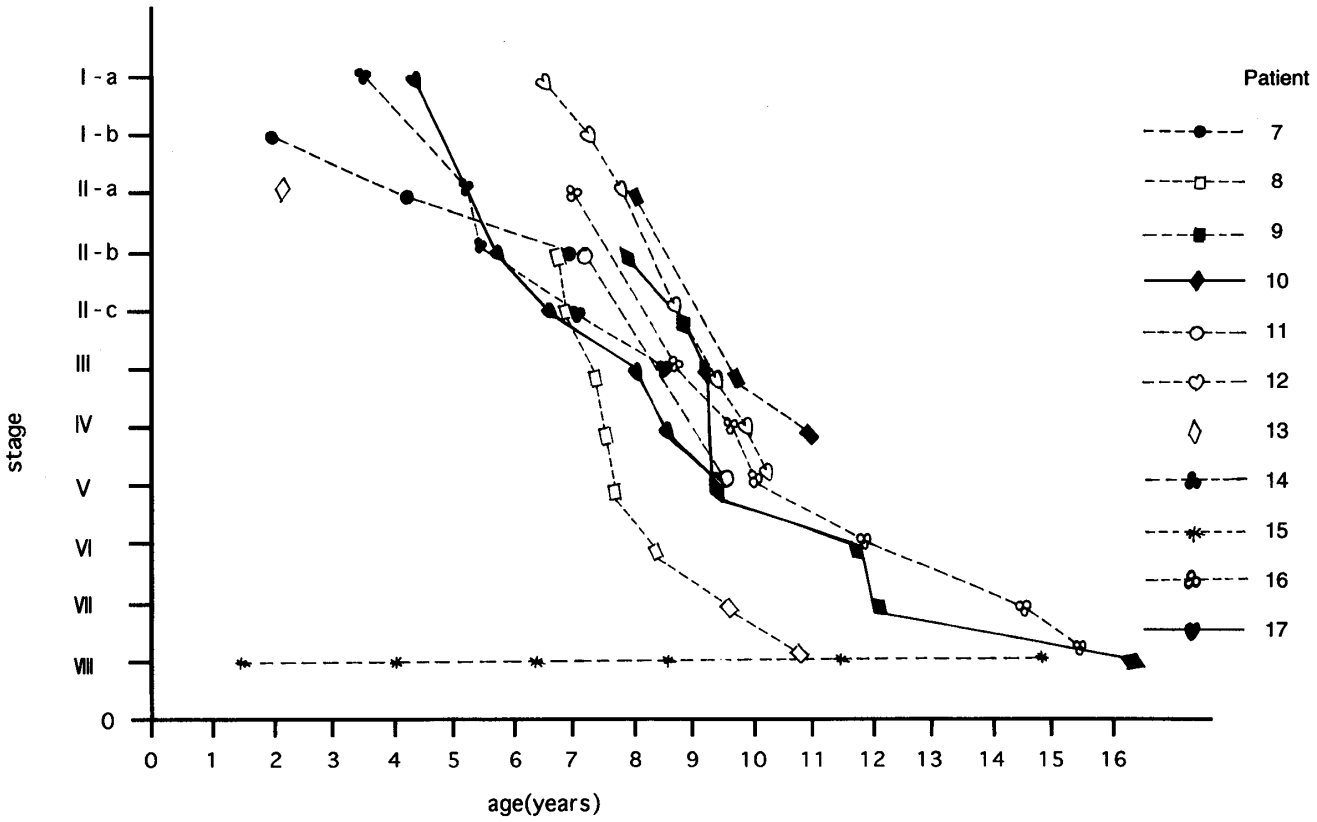


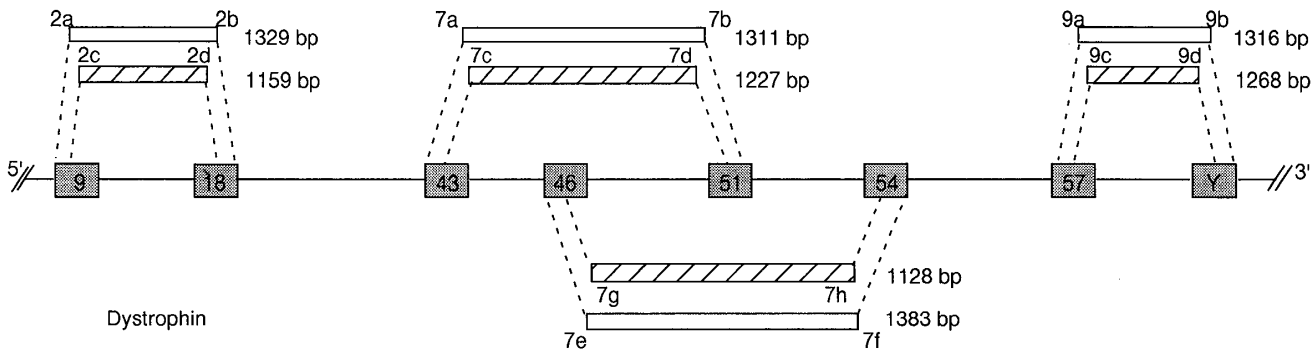
Fig. 1 Relation between age and muscle degeneration in DMD

