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Thesis :

Analysis of the CTG Trinucleotide Repeat Expansion in Patients with Congenital Myotonic Dystrophy

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ABSTRACT **Myotonic dystrophy (Dystrophia myotonica, DM) is caused by an abnormal expansion of an unstable CTG trinucleotide repeat in the 3' untranslated region of mRNA encoding a putative serine/threonine protein kinase. (1) We analyzed 59 patients with DM (28 congenital DM families : 27 families with maternal transmission and 1 paternal transmission) and 27 subjects with normal controls to evaluate their CTG repeat size between DM patients and normal controls, and to examine a correlation between the clinical characteristics of congenital DM (CDM) and CTG repeat expansions. Analysis was on the basis of the Southern blot and polymerase chain reaction (PCR) methods, and by direct sequencing of PCR amplified CTG repeat. Analysis of the intergenerational differences in the CTG repeat size for mother-child pairs showed a positive correlation ($y=1.0384x+1265.2$, $r^2=0.311$). In addition to the strong parental bias, this group showed genetic anticipation. There was a significant correlation of the CTG repeat expansion with disease severity. The largest CTG repeat expansion (2293 CTG repeats) on average was disclosed to patients with severe CDM, and the smallest (129 CTG repeats), to patients with subclinical DM. The mutant allele of an asymptomatic father in the paternally transmitted pedigree revealed 75 CTG repeats, demonstrating that he was a DM protomutation carrier. (2) We have analyzed the amplification of the CTG repeat of DNAs extracted from skeletal muscles and lymphocytes in five CDM patients. The amplification from skeletal muscles showed an increase of about 1.5-kb to 3.5-kb larger than that from leukocytes in all patients. A patient with severe CDM had an abnormal enlarged and blurred band of 12.4-kb which demonstrated slight somatic cell heterogeneity in different organs.**

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Key words : Myotonic dystrophy (Dystrophia myotonica, DM), Congenital form, Trinucleotide (CTG) repeat mutation, Parental bias, Anticipation

1. Introduction

Myotonic dystrophy (Dystrophia myotonica, DM) is an autosomal dominant, multisystemic disorder characterized by myotonia, progressive muscle weakness and atrophy, cardiac conduction disturbances, cataracts, and abnormal glucose intolerance[1]. DM is the most common form of adult muscular dystrophy, with an estimated incidence of 1 in 8,000. The clinical features of DM are markedly variable, ranging from neonatal mortality (congenital DM, CDM) to a complete absence of symptoms. The age at onset and severity of the symptoms show extreme variation even among family members. The rare CDM is associated with severe hypotonia, feeding difficulty, neonatal respiratory distress, developmental

§ This thesis is based on the five papers that are indicated in the list of original pubications. The publishers granted permission for use the citations in the tables.

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motor delay and mental retardation, distinct from the adult DM. Most children with CDM are the offspring of affected mothers[2]. The phenomenon of anticipation, earlier age of onset and increasing severity of the disease in successive generations, always occurs in DM pedigrees[3].

The gene responsible for DM (DM kinase gene) has been mapped on human chromosome 19q13.3. The complete sequence of the DM kinase gene is within a 14-kb genomic region contained in five contiguous *Bam*HI fragments and constituted of fifteen exons[4]. The full length of mRNA of the DM kinase gene is estimated at approximately 3.4-kb and encodes a 69-kDa translational product of 629 amino acids which has no homology to known protein sequences. The molecular basis of DM mutation is an unstable trinucleotide (CTG) repeat, located in the 3' end of a transcript encoding a putative serine/threonine protein kinase[5-7]. The number of expansions varies markedly even in normal populations from 5 to 30, but in DM patients it ranges from 50 to several thousand copies[8]. In asymptomatic DM, the premutation stage, CTG repeat varies from 35 to 50[9]. Trinucleotide repeat mutation similar to DM have been identified in Fragile X mental retardation syndrome[10], spinal and bulbar muscular atrophy (Kennedy's disease)[11], FRAXE mental retardation[12], Huntington's disease[13], spinocerebellar ataxia type 1[14], dentatorubral-pallidoluysian atrophy[15], Machado-Joseph disease[16], and Friedreich's ataxia[17].

At first, we analyzed 59 DM patients (28 CDM families: 27 families with maternal transmission and 1 paternal transmission) and 27 normal controls to evaluate CTG repeat size between DM patients and normal controls, and to clarify correlations between the clinical characteristics of CDM and CTG repeat expansions on the basis of Southern blot analysis and polymerase chain reaction (PCR). We identified the precise CTG repeat number and performed direct sequencing of PCR product of minimal expanded allele in paternal transmitted family. Secondly, we described a comparison of the CTG repeat amplification of genomic DNAs from leukocytes and skeletal muscles in five unrelated CDM patients, based on Southern blot analysis. Furthermore, we have examined the genomic DNAs from various tissues in a severely affected neonate with CDM.

2. List of original publications

This study is based on the following publications. This thesis consists of those five papers.

1. Tachi N, Ohya K, Chiba S, Sato T. Unstable DNA in a patient with a severe form of congenital myotonic dystrophy. J Neurol Sci 1993, 119: 180-182.
2. Ohya K, Tachi N, Chiba S, Satoh T, Kon S, Kikuchi K, Imamura S, Yamagata H, Miki T. Congenital myotonic dystrophy transmitted from an asymptomatic father with a DM-specific gene. Neurology 1994, 44: 1958-1960.
3. Ohya K, Tachi N, Kon S, Kikuchi K, Chiba S. Somatic cell heterogeneity between DNA extracted from lymphocytes and skeletal muscle in congenital myotonic dystrophy. Jpn J Hum Genet 1995, 40: 319-326.
4. Tachi N, Ohya K, Chiba S, Sato T, and Kikuchi K. Minimal somatic instability of CTG repeat in congenital myotonic dystrophy. Pediatr Neurol 1995, 12: 81-83.
5. Ohya K, Tachi N, Sato T, Kon S, Kikuchi K, Chiba S. Detection of the CTG repeat expansion in congenital myotonic dystrophy. Jpn J Hum Genet 1997, 42: 169-180.

