severe weakness and absence of early contractures as seen in our patient and his brother.

Indeed the presence of RBs in RBM, and the retrospective identification of RBs in RSS patient reported here and SPM patient (unpublished data) suggests that *FHL1* is the causative gene for a variety of clinical disorders with RBs as the common diagnostic pathological finding. On the basis of our results, *FHL1* can be one of the causative genes for RSS.

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Mutational Analysis of Fukutin Gene in Dilated Cardiomyopathy and Hypertrophic Cardiomyopathy

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Background Mutations in *FKTN* encoding for fukutin cause Fukuyama-type congenital muscular dystrophy characterized by severe muscle wasting and hypotonia with mental retardation. Fukuyama-type congenital muscular dystrophy is a recessive genetic trait. *FKTN* mutations in patients with dilated cardiomyopathy (DCM) have been investigated by our research group. The patients showed hyper-CKemia with mild or no muscle weakness and without mental retardation, suggesting that the clinical spectrum of *FKTN* mutations are wider than previously thought. The current study was designed to further explore the association of *FKTN* mutations with DCM or hypertrophic cardiomyopathy (HCM).

Methods and Results A total of 172 patients with DCM, 144 patients with familial HCM and 384 control individuals were analyzed for *FKTN* mutations. There was a DCM patient who was a compound heterozygote of a 3-kb insertion mutation and a missense mutation Cys101Phe. The patient showed hyper-CKemia with mild muscle involvement and no brain involvement. In contrast, 2 other DCM patients and 3 controls were heterozygous for the insertion mutation and normal allele, showing that the heterozygous insertion mutation itself was not associated with DCM. No mutation was found in the HCM patients.

Conclusions These observations indicated that the compound heterozygous *FKTN* mutation was a rare cause of DCM. Hyper-CKemia might be indicative of *FKTN* mutation in DCM.

Key Words: Cardiomyopathy; Genes; Genetics; Muscles

Idiopathic cardiomyopathy (ICM), which is mainly classified into 2 clinical phenotypes; hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM), is a primary heart muscle disorder caused by functional abnormalities in the cardiomyocytes and a major cause of sudden cardiac death and progressive heart failure.[1] Although the etiology of ICM has not been completely elucidated, recent molecular genetic studies have shown that ICM can be caused by a variety of genetic abnormalities.[1] Inheritance of familial HCM is usually autosomal dominant, whereas that of familial DCM is autosomal dominant, autosomal recessive, or X-linked recessive, ie, various type of disease inheritance can be found in DCM cases.[2,3] It also should be noted that causative gene mutations could be found not only in familial cases but also in sporadic cases, indicating that the absence of family history cannot exclude a possibility of causative gene mutation in ICM cases.[3] In addition, mutations in muscular dystrophy-causing genes might also lead to ICM phenotype, as exemplified that titin/connectin gene (*TTN*) mutations were found in patients with HCM,[4] DCM[5] or tibial musclular dystrophy and limb-girdle type muscular dystrophy (LGMD)[6] and that Tcap gene (*TCAP*) mutations were found in HCM and DCM,[7] as well as in LGMD.[8] These observations indicate that there is an etiological overlap between ICM, and the skeletal muscle disorders.[9]

Mutations in *FKTN* encoding for fukutin cause Fukuyama-type congenital muscular dystrophy (FCMD; MIM253800), the second most common muscular dystrophy in Japan after Duchenne muscular dystrophy. FCMD is an autosomal recessive disease manifested with severe muscle wasting and mental retardation.[10,11] The majority of the FCMD patients were homozygous for a 3-kb insertion in the 3' non-coding region of *FKTN*, whereas a small population of FCMD patients were compound heterozygotes of the 3-kb insertion and a missense mutation.[12,13] In addition, we recently identified compound heterozygotes of the insertion and a missense mutation in 2 sibling cases and 2 sporadic cases of DCM, who manifested with minimal muscle weakness and elevated serum creatine kinase (CK) concentration, hyper-CKemia, but not mental retardation.[14] However, it remains unknown whether *FKTN* mutation can be associated with ICM not accompanied by signs of muscular dystrophy and in which type of ICM patients who should be examined for *FKTN* mutations as a disease-causing gene.

In the present study, we searched for *FKTN* mutations in a large panel of patients with DCM or HCM. We found a compound heterozygote of *FKTN* mutations in 1 out of 172 DCM patients, who also had mild muscular dystrophy and hyper-CKemia.

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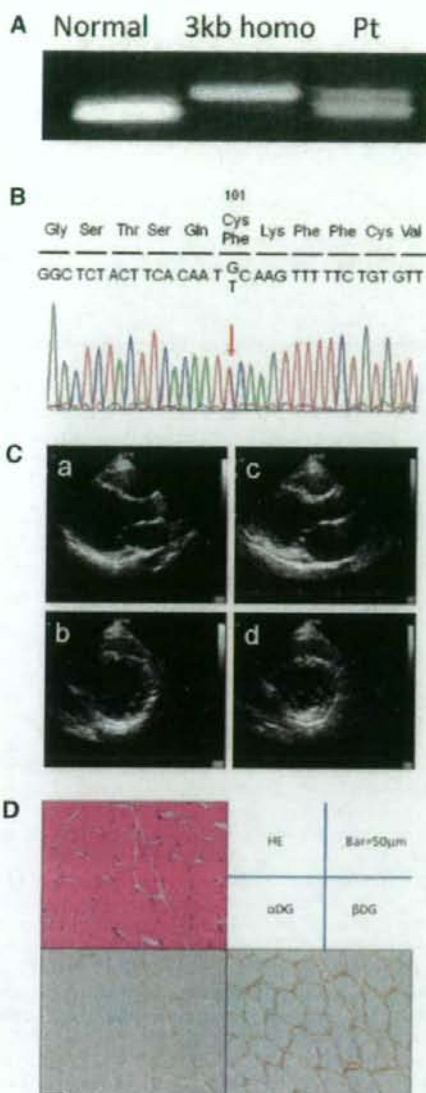

Fig 1. Gene and histochemical analyses of the dilated cardiomyopathy (DCM) patient carrying the *FKTN* mutation. (A) Detection of 3-kb insertion. Left, normal individual without mutation; middle, Fukuyama-type congenital muscular dystrophy patient carrying homozygous 3-kb insertion; right, the DCM patient with *FKTN* mutation. The patient showed both normal and insertion bands. (B) Direct sequencing data from the DCM patient. Polymerase chain reaction products containing exon 4 of *FKTN* gene from the patient were directly sequenced. Nucleotide sequences are shown along with predicted amino acid sequences. An arrowhead indicates the mutation resulting in TGC (Cys) to TTC (Phe) change. (C) Echocardiography of the patient. Endodiastolic (a, b) and endosystolic (c, d) data for sagital (a, c) and vertical (b, d) views showing left ventricular dilation. (D) Hematoxylin and eosin staining (HE) and immunohistochemical analysis. On HE, only mild variation in fiber size was found. Immunohistochemical analysis using a monoclonal antibody VIA4-1 that recognizes heavily-glycosylated form of α-dystroglycan (αDG), showed reduced sarcolemmal staining, whereas the staining of β-dystroglycan (βDG) using monoclonal antibody 43DAG1/8D5 showed no abnormality. Bar=50 μm.

