congenital myopathy defined by type 1 fiber hypotrophy of 12% or more than type 2 fibers, and with the absence of structural abnormalities within myofibers [2]. Type 1 fiber predominance is also commonly seen. Clinically, CFTD patients show hypotonia, facial muscle weakness, and severe respiratory insufficiency at birth. Long face, high-arched palate, and joint contractures are often seen. CFTD is a genetically heterogeneous disorder and mutations in the genes for tropomyosin 3 (*TPM3*; OMIM 191030), α-skeletal muscle actin 1 (*ACTA1*; OMIM 102610), and selenoprotein N1 (*SEPN1*; OMIM 606210) have been identified [3, 4, 8]. Reportedly *TPM3* mutations are the most common ones and observed approximately in 20–25% of the CFTD patients [4]. *ACTA1* mutations were identified in 6% of CFTD [8], and only one family was reported having an *SEPN1* mutation [3].

Although the muscle pathology features of CDM seem to be well defined, our experience with one CDM patient who was previously diagnosed as CFTD made us hypothesize that CDM may have features other than the presently defined ones, both in terms of muscle pathology and clinical characteristics. In this study, we looked for CDM patients among patients who presented with CFTD. We also performed clinical and pathological analysis to find out whether patients with CDM can be distinguished from CFTD.

Materials and methods

Patients

All clinical materials used in this study were obtained for diagnostic purposes and with informed consent. This work was approved by the Ethical Committee of National Center of Neurology and Psychiatry (NCNP). In this study, we chose muscle specimens from patients younger than 1 year of age. From the muscle repository of NCNP, there were 28 unrelated patients who were pathologically diagnosed as CFTD. Twenty CDM patients, who had symptomatic family members and whose diagnosis was genetically confirmed, were also used for comparison.

Histochemistry

Biopsied skeletal muscles were frozen with isopentane cooled in liquid nitrogen. Serial frozen sections of 10 μm thickness were stained with hematoxylin and eosin (H & E), modified Gomori-trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), and ATPases (pH 10.6, pH 4.6 and pH 4.3). For each muscle specimen, the mean fiber diameter was determined by obtaining the shortest anteroposterior diameter of 100 each of type 1 and type 2 (A + B) fibers using ATPase stains. The myofiber diameter was used to calculate the fiber size disproportion (FSD). FSD was computed as: difference of type 2 fiber diameter (mean) and type 1 fiber diameter (mean) divided by type 2 fiber diameter (mean) × 100%.

Genetic analyses

Genomic DNA was extracted from peripheral lymphocytes or frozen muscle specimens using standard protocol. To examine CTG repeat expansion in *DMPK*, triplet repeat primed PCR was performed as described previously [12]. The presence of the expanded CTG repeats was examined by Gene Mapper using ABI PRISM 310 automated sequencer (Applied Biosystems Japan Co., Ltd, Japan). To know the approximate number of triplet repeats, we performed Southern blotting analysis using PCR-amplified CTG repeats because of the limited amounts of muscle specimens [10]. The primer sequences used in this study are F: 5′-CGAACGGGGCTCGAAGGGTCCTTGTAGC CG-3′, and R: 5′-TCTTTCTTTACCAGACACTAGGG-3′.

The PCR products were electrophoresed with 1% of Seakem HGT agarose gel (Cambrex Bio Science Rockland Inc., ME, USA), transferred to Hybond-XL (GE Healthcare, UK) for overnight, hybridized with ^{32}P-labeled probes of $(CTG)_{10}$ oligonucleotide at 65°C for overnight, and detected using BAS2500 (Fuji Film, Japan). By using genomic DNA from a CDM patient with known CTG repeat number, we confirmed that this PCR-based method can detect the corresponding size of the CTG repeats using genomic DNA. For mutation screening of *ACTA1* and *TPM3*, all exons and their flanking intronic regions were amplified by PCR and directly sequenced by an ABI PRISM 3100 automated sequencer (Applied Biosystems). Primer sequences are listed in the Supplemental Table.

Statistical analyses

All data are presented as means ± SD. Comparisons among groups were done by using Student's *t* test and analysis of variance (ANOVA) as appropriate. Statistical significance was considered when *p* value was less than 0.05.