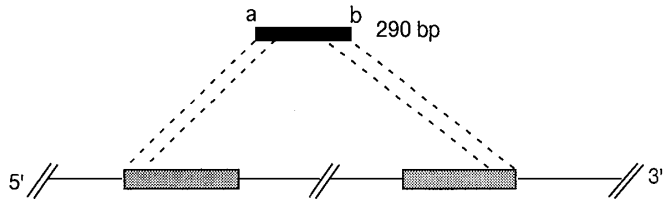
The stages are designated as I-VIII according to the classification standard devised by the Report of Research on Clinical and Immunology of Muscular Dystrophy in 1980[13]. I-a: capable of climbing up stairs without rail or hand support, I-b: climbing up stairs without a rail but must have hands on knees, II-a: climbing up stairs with one hand on the rail, II-b: climbing up stairs with one hand on the rail, and the other hand on the knee, II-c: climbing up stairs with both hands holding the rail, III: capable of standing up from a sitting position, IV: capable of walking, V: capable of dropping down on all fours, VI: unable to drop down on all fours, but able to crawl, VII: able to maintain a sitting posture, VIII: unable to maintain a sitting posture. The different symbols indicate patient Nos. 7-17.

Table 2 Comparison of muscle dystrophin mRNA in the DMD/BMD patients and controls

Diagnosis	Patient No.	n	Quantity of dystrophin mRNA (OD)	mean±SD (OD)	t-test
BMD	1	4	1.22	0.68±0.207	p=0.245
	2		0.24		
	3		0.74		
	5		0.50		
DMD	10	3	0.40	0.32±0.144	>0.05
	11		0.04		
	13		0.52		
DMD+BMD	1, 2, 3, 5, 10, 11, 13	7		0.523±0.143	t=2.797
					p=0.0174
Controls		6		9.375±3.494	<0.05

OD : optical density.





a

Primer used for detecting three regions of dystrophin gene

7a: GCAACGCCTGTGGAAAGGGTG.....6404-6424
7b: GTCACCCACCATCACCCTCTG.....7694-7714
}(E43-51)

7c: CAGGAAGCTCTCTCCCAGC.....6431-6449
7d: GGTAAGTTCTGTCCAAGCCCGG.....7636-7657

7e: GGAG GAAGCAGATAACATTGCT.....6829-6851
7f: TGAATGCTTCTCCAAGAGGC.....8210-8229
}(E46-53)

7g: GAACCTCGAAAAGAGCAGCAAC.....6923-6945
7h: GCATCTACTGTATAGGGACCC.....8031-8051

2a: CGATTCAAGAGCTATGCCTAC.....1092-1111
2b: GCCGAGTAATCCAGCTGTGAAG.....2399-2419
}(E9-18)

2c: CCTCTGACCCTACACGGAGC.....1134-1153
2d: CAGTTATATCAACATCCAA CC.....2376-2396

9a: GGGCCTTCAAGAGGGAATTG.....8754-8773
9b: CCAGTCTCATCCAGTCTAGG.....10051-10070
}(E57-Y*)

9c: CTAAAGAACCTGTAATCATG.....8778-8797
9d: GGGCCGCTTCGATCTCTGGC.....10027-10046

.....(GAPDH).....
Ga: AGTCAGCCGCATCTTCTTT TGC.....
Gb: CTCCTGGAAGATGGTGATGGA.....

b

Ey*: represents the immediately subsequent exon

Fig. 2 **a:** Schematic diagram of the positions of the 4 sets of primers for dystrophin mRNA used for the nested PCR reaction. The exon borders shown were determined by Koenig et al[15] and Roberts et al[12]. Empty bars indicate PCR primers, and shaded bars nested PCR primers. The extents of the primers flanking the genomic deletions of E43-51 and E46-54 are illustrated in the central portion of the dystrophin gene. The extents of the primers corresponding to the upstream and downstream regions of the dystrophin cDNA of exons 9-18 and exon 57-N 10046 are shown on both sides. A house-keeping gene, i.e. the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer, is indicated by the blackened bar below the dystrophin gene. The primers are designated as a, c, F (i.e. forward), and c, d, R (i.e. reverse).
b: Representation of the sequences of the primers described above.

guanidinium thiocyanate-phenol-chloroform RNA extraction[15]. Peripheral lymphocyte mRNA from patient Nos. 10 and 17 was also prepared employing the same procedure. The RNA was stored as an ethanol precipitate at −40°C until use. The reagents and equipment were used under RNase-free conditions

Construction of the primers

As the hot spot dystrophin DNA deletions in our DMD/BMD patients involved exons 44 to

53, two sets of primers were constructed to determine the deleted region (Fig. 2); a 7a-7b outer set and a 7c-7d inner set for the 5′ and 3′ ends, respectively[12] (corresponding to exons 43-51), spanning N 6431-7658 of the cDNA sequence, and 7e-7f and 7g-7h (corresponding to exons 46-54) spanning N 6923-8051. We also used two other primer sets, one of which spanned the 5' end (N 1134-2396)[12] (exons 9-18). The other set of primers was for the 3′ end (N 8778-10046)[12] (exon 57 to 3′ region). These primers were used to screen the regions upstream and downstream of the dystrophin cDNA. Primers 7e, 7f, 7g and 7h were designed as part of the present study. For quantification of the transcripts, a house-keeping gene, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, was used as an internal control for RT-PCR. The Ga and Gb primers were previously described by Oppenheim et al[16] (Fig. 2a, b).