Methods

Study Population

We studied 172 genetically unrelated Japanese patients with DCM and 144 patients with familial HCM. Among the DCM patents, family history was not found in 100 patients (sporadic cases), whereas apparent family history was found in 72 patients; 4 were probands of sibling cases (possible autosomal recessive cases) and 68 patients were probands of DCM families, in which disease was inherited as a autosomal dominant genetic trait. In addition, family history consistent with autosomal dominant inheritance was found in all HCM patients. The patients were diagnosed based on medical history, physical examination, 12-lead electrocardiogram (ECG), echocardiography, and other special tests if necessary. Diagnostic criteria for DCM and HCM were described previously.[7,15] These patients had been investigated for mutations in the known disease genes for ICM, such as sarcomere genes and Z-disc component genes,[3] and no disease-causing mutations were identified. All patients showed no sign of brain involvement, ie, typical FCMD cases were clinically excluded. Control subjects were 384 unrelated healthy Japanese individuals selected at random. After acquiring informed consent, blood samples were obtained from each participant. The research protocol was approved by the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University and that of National Institute of Neuroscience, National Center of Neurology and Psychiatry.

Mutational Analysis of FKTN in ICM

Genomic DNA extracted from peripheral blood was subjected to polymerase chain reaction (PCR). To detect the 3-kb insertion in *FKTN*, we carried out PCR in all participants using 2 primer sets as described previously.[14,16] Entire exons and their flanking regions of *FKTN* were directly sequenced on both strands by using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems Foster City, CA, USA) as reported previously.[14]

Immunohistochemical Analysis

Monoclonal anti-α-DG (VIA4-1, Upstate Biotechnology, Lake Placid, NY, USA) and monoclonal anti-β-DG (43DAG1/8D5, Novocastra Laboratories, Newcastle upon Tyne, UK) were used for immunostaining of biopsied skeletal muscle samples as described previously.[14]

Results

The 3-kb insertion mutation was searched in 172 DCM patients and 144 HCM patients. We found that 3 patients (all were sporadic DCM cases) carried the insertion mutation in the heterozygous state. This 3-kb insertion was not detected in other patients, but was identified in 3 out of 384 controls. We then sequenced all exons and adjacent introns of *FKTN* in the 3 sporadic DCM patients carrying the 3-kb insertion (Fig 1A) and found a missense mutation (c.302G>T, p.Cys101Phe) in one case (Fig 1B), suggesting that this patient was a compound heterozygote of *FKTN* mutations.

The patient was a 19-year-old female who manifested with exertional dyspnea and mild muscular weakness at neck and proximal extremities along with bilateral calf hypertrophy. She had shown hyper-CKemia (6,570 IU/L) without any muscle symptoms from the age of 17 years. Since then, she was followed up by physicians as a result of

the hyper-CKemia of unknown etiology. Diffuse left ventricular hypokinesis with left ventricular ejection fraction (LVEF) of 38% was observed at the age of 18 years, along with diffuse muscle atrophy and mild necrosis-regeneration process in biceps brachii muscle biopsy. She felt excertional dyspnea from the age of 18 years and when she was 19 years old, her ECG showed incomplete right bundle branch block, and her echocardiogram showed systolic dysfunction with ventricular dilatation (LVEF, 41%; left ventricular end-diastolic diameter, 53 mm; left ventricular end-systolic diameter, 43 mm; fractional shortening, 20%), whereas no ventricular hypertrophy was observed (inter ventricular septum, 6 mm; posterior wall, 7 mm). Biochemical analysis showed that she had hyper-CKemia (2,485 IU/L). Immunohistochemical analysis of biopsied muscle sample showed marked decrease of α-dystroglycan staining, whereas distribution and expression of β-dystroglycan was not changed (Fig 1B). This finding was consistent with *FKTN* mutations[14] albeit that no family history of DCM or muscle disease was evident with her. From these observations, she was finally diagnosed as LGMD manifested with mild DCM phenotype.

In addition, we sequenced the entire coding regions and adjacent introns of *FKTN* from 72 patients with familial DCM (4 consistent with recessive inheritance and 68 with dominant inheritance). The sequencing analyses showed 2 variations, 1 non-synonymous change in exon 5 (c.608G>A, p.Arg203Gln) and 1 synonymous change in exon 8 (c.1026C>A, p.Leu342Leu), in several patients. However, both variations were reported to be polymorphisms in the SNP database (rs34787999 and rs17309806, respectively), suggesting that these were polymorphisms not related with DCM.

Discussion

The 3-kb insertion into the 3'-untranslated region of the *FKTN*, which has been derived from a single ancestral founder and causes a significant reduction of *FKTN* mRNA, could cause FCMD in homozygous states or in compound heterozygous states with another point mutation[12,13] FCMD is one of the most severe congenital muscular dystrophy in combination with brain malformation, principally cerebral and cerebellar cortical dysplasia[10,11] In contrast to the severely affected skeletal muscle, cardiac muscle involvement is quite rare in FCMD patients. However, we recently showed that the compound heterozygous mutations could also be associated with DCM accompanied by minimal limb girdle muscle involvement and normal intelligence[14] These observations implied the wide phenotypic spectrum of the *FKTN* mutations. In the current study, we identified a patient carrying the 3-kb insertion and a missense mutation, who manifested with DCM and mild skeletal muscle phenotype. Clinical phenotype of the patient in this study was similar to those reported previously[14] further supporting that the compound heterozygous mutation was associated with DCM. Because we have not examined her parents for the *FKTN* mutations, we could not formally exclude a possibility that these 2 mutations were in *trans* and not in *cis*. However, if the mutations were in *cis*, this patient should have one normal allele and the other non-expressing allele due to the 3-kb insertion, which is in a similar situation as the heterozygote of the 3-kb mutation; the situation not causing any disease phenotypes as discussed below.

The 3-kb insertion was also found in 2 other sporadic DCM cases, but these patients did not carry any additional *FKTN* mutations nor did they show hyper-CKemia, indicating that heterozygote of the insertion mutation and normal allele did not manifest with cardiomyopathy or muscle diseases. In addition, we identified 3 heterozygous carriers of the 3-kb insertion in 384 Japanese controls (0.78%), and this carrier frequency was similar to those previously reported by 2 other groups (6 in 676; 0.89%[16] and 15 in 2,814; 0.53%[17]). In this study, we investigated familial HCM patients for *FKTN* mutations even though the disease was inherited as an autosomal dominant trait as in the most cases of familial DCM. Because mutations in the muscular dystrophy genes such as *TTN* and *TCAP* cause skeletal muscle disease as the autosomal recessive trait and cardiomyopathy (HCM or DCM) as the autosomal dominant trait, we had not been able to exclude a possibility of *FKTN* mutations in autosomal dominant cases. However, no *FKTN* mutation was found in the patients with familial HCM as in familial DCM, examined in this study. These observations suggest that *FKTN* mutations should be considered as a cause of DCM, albeit not a major cause, especially in the sporadic cases or sibling cases.

What was the characteristic feature of DCM caused by *FKTN* mutations? The patient carrying the causative *FKTN* mutations showed hyper-CKemia before manifesting with cardiomyopathy and skeletal muscle symptoms. All the patients carrying the compound heterozygous mutations in the previous study had elevated serum CK concentrations, although they showed no or minimal skeletal muscle phenotypes[14] The hyper-CKemia can also be found in the patients carrying *FKTN* mutations affected with FCMD[18] or LGMD[19,20] These observations are in good agreement with the association between the *FKTN* mutations and hyper-CKemia. In our cohort of DCM patients, we identified disease-causing mutations in 4 sporadic DCM patients who showed continuously hyper-CKemia. One was the patient carrying the *FKTN* mutations reported here, whereas the other 3 patients had abnormalities in the dystrophin gene (DMD) with a deletion of exon 3, exon 44, or exons 45–51. The DCM patients with DMD mutations showed elevated serum CK concentrations of approximately 500–1,000 IU/L. The finding was in part consistent with that DCM patients carrying DMD mutations were reported to show hyper-CKemia even though they had no or minimal symptoms of muscle involvement[21,22] These observations suggested that hyper-CKemia in patients with DCM might be an indicative sign of *FKTN* or *DMD* mutations.

In summary, we have investigated *FKTN* mutations in a large panel of patients with DCM or HCM and found that a sporadic DCM case with hyper-CKemia was a compound heterozygote of *FKTN* mutations.