3. Materials and Method

3. 1 Materials

3. 1. 1 Patients and Blood samples for DNA analysis. Peripheral blood leukocytes were obtained from 59 DM patients (28 DM families: 27 families with maternal transmission and 1 paternal transmission; 16 severe CDM patients, 16 CDM patients, 23 adult DM patients, and 4 subclinical DM patients;

25 mother-child pairs and 2 father-child pairs) and 27 normal controls. Peripheral blood leukocytes from the patients were taken at the time of this study. CDM was diagnosed based on clinical features and muscle pathology. We classified the patients into the following four groups on the basis of clinical manifestations and age of onset. (1) Severe CDM: These patients were characterized by clinical features such as the presence of polyhydramnios and reduced fetal movements, marked hypotonia with facial weakness at birth, neonatal respiratory distress, and feeding difficulty during the neonatal period. (2) CDM: They were characterized by facial weakness, generalized hypotonia at neonatal period, delayed motor development, and mental retardation. (3) Adult DM: They were characterized by myotonia at onset of adult life, progressive muscle weakness and atrophy. (4) Subclinical DM: They were asymptomatic DM carriers with only cataracts.

3. 1. 2 Patients and tissue samples for DNA analysis. We studied five patients with CDM whose mother had been diagnosed as adult DM. Peripheral blood leukocytes from the patients were taken at the time of this study, but the muscle samples had been taken 1 to 8 years earlier, frozen in liquid nitrogen and stored at −80°C until the analyses reported here. All muscle samples were taken from the quadriceps femoral muscle. We obtained postmortem tissues, including liver, diaphragm, urinary bladder, small intestine, tongue, uterus, and cerebral cortex, from a severely affected neonate with CDM, who died of respiratory failure at one month of age.

3. 2 Method

3. 2. 1 Southern Blot Analysis. Genomic DNAs were prepared from peripheral blood leukocytes by the standard procedures[18]. Seven micrograms of genomic DNA was digested with appropriate restriction endonucleases (*Eco*RI, *Bam*HI, and *Bgl*I), separated by electrophoresis on 0.8% agarose gel, and transferred onto nylon membranes using a vacuum transfer apparatus (VacuBlot™ Transfer System, American Bionetics, California). After prehybridization, digested DNA was hybridized to radiolabeled p5B 1.4 for 24 hours. The filters were washed to a stringency of 0.1×SSC containing 0.1% SDS and autoradiography was performed at −80°C for 2 to 5 days. The probe p5B1.4[19] (Fig. 1), supplied by Dr. Keith Johnson, is a 1.4-kb *Bam*HI fragment from cDNA25[6] subcloned into pBluescript SK+. This probe was labeled by random priming.

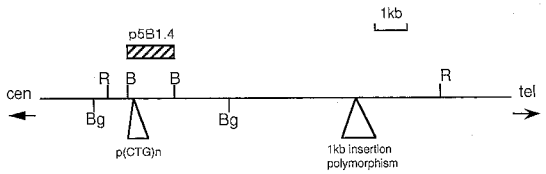

Fig. 1 A restriction map of the region containing the CTG polymorphism, showing the position of p5B1.4.
restriction sites: Bg=*Bgl*I; R=*Eco*RI; B=*Bam*HI.

3. 2. 2 PCR amplification. PCR amplification was performed using CTG region flanking primers[5,7]. The cycling conditions were as follows: initial denaturation at 95°C for 3 minutes, 35 cycles of 95°C for 1.5 minutes, 65°C for 1 minute and 72°C for 2 minutes, followed by a final stage at 72°C for 7 minutes. PCR products were electrophoresed on 2% agarose, blotted onto nylon membrane, and probed with a γ-[^{32}P]adenosine triphosphate end labeled $(CTG)_{10}$ oligonucleotides. Membrane was washed in 6×SSC containing 0.1% SDS and exposed to Kodak X-ray film. CTG closely flanking primers were as follows:

primer #101 (26 mer): 5'-CTTCCCAGGCCTGCAGTTTGCCCATC-3'
primer #102 (27 mer): 5'-GAACGGGGCTCGAAGGGTCCTTGTAGC-3'
primer #406 (22 mer): 5'-GAAGGGTCCTTGTAGCCGGGAA-3'
primer #409 (20 mer): 5'-GGAGGATGGAACACGGACGG-3'.

3. 2. 3 Identification of the CTG repeat number. Primer #101 (1 microgram) was radioactively end-labeled with γ-[^{32}P]adenosine triphosphate by T4 polynucleotide kinase. Radiolabeled primer #101 and

primer #102 were added to each PCR sample. PCR running conditions were above mentioned. The PCR product was run on a 6% denaturing polyacrylamide gel. The polyacrylamide gel was dried and exposed to Kodak X-ray film for one to two days.

3. 2. 4 Direct sequencing of PCR product. DNA fragments containing the CTG trinucleotide repeats in leukocytes were amplified by PCR using the CTG repeat closely flanking primers, purified by electrophoresis through 2% agarose gel. Primer #409 was biotinylated at the 5' end. Biotinylated single stranded DNA was prepared by Dynabeads™ M-280 Streptavidin (Dynal®, Norway) procedures and sequencing was performed according to Sanger's dideoxy chain termination method using a Sequenase kit Ver. 2™ (USB, USA) and ^{35}S-dATP. Nucleotide sequences were obtained by 6% polyacrylamide electrophoresis followed by drying and exposed to Kodak X-ray film for 24 hours.