Reverse transcription

Five hundred ng of total muscle RNA (2.5 μl) was denatured with 50 ng of primer Xb (X; 2, 7 or 9) or 7f (for the sequence, see Fig. 1) at 65°C for 10 min, and then chilled on ice. Four microliters of 5× reverse transcriptase buffer, 2 μl of 100 mM DTT (dithiothreitol), 200 units of MMLV (Moloney murine leukemia virus) reverse transcriptase (GIBCO BRL, Gaithersburg, U.S.A.), 5 μl of 5 mM dNTPs (TAKARA, Otsu-shi, Japan), and 25 units of RNase inhibitor (GIBCO BRL, Gaithersburg, U.S.A.) were added to give a total volume of 20 μl. The reaction was performed at 42°C for 1 hour.

Nested PCR (master mix method)

A 30 μl mixture comprising 5 μl of 10× Ex taq PCR buffer (TAKARA, Otsu-shi, Japan) and 500 ng of primer DMD Xa (X; 2, 7 or 9) or 7e, 450 ng of DMD Xb (X; 2, 7 or 9) or 7f, and 1.25 units of Ex taq polymerase was added to the RT mixture. Twenty cycles of PCR (93°C for 1 min, 55°C for 1 min, and 72°C for 2 min) were performed, followed by incubation at 72°C for 5 min. One microliter of the PCR products was added to a 50 μl mixture comprising 5 μl of 10× Ex taq PCR buffer, 4 μl of 5 mM dNTPs, 500 ng of primer DMD Xc (X; 2, 7 or 9) or 7g, 500 ng of primer DMD Xd (X; 2, 7 or 9) or 7h, and 1.25 units of Ex taq polymerase. The PCR reaction was repeated as above. Eight microliters of the final products was electrophoresed in a 1% agarose gel, followed by staining with ethidium bromide.

Quantitative PCR

For quantification of the amplified transcripts, an in vitro semi-nested RT-PCR procedure was used, using a house-keeping gene, the GAPDH gene, as described elsewhere[16], in four BMD and three DMD patients. The same amount of cDNA (2 μl) was mixed with 500 ng of dystrophin genes 7a and 7b, and 7e and 7f, respectively, under the conditions described above, and 30 PCR cycles were carried out. The 3′ primers of 7d, 7h and Gb were then end-labeled with ^{32}P. The hot PCR reaction conditions were the same as above, that is within the exponential part of the curve (25 cycles). The amplified products were electrophoresed in 2.5% agarose gels, which were then dried and exposed to imaging plates (Fuji Photo Film Co., Tokyo, Japan). The target and internal standard bands identified were examined for radioactivity, expressed as counts per minute (cpm) of photo-stimulated luminescence, with a Bioimaging Analyzer, BAS 2000 (Fuji Photo Film Co., Tokyo, Japan). The quantity of dystrophin mRNA was presented as the optical density (OD), and the results were statistically analyzed by Student's unpaired t-test.

Direct sequencing

The PCR products were extracted from the 1% agarose gels by excising the individual bands, followed by transfer to a NANOSEP MF 0.45 μl tube (Pall Filtron, Massachusetts, U.S.A.), centrifugation at 14,000 cpm, ethanol precipitation of the filtered solution twice, and then direct sequencing with an ALF sequencer (Amersham Pharmacia Biotech, Tokyo, Buckinghamshare England) using a thermal sequence fluorescence labeled primer cycle sequencing kit (Amersham Pharmacia Biotech,

Buckinghamshare, England). The forward primers that fluoresced were used for PCR.

Results

Quantification of muscle dystrophin gene transcription

The dystrophin transcripts from four BMD and three DMD patients were found to be decreased as compared with in six control subjects, and the optical densities of the muscle transcripts were found to be drastically decreased ($p<0.05$) on quantitative analysis using semi-nested-PCR. There was, however, no statistically significant difference between the four BMD and three DMD patients (Table 2).

Analysis of lymphocyte DNA and muscle transcripts

Lymphocyte DNA data revealed in-frame deletions in the six BMD patients and out of frame deletions in the eleven DMD patients. The total muscle RNA samples obtained from the 17 patients were sufficient for reverse transcription of muscle mRNA. BMD patient Nos. 2 and 3 showed deletion of exons 45-48, Nos. 4 and 5 of exons 45-47 (Fig. 3), and No.6 of exons 45-53. Patient Nos. 9 and 10, who had DMD, showed an exon 45 genomic DNA deletion. Revertant fibers were also confirmed by muscle dystrophin staining in the latter two cases (Table 3). Patient Nos.14, 15 and 16 had deletion of exons 48-52 in lymphocyte DNA, but the transcript size in patient No. 16 was about 200 bp larger (Fig. 3). The muscle transcripts from patients Nos. 1-15 were consistent with the lymphocyte DNA deletion. In spite of that the transcript of GAPDH, which was the internal control, was obtained from patient No. 17, no dystrophin transcript was amplified despite repeated efforts (Table 3), and the same results were obtained for lymphocyte transcript. The peripheral lymphocyte mRNA from patient No. 10 showed 175 bp and 480 bp transcripts, which were consistent in terms of size with deletion of exons 45-48 and exon 45, respectively (Fig. 4).

