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Congenital muscular dystrophy with glycosylation defects of α-dystroglycan in Japan

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Abstract

Glycosylation defects of α-dystroglycan (α-DG) cause various muscular dystrophies. We performed clinical, pathological and genetic analyses of 62 Japanese patients with congenital muscular dystrophy, whose skeletal muscle showed deficiency of glycosylated form of α-DG. We found, the first Japanese patient with congenital muscular dystrophy 1C with a novel compound heterozygous mutation in the fukutin-related protein gene. Fukuyama-type congenital muscular dystrophy was genetically confirmed in 54 of 62 patients. Two patients with muscle–eye–brain disease and one Walker-Warburg syndrome were also genetically confirmed. Four patients had no mutation in any known genes associated with glycosylation of α-DG. Interestingly, the molecular mass of α-DG in the skeletal muscle was similar and was reduced to ~90 kDa among these patients, even though the causative gene and the clinico-pathological severity were different. This result suggests that other factors can modify clinical features of the patients with glycosylation defects of α-DG.

Keywords: α-dystroglycan (α-DG); Fukuyama-type congenital muscular dystrophy (FCMD); Congenital muscular dystrophy 1C (MDC1C); Muscle-eye-brain disease (MEB); Walker-Warburg syndrome (WWS); Glycosylation; Fukutin; FKRP; POMGnT1; POMT1; LARGE

1. Introduction

Recent advances demonstrated that glycosylation defects of cell surface membrane protein, α-dystroglycan (α-DG) cause a group of muscular dystrophy, including Fukuyama-type congenital muscular dystrophy (FCMD), muscle–eye–brain disease (MEB), Walker-Warburg syndrome (WWS), congenital muscular dystrophy 1C (MDC1C) and its allelic limb-girdle muscular dystrophy (LGMD) 2I, and congenital muscular dystrophy 1D (MDC1D) [1–8]. Some of these forms are associated with neuronal migration disorder in brain and ocular abnormalities, and others with normal brain and eyes. Characteristically, they all show abnormally glycosylated α-DG with preserved core structure in the muscle sarcolemma [9]. From this result, the responsible gene products of these diseases are thought to have a role in the glycosylation process of α-DG. In fact, mutations in the glycosyltransferase genes of protein *O*-mannose β 1,2-*N*-acetylglucosaminyltransferase 1 (*POMGnT1*) and protein *O*-mannosyltransferase 1 (*POMT1*) have been identified in patients with MEB and WWS, respectively [3,4]. In addition, other responsible gene products of fukutin, fukutin-related protein (FKRP), and LARGE are also predicted to have structural similarity to glycosyltransferases [10].

In Japan, FCMD is the most common form of congenital muscular dystrophy (CMD) [11], whereas merosin-deficient

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CMD (MDC1A), which is common in European countries, MEB, and WWS were rarely seen [12,13]. Patients with MDC1C and MDC1D have not been identified yet in Japan. To know more about the CMD patients with glycosylation defects of α-DG in Japan, we performed detailed genetic and clinico-pathological analyses on 62 patients.

2. Materials and Methods

2.1. Clinical materials

All clinical materials were obtained for diagnostic purposes with informed consent. We analyzed a total of 62 patients whose limb-muscle specimens showed altered glycosylation of α-DG. The clinical diagnoses of the 62 patients are shown in Table 1. The muscle samples were flash-frozen in isopentane chilled with liquid nitrogen.

2.2. Immunohistochemistry, immunoblotting, and laminin overlay assay

The following antibodies were used for immunohistochemical and immunoblotting analyses: monoclonal anti-α-DG (VIA4-1, Upstate Biotechnology), polyclonal goat anti-α-DG (GT20ADG) [9], monoclonal anti-laminin α2 chain (5H2, Chemicon), polyclonal anti-laminin-1 (Sigma), and monoclonal anti-β-DG (43DAG1/8D5, Novocastra Laboratories). The detailed techniques of the immunohistochemistry, immunoblotting and laminin overlay assay have been described previously [1,9].