Results

4. 1 Southern blot analysis and PCR amplification. *Eco*RI-digested DNAs probed with p5B1.4 (Fig. 2) showed an *Eco*RI polymorphism with 9.8- and 8.6-kb alleles in normal controls. In pedigree 1, both the asymptomatic maternal grandmother (Subject 1) and the mother's unmarried younger sister (Subject 5) had 8.6- and 9.8-kb heterozygous alleles, and the father (Subject 2) had 8.6-kb homozygous alleles. They had no CTG repeat expansions (normal). Mother (Subject 3) had adult DM; she had 9.8-kb and expanded smearing 14.2-kb alleles (about 1400 CTG repeats). The girl suffered with typical severe CDM (Subject 4); she had 9.8-kb and expanded discrete 17.8-kb alleles (about 2800 CTG repeats). In another two CDM families (Pedigrees 2 and 3), the adult DM patient (Subject 6) had normal alleles and expanded alleles, and the CDM patients (Subject 7, 8, 9, and 11) had normal alleles and larger expanded repeat than adult DM patient. Lane 10 (Subject 10) had 8.6-kb homozygous alleles (normal). *Bam*HI-digested fragments (Fig. 3) can detect smaller allelic expansions of about 200- bp (65-70 CTG repeats). Patients in whom allelic expansion is ambiguous can be diagnosed. Southern blot analysis of *Bam*HI-digested DNA showed a single band of 1.4-kb in normal populations. In pedigree 1, the expanded CTG repeats were detected as smears in the mother with the adult form of

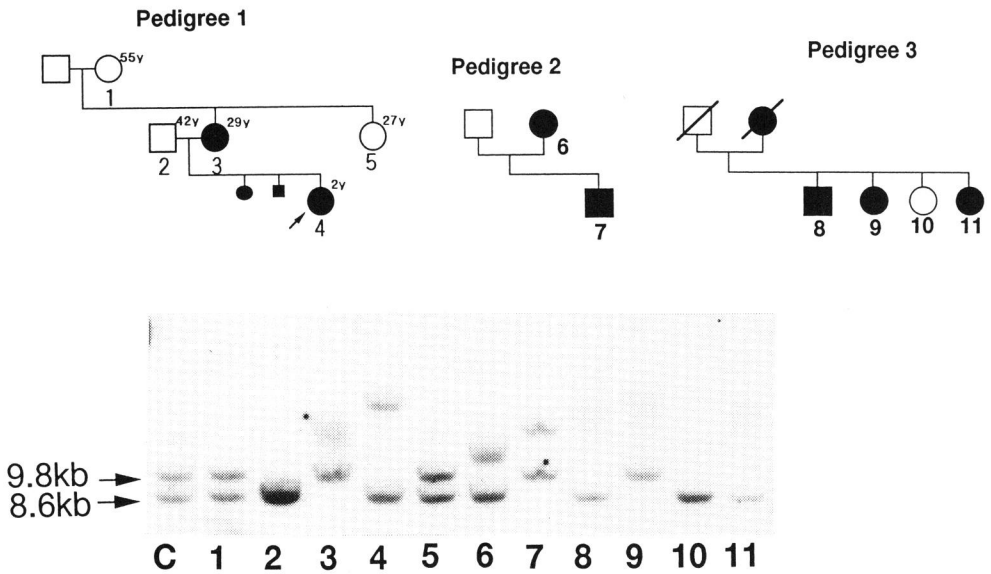

Fig. 2 Southern blot analysis of three families with CDM in *Eco*RI fragments.

DM (Subject 3). A girl with severe CDM (Subject 4) had a normal band of 1.4-kb plus a larger discrete expanding band of 9.2-kb. No expansion was detected in the asymptomatic grandmother (Subject 1), the father (Subject 2), and the aunt (Subject 5). In pedigree 4, the expanded CTG repeat was detected as a smear in the mother with the adult form of DM (Subject 6). A girl with CDM (Subject 8) had a normal band of 1.4-kb plus a larger discrete expanding band. No expansion was detected in the asymptomatic older brother (Subject 7). Table 1 summarizes the characteristics and results of CTG repeat expansions of CDM patients on Southern blotting. All DM patients had variable CTG repeat expansions, and healthy individuals had no expansions.

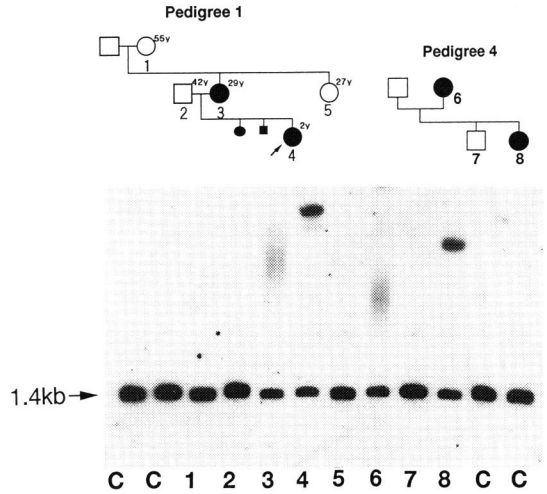

Fig. 3 Southern blot analysis of two families with CDM in *Bam*HI fragments.