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Nuclear changes in skeletal muscle extend to satellite cells in autosomal dominant Emery-Dreifuss muscular dystrophy/limb-girdle muscular dystrophy 1B

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Regeneration

ABSTRACT

Autosomal forms of Emery-Dreifuss muscular dystrophy (AD-/AR-EDMD) and limb-girdle muscular dystrophy type 1B (LGMD1B) are caused by mutations in the gene encoding A-type lamins (*LMNA*). A-type lamins are major components of nuclear lamina and known to have important roles in maintaining nuclear integrity. *LMNA* mutations are also suggested to cause reduced myogenic differentiation potentials, implying that satellite cell nuclei in AD-EDMD/LGMD1B are likewise affected. We examined nuclear changes of skeletal muscles including satellite cells from four patients with AD-EDMD/LGMD1B by light and electron microscopy. We found that 92.5 ± 5.0% of myonuclei had structural abnormalities, including shape irregularity and/or chromatin disorganization, and the presence of peri-/intranuclear vacuoles. Chromatin changes were also observed in 50% of the satellite cell nuclei. Increased number of Pax7-positive nuclei, but fewer number of MyoD-positive nuclei were seen on immunohistochemical analyses, suggesting functional alteration of satellite cells in addition to the nuclear morphological changes in AD-EDMD/LGMD1B.

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1. Introduction

Autosomal dominant and recessive forms of Emery-Dreifuss muscular dystrophy (AD- and AR-EDMD, respectively) are caused by the mutations in the *LMNA* gene that encodes A-type lamins, and are clinically characterized by muscle weakness with humeroperoneal distribution, early onset joint contractures and dilated cardiomyopathy with conduction defects [1]. *LMNA* mutations can also cause limb-girdle muscular dystrophy type 1B (LGMD1B), which has the clinical feature of proximal dominant muscle weakness with cardiac involvement [2].

Lamins form the nuclear lamina meshwork at the inner nuclear membrane and are suggested to have an important role in the maintenance of nuclear architecture. *LMNA* mutations or *Lmna* knockout are proven to cause nuclear dysmorphism and increase the fragility against mechanical stress in cultured cell analyses [3,4], supporting the notion that the nuclear lamina holds nuclear integrity. From this concept, myonuclei are presumably affected when *LMNA* is mutated since the skeletal muscle is frequently exposed to strong mechanical stress through the process of muscle contraction and relaxation. *LMNA* mutations were also shown to cause altered differentiation potential and kinetics of skeletal muscle cells by *in vitro* cell analyses [5].

In this study, we examined the morphological changes of myonuclei, and also nuclei of satellite cells in the skeletal muscles from four patients with genetically confirmed AD-EDMD/LGMD1B by using light and electron microscope. Further, we also counted the number of the myonuclei expressing Pax7 and MyoD to evaluate the differentiation potential of satellite cells in AD-EDMD/LGMD1B.

2. Patients and methods

2.1. Patients

All clinical materials used in this study were obtained for the diagnostic purposes with informed consent. Clinical information of four patients with AD-EDMD/LGMD1B is summarized in Table 1. In brief:

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Patient 1 is a 4-year and 5-month-old girl who was clinically suspected to have LGMD. She presented with lordosis and waddling gait from the age of 2 years. On physical examination, she showed proximal limb muscle weakness with wasting and calf hypertrophy. Serum creatine kinase (CK) level was elevated to 1408 IU/L (normal: 51–197 IU/L). Sequence analysis of *LMNA* revealed a heterozygous mutation of c.1357C > T (p.R453W) in exon 7.

Patient 2 is a 5-year and 8-month-old girl, who was noticed to have limited ankle flexion and elevation of serum CK level at the age of 2 years. On admission, she showed generalized muscle wasting, lordosis, and ankle/knee joint contractures. Serum CK levels were elevated from 900 to 1500 IU/L. The presence of a heterozygous mutation of c.746G > A (p.R249Q) in exon 4 of *LMNA* confirmed the diagnosis of EDMD.

Patient 3 is an 8-year and 1-month-old girl who had muscle weakness and delayed motor milestones. She walked without support at the age of 1 year and 4 months, but gradually lost independent ambulation at around 3 years of age. Scoliosis was noted on physical examination. Serum CK level was 351 IU/L. Echocardiography showed mildly decreased cardiac motility. Genetic analysis revealed a heterozygous missense mutation of c.875T > C (p.L292P) in exon 5 of *LMNA*.

Table 1
Clinical and genetic information of four patients with AD-EDMD/LGMD1B.

	Patient 1	Patient 2	Patient 3	Patient 4
Age/Sex	4 years/F	5 years/F	8 years/F	52 years/F
Age at onset	2 years	2 years	1 year 4 months	6 years
Skeletal muscle symptoms	Prox. limb weakness Lordosis Calf hypertrophy	Prox. limb weakness Lordosis	Prox. limb weakness Scoliosis Loss of ambulation	Prox. limb weakness Facial/neck weakness
Joint contracture	None	Ankles & knees	None	None
Cardiac symptoms	None	None	Wall motion decrease on echocardiography	Sick sinus syndrome + ventricular tachycardia → pacemaker insertion
Serum CK (IU/L)	1408	900–1500	351	117
LMNA mutation	p.R453W	p.R249Q	p.L292P	p.D511fs

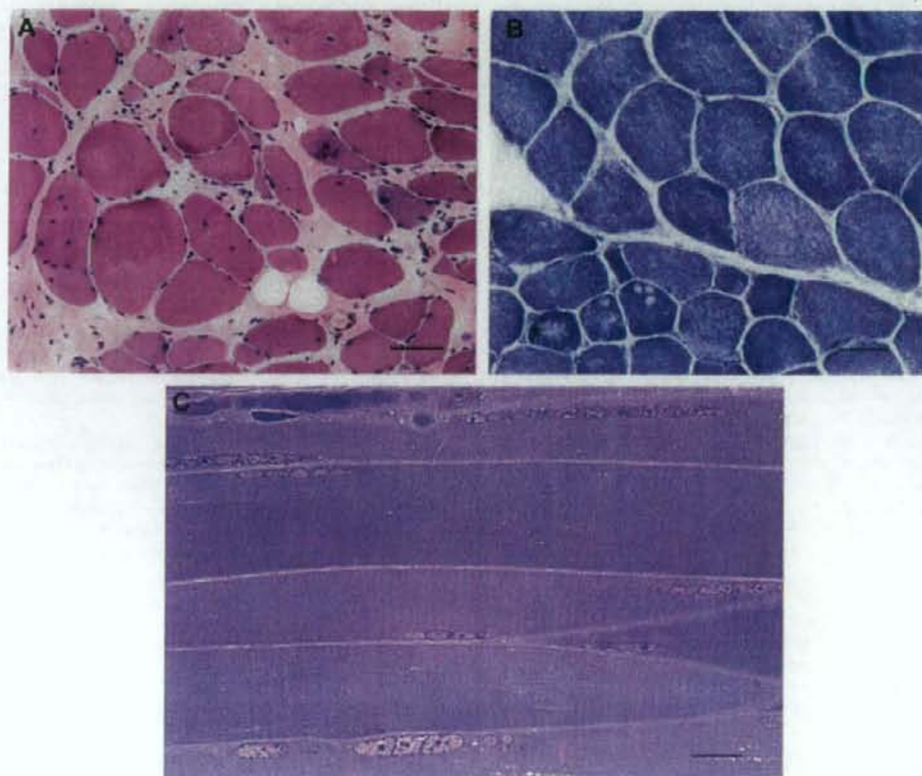

Fig. 1. Light microscopic observation of skeletal muscle. (A) H&E staining in patient 3 shows that the number of nuclei within one fiber is markedly increased. (B) NADH staining in patient 4 shows well-preserved myofibril organization in many fibers, whereas a few fibers have core/mini core-like structure. (C) Semi-thin section in patient 1 stained by toluidine blue contains nuclear chains of variable size in subsarcolemmal regions. Bar = 100 μm (A and B), 50 μm (C).