Sequencing evidence of DNA deletion boundary region transcription

The boundary of the deleted mRNA in each case determined by sequencing of the RT-PCR products derived from the deletion-containing region (exons 43-53) of muscle mRNA, obtained using primers 7c and 7d[12] or 7g and 7h (Table 4), was confirmed. In patient No.10, when we sequenced the two transcripts from lymphocyte mRNA, two deletion types involving exon 45 and exons 45-48 were confirmed (Fig. 4-a, b). However, only the exon 45 deletion was detected in muscle. In patient No. 16, whose genomic DNA deletion encompassed exons 48-52, amplification of the transcript clarified that the 5′ region of exon 48 was present and that there was a 15 bp deletion in the 3′ region of exon 48. The latter caused a transition from C to A at nucleotide 7301 (Fig. 5). The transcript subsequently spliced to exon 53.

Transcripts of the 5′ and 3′ regions of dystrophin mRNA and motor function

Analysis of the transcripts of exons 9-18 and exon 57 to N 10046[12] to clarify the transcription in the upstream and downstream regions of the dystrophin gene, using RT-PCR, revealed the following: sixteen upstream transcriptional products were obtained, the exception being patient No. 17. Patient Nos. 8, 9, 12, 14, 15 and 17 showed no transcripts downstream from the detected region (Table 4).

The wheelchair-bound age was 108.7 ± 9.2 m in the group with no transcript in the downstream region of the dystrophin gene (patient Nos. 8, 9, 12, 14 and 17), while the group with positive one (patient Nos. 7, 10, 11, 13 and 16) it was 116.3 ± 2.3 m. There was no significant difference between the two groups.

Thus, the clinical features were not affected by the absence of a transcript of the downstream region of the dystrophin gene.

Discussion

Confirmation of the effect of dystrophin gene transcription on muscle mRNA

Screening for DMD/BMD involves genomic DNA and immunochemical analysis. The reading frame theory of Monaco et al[9] was found to

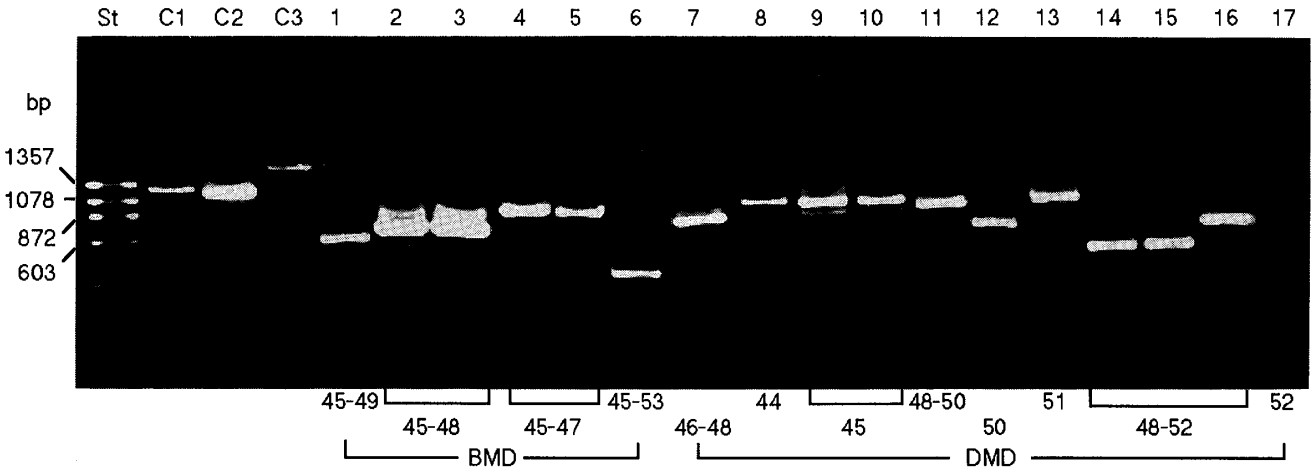


Fig. 3 Amplification of dystrophin mRNA corresponding to the DNA deletions detected in the muscle biopsy specimens
C1-C3 are controls for the three primers of the amplified region of the deleted DNA. The designations are: C1; for exons 43-51, C2; for exons 46-54, C3; for exons 43-54. Numbers 1-17 above the picture are those the patients in Table1. The numbers below the picture are deleted exon extention in lymphocyte DNA. The number on the right side of the picture are the sizes of the $\phi \times 174$ marker.

Table 3 Comparison of dystrophin DNA and mRNA

Patient No.	Pheno-type	Dystrophin staining	Deleted exon in lymph. DNA	Deleted exon in muscle mRNA	Deleted exon in lymphocyte mRNA	Consistency
1	B	F	45-49	45-49	ND	+
2	B	F	45-48	45-48	ND	+
3	B	F	45-48	45-48	ND	+
4	B	F	45-47	45-47	ND	+
5	B	F	45-47	45-47	ND	+
6	B	F	45-53	45-53	ND	+
7	D	—	46-48	46-48	ND	+
8	D	—	44	44	ND	+
9	D	—(R)	45	45	ND	+
10	D	—(R)	45	45	45/45-48	+(somatic mosaicism)
11	D	—	48-50	48-50	ND	+
12	D	—	50	50	ND	+
13	D	—	51	51	ND	+
14	D	—	48-52	48-52	ND	+
15	D(C)	—	48-52	48-52	ND	+
16	D	—	48-52	E48(N 7304)*49-52	ND	—
17	D	—	52	unable to be amplified	unable to be amplified	transcription disturbance

* : 5′ region of exon 48 detected, and a 15 bp deletion (from N 7304 to N 7318) in the 3′ region of exon 48 with subsequent splicing to exon 53, ND : not done.