Table 1 *Summary of the profile of patients with congenital myotonic dystrophy.*

Case	Age	Sex	Clinical severity	CTG repeat size (leukocyte)	Prognosis	Transmission	Severity of mothers	Mother's CTG repeat size
1	1 y	F	SCDM	7.8 kb	alive	maternal	MS-DM	4.2 kb
2	1 m	F	SCDM	5.6 kb	alive	maternal	adult DM	1.5 kb
3	1 m	F	SCDM	9.0 kb (tissue)	dead	maternal	MS-DM	4.0 kb
4	3 y	M	SCDM	6.0 kb	alive	maternal	adult DM	2.0 kb
5	6 m	M	SCDM	7.2 kb	alive	maternal	adult DM	n. d.
6	6 m	F	SCDM	7.2 kb	alive	maternal	MS-DM	2.9 kb
7	2 y	F	SCDM	5.8 kb	alive	maternal	adult DM	2.2 kb
8	1 m	M	SCDM	10.3 kb	dead	maternal	adult DM	2.1 kb
9	10 y	M	CDM	4.0 kb	alive	maternal	adult DM	1.3 kb
10	10 y	M	CDM	5.9 kb	alive	maternal	adult DM	1.9 kb
11	22 y	M	CDM	5.3 kb	alive	maternal	adult DM	1.3 kb
12	14 y	F	CDM	4.0 kb	alive	maternal	adult DM	1.0 kb
13	6 y	F	CDM	5.5 kb	alive	maternal	MS-DM	3.0 kb
14	17 y	F	CDM	7.1 kb	alive	maternal	adult DM	0.8 kb
15	3 m	F	SCDM	7.0 kb	alive	maternal	adult DM	2.7 kb
16	10 m	F	SCDM	6.6 kb	alive	maternal	adult DM	1.2 kb
17	1 m	F	SCDM	7.2 kb	alive	maternal	adult DM	3.0 kb
18	3 y	M	CDM	3.8 kb	alive	maternal	adult DM	1.8 kb
19	5 y	M	CDM	3.5 kb	alive	maternal	adult DM	1.6 kb
20	10 y	F	CDM	2.5 kb	alive	maternal	adult DM	1.6 kb
21	1 y	M	SCDM	6.5 kb	alive	maternal	subclinical DM	0.4 kb
22	6 y	M	CDM	3.2 kb	alive	maternal	subclinical DM	0.5 kb
23	3 y	F	CDM	4.9 kb	alive	maternal	adult DM	1.2 kb
24	2 y	F	CDM	5.4 kb	alive	maternal	adult DM	1.0 kb
25	5 y	M	CDM	4.3 kb	alive	maternal	adult DM	1.0 kb
26	6 y	M	CDM	5.0 kb	alive	patarnal	normal	none
27	2 y	F	CDM	3.3 kb	alive	patarnal	normal	none
28	10 y	M	SCDM	6.0 kb	alive	maternal	adult DM	n. d.
29	1 y	M	CDM	3.8 kb	alive	maternal	adult DM	n. d.
30	3 y	F	SCDM	6.2 kb	alive	maternal	adult DM	n. d.
31	9 m	F	SCDM	6.8 kb	alive	maternal	adult DM	n. d.
32	1 y	M	SCDM	5.8 kb	alive	maternal	adult DM	n. d.

SCDM, severe CDM; MS-DM, multisystemic adult DM; n. d., not done.

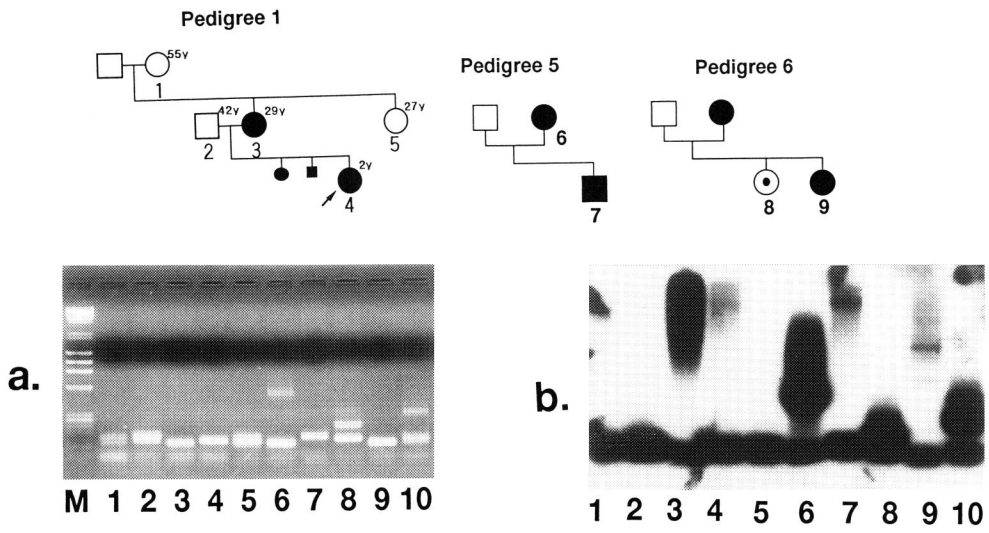

Fig. 4 **a)** Ethidium bromide-stained 2% agarose gel banding pattern of PCR-amplified CTG repeats region in CDM pedigrees.
b) Southern blot analysis probed with 5' end radiolabeled $(CTG)_{10}$ oligonucleotides in CDM pedigrees.