2.3. Genetic analyses of fukutin, FKRP, POMGnT1, POMT1, and LARGE

DNA was isolated from skeletal muscle or peripheral lymphocytes using a standard technique.

To detect the 3-kb retroransposal insertion in *fukutin*, the genomic PCR was performed using two primer sets; one is designed to amplify a 375 bp product containing a part of retrotransposal insertion and the other is designed to amplify a normal 157 bp fragment (the primers were designed by Dr Toda, Osaka University). All exons and their flanking intronic regions of *fukutin* [14] were directly sequenced in patients without homozygous retrotransposal insertion using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems).

Mutation analysis of *FKRP* was performed using the primers reported elsewhere [15].

Mutation analysis of *POMGnT1*, *POMT1*, and *LARGE* was performed by directly sequencing all exons and their flanking introns. The information on primer sequence and PCR conditions is available upon request. To detect the mutation in exon 11 of *POMGnT1* in patient 2, primers F (5′-CATTCACCTCTGTGGGTAAGC) and R (5′-AGGCC TTCACATTTCACAGC) were used.

Table 1
Clinical and genetic diagnosis of 62 patients

Genetic diagnosis		Clinical diagnosis	
FCMD	54	FCMD	53
		MEB	1
MEB	2	FCMD	1
		CMD	1
WWS	1	WWS	1
MDC1C	1	FCMD	1
Unknown	4	MEB	1
		WWS	3

2.4. Single-strand conformation polymorphism (SSCP) analysis of FKRP

To exclude the possibility of polymorphism, we performed SSCP analysis for the missense mutation identified in *FKRP* in patient 1, using Gene Gel Excel (Pharmacia Biotech). The amplified genomic DNA fragments using a set of primers (4-2F and 4-2R [15]) including the site of the missense mutation was electrophoresed for 600 mA at 10 °C in a Gene Phor Electrophoresis Unit (Pharmacia Biotech). One hundred chromosomes from healthy individuals were analyzed as control.

3. Results

We found the first patient with MDC1C (patient 1) in the oriental countries. *Fukutin* mutations were found in 87% of the patients examined, and two MEB (patients 2 and 3) and one WWS (patient 4) were genetically confirmed. Four patients had no mutation in the known genes associated with glycosylation defects of α-DG (Table 1).

3.1. Clinical features of the patients

Patient 1 (MDC1C) was a Japanese girl and first admitted to a hospital at 12-months old. She was the first child of nonconsanguineous healthy Japanese parents. From at birth, left eye strabismus was seen, and the floppiness and delayed motor milestones became apparent in growing. She was able to sit at 7 months, but unable to crawl or stand up at 12 months of age. She spoke some meaningful words, and no mental retardation was observed. Serum creatine kinase (CK) level was 6429 IU/l. Muscle biopsy was performed at 12 months of age and showed dystrophic changes with marked variation in fiber size, active necrotic and regenerating process, and dense interstitial fibrosis (Fig. 1A). She was diagnosed to have FCMD. At 6-years-old, generalized muscular atrophy was marked, but she could move by herself using her wheelchair. Facial and calf muscles were mildly hypertrophic and high-arched palate was seen. Tongue hypertrophy was not apparent. Cardiac dysfunction was not detected from the chest radiograph or electrocardiogram. Joint contractures