be applicable to 92% of 258 DMD and BMD patients[10], and was also confirmed at the protein level by Hoffman et al[3]. Genetic information carried by DNA is expressed in two stages: transcription of one DNA strand into mRNA, followed by translation of the mRNA into a protein. Thus, analysis of muscle mRNA is useful for determining the effect a genotypic alteration has on the transcript structure. Messenger RNA analysis also allows confirmation of reading frame mutations in cases in which analysis at the genomic level is inconclu-

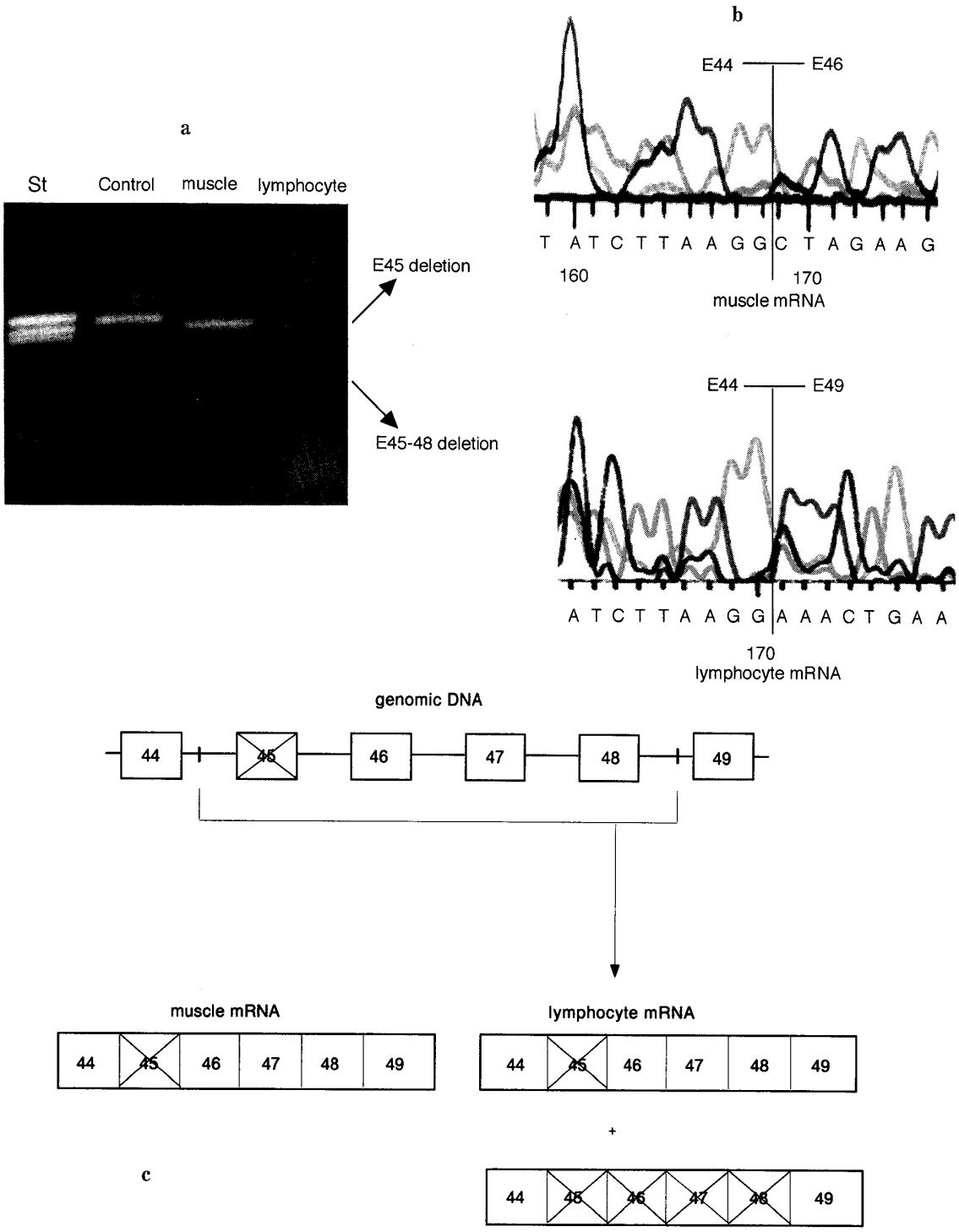


Fig. 4 **a:** Amplification of the RT-PCR products from controls, muscle transcripts and lymphocyte transcripts, in a 1% agarose minigel, is illustrated. **b:** The two graphs beside the picture present sequencing evidence of two distinct transcript patterns for two peripheral lymphocytes. **c:** Schematic representation of different types of transcription, one in muscle and the other in lymphocytes, in patient No. 10. The same deletion of exon 45 was seen in muscle transcripts and genomic DNA, while alternative splicing produced two different deletion patterns i.e. deletions of exon 45 and exons 45-48, was demonstrated on lymphocyte transcription. These results are consistent with somatic mosaicism.