Ethidium bromide staining of the PCR- amplified CTG repeat region ,using flanking primers #101 and #102, (Fig. 4. a) indicated that the grandmother (Subject 1) and the aunt (Subject 5) in pedigree 1 had two normal alleles of 125-base pairs (bp) and 149-bp (5 and 13 CTG repeats). The father (Subject 2) had two normal alleles of 134- bp and 155-bp (8 and 15 CTG repeats). PCR-amplified DNA in the mother (Subject 3) and the girl (Subject 4) showed only the normal alleles of 125-bp and 134-bp, respectively. In another two CDM families (Pedigrees 5 and 6), the adult DM patient (Subject 6) had normal allele plus expanding band of 130 CTG repeats and the CDM patients (Subject 7, 9) had only normal allele. Subclinical Patient (Subject 8) had normal allele of 149-bp (13 CTG repeats) and minimal expanded allele of 269-bp (53 CTG repeats). Lane 10 showed subclinical DM patient. In Southern blot analysis probed with 5' end radiolabeled $(CTG)_{10}$ oligonucleotides (Fig. 4. b), PCR products from the asymptomatic grandmother (Subject 1) and the aunt (Subject 5) in pedigree 1 showed a hybridized signal only above the normal alleles, while PCR- amplified DNA from the mother with adult DM (Subject 3) showed two distinct hybridized signals above both a normal allele and a mutant allele. The girl (Subject 4) had both a normal allele signal and a faint smearing of the hybridization signal above an expanded mutant allele. On the basis of the clinical features presented, the results of Southern blot analyses of *Eco*RI, and *Bam*HI-digested DNA, and PCR amplification analyses of CTG repeat regions, the girl (Subject 4) in pedigree 1 was confirmed as having the CDM. The asymptomatic grandmother (Subject 1) and the girl's aunt (Subject 5) were confirmed to be normal. In another two CDM families (Pedigrees 5 and 6), the adult DM patient (Subject 6) had two distinct hybridized signals above both a normal allele and a mutant allele. The CDM patients (Subject 7, 9) had both a normal allele signal and a faint smearing of the hybridization signal above an expanded mutant allele.

4. 2 Intergenerational differences in CTG repeat size of mother-child pairs (Fig. 5) : Analysis of the intergenerational differences in CTG repeat size for mother-child pairs showed a positive correlation ($y=1.0384x+1265.2$, $r^2=0.311$, $n=25$). The present group of CDM showed a strong parental bias; all 27 pedigrees except one showed transmission by the affected mother. They showed genetic anticipation: earlier age of onset and increasing disease severity in successive generations.

4. 3 Paternal transmission : We previously reported the first CDM patients in a family in whom the transmission was paternal and showed the anticipation in both phenotype and genotype which was characteristic of DM, identified by DNA analysis[20]. In Southern blot analysis of the *Eco*RI-digested DNAs probed with p5B1.4 (Fig. 6. a), both the father (Subject 1) and mother (Subject 2) in pedigree 7 had 8.6- and 9.8-kb alleles, but no distinct expansion was detected. The asymptomatic older brother of the proband (Subject 3) had 8.6- and 11.5-kb alleles (about 500 CTG repeats). Patients 1 (Subject 4) had alleles of 9.8- and 15.0-kb (about 1600 CTG repeats), and patient 2 (Subject 5), 8.6- and 13.0-kb (about 1100 CTG repeats). The father had a minimal expanding band of 1.6-kb in *Bam*HI-digested DNA (Figure 6. b). In determining the accurate number of CTG repeat of minimal expanded mutant alleles, PCR amplification using CTG repeat flanking primers and direct sequencing of PCR products were performed (Fig. 7). The mother (Subject 2) had two normal alleles of 12 and 23 CTG repeats. PCR-amplified DNA in patient 1 (Subject 4), patient 2 (Subject 5), and the asymptomatic brother (Subject 3) showed an only normal allele of 12, 23, and 12 CTG repeats, respectively. The father (Subject 1) had a normal allele of 11 CTG repeats and a mutant allele of 75 CTG repeats. A mutant allele of the father by direct sequencing confirmed minimal expanded CTG repeats (Fig. 8).

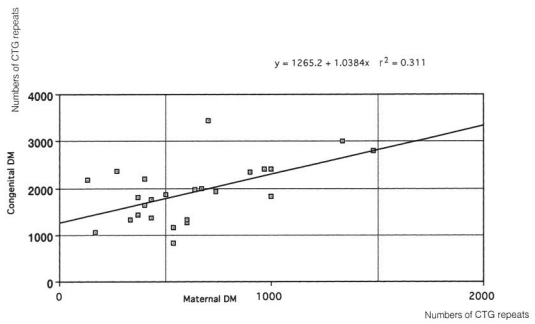

Fig. 5 The intergenerational differences in CTG repeat size of mother-child pairs.

4. 4 Correlation between the number of CTG repeat and the age of onset : There were significant correlations between the number of CTG repeats and both the age of onset and disease severity (Fig. 9). The largest CTG repeat expansion on average belonged to the CDM group, and the smallest, to the