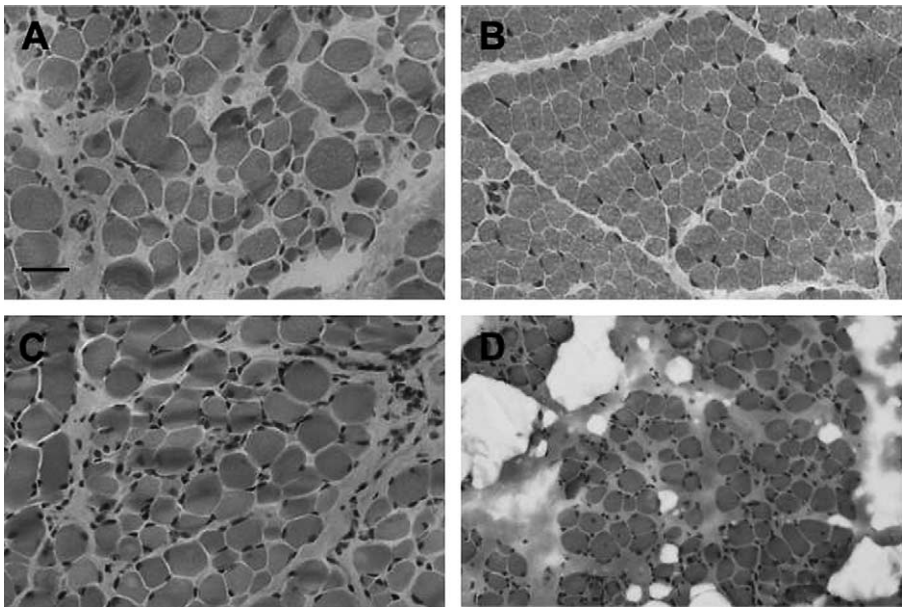

Fig. 1. Hematoxylin and eosin staining of skeletal muscles. Patient 1 (MDC1C; A), patient 3 (MEB; C), and patient 4 (WWS; D) show severe dystrophic changes with marked variation in fiber size, necrotic and regenerating fibers, and dense fibrosis in endomysium, whereas the pathological changes in patient 2 (MEB; B) is very mild, showing only mild caliber changes of muscle fibers. Bar = 50 μm.

were seen in elbows, knees, and ankles. Brain magnetic resonance image (MRI) at age 5 years showed some cerebellar cysts and disorganized formation of cerebellar folia, but no structural abnormality was found in the cerebrum and the brain stem (Fig. 2A and B). Her intelligence was normal at age of 6.

Patient 2 (MEB-1) was a child of nonconsanguineous Japanese parents, and he had healthy sister and brother. He was delivered after uneventful pregnancy, and he was noted to be floppy at 4 months. At 6 months he was not able to control his head, and serum CK level was elevated to 6900 IU/l. Computed tomography of the brain showed

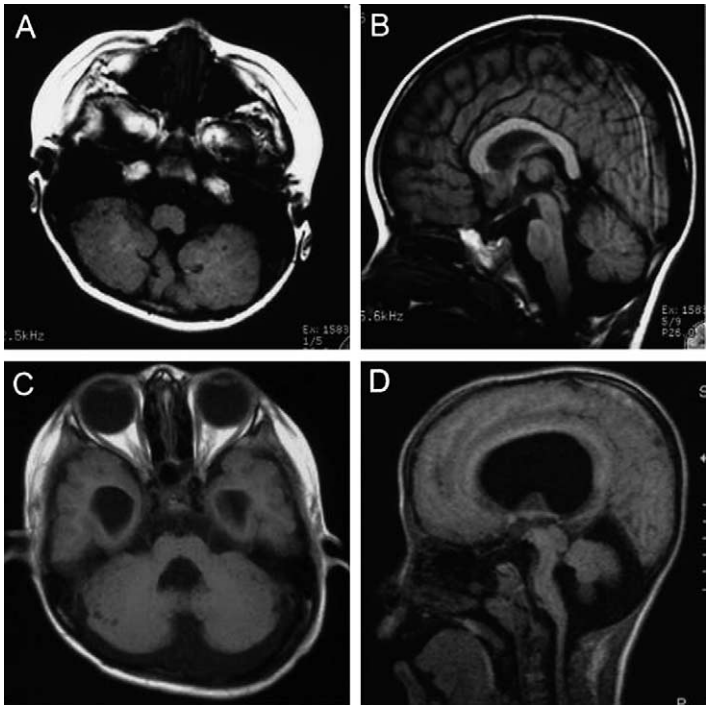

Fig. 2. Brain MRI of patient 1 (A and B) and patient 2 (C and D). T1 weighed images of patient 1 show multiple cerebellar cysts and dysmorphic cortical structures in bilateral cerebellar hemisphere (A). No abnormal findings are seen in cerebrum and brain stem (B). T1 weighed images of patient 2 show multiple cerebellar cysts (C), atrophic brain stem and markedly dilated lateral and third ventricles (D). Typical type II lissencephaly was seen in cerebrum (data not shown).