Patient 4 is a 52-year-old female, whose family history was suggestive of an autosomal dominant inheritance because her mother and her mother's sister had muscle weakness and cardiac enlargement/arrhythmia. She had been a slow runner since childhood and waddling gait was noticed at the age of 6 years. She felt difficulty in climbing stairs from 35 years of age. A diagnosis of sick sinus syndrome with ventricular tachycardia was given at 46 years whereby a pacemaker was inserted. Serum CK level was 117 IU/L. Mutation analysis showed a novel heterozygous frame-shift mutation of c.1527-1529TAC < AA (p.D511fs) in exon 9 of *LMNA*.

For comparison of histochemical and immunohistochemical analyses, muscle specimen from age-matched patients with Duchenne/Becker muscular dystrophy (DMD/BMD) ($n = 4$) and normal controls ($n = 4$) were also analyzed.

2.2. Histochemical analyses

Biopsied muscle specimens were flash-frozen in isopentane chilled with liquid nitrogen. Serial 10 μm-thick frozen sections were analyzed with a set of histochemical staining including hematoxylin and eosin (H&E), modified Gomori trichrome, nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome *c* oxidase and myosin ATPase. On ATPase staining, the ratio of type 2C fibers was considered in the patients with AD-EDMD/LGMD1B, and age-matched Duchenne/Becker muscular dystrophy (DMD/BMD) and normal controls.

2.3. Immunohistochemical analysis

Serial 6 μm-thick frozen muscle sections were fixed with acetone. Immunostaining was performed using standard method. To exclude other diagnosable muscular dystrophies, we used antibodies against dystrophin (Dys1, Dys2, and Dys3 from Novocastra Laboratories, Newcastle upon Tyne, UK), α-dystroglycan (Upstate Biotechnology, Lake Placid, NY), β-dystroglycan (Novocastra Laboratories), dysferlin (Novocastra Laboratories), α-, β-, γ-, δ-sarcoglycan (Novocastra Laboratories), caveolin-3 (Novocastra Laboratories), laminin-α2 chain (Chemicon, Temecula, CA), collagen VI (Abcam, Cambridge, UK), emerin (Novocastra Laboratories), and lamin A/C (Abcam, Tokyo, Japan).

For the satellite cell analyses, primary antibodies against Pax7 (Developmental Studies Hybridoma Bank, The University of Iowa, IA), MyoD (Santa Cruz Biotechnology, CA), and laminin α2 (Chemicon) were used. The stained sections were mounted together with DAPI for nuclear localization, and examined under immunofluorescence microscope (Carl Zeiss). In this study, immunopositive nuclei for Pax7 or MyoD located beneath the laminin α2-positive basal lamina are defined as Pax7-positive or MyoD-positive. From each specimen, 200–300 nuclei were recruited for analyses. The number of Pax7-positive or MyoD-positive nuclei was counted and compared with the total number of myonuclei to obtain the ratio of Pax7 and MyoD-positive nuclei per 100 myonuclei.

To detect DNA fragmentation, the TUNEL method was performed on frozen muscle specimens by using in situ apoptosis detection kit (Takara, Shiga, Japan) according to the manufacturer's instruction.

2.4. Electron microscopic observation

Muscle specimens were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. After shaking with a mixture of 4% osmium tetroxide, 1.5% lanthanum nitrate, and 0.2 M s-collidine for 2–3 h, samples were embedded in epoxy resin. Semi-thin sections (1 μm-thickness) were stained with toluidine blue. Ultrathin sections of 50 nm thickness were stained with uranyl acetate and lead citrate, and then examined under H-600 transmission electron microscope (Hitachi, Japan) at 75 kV. We examined morphological changes of more than 100 myonuclei in each patient and 100 myonuclei in one DMD patient as a control. We also examined 20 satellite cell nuclei in each patient. Myonuclei in significantly degenerated myofibers were excluded from the count. We calculated the frequency of myonuclei showing markedly irregular membrane contours and nuclear chains. We also counted the number of nuclei having only thin heterochromatin (scanty heterochromatin) beneath the nuclear membrane. Mean percentage ± standard deviation (SD) of abnormal nuclei was calculated. To detect the euchromatin changes, intensities of photocopied euchromatin regions in randomly sampled 40 nuclei of each patient and a DMD control were measured by using Photoshop. We

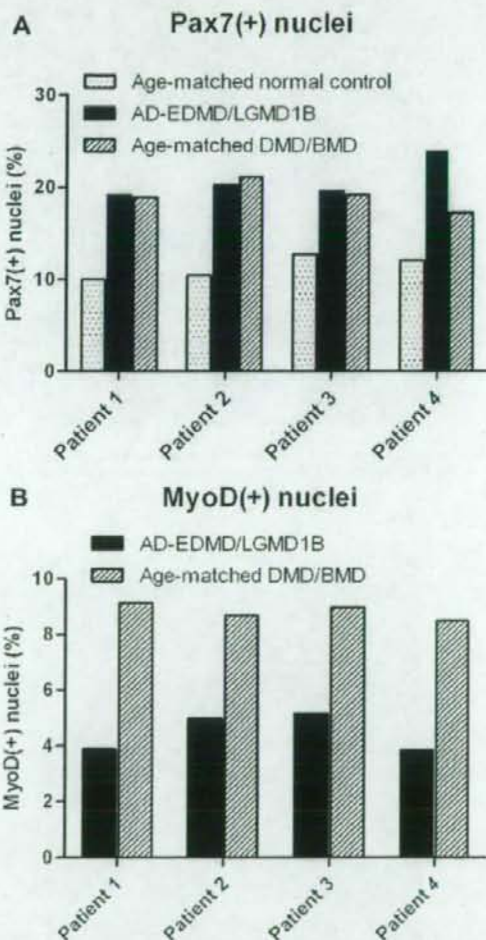

Fig. 2. Graphs showing the ratio of Pax7-positive (A) and MyoD-positive nuclei (B) in the patients with AD-EDMD/LGMD1B, and age-matched DMD/BMD and normal controls. (A) The ratio of Pax7-positive nuclei is increased both in AD-EDMD/LGMD1B and DMD/BMD patients compared with normal controls. (B) MyoD-positive nuclei in AD-EDMD/LGMD1B patients are much less than in DMD/BMD patients.

randomly chose three spots of 0.09 μm^2 in euchromatin region per each nucleus and calculate the mean intensity. To normalize the intensity of each photocopy, we used the mean intensity of three spots in A-band (excluded M-line region) of well-preserved sarcomere close to the nucleus as control. We defined as 'coarse euchromatin' when the nucleus has higher intensity (less electron dense) of euchromatin than mean plus SD of euchromatin intensity in DMD muscle. *P*-value was calculated using One-way ANOVA test.