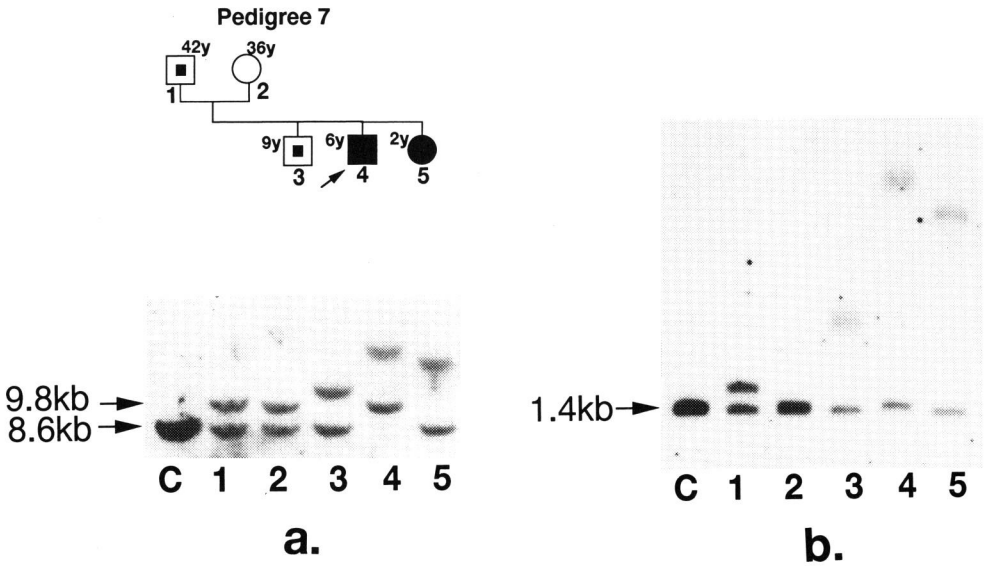

Fig. 6 **a)** Southern blot analysis in the paternally transmitted family pedigree in *Eco*RI fragment.
b) Southern blot analysis in *Bam*HI fragments.

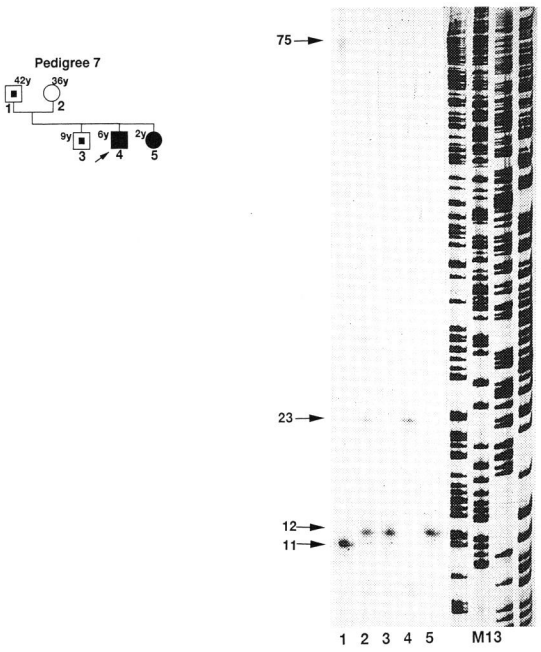

Fig. 7 Identification of PCR-amplified CTG repeats in the paternally transmitted family pedigree.

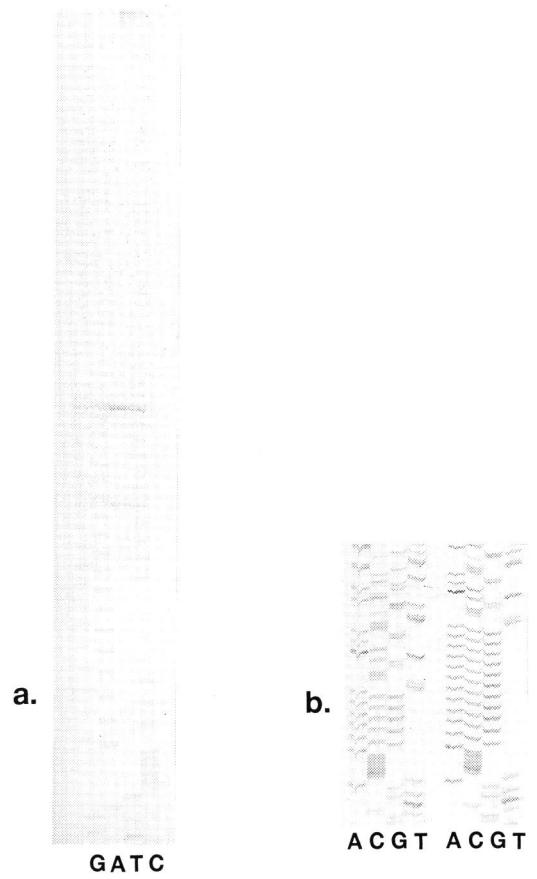

Fig. 8 Direct sequencing of mutant allele of DM protomutation carrier in the paternally transmitted family pedigree (Pedigree 7) normal alleles.
a) shows minimal expansion of the CTG repeats, and b) shows the 5 and 11 copies of normal alleles.

subclinical DM group. 1) The patients suffering severe CDM had 2293±404* CTG repeats. 2) CDM had 1445±400* CTG repeats. 3) Adult DM had 581±234* CTG repeats. 4) Subclinical DM had 129±98* CTG repeats. (The mark "*" shows mean±SD. There was a significant difference in repeat size among the four groups: $p<0.001$)

4. 5 Southern blot analysis using skeletal muscles. Southern blot analysis using *Bgl*I-digested DNAs probed with p5B1.4 showed a single band of 3.4-kb in normal controls (Fig. 10). Patients 1, 2, 3, and 4 had additional expanding bands ranging from 4.1-kb to 5.5-kb in leukocytes and 5.5-kb to 7.5-kb in skeletal muscle. All five patients had a larger expanded band, ranging from 1.5-kb to 3.5-kb, in skeletal muscle than in leukocytes, which suggested that the somatic cell heterogeneity between skeletal muscle and leukocytes was established during the early stage of childhood[21].

4. 6 Somatic cell heterogeneity in various organs. Various tissues from autopsied patient with severe CDM[22] showed an abnormal enlarged and blurred band of 12.4-kb on Southern blot analysis with *Bgl*I digestion. These findings disclosed a slight somatic cell heterogeneity[21),23] (Fig. 11).