Table 4 Muscle dystrophin mRNA data for the 17 DMD/BMD patients

Patient No.	Pheno-type	Deleted exons in muscle mRNA	Muscle cDNA del. boundary	Detection of three regions in dystrophin gene		
				E9-18	E43-53	E57-Y
1	B	E45-49	6648-7405	+	+ (truncated)	+
2	B	E45-48	6648-7321	+	+ (truncated)	+
3	B	E45-48	6648-7321	+	+ (truncated)	+
4	B	E45-47	6648-7117	+	+ (truncated)	+
5	B	E45-47	6648-7117	+	+ (truncated)	+
6	B	E45-53	6648-8077	+	+ (truncated)	+
7	D	E46-48	6624-7321	+	+ (truncated)	+
8	D	E44	6299-6647	+	+ (truncated)	−
9	D	E45	6648-6823	+	+ (truncated)	−
10	D	E45	6648-6823	+	+ (truncated)	+
11	D	E48-50	7118-7517	+	+ (truncated)	+
12	D	E50	7406-7517	+	+ (truncated)	−
13	D	E51	7518-7747	+	+ (truncated)	+
14	D	E48-52	7118-7868	+	+ (truncated)	−
15*	D(C)	E48-52	7118-7868	+	+ (truncated)	−
16	D	E48(N7304)-52	7307-7868	+	+ (truncated)	+
17	D	unable to be amplified	unable to be amplified	−	−	−

+ : positive transcript, − : negative transcript, * : clinical features resembling those of congenital muscular dystrophy (CMD).

sive. Hoffman et al[17] investigated muscle dystrophin mRNA in mice and humans, and demonstrated this mRNA accounts for approximately 0.01 to 0.001% of total cardiac and skeletal muscle mRNA. Chelly et al[18] studied muscle transcripts quantitatively in eight DMD and three BMD patients, finding that the amount of truncated transcripts in DMD cases who had a frameshift deletions was less than 10% of the control level, while it was only slightly decreased without a frameshift deletion, but did not present statistical data.

In our study, the amounts of residual dystrophin transcripts were markedly decreased in four BMD cases who had an in-frame deletion and three DMD cases who had a frame shift deletion, compared to in six controls ($p<0.05$). There was, unexpectedly, no significant difference between the three DMD and four BMD cases, despite that the residual dystrophin immunostaining was more prominent and the clinical severity milder in the BMD than in the DMD cases. The results suggested that the three DMD cases (patient Nos. 10, 11 and 13) had relatively the same amounts of transcripts as the BMD cases quantitatively. Qualitatively, all three DMD cases showed truncated transcripts and the downstream region of the dystrophin gene was amplified, as shown in Table 4. Thus, we presumed that the intact transcription in the three DMD patients was correlated with our results.

We detected an inconsistency in the deletion range between lymphocyte DNA and muscle mRNA. Patient No. 16 had the same deletion pattern, i.e. exons 48-52, in lymphocyte DNA as patient Nos. 14 and 15. However, the PCR product in patient 16 was larger than those in patient Nos. 14 and 15. Thus, the transcript in patient No. 16 apparently differed from those in patient Nos. 14 and 15. The sequencing results confirmed the existence of a part of exon 48, with deletion of 15 bps of the 3′ end of exon 48 (N 7304 to N 7318), subsequent deletion of exons 49-52 with alternative splicing to exon 53, and a missense mutation causing a transition from C to A at N 7301 (Fig. 5). The reason for the apparent genomic DNA deletion of exon 48, despite the partial existence of exon 48, was a deletion of intron 48, where the primer was constructed. When the transcript of exons 46-53 was amplified, a part of exon 48 was detected

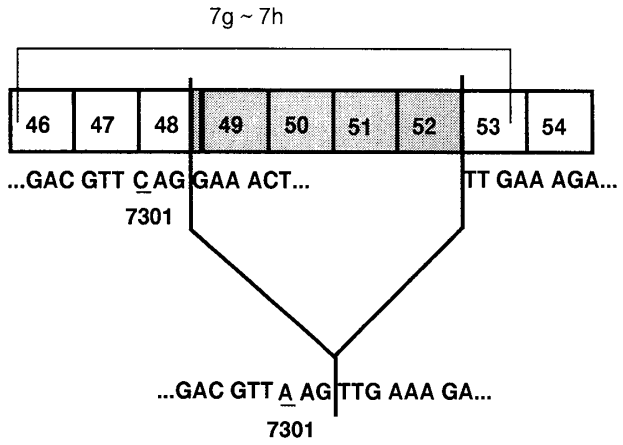


Fig. 5 Illustration of alternative splicing in muscle mRNA from patient No. 16
The thin line shows the extension of primers 7g and 7h, which were used for RT-PCR for amplification of the muscle mRNA. The boxes indicate exons 46-54 mRNA. The shaded parts of exons are deletions which were detected by RT-PCR. Bold lines and sequences of amino acid illustrate alternative splicing boundaries, and a missense mutation causing a transition from C to A at site 7301 is also indicated.

as a larger band.

In the case of patient No. 17, who had DMD with an exon 52 deletion in lymphocyte DNA, the mother was confirmed to be a carrier by Southern blotting, showing an exon 52 deletion. His motor function deterioration was less than patient No. 8, but worse than that of patient Nos. 10, 11, 12 and 16, and he had mental retardation, and muscle dystrophin transcripts were not amplified on RT-PCR. A transcriptional disturbance in muscle was assumed, which was supported by lymphocyte transcription with no amplified products. There were four patients who had mental retardation, in three of whom dystrophin transcripts were obtained, the exception being patient No. 17. Thus, we concluded that the intact transcription had no influence on the mental state in the DMD patients, but that affected transcription was one of the possible reasons why patient No. 17 showed a relatively severe phenotypes in the motor and mental states.