Table 2
Clinical summary of the four patients with MDC1C, MEB, and WWS

Patient no.	1 (MDC1C)	2 (MEB-1)	3 (MEB-2)[a]	4 (WWS)[b]
Age at biopsy	1 y/o	6 mo/o	1 y/o	3 y/o
Sex	F	M	F	M
Gene mutation	*FKRP* 266C>G, 1169–1170delGC	*POMGnT1* 1106insT	*POMGnT1* 900G>A, 1077insG	*POMT1* 1260_1262del CCT
Mental retardation	–, DQ 110	+	+	+
Speech	Sentences	No words	No words	No words
Hydrocephalus	–	–	–	+
Type II lissencephaly	–	+	+	+
Cerebellar cysts	+	+	+	+
Brain stem hypoplasia	–	+	+	+
Eye symptoms	Strabismus	Cataract, retinal dysplasia	Myopia, retinal dysplasia	Corneal clouding
Maximum motor function	Sitting, move on buttocks	Sitting, roll over	Bed ridden	Bed ridden
CK (IU/l)	6429	6900	8019	600–31,000
Muscle pathology				
Necrosis	Occasional	Few	Occasional	Occasional
Fibrosis	Marked	Very mild	Marked	Marked

[a] Previously reported as SI [12].
[b] Previously reported [13].

dilated lateral ventricles and diffuse periventricular lucency of white matter. Brain MRI at age 10 years showed markedly dilated ventricles, type II lissencephaly, cerebellar cysts, and flat brain stem (Fig. 2C and D). Examination of eyes showed bilateral retinal degeneration. His eye problems had been progressive, and at age 10 years he received operation for bilateral cataracts. Bilateral optic nerve atrophy and detachment of retina of right eye were also found. A muscle biopsy taken at 6 months of age showed only mild dystrophic changes with a few necrotic and regenerating fibers. No marked fibrous tissue involvement was seen (Fig. 1B). He was suggested to have CMD. He was able to turn over at 22 months, but further motor development was not obtained. At age 11, he was wheel chair bound, and could move by rolling over on the floor. Contractures were seen in bilateral knee and ankle joints. In comparison with his motor function, mental retardation was severe. He could not speak any meaningful words, and could only express his pleasure or sad feelings by facial expression in response to his parents' voice.

The clinical and genetic description of patient 3 (MEB-2) was previously reported (patient SI [12]), and they are summarized in Table 2.

We recently reported the clinical features and the result of genetic analysis of Patient 4 (WWS) [13], and they are summarized in Table 2.

3.2. Patients with unknown cause

No mutation was identified in four of 62 patients who were clinically diagnosed to have MEB or WWS (Table 1). All four patients showed severe mental retardation, hypotonia from early infancy, and eye involvements. Brain MRI displayed type II lissencephaly, enlarged lateral ventricles, and hypoplastic brainstem and cerebellum. In the skeletal muscles, three patients who were clinically diagnosed as WWS showed severe dystrophic changes with marked fibrous tissue involvement. However, one patient who was clinically diagnosed as MEB showed only mild myopathic changes in his muscle.

3.3. Genetic analyses for fukutin, FKRP, POMGnT1, POMT1, and LARGE

Among 62 patients served for genetic analyses, 54 patients (87%) had 3-kb retrotransposal insertion homozygously or heterozygously (Table 3). Twelve patients had this insertion heterozygously, and we performed sequence analysis of all exons and their flanking region of *fukutin*. We identified point mutations in *fukutin* in seven patients, including one novel mutation (Table 3), but no mutation was found in the remaining five patients with 3-kb insertion in one allele.

We performed mutation screening in *FKRP*, *POMGnT1*, *POMT1*, and *LARGE* on eight patients who had no retrotransposal insertion in *fukutin*. We found a patient (patient 1) with a novel compound heterozygous mutation in *FKRP* (266C>G transversion which generate P89R, and 1169_1170 del GC which causes premature termination)

Table 3
Results of mutation analysis of *fukutin*

3-kb insertion	*Fukutin* mutation (amino acid change)	No. of patients
Homozygote	42	
Heterozygote	12	
	250C>T (R47X)	3
	626A>G (H172R)	1
	859T>G (C250G)	1
	1025G>A (W305X)[a]	1
	1169T>A (L353X)	1

[a] A novel mutation.