Discussion

DM patients in our studies showed a quite variability in phenotype, ranging from a severe CDM that is frequently fatal after birth to a complete absence of symptoms[24]. The phenomenon of anticipation, which is characteristic of DM, was observed in all the present pedigrees. The severity of CDM

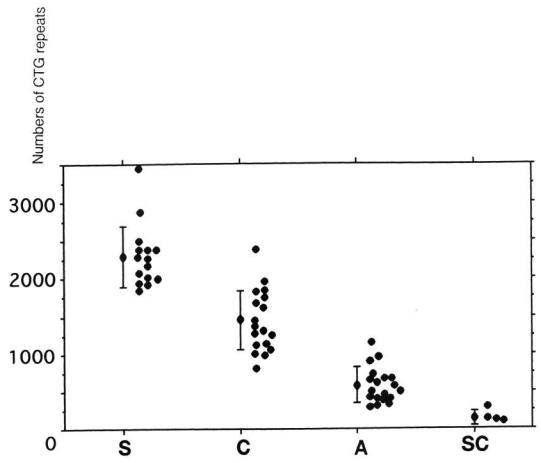

Fig. 9 Correlation between the numbers of CTG repeat and the age of onset of DM.

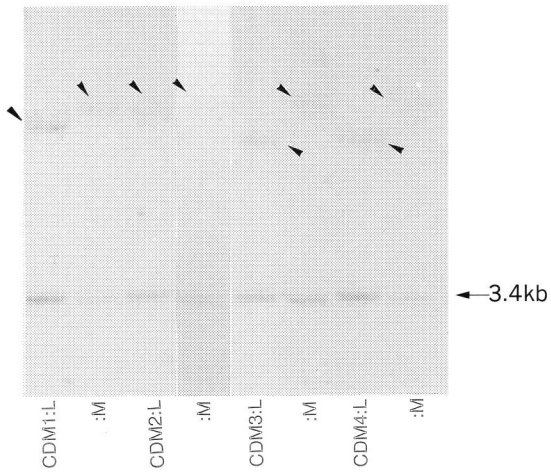

Fig. 10 The amplification of the CTG repeat in *Bgl*I fragments from lymphocytes (L) and skeletal muscles (M) in four patients with CDM.

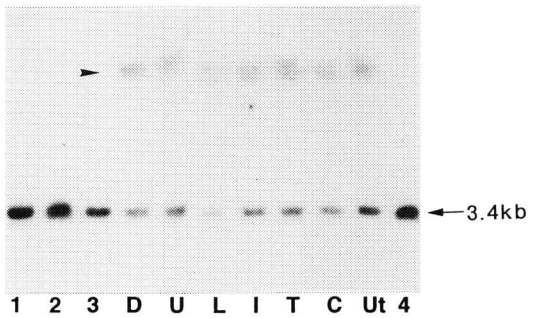

Fig. 11 The amplification of the CTG repeat in *Bgl*I fragments from various tissues in an autopsied patient with severe CDM.

confirmed to correspond to the length of CTG repeat amplification in mutant genes from peripheral blood leukocytes in our study. Mahadevan *et al.* suggested that a blurred or smeared appearance of expanded alleles seen on Southern blots in lymphocytes indicated somatic cell heterogeneity in the size of the expanded alleles[7]. We also observed smeared expanded bands in several DM patients on Southern blots. In PCR analysis, we detected smeared bands of mutant alleles in all DM patients. Our molecular data in CDM were consistent with the results of previous reports[7),8),25]. Yamagata *et al.* reported the characteristic relationships between CTG repeat mutations and clinical phenotypes in Japanese DM patients[25]. We emphasize that our study disclosed the correlations between expanded CTG repeat size and its clinical features in Japanese CDM patients.

The transmission of CDM was previously considered exclusively maternal. Less than 10% of affected mothers gave birth to CDM infants. The risk of the baby being congenitally affected inherited from multisystemic DM mothers at the time of the pregnancy is approximately 80%[27]. Although we observed the infants born to four multisystemic DM mothers were all severe or lethal congenitally affected DM babies, their mothers had no understandings of high risk for bearing CDM offsprings[24]. The CTG repeat expansion in DM mutations enables us to explain the genetic anticipation and phenotypic variability in DM, and to diagnose DM, even in patients who were asymptomatic or showed few of the classical DM signs. The identification of the DM mutation and the ability to perform a direct DNA analysis will enable physicians to provide families more accurate risk estimates and information for genetic counseling. Moreover, a DNA analysis will be especially useful for prenatal diagnosis.

O'Hoy *et al.* reported a reduction in the size of the DM-specific expansive allele in some DM patients transmitted paternally[28]. Maternal transmission of the unstable CTG repeat in CDM is as-

sociated with variable increased amplification, whereas, paternal transmission of the CTG repeat is associated with reduced amplification. There are sex differences in maternal and paternal transmission, similar to those in the Fragile X syndrome. Although DM is an autosomal dominant disease, the previously reported cases of CDM were said to be inherited only maternally. In an early report, Harper *et al.* suspected that maternal intrauterine factors might be related the mechanism for the external maternal transmission of CDM[29]. Harper reported that differential DNA methylation of maternal and paternal alleles (so-called, genomic imprinting) had occurred in neonates who inherited the DM gene from the mother[1]. Shaw *et al.* detected no DNA methylation differences between paternally and maternally derived alleles in CDM and adult onset DM families[30]. We previously presented the first known case of the CDM via paternal transmission[20]. Some DM patients have a very mild clinical signs associated with minimal amplification of the $(CTG)_n$ repeat, ranging from 50 to 80 CTG repeats. This class of DM mutation was called a DM protomutation carrier[31]. In our study, a mutant allele of the father reveals 75 CTG repeats. The asymptomatic father was a DM protomutation carrier. We speculate that the changes in CTG repeat numbers may occur during oogenesis and spermatogenesis. Analysis of CTG repeats in patient's father's sperm may shed some light on paternal transmission of DM mutations.