Results

Genetic analyses

By using triplet repeat primed PCR, expanded CTG repeats in *DMPK* were detected in 4 of 28 (14%) unrelated patients who were pathologically diagnosed as CFTD (Figs. 1, 2a). This diagnosis of CDM was further confirmed by Southern blotting analysis, wherein all four patients had more than 1,000 CTG repeats (Fig. 2b). We also identified three heterozygous *ACTA1* mutations (p.Gly48Cys, p.Leu221-Pro, and p.Pro332Ser) in four unrelated CFTD patients. Two mutations of p.Leu221Pro and p.Pro332Ser have already been reported [8], whereas the p.Gly48Cys mutation observed in two patients was a novel one. The Gly48 is a highly conserved amino acid among several species. Two unrelated CFTD patients had the same heterozygous mutation p.Arg168Cys in *TPM3*, which was previously reported in CFTD patients [4].

Clinical findings

We compared the clinical findings among 4 CFTD patients with CTG expansion and 6 CFTD patients with *ACTA1* or *TPM3* mutations, and compared the clinical features with 20 patients genetically confirmed as CDM (Table 1). In terms of family history, none of the four CFTD patients with CTG expansion had a positive family history. This is in stark contrast with the typical picture in CDM patients, as all of them had at least one symptomatic family member. Hydramnios and premature delivery were seen in more than 50% of the CFTD patients with CTG expansion and CDM, but none of CFTD patients with *ACTA1* or *TPM3* mutations. Hypotonia and respiratory insufficiency at birth were seen in all groups except for two patients with *TPM3* mutation.

Muscle pathological findings

As muscle pathology can have drastic changes according to the gestational age of infantile patients, we adjusted the age by setting the full-term day (37 weeks of gestation) as putative birthday. After adjustment, the age at biopsy of the CDM patients ranged from −7 to 43 weeks, and those of the four CFTD patients with CTG expansion were from 21 to 42 weeks.

Congenital fiber type disproportion is defined as a congenital myopathy wherein FSD is higher than 12%, but with no associated structural abnormalities within the myofibers [2]. In this study, FSD in CDM, CFTD with CTG expansion, CFTD with *ACTA1* mutation, and CFTD with *TPM3* mutation was calculated to be 7.2 ± 6.8% (mean ± SD), 23.0 ± 5.0, 47.5 ± 4.0, and 52.0 ± 9.9%, respectively (Fig. 3). FSD was significantly ($p < 0.05$) higher in CFTD with *ACTA1* or *TPM3* mutations as compared to the CFTD patients with CTG expansion and CDM.