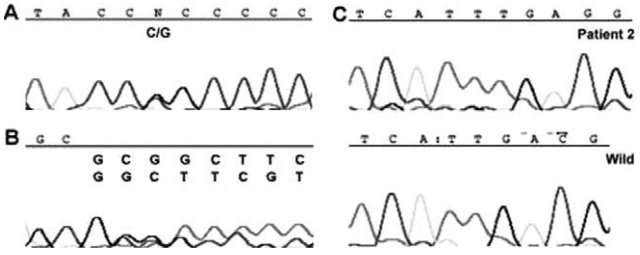

Fig. 3. Electropherograms of the sequence analysis of *FKRP* and *POMGnT1*. A novel compound heterozygous mutation in *FKRP* was identified in patient 1 with heterozygous 266C>G (A) and 1169_1170 del GC (B). Patient 2 shows homozygous 1106 ins T in *POMGnT1* (C, Patient 2).

(Fig. 3A and B). The SSCP analysis of *FKRP* showed that only this patient but not 50 healthy individuals had a mobility shift (data not shown). Two patients had mutations in *POMGnT1*. A homozygous 1106 ins T in exon 11, which generates D338fs (Fig. 3C) was seen in patient 2. Mutations of *POMGnT1* in patient 3 and *POMT1* in patient 4 were reported previously [12,13]. The remaining four patients had no mutation in the all genes examined including *LARGE*.

3.4. Immunostaining and immunoblotting analyses

In all muscles from FCMD patients, marked reduced membrane staining was seen using α-DG (VIA4-1) antibody, which recognize glycosylated form of α-DG, while β-DG immunoreaction was well preserved as previously described [1]. Immunostaining with anti-GT20ADG antibody that recognize the core region of α-DG showed positive membrane staining [9]. All eight CMD patients other than FCMD showed similar immunoreactions to those of FCMD muscles, including patient 1 with FKRP mutations (Fig. 4). Immunoreaction of laminin α2 chain in patient 1 show very mild reduction (Fig.4C). Reduced staining of laminin α2 chain was marked in FCMD and WWS patients [13], however, in the two MEB patients, nearly normal immunoreaction was seen (Fig. 4C) [12]. On immunoblotting analysis, barely detectable level of glycosylated form of α-DG was seen using VIA4-1 antibody in all the patients (Fig. 5A), whereas the GT20ADG antibody recognized more migrating bands in ~90 kDa in all patients we examined independent on the causative gene (Fig. 5B). Laminin overlay assay revealed no detectable binding