The direct mechanism of CTG repeat expansion in the DM gene is still unknown for the present. But, the association of the insertion and deletion polymorphism located in intron 8 of the DM gene and $(CTG)_n$ repeat size was closely correlated in Caucasian and Japanese populations[32-33]. In DM patients, the expanded $(CTG)_n$ repeat is associated with the 1-kb insertion. The $(CTG)_{11-13}$ repeat was almost always associated with the deletion and the $(CTG)_5$, $(CTG)_{10}$, and $(CTG)_{19-30}$ repeats were associated with the insertion. The $(CTG)_{11-18}$ allele were stable and had no increasing change of repeats in successive generation, but $(CTG)_5$, $(CTG)_{19-30}$ repeat alleles were quite unstable, almost always expanding in successive generations[34]. South African Negroids had a $(CTG)_n$ allelic distribution that was significantly different from that in Caucasian and Japanese populations: the $(CTG)_{>19}$ repeat was very rare. Those findings explained the absence of DM patients in South Africa[35]. Recently, Wong *et al.* reported that the size of expanded CTG repeat from the same adult DM patients showed a clear tendency to increase over 2- to 5-years intervals, suggesting an association of progressive shift to larger expansion size with advancing age (postnatal instability of CTG repeats)[36]. Therefore, our results require the verification of the CTG repeat expansion by prospective study with the same subjects after several years. Accumulations of new findings associated with CTG repeat expansion in DM gene study may become gradually the mechanism of CTG repeat expansion clear.

The disease severity of CDM generally seems to correspond to the length of CTG repeat amplification in mutant genes from lymphocytes, but this correlation is weaker in the alleles of other organs such as skeletal muscle. Anvret *et al.* were the first to report that the expansions of CTG repeat in muscle from patients with adult form of DM were much larger than those in lymphocytes. They demonstrated that the expansions seen in muscle from adult DM were stable over a significant time period (10-15 years) and were useful to predict the progression of DM symptoms[37]. Thornton *et al.* reported that the DM-specific expansive alleles in skeletal muscle from adult DM were 2- to 13-fold the size of those in lymphocytes; different muscles were generally consistent with each other within each patient[38]. Ashizawa *et al.* also reported move expanded CTG repeat in skeletal muscle from adult DM patients than in lymphocytes[39]. Thornton *et al.* and Ashizawa *et al.* demonstrated that the size of CTG repeat amplifications in affected tissues, such as skeletal muscle, correlated better with DM severity than the repeat in lymphocytes did. They concluded that the somatic instability of the repeat might cause substantial somatic cell heterogeneity. Lavedan *et al.* reported that, as observed in leukocytes, various tissues of a 20-wk-old fetus carrying the DM mutation showed discrepancy in the size of expand-

ed CTG repeat under Southern blot analysis, reflecting the mitotic instability in all fetal tissues[40]. Our data are also consistent with the results of Thornton *et al.* and Ashizawa *et al.*, and demonstrate that the extent of the expanded CTG repeat in skeletal muscle from CDM patients was always larger than that of in leukocytes. It is likely that somatic cell heterogeneity is established during the early stage of childhood. Furthermore, we displayed somatic cell heterogeneity within different organs except for leukocytes from the same CDM patient, and minimal degree disparity among the expansions of CTG repeats of various tissues[21-22].

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先天性筋緊張性ジストロフィーにおけるCTG塩基繰り返し配列の増大に関する研究

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筋緊張性ジストロフィー（DM）の遺伝子異常はmRNAの3'側非翻訳領域にあるCTGの繰り返し配列の増大にある．DM患者59人(28 DM家系：母由来遺伝が27家系，父母由来不明が1家系で，重症CDM児16人，CDM 16人，成人型DM患者23人，軽症DM 4人)を対象として遺伝子解析を行った．DM患者の末梢血白血球，5人のCDM児の骨格筋と1人の重症CDM児の各種剖検組織からゲノムDNAを抽出した．方法は，p5B1.4をプローブとしてSouthern Blot法およびPCR法を行った．Direct sequencing法をもちいてPCR法で増幅したPCR産物が増大したCTGリピートを含んでいること確認した．

(1)全てのDM患者の末梢血白血球で，CTGリピートの増大を認めた．臨床的に母親由来の遺伝を示したCDM27家系では，分子遺伝学的にも母親由来遺伝(母親が成人型DM）であった．父母由来が不明であった1家系では，父親由来の遺伝で，父親のCTGリピート数は75コピーと軽度の増大であった．DM家系では，表現促進(anticipation)を示した．重症度によりDMを分類したとき，各群においてCTGリピートサイズに有為差を認めた．(2) PCR法をもちいることで，200 bpまでのCTGリピートの増大を検出することが可能であった．これにより，DMの保因者診断が可能となった．(3) 5人のCDM児の骨格筋のCTGリピート数は全例末梢血白血球より抽出したゲノムDNAより1.5-kbから3.5-kbの増大を認めた．(4)重症CDM児の剖検組織（肝，横隔膜，膀胱，小腸，舌，子宮，大脳皮質）からえられたゲノムDNAでは9.0-kbと著明に増大したCTGリピートを認めた．さらに，各臓器間で軽度のsomatic heterogeneityを認めた．