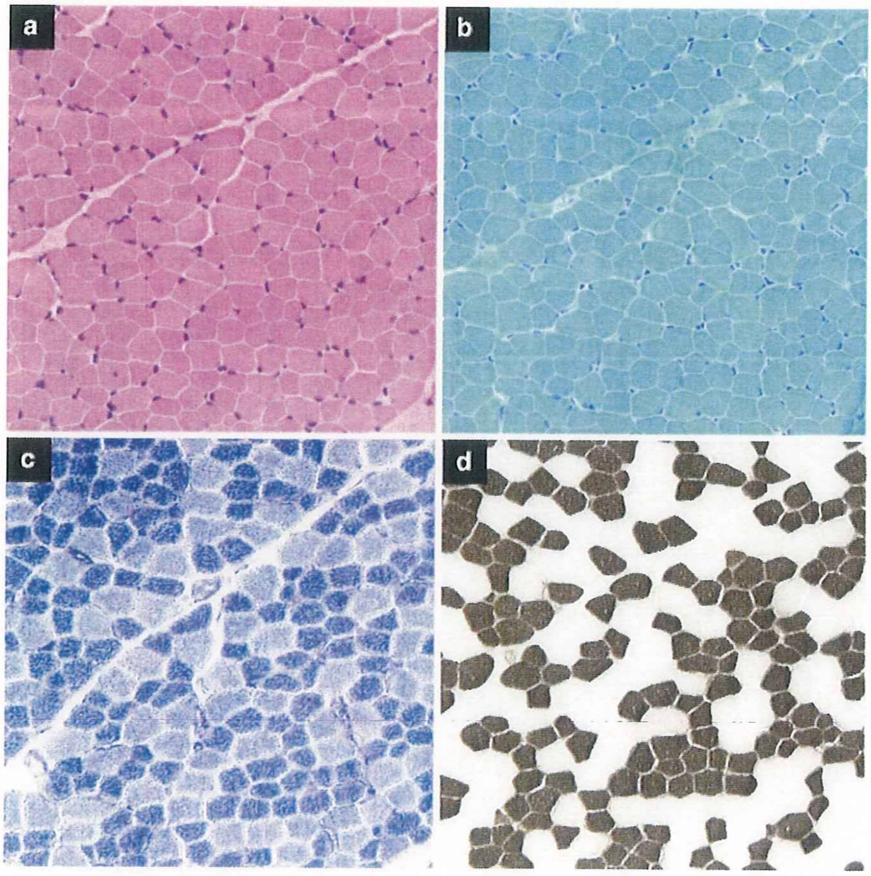

Fig. 1 Muscle pathology of a 42-week-old CFTD patient with CTG expansion. **a** Hematoxylin and eosin, **b** modified Gomori trichrome, **c** NADH-TR, and **d** ATPase (pH 4.4) stain. Type 1 fiber atrophy (FSD [(mean type 2 fiber diameter) − (mean type 1 fiber diameter)/mean type 2 fiber diameter × 100] = 26%), type 1 fiber predominance (65%), and only 1% of type 2C fibers with no peripheral halo is seen. *Bar* 50 μm