3. Results

3.1. Histochemical analyses

Biopsied muscles from all four patients showed marked variation in fiber size, a few necrotic and regenerating fibers and mild to moderate endomysial fibrosis, which were compatible with the diagnosis of muscular dystrophy. The number of muscle fibers with internalized nuclei was increased. Interestingly, the number

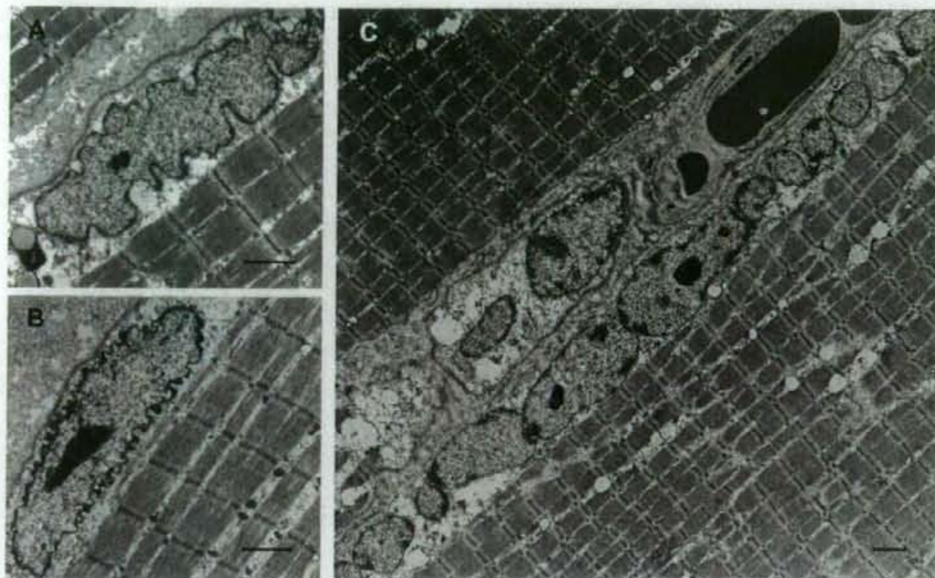

Fig. 3. Changes in the shape of myonucleus from patient 4 (A and B) and patient 1 (C). (A and B) Nuclear contours are irregular showing serpentine and sawtooth-like features. (C) A nuclear chain is identified in subsarcolemmal region, which is corresponded to those in the semi-thin section of Fig. 1C. Bar = 1 μm.

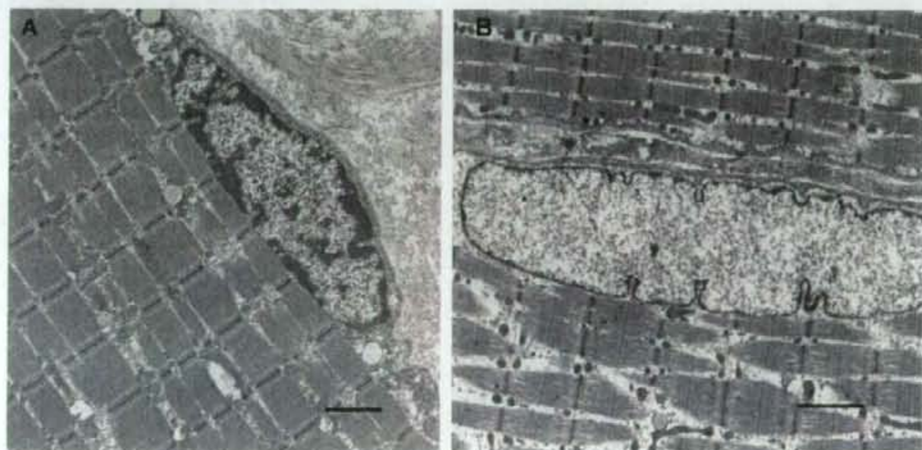

Fig. 4. Chromatin organization in myonuclei from one DMD control (A) and patient 4 (B). (A) Myonucleus of DMD has abundant heterochromatin and condensed euchromatin. (B) In myonucleus of the patient with AD-EDMD/LGMD1B, heterochromatin was scanty or nearly absent, and euchromatin reveals reduced density and coarse texture. Bar = 1 μm.

of nuclei contained in one muscle fiber was markedly increased, sometimes exceeding ten in number (Fig. 1A). The organization of intermyofibrillar networks was not disrupted in three of the patients, but in a restricted area of muscle in patient 4, myofibril disorganization was seen in a few fibers on NADH-TR showing core/minicore-like structures (Fig. 1B). On ATPase staining, type 2C fibers comprised 7.8 ± 1.5% in the patients with AD-EDMD/LGMD1B, and this value was slightly higher than in age-matched DMD/BMD controls (6.8 ± 0.9%). In age-matched normal controls, type 2C fibers were less than 1%.

Longitudinal semi-thin sections from epoxy resin-embedded blocks also showed nuclei in the center of myofibers, as well as subsarcolemmal regions. Some myonuclei had pale-colored nucleoplasm making nucleoli more distinct. Notably, several smaller-sized nuclei were arranged in a row giving a feature of the 'nuclear chain' on longitudinal sections, and most of them were located in the subsarcolemmal regions (Fig. 1C).

3.2. Immunohistochemical analysis

The immunostaining of various antibodies as described above excluded all other diagnosable muscular dystrophies. In all four patients with AD-EDMD/LGMD1B and DMD patients examined, only few TUNEL-positive myonuclei were seen (data not shown). This result suggests that apoptotic nuclear change is rare in AD-EDMD/LGMD1B.

In satellite cell analyses, the ratio of Pax7-positive nuclei in the patients with AD-EDMD/LGMD1B, DMD/BMD patients, and normal controls were 20.7 ± 2.1%, 19.1 ± 1.6% and 11.3 ± 1.3%, respectively (Fig. 2A). The ratio of Pax7-positive nuclei in each patient with AD-EDMD/LGMD1B were similar to those in the age-matched DMD/BMD controls except for patient 4 who showed more increased number of Pax7-positive nuclei. In contrast, the ratio of MyoD-positive nuclei was lower in AD-EDMD/LGMD1B (4.5 ± 0.7%) than in DMD/BMD (8.8±0.3%) (Fig. 2B).

3.3. Electron microscopic observation

Abnormalities in the nuclear morphology were the major findings on electron microscope in all four patients with AD-EDMD/LGMD1B, and which were virtually absent in the DMD patient examined. We have focused on the changes in nuclear shape and chromatin organization, and the presence of peri- and intranuclear vacuoles.

The shape of myonuclei was altered in all four patients with AD-EDMD/LGMD1B; 17.3 ± 11.1% of myonuclei displayed markedly irregular membrane contours, such as serpentine or sawtooth-like features (Fig. 3A and B). In addition, nuclear chains corresponding to those seen in semi-thin sections were identified in subsarcolemmal regions, accounting for 18.5 ± 6.4% of the myonuclei (Fig. 3C). In contrast, almost all myonuclei in the DMD patient had smooth-contoured nuclear membranes albeit mild indentation.

Another nuclear abnormality noted was chromatin disorganization in the myonuclei. In the myonuclei of DMD patient, heterochromatin appears abundant in the nuclear periphery and euchromatin is well-condensed in the nuclear interior (Fig. 4A). On the other hand, 64.3 ± 18.5% of the myonuclei in AD-EDMD/LGMD1B had only thin layer of heterochromatin under the inner nuclear membrane (scanty heterochromatin), and some of them showed totally absent heterochromatin (Fig. 4B). In addition, euchromatin appeared coarse and had reduced electron density as compared with myonuclei in DMD (Fig. 4B). Mean euchromatin intensities in all four patients were significantly higher than DMD ($P < 0.001$) (Supplemental Fig.), and 75.0 ± 16.7% of nuclei had higher euchromatin intensity than mean + SD of DMD.

More importantly, chromatin organization of the satellite cell nuclei were also affected (Fig. 5). Although only twenty satellite cells were observed in each patient, nearly a half of them contained the nuclei with scanty heterochromatin, and less condensed and coarse euchromatin, features of which are quite similar to those seen in the myonuclei. In spite of chromatin disorganization, however, the nuclear shape was well preserved in the satellite cells.

Another finding associated with the myonuclei of the four patients is the presence of peri- and intranuclear vacuoles, which were observed in ~10% of the myonuclei. Most of these vacuoles were variable in size and contained various materials within, but the larger intranuclear vacuoles were mostly empty (Fig. 6). All the myonuclei with vacuoles were basically abnormal in shape and/or chromatin organization as shown in Fig. 6.