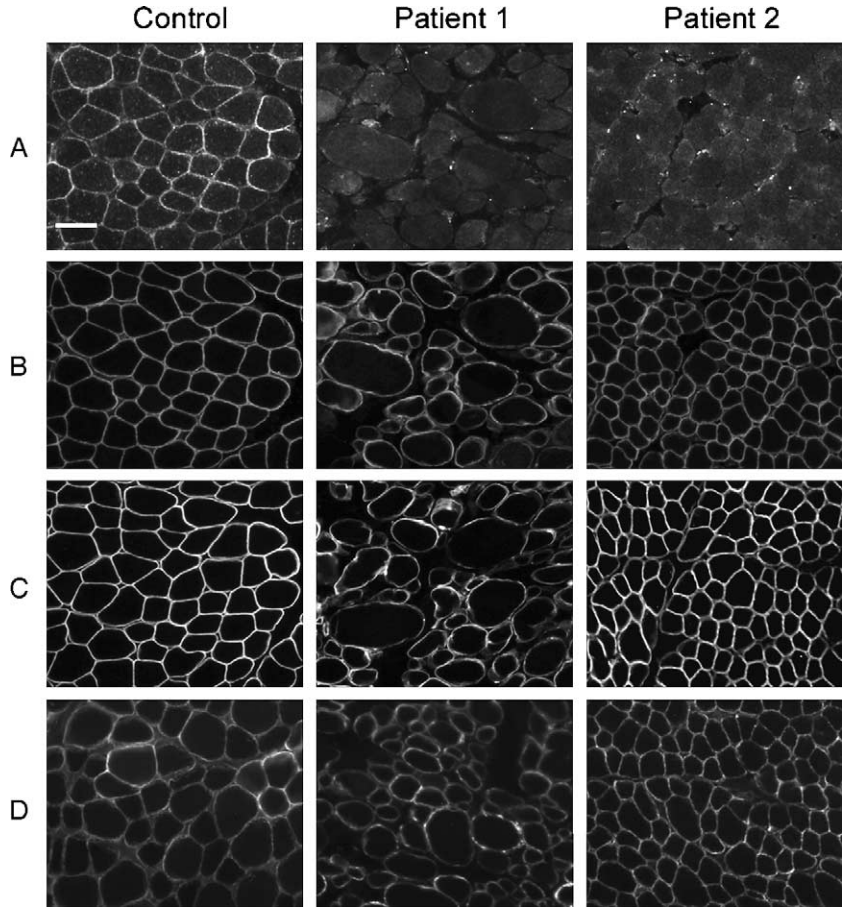

Fig. 4. Immunohistochemical analysis. Skeletal muscles from control, patient 1 (MDC1C), and patient 2 (MEB) are immunostained with antibodies against α-DG (VIA4-1; A), β-DG (B), laminin α2 chain (C), and core antibody against α-DG (GT20ADG; D). Immunoreaction for VIA4-1 is markedly reduced in patient 1 and patient 2, while β-DG is normally expressed in the sarcolemma. α-DG core protein is preserved by GT20ADG in both patients. Bar=50 μm.

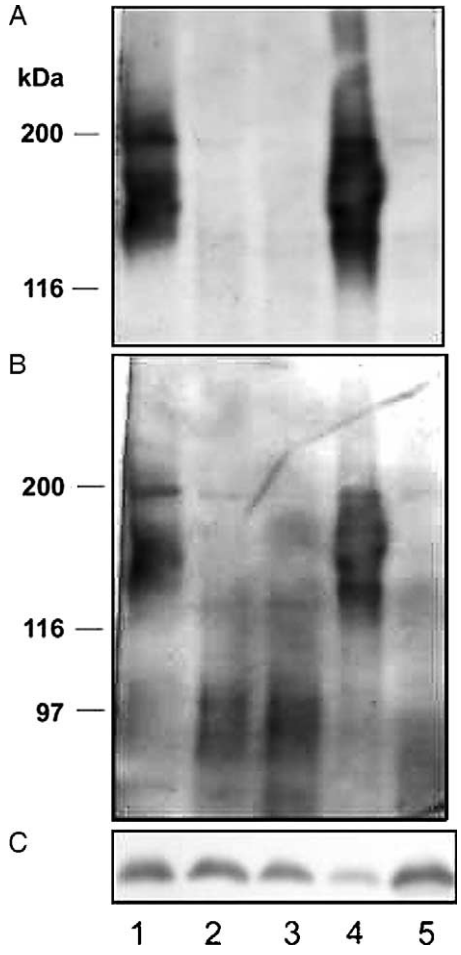

Fig. 5. Immunoblotting analysis. Skeletal muscle homogenates from control (lane 1), patient 1 (MDC1C; lane 2), FCMD (lane 3), control (lane 4), and patient 2 (MEB; lane 5) were blotted with VIA4-1 (A), GT20ADG (B), and β-DG (C) antibodies. FCMD, patient 1, and patient 2 showed barely detectable glycosylated form of α-DG by VIA4-1, whereas decreased sized bands (∼90 kDa) were detected by GT20ADG.

product in the muscles with glycosylation defects of α-DG (data not shown).