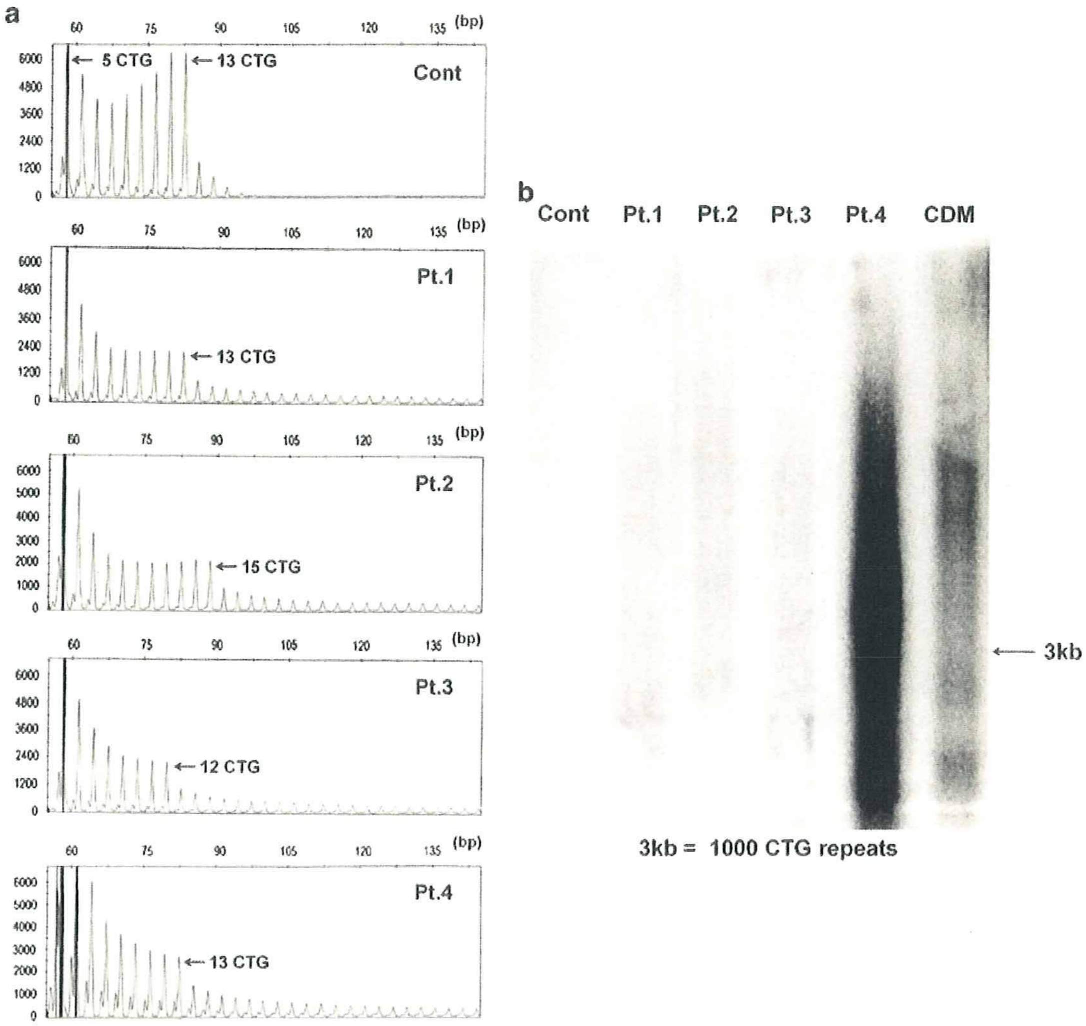

Fig. 2 Genetic analyses. **a** Triplet repeat primed PCR. Control (*Cont*) has 5 and 13 CTG repeats. The four CFTD patients (Pt.1, Pt.2, Pt.3, and Pt.4) have the ladder pattern that represents a large CTG allele together with higher peaks that show normal-sized allele (*arrows*). **b** Southern blotting analysis using PCR products. Four CFTD patients (Pt.1, Pt.2, Pt.3, and Pt.4) and one genetically confirmed CDM showed smear band larger than 3 kb corresponding to 1,000 CTG repeats, whereas a control (*Cont*) has no detectable band

Table 1 Clinical summary of the patients

Pathological diagnosis	CDM	CFTD	CFTD	CFTD
Gene mutation	CTG expansions in *DMPK*	CTG expansions in *DMPK*	*ACTA1*	*TPM3*
Number of patients	20	4	4	2
Hydramnios	65% (13/20)	50% (2/4)	0% (0/4)	0% (0/2)
Premature delivery (<37w)	50% (10/20)	50% (2/4)	0% (0/4)	0% (0/2)
Hypotonia at birth	100% (20/20)	100% (4/4)	100% (4/4)	0% (0/2)
Respiratory insufficiency at birth	95% (19/20)	75% (3/4)	75% (3/4)	0% (0/2)
Symptoms seen in family	100% (20/20)	0% (0/4)	0% (0/4)	0% (0/2)

In addition to FSD, we also checked other features in pathology that define either CFTD or CDM. Type 1 fiber predominance is a notable pathological finding observed in CFTD, and all our CFTD patients, including those with CTG expansion, showed type 1 fiber predominance. The mean composition of type 1 fibers in CDM, CFTD with

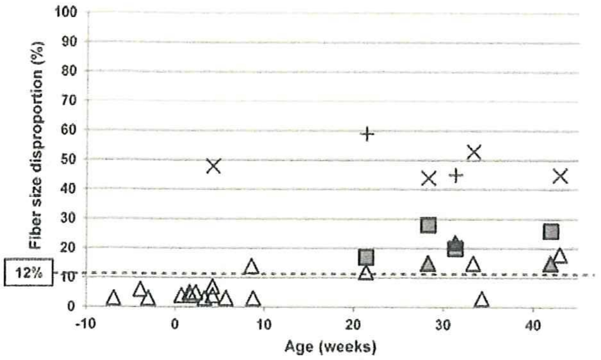

Fig. 3 Fiber size disproportion (*FSD*) of each patient. CFTD with CTG expansion (*filled square*; $N = 4$), CDM (*open triangle*; $N = 17$), CDM with similar pathological findings to CFTD (*filled triangle*; $N = 3$), CFTD with *ACTA1* mutations (*multi symbol*; N = 4), and CFTD with *TPM3* mutations (*plus*; $N = 2$). *Dot line* at 12% of FSD is the lowest FSD by the definition of CFTD

CTG expansion, and CFTD with *ACTA1* or *TPM3* mutations was 19.6 ± 16.3, 58.2 ± 6.2, 57.8 ± 2.0, and 65.5 ± 12.0%, respectively (Fig. 4). On the other hand, the presence of numerous immature type 2C fibers with peripheral halo is a characteristic finding in CDM. A markedly increased number of type 2C fibers were actually observed in CDM especially in patients younger than 10 weeks of adjusted age (Fig. 5). The frequency of type 2C fibers was inversely correlated to age of patients, while the number of type 1 fibers was directly proportional to age of patients. In other words, type 2C fibers were increased among younger age, while type 1 fiber predominance is seen more among older patients. Peripheral halo was observed in 14 of 20 (70%) CDM patients even in a 43-week-old patient. In CFTD patients with CTG expansion, type 2C fibers accounted for less than 20% and in CFTD with *ACTA1* or *TPM3* mutations, only a few type 2C fibers were seen. No peripheral halo was seen in either group. The increased number of fibers with internally located nuclei is another characteristic pathological finding of myotonic dystrophy. In our series, fibers containing internal nuclei were variably increased up to 26% in CDM patients, whereas less than 2% of fibers contained internal nuclei in the CFTD patients with CTG expansion or mutation in *ACTA1* or *TPM3*. The number of the fibers with internal nuclei is relatively correlated to the number of immature fibers in CDM, which may reflect immaturity of the fibers as described previously [6, 11].