The perinuclear vacuoles were observed in close proximity to the myonuclei; for example, in the poles or in deeply indented por-

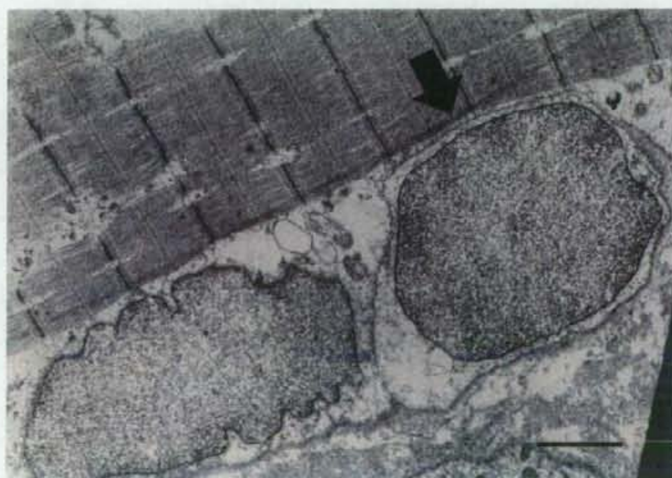

Fig. 5. Chromatin disorganization of satellite cell nuclei from patient 4. Arrow indicates the satellite cell having abnormal chromatin texture of nucleus, which is similar to that seen in myonucleus. Bar = 1 μm.

tion of the myonuclei (Fig. 6A and B). Several perinuclear vacuoles were almost attached to the myonuclei, so that the border of each membrane was indistinguishable (Fig. 6A). Specifically, the outer nuclear membrane of some with perinuclear vacuoles was partially separated from the inner membrane giving a visible gap between the two membranes (Fig. 6B).

Intranuclear vacuoles were observed in obviously degenerated myonuclei which have highly condensed chromatin structures (Fig. 6C and D). Of note, the larger intranuclear vacuoles occupied a significant portion of the intranuclear area leaving only a little space of compact chromatin, and these myonuclei were usually placed in the markedly degenerated myofibers (Fig. 6D). Nevertheless, general myofibrillar organization was relatively well preserved in most part of myofibers, except for only limited regions close to the altered myonuclei showing a Z-line streaming or myofibril derangement (Fig. 7).

Overall, 92.5 ± 5.0% of the myonuclei had abnormality in shape and/or chromatin organization. However, the proportion of abnormal myonuclei was similar from the youngest to the adult patient, with no correlation to the patients' age at biopsy, clinical severity or the location of mutations in *LMNA* (Fig. 8).

4. Discussion

We characterized the nuclear changes in the skeletal muscles of AD-EDMD/LGMD1B caused by *LMNA* mutations. The nuclear lamina is expected to function as a scaffold of the nucleus, thus nuclear fragility due to *LMNA* mutations is regarded as one of the patho-

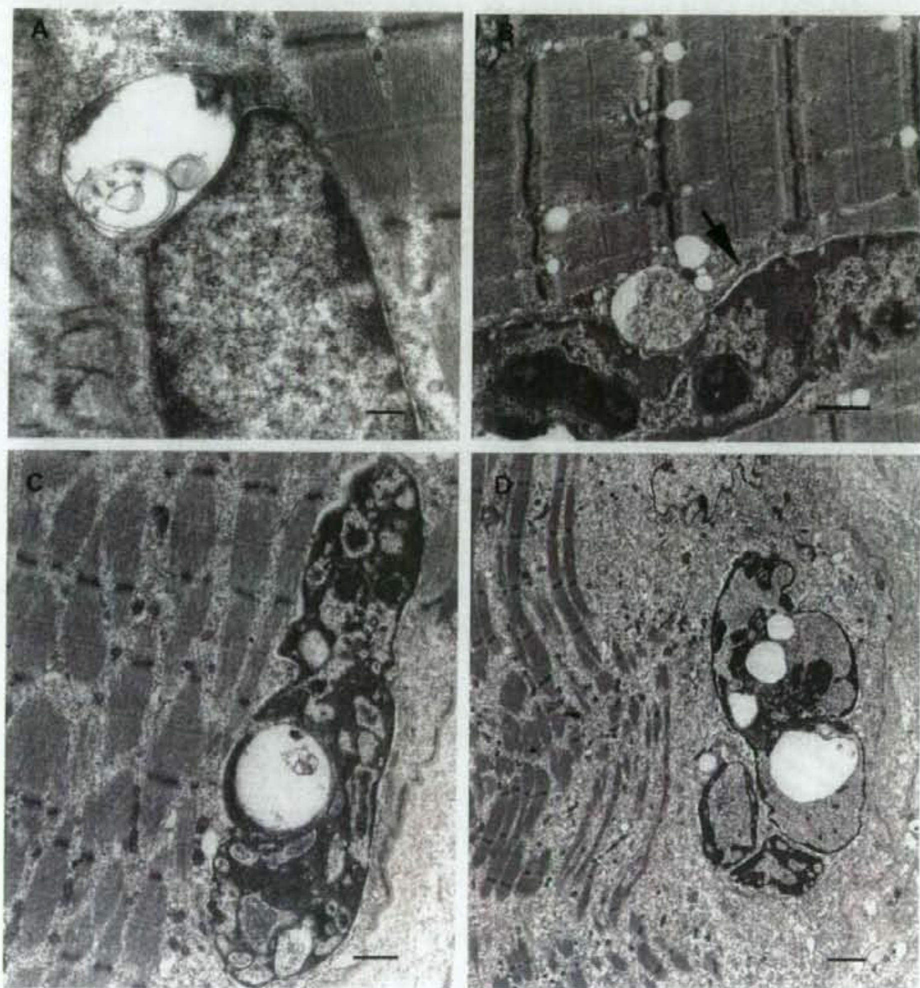

Fig. 6. Perinuclear and intranuclear vacuoles of myonuclei in patient 1 (A and B), patient 4 (C) and patient 3 (D). Vacuoles contain various materials (A, B and C), but larger intranuclear vacuoles are empty (D). (A) Nuclear membrane is difficult to be distinguished from the membrane of perinuclear vacuole. (B) Inner and outer nuclear membranes are partially separated from each other, as indicated by an arrow, in the myonucleus with perinuclear vacuoles. (C) Intranuclear vacuole is seen in quite degenerated myonucleus with highly condensed chromatin. (D) Intranuclear vacuoles occupy significant portion of intranuclear area leaving only small spaces for disorganized chromatin. Myofibrils surrounding the myonucleus were markedly degenerated. Bars = 0.2 μm (A), 0.5 μm (B and C), 1 μm (D).

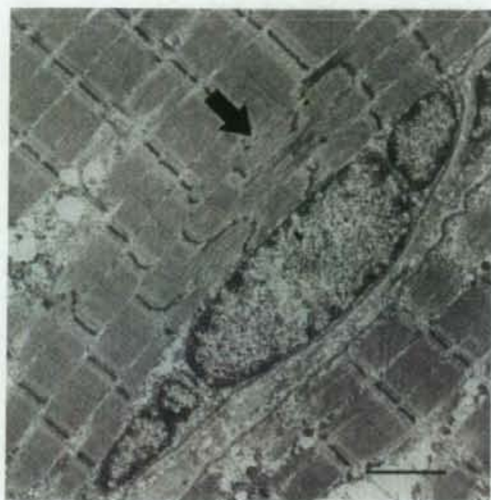

Fig. 7. In a few fibers in patient 4, myofibrils were disorganized in the region close to the altered myonucleus showing Z-line streaming as indicated by an arrow. Bar = 1 μm.

mechanism in laminopathy. In support of this concept, nuclear dysmorphism and vulnerability to mechanical stress were reported in cultured fibroblasts from *Lmna*-null mice [3,4] and the patients with *LMNA* mutations [6,7]. In this study, we demonstrated that the myonuclei of AD-EDMD/LGMD1B patients had markedly irregular nuclear membrane contours. Our findings are in agreement to previous reports which showed that the AD-EDMD/LGMD1B patients had irregularly shaped nuclei, such as convoluted, segmented and fragmented features [8–10]. These data with myonuclei provide additional evidence of nuclear fragility induced by *LMNA* mutations.