Possibility of existence of somatic mosaicism

Dystrophin mRNA is mainly detected in muscle and cardiac tissues, at significant levels, while low levels of transcription (of 1 copy/1000 cells) have been reported in lymphocytes[12)19)] and brain[20)]. Patient Nos. 9 and 10 were both diagnosed as having DMD, and both had an exon 45 deletion which caused a frameshift, while dystrophin immunostaining with DYS 1-3 revealed revertant fibers. In patient No. 10, who had an exon 45 deletion in muscle mRNA, we conducted RT-PCR using peripheral blood lymphocyte mRNA, and found two fragments. We analyzed the fragments with a sequencer. A deletion of exons 45-48 was detected simultaneously with that of exon 45 (Fig. 4c).

The mechanism restoring the correct reading frame in revertant fibers has been described by Hoffman et al[21)], Klein et al[22)], Sherratt et al[23)] and Winnard et al[24)]. A small proportion of transcripts was identified in which one or more exons were skipped adjacent to the deletion, and Roberts et al[12)] have shown that alternative splicing of a nonsense mutation at the 5′ or 3′ end of the reading frame creates a cryptic splice site or causes complete exon skipping. Other mechanisms have been proposed to account for the restored reading frame in revertant fibers, including somatic reversion or suppression[21)22)25)]. Another site deletion, presumably next to the deleted exon, has been suggested to change the original deletion to an in-frame deletion. In the present study, patient No. 10 did not have a mutation at either the 5′ or 3′ end of exon 44 or 49, but did have deletions of both exon 45, i.e. a frameshift deletion, and exons 45-48, i.e. an in-frame deletion, in lymphocyte mRNA. The former deletion pattern disrupted the reading frame while the latter one restored the reading frame, resulting in the appearance of scattered dystrophin-positive fibers. Patient No. 10 was a sporadic case. Different mutated transcripts were found in muscle and lymphocytes, which are derived from the mesoderm. As he exhibited excellent school achievement, his brain dystrophin mRNA, which was derived from the ectoderm, was predicted to be normal. Thus, we concluded

that this case had somatic mosaicism. This is the second case confirmed to have somatic mosaicisim by us[25].

Patient No. 6 who had a BMD, showed an autistic tendency and mental retardation, and his current age was 3y7m. His motor function level at initial visit to our clinic was I-a, but he could not complete jumping. His muscle dystrophin immunostaining was faint and his muscle mRNA was consistent with lymphocyte DNA, which had a deletion of exons 45-53, i.e. in-frame deletion. We assumed the reason for his relatively affected mental and motor functions, in spite of his BMD genotypes was somatic mosaicisim in the brain and muscle, like in patient No. 10, or somatic reversion in the brain tissue.

Transcription of the 5′ and 3′ ends of the dystrophin gene

We have also studied the upstream and downstream regions of the dystrophin gene. In all six BMD cases, both the 5′ and 3′ regions of the transcripts were obtained. Koenig et al[10] and England et al[26] proposed that internal deletions in BMD can result in the loss of most of the amino terminal and central rod domains, with preservation of partial functions and perhaps relatively stable dystrophin molecules. As frameshift deletions in DMD result in termination codons, translation may be blocked, such that the terminal region of the dystrophin gene is undetectable. In our study on DMD cases, upstream transcripts were found in all DMD and BMD patients, except patient No. 17. However, in six DMD cases, transcripts of the 3′ end of the DMD gene were not obtained. The genomic DNA deletions involved exons 44, 45, 50, 48-50, 48-52, and 52 distributed in patient Nos. 8, 9, 12, 14 and 17, respectively.

In conclusion, our results showed that the results of transcript analysis did not always reflect the clinical phenotype of DMD/BMD. However, we detected the existence of somatic mosaicism and splicing mutations, different from the results obtained on genomic DNA analysis. As messenger RNA transmits the DNA information to a protein, analysis of the transcripts of the dystrophin gene is important for clarifying the step of dystrophin protein synthesis, which is causative of DMD/BMD, and to elucidate the molecular pathology of DMD/BMD.

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References

1) **Kunkel LM, Hejtmancik JF, Caskey CT:** Analysis of deletions in DNA of patients with Becker and Duchenne muscular dystrophy. Nature **322**: 73-77, 1986
2) **Koenig M, Hoffman EP, Bertelson CJ et al:** Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA, and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell **50**: 219-228, 1987
3) **Hoffman EP, Kunkel LM:** Dystrophin abnormalities in Duchenne/Becker muscular dystrophy. Neuron **2**: 1019-1029, 1989
4) **Hoffman EP, Knudson CM, Campbell KP et al:** Subcellular fraction of dystrophin to the triads of skeletal muscle. Nature **330**: 754-758, 1987
5) **Koenig M, Monaco AP, Kunkel LM:** The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell **53**: 219-228, 1988
6) **Sugita H, Arahata K, Ishiguro T et al:** Negative immunostaining of Duchenne muscular dystrophy (DMD) and mdx mouse muscle surface membranes with antibodies against a synthetic peptide fragment predicted from DMD cDNA. Proc Jpn Acad **64**: 210-212, 1988
7) **Arahata K, Ishiura S, Ishiguro T et al:** Immunostaining of skeletal and cardiac muscle surface membranes with antibodies against Duchenne muscular dystrophy peptide. Nature **333**: 861-863, 1988
8) **Beggs AH, Koenig M, Boyce FM et al:** Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. Hum Genet **86**: 45-48, 1990
9) **Monaco AP, Bertelson CJ, Liechti S et al:** An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics **2**: 90-95, 1988
10) **Koenig M, Beggs AH, Moyer M et al:** The molecular basis for Duchenne versus Becker muscular dystrophy: Correlation of severity with type of deletion. Am J Genet **45**: 498-506, 1989
11) **Hoffman EP, Hudecki MS, Rosenberg PA et al:**