Of the 20 CDM patients, 3 showed pathological findings similar to CFTD with CTG expansion. The ages of these three patients were 29, 32 and 42 weeks, respectively. FSD was 15–21%, with less than 20% of type 2C fibers and no peripheral halo. In these patients, the clinical diagnosis of CDM was made based upon the presence of the symptomatic family member.

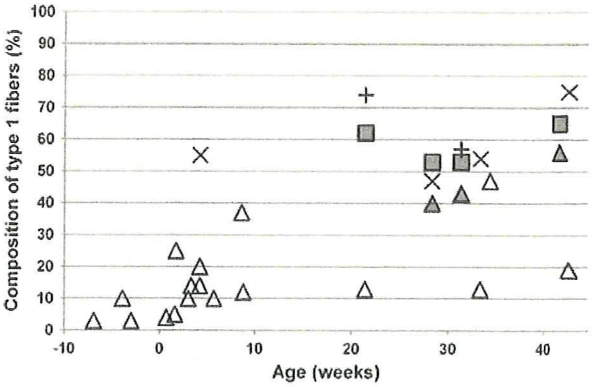

Fig. 4 Composition of type 1 fibers in each patient. *Filled square* CFTD with CTG expansion, *open triangle* CDM, *filled triangle* CDM with similar pathological findings to CFTD, *multi symbol* CFTD with *ACTA1* mutations, and *plus* CFTD with *TPM3* mutations

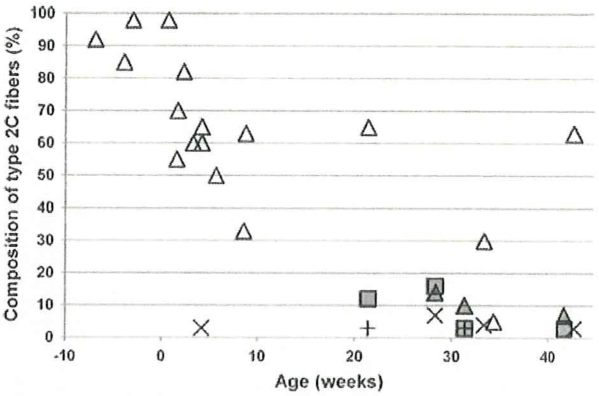

Fig. 5 Composition of type 2C fibers in each patient. *Filled square* CFTD with CTG expansion, *open triangle* CDM, *filled triangle* CDM with similar pathological findings to CFTD, *multi symbol* CFTD with *ACTA1* mutations, and *plus* CFTD with *TPM3* mutations

Discussion

In this study, we identified 4 of 28 patients (14%) who have CTG expansion in *DMPK* but were pathologically diagnosed as CFTD. Clinical symptoms of CFTD and CDM are quite similar during neonatal stage, including hypotonia and respiratory insufficiency. However, most of CDM patients are readily diagnosed by the presence of symptomatic family members, typically the mother. In fact, all CDM patients in our series had symptomatic family members and 75% of the mothers had the diagnosis of myotonic dystrophy. In contrast, no notable clinical symptoms were recorded in the mother of the CFTD patients with CTG expansion, and we could not examine the repeat size of the mothers. No marked difference in the size of CTG repeats was seen between CFTD patients with CTG expansion and CDM.

Among the CDM patients we examined, three patients showed pathological findings similar to those observed in CFTD with CTG expansion. They showed a small number of type 2C fibers, no peripheral halo, and hypotrophy of type 1 fibers (FSD >15%). The diagnosis of CDM was done from the typical clinical symptoms of myotonic dystrophy observed in the family member. Interestingly the ages of these three patients were over 29 weeks. Consistently, the ages of the patients who have CFTD with CTG expansion ranged from 21 to 42 weeks. These results suggest that CFTD pathology may be seen in this age range of CDM patients.

We identified four patients with mutations in *ACTA1* and two in *TPM3*. FSD in these patients was over 45% and significantly higher than that observed in CFTD with CTG expansion. This finding is also consistent with a previous report of CFTD patients with *TPM3* mutations whose muscle showed higher than 50% of FSD [4]. From these results, CDM should be considered for the patients whose muscle shows CFTD with FSD lower than 40%. In our series, only 4 (14%) and 2 (7%) of 28 patients had the mutations respectively in *ACTA1* and *TPM3*, leaving 18 (65%) patients still genetically uncharacterized and suggesting that defects in these genes may not be the major causes of CFTD in Japan. Further studies are necessary to elucidate such causes.

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