The presence of nuclear chain, which has not been previously described in AD-EDMD/LGMD1B, is worth mentioning, as it has been described almost as a pathognomonic feature of myotonic dystrophy [11], although nuclear chains can be seen in other neuromuscular disorders. The difference on these nuclear chains in AD-EDMD/LGMD1B is that they are found mostly in the subsarcolemmal area, while those in myotonic dystrophy were usually seen in the center of myofibers. Although the comparison is difficult because nuclear chains observed in myotonic dystrophy have not been well characterized by electron microscope, each disorder might have different mechanism forming nuclear chains. Similar nuclear chains were seen in the skeletal muscles of X-linked recessive EDMD (X-EDMD) under light microscope (data not shown). X-EDMD is caused by the mutations in the gene encoding emerin (*EMD*), which is another nuclear envelope protein [12] that directly associates to the nuclear lamina [13]. Based on these findings, nuclear chain formation, together with irregular nuclear shape, can be thought to result from the fragility of nuclear envelope.

Chromatin disorganization characterized by scanty heterochromatin and less condensed euchromatin, was also highlighted in our study. Previously, Sabatelli et al. have described the absence of heterochromatin and de-condensed euchromatin in about 10% of the myonuclei in AD-EDMD/LGMD1B [8]. Fidzianska and Hausmanowa-Petrusewicz also reported dark and dense heterochromatin in the myonuclei of AD-EDMD [9]. These chromatin changes observed in AD-EDMD/LGMD1B could correspond to the fact that nuclear lamins have an important role in the chromatin organization [14].

The presence of peri-/intranuclear vacuoles is a novel finding in this study, and is indeed a constant feature in all AD-EDMD patients we examined. We have previously reported similar perinuclear vacuoles in emerin-deficient mice [15], suggesting that these vacuoles can be one of the common characteristic pathological features in nuclear envelopathies including laminopathy and emerinopathy. Because perinuclear vacuoles were closely associated with nuclear membrane, and intranuclear vacuoles were found in quite distorted myonuclei having irregular shape and compact chromatin, we hypothesize that these could be secondary changes to the altered nuclear envelope. However, further experiment should be performed to characterize how these vacuoles are formed and how they contribute to the overall pathomechanism of laminopathy and emerinopathy.

Notably, we did not find any correlation of the degree of nuclear changes with the patients' age at biopsy, clinical severity and the location of mutations in the gene. Furthermore, even in the youngest patient the nuclear changes in shape and chromatin organization were already developed in a significant portion of the myonuclei as seen in Fig. 8, which can imply that the nuclear abnormality in patients with *LMNA* mutations occur at a much younger age than expected.

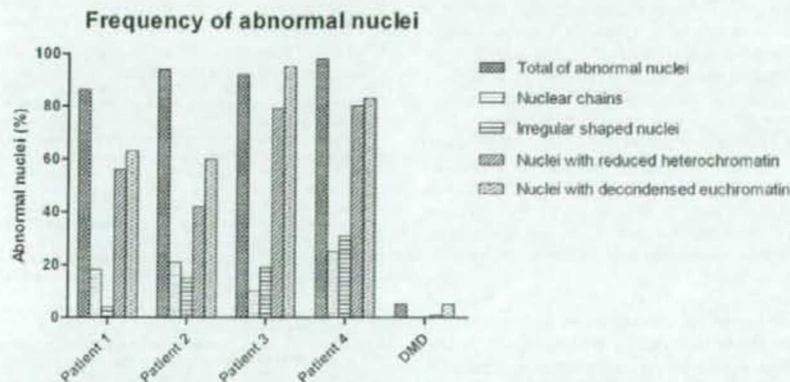

Fig. 8. The frequency of myonuclear abnormalities in four patients with AD-EDMD/LGMD1B and one DMD patient. Various kinds of nuclear changes are observed in high frequency in muscles from all four patients with AD-EDMD/LGMD1B. These changes are not correlated with the patients' age at biopsy, clinical severity and the location of mutations in the gene. Nuclear changes in a DMD muscle used as control are rare.

Recent reports suggested that decreased differentiation potential might be associated with muscular dystrophy caused by *LMNA* mutations. Favreau et al. reported that C2C12 myoblasts expressing the mutant (R453W) lamin A failed to form multinucleated fibers and were unable to express the myogenic transcriptional factor, myogenin, at proper timepoint during differentiation process [5]. Moreover, the mutant lamins induce the persistence of hyperphosphorylated Rb, preventing cell cycle arrest. Similarly, Bakay et al. revealed that the Rb-MyoD pathway of muscle regeneration is disrupted in nuclear envelope muscular dystrophies [16]. Furthermore, in $Lmna^{-/-}$ mouse, satellite cells showed delayed differentiation kinetics due to loss of A-type lamins [17]. Collectively these data raise the possibility that the skeletal muscle satellite cells in AD-EDMD/LGMD1B are likewise affected. As described in electron microscopic observation, the satellite cell nuclei show disorganized chromatin, and are indeed affected by *LMNA* mutations. The chromatin changes in satellite cell nuclei and myonuclei appeared to be quite similar, further providing the evidence that mutant lamins can influence chromatin organization even in the satellite cell nuclei.

A large number of type 2C fibers indicate the presence of immature fibers to be further differentiated. High composition of type 2C fiber in DMD/BMD muscles can be reflective of active regeneration following to muscle fiber necrosis. On the other hand, similar or rather higher composition of type 2C fibers with fewer necrotic fibers in the skeletal muscles of AD-EDMD/LGMD1B than DMD/BMD might indicate delayed completion of regeneration process. Altered chromatin organization observed in the satellite cells might have certain effects on transcriptional regulation in AD-EDMD/LGMD1B.

To evaluate the satellite cell functions, we examined Pax7 and MyoD expression in skeletal muscles. The number of Pax7-positive nuclei was increased to the similar extent in both AD-EDMD/LGMD1B and DMD/BMD compared with the age-matched normal controls. On the other hand, MyoD-positive nuclei were less pooled in AD-EDMD/LGMD1B than DMD/BMD.

In response to stimuli such as muscle injury, mechanical loading and denervation, satellite cells proliferate and a part of them terminally differentiate into myogenic cells [18]. The quiescent satellite cells express the transcriptional factor of Pax7, and when activated they proliferate and co-express Pax7 and MyoD, and after this, muscle differentiation is thought to progress with the down-regulation of Pax7 [19]. Increased number of both Pax7-positive and MyoD-positive nuclei observed in DMD/BMD patients indicates the activation and proliferation of satellite cells, as reported in dystrophin-deficient *mdx* mice [20]. Different from the condition of DMD/BMD, much smaller population of MyoD-positive nuclei in AD-EDMD/LGMD1B might reflect insufficient differentiation/regeneration process of skeletal muscle, even with similarly increased Pax7-positive satellite cells. These results are also consistent with the previous data which demonstrated markedly reduced (<60%) level of MyoD but preserved levels of Pax7 and MEF2 in $Lmna^{-/-}$ myoblasts [17]. In AD-EDMD/LGMD1B patients, the genes associated with muscle differentiation might also be affected resulting in delayed muscle regeneration. Further examination is needed to elucidate the roles of chromatin disorganization in the gene expression related to muscle differentiation.