Dystrophin characterization in muscle biopsy specimens from Duchenne and Becker muscular dystrophy patients. N Engl J Med, 318: 1363-1368, 1988
12) **Roberts RG, Barby TFM, Manners E et al:** Direct detection of dystrophin gene rearrangements by analysis of dystrophin mRNA in peripheral blood lymphocytes. Am J Hum Genet **49**: 298-310, 1991
13) **Fukuyama Y, Takayanagi T, Nojima M:** Research in progression of functional disabilities in muscular dystrophy patients. *In* The 1980 Annual Report of the Research Group on the Immunologically Compromised Neurological Disease Sponsored by the Ministry of Health and Welfare: 4-7, 1980
14) **Saito K, Osawa M, Kondo E et al:** Genomic deletion study of the dystrophin gene in congenital muscular dystrophy. J Tokyo Wom Med Coll **63**: 26-35, 1993
15) **Chomczynski P, Sacchi N:** Single-step method of RNA isolation by acid guanidinum thiocyanate-phenol-chloroform extraction. Anal Biochem **162**: 156-159, 1987
16) **Oppenheim JJ, Zachariae COC, Mukaida N et al:** Properties of the novel proinflammatory supergene "intercrine" cytokine family. Ann Rev Immunol **9**: 617-648, 1991
17) **Hoffman EP, Monaco AP, Feener CC et al:** Conservation of the Duchenne muscular dystrophy gene in mice and humans. Science **238**: 347-350, 1987
18) **Chelly J, Gilgenkrantz H, Lambert M et al:** Effect of dystrophin gene deletions on mRNA levels and processing in Duchenne and Becker muscular dystrophy. Cell **63**: 1239-1248,1990
19) **Chelly J, Kaplan JC, Maire P et al:** Transcription of the dystrophin gene in human muscle and non-muscle tissues. Nature **333**: 858-860, 1988
20) **Bies RD, Phelps SF, Cortez MD et al:** Human and murine dystrophin mRNA transcripts are differentially expressed during skeletal muscle, heart, and brain development. Nucleic Acids Res **20**(7): 1725-1731, 1992
21) **Hoffman EP, Morgan JE, Simon CW et al:** Somatic reversion/suppression of the mouse mdx phenotype in vivo. J Neurol Sci **99**: 9-25, 1990
22) **Klein CJ, Coovert DD, Bulman DE et al:** Somatic reversion/suppression in Duchenne muscular dystrophy (DMD): Evidence supporting a frame-restoring mechanism in rare dystrophin-positive fibers. Am J Hum Genet **50**: 950-959, 1992
23) **Sherratt TG, Vulliamy T, Dubowitz V et al:** Exon skipping and translation in patients with frame-shift deletions in the dystrophin gene. Am J Hum Genet **53**: 1007-1015, 1993
24) **Winnard AV, Mendell JR, Prior TW et al:** Frame-shift deletions of exons 3-7 and revertant fibers in Duchenne muscular dystrophy: Mechanisms of dystrophin production. Am J Hum Genet **56**: 158-166, 1995
25) **Saito K, Ikeya K, Kondo E et al:** Somatic mosaicism for a DMD gene deletion. Am J Med Genet **56**: 80-86, 1995
26) **England SB, Nicholoson LVB, Johnson MA et al:** Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. Nature **343**: 180-182, 1990

ジストロフィン遺伝子部分欠失を示すDMD/BMD患者における骨格筋のジストロフィンmRNAの分析

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Duchenne型（DMD）およびBecker型（BMD）筋ジストロフィー患者におけるジストロフィン遺伝子部分欠失がmRNAレベルではどのようになっているのかを明らかにするため、RT-PCRを用いて，欠失を示すDMD 11例およびBMD 6例について骨格筋のジストロフィンmRNAの分析を行った．さらに，DMD 3例とBMD 5例について，semi-nested PCRによりジストロフィンmRNAを定量した．コントロールに比較して，DMD/BMD患者のジストロフィンmRNAはいずれも有意に減少していたが（$p<0.05$），DMDとBMDとの間には有意差が認められなかった．DMD/BMD患者17例について，ジストロフィン遺伝子の三つの領域（欠失を含む領域，欠失の上流側，下流側）をそれぞれ増幅した．欠失を含む領域の転写産物が得られ，その塩基配列を分析した．16例において，DMD 1例では他のDMDに比べ、臨床的重症度に差はなかったが，RT-PCR産物は得られず，転写における障害と推測された．他のDMD 1例では，リンパ球のDNAの欠失と骨格筋のmRNAにおける欠失領域の不一致を認めた．このことはDNA診断において，プライマー領域の欠失により，欠失していないexonを欠失していると誤る可能性を示唆した．別のDMD1例では，骨格筋のmRNAとリンパ球のmRNAの欠失領域に不一致があり，これはsomatic mosaicismによるものと考えられた．DMD/BMDの患者におけるジストロフィン遺伝子欠失の分子レベルにおけるメカニズムを解明するため，mRNAの分析は重要と考えられる．