4. Discussion

In this study, 86% of the patients with glycosylation defects of α-DG were genetically confirmed as FCMD, reflecting the most common form of CMD in Japan. The FCMD patients show severe muscular dystrophy with central nervous system involvements, but relative broad spectrum of clinical symptoms is known. A small number of FCMD patients can walk at some point and speak meaningful sentences, while some patients show severely affected brain and eye malformations mimicking to Walker-Warburg phenotypes [11,14]. One FCMD patient in our series with a compound heterozygous mutation (a 3-kb insertion and a Arg47stop) showed severe muscular dystrophy, hydrocephalus and retinal degeneration, and clinically diagnosed as MEB/WWS. Despite of the different clinical severity, the positive muscle fibers of glycosylated form of α-DG (VIA4-1) was very few in all FCMD patients. The molecular mass of α-DG detected by the antibody for core region (GT20ADG) were also equally reduced to ∼90 kDa in all FCMD patients examined (data not shown). These results imply additional factor(s) to determine the clinical severity of FCMD.

Here, we report the first patient with MDC1C in the oriental countries. Patients with *FKRP* mutations are known to show wide variety of clinical spectrums from LGMD2I, MDC1C to severe MEB/WWS phenotypes. Recently genotype–phenotype correlations in *FKRP* mutations were reported [15]. Patients with MDC1C phenotype have a compound heterozygous mutation between a null and a missense mutation or carried two missense mutations, while common Leu276Ileu mutation was constitutionally seen in LGMD2I. Our patient had novel mutations in a combination of frameshift and missense mutations. Clinically, the patient showed severe muscle weakness from early infancy, marked elevation of serum CK level, calf hypertrophy, and normal intelligence; and those are consistent with MDC1C. Further, the structural abnormality in the cerebellum was seen on brain MRI including disorganized folia and multiple cysts, those are commonly observed in FCMD/MEB. Unlike the other forms of CMD with glycosylation defects of α-DG, central nervous system involvements are rare in the patients with *FKRP* mutations [4,5]. Only a few patients with mental retardation and cerebellar cysts were reported from Turkey and Tunisia [16,17]. More recently, two patients with MEB and WWS phenotypes caused by *FKRP* mutations were reported [18]. Although the brain involvement in MDC1C is quite rare, these reports suggest the possibility that FKRP may play some roles in normal development of the brain, especially in the cerebellum.

Skeletal muscles from patients with *FKRP* mutations show variable levels of reduction of α-DG, from nearly normal in LGMD2I to almost absent in MDC1C by using anti-α-DG (VIA4-1) antibody [15]. Our MDC1C patient displayed almost absent glycosylated form of α-DG recognized by VIA4-1, which is similar to the other severe forms of CMD with glycosylation defects of α-DG. Further, we found the preserved core peptide of α-DG in the skeletal muscle. Surprisingly, molecular mass of α-DG recognized by the core antibody was quite similar to the other related diseases including FCMD, MEB, and WWS, although brain involvement was limited. From this result, the functions of FKRP on the glycosylation process of α-DG seems to be similar in the skeletal muscle to the other related gene products for glycosylation defects of α-DG.

The broader clinical spectrum of MEB was reported recently, though MEB pedigree in Finland shows uniform clinical features [12,19]. Two genetically confirmed MEB patients identified in this study (patients 2 and 3) showed similar brain and eye involvements, but interestingly, histological findings of skeletal muscles were quite different. Patient 2 showed only mild dystrophic changes,

while patient 3 showed active necrotic and regenerating process with severe endomysial fibrosis. No difference was seen in the expression patterns and the molecular mass of α-DG detected by the core antibody. Both patients showed nearly normal expression of laminin α2 chain around muscle fibers. The homozygous insertion mutation identified in patient 2 was located in the catalytic domain, while the mutations in patient 3 was a missense mutation in the stem domain and one base pair insertion in the catalytic domain. Previous report showed that all mutant POMGnT1s uniformly lost their enzyme activities [20]. These results imply that additional factor(s) other than enzyme activity of POMGnT1 may play a role in determining disease severity in both brain and skeletal muscle.

Genetic analysis revealed that no mutation was identified in the four patients in our series, which strongly suggest that there still remain other related genes for glycosylation process of α-DG. Careful observation of clinical and pathological findings should help to clarify the precise pathomechaisms of CMD.

Acknowledgements

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