In conclusion, our results demonstrate that *LMNA* mutations can make various kinds of nuclear abnormalities both in myonuclei and satellite cell nuclei, further supporting the role of A-type lamins in the maintenance of nuclear integrity. In addition, our results show that the skeletal muscle regeneration might be delayed due to decreased differentiation potential of the satellite cells in AD-EDMD/LGMD1B.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2008.09.018.

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Clinical/Scientific Notes

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NOVEL *FHL1* MUTATIONS IN FATAL AND BENIGN REDUCING BODY MYOPATHY

Reducing body myopathy (RBM) is a rare disorder characterized pathologically by the presence of intracytoplasmic inclusions strongly stained by menadione-NBT (nitroblue tetrazolium) staining in the absence of the substrate α-glycerophosphate. The causative gene for RBM was recently identified as *FHL1* on chromosome Xq27 encoding four and a half LIM domains 1.[1] FHL1 is a 32 kDa protein, composed of four LIM domains preceded by a single N-terminal zinc finger. FHL1 is highly expressed in skeletal muscle and heart. Here, we searched for *FHL1* mutations in three sporadic cases[2-4] and one familial case[5] of RBM we previously reported.

Methods. All clinical materials used in this study were obtained for diagnostic purpose with informed consent. Patient 1 and patient 2 have fatal infantile form,[2,3] and patient 3 has adult-onset form.[4] Patients 4 (son) and 5 (his mother) had familial cases.[5] We directly sequenced all exons and their flanking intronic regions of *FHL1* in the five RBM patients and 250 Japanese controls. Frozen muscle specimens were examined by immunohistochemistry and immunoblotting using standard technique.

Results. We identified four novel mutations in *FHL1*: a heterozygous missense mutation of c.449G>A (p.C150Y) in patient 1 and c.302G>T (p.C101F) in patient 2, an in-frame 9 bp deletion at c.304-312delAAGGGGTGC (p.102-104delKFC) in patient 3, and a hemizygous mutation c.310T>C (p.C104R) in patient 4. The mother (patient 5) had the same mutation in heterozygous mode. All mutations we identified are located in the second LIM domain of FHL1 (figure e-1 on the *Neurology*® Web site at www.neurology.org).

Immunohistochemical analysis of patients' muscles showed strong immunoreactive depositions of FHL1, α5-integrin, myosin heavy chain-slow (MyHC-slow), ribosomal proteins, and nucleolar protein coilin (figure). Protein amount of FHL1 was significantly reduced in patients 2 and 4 with less reduction in patient 5 after normalization to actin level. In contrast, patient 3 showed mild increase in FHL1 (figure).

Supplemental data at www.neurology.org

Discussion. All our RBM patients, with a wide range of clinical phenotypes, fatal infantile (patient 1 and 2), benign childhood (patient 4), and adult-onset (patients 3 and 5), had novel *FHL1* mutations, confirming the recent report that *FHL1* is the causative gene for RBM.[1] All the mutations identified in RBM patients affects the cysteine or histidine residues located within the second LIM domain of FHL1, indicating their irreplaceable role in stabilizing FHL1 (figure e-1). Phenotypic severity may depend on how the altered residue affects the zinc binding sites and resulting disruption of the structure and function of the LIM domain.

In this study, clinical severity is correlated with the amount of the FHL1 protein. Nevertheless, previously reported fatal RBM patients show increased FHL1 amount.[1] Since RBM shows asymmetric muscle involvement and focal pathologic changes in the same muscle specimen (figure), the decrease or increase of FHL1 amount may depend on the degree of affection of the biopsied part of the muscle. We should also consider the degree of protein degradation/turnover.

Here we showed that MyHC-slow is aggregated in patient muscles. It was reported that both overexpression and underexpression of FHL1 were associated with the failure of myosin to assemble into thick filaments. Aggregation of myosin was also noted in FHL1 knockdown cells. In RBM muscles, mislocalization of myosin filaments and the sarcomeric disassembly may be caused by FHL1 dysfunction. Surprisingly, α5-integrin was also highly aggregated in RBM patients although normally α5-integrin is expressed in myoblasts and during primary myogenesis, and is downregulated in mature muscle. FHL1 was reported to induce $\alpha5\beta1$-integrin-dependent myocyte elongation. Whether or not there is a correlation between α5-integrin aggregation and the suggested role of FHL1 in integrin signaling and regulation of cytoskeletal dynamics during muscle differentiation is not clear.

To date, only 6 families and 16 sporadic patients with RBM have been reported. However, RBM patients may be overlooked and underestimated, since reducing bodies can be observed in selective parts of the muscle, as shown in the figure. Furthermore, menadione-

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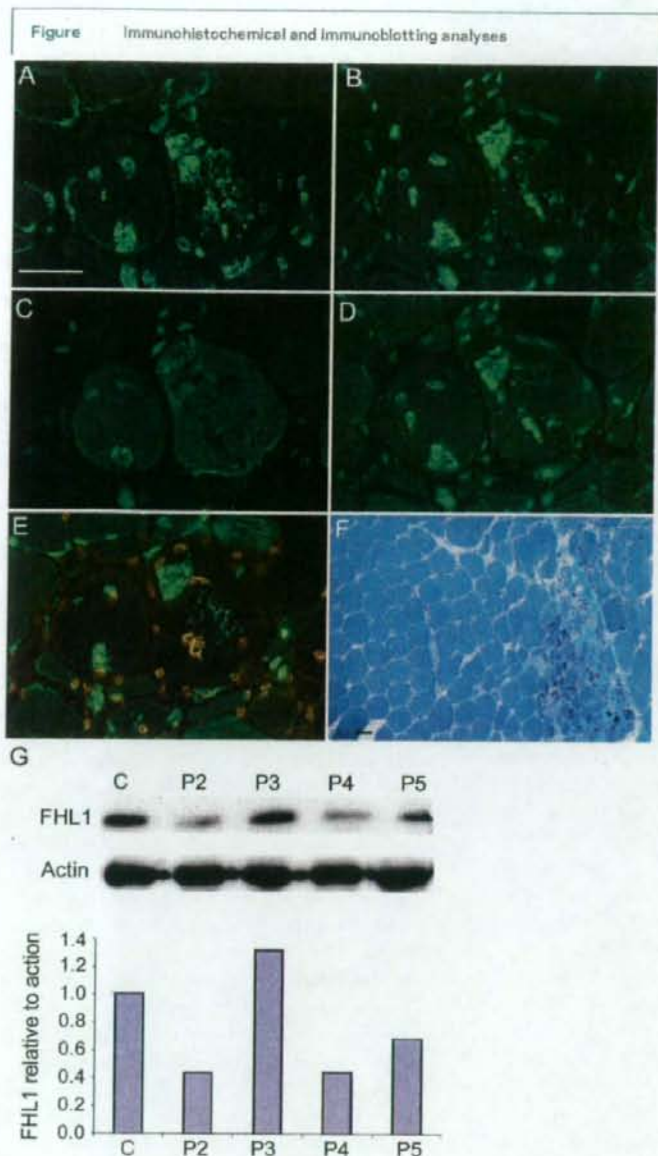

(A–E) Immunohistochemical analysis of patient 3 was performed using antibodies against FHL1 (AVIVA), α5-integrin (Chemicon), slow myosin heavy chain (MyHC-slow; Novocastra), ribosomal protein L28 (Santa Cruz), coilin (Sigma), and lamin C (see reference e-1 at www.neurology.org). Abnormal accumulation of FHL1 (A), α5-integrin (B), MyHC-slow (C), and ribosomal proteins (D) are seen. Double immunostaining of coilin (green) and lamin C (orange) revealed intracytoplasmic and perinuclear accumulation of coilin (E). These findings may be characteristic for reducing body myopathy (RBM) as it was observed in patients 2, 4, and 5 (fatal and benign RBM) but not seen in muscle specimens from a healthy control or diseased controls. Because of the limited amounts of the specimens, we could not examine in patient 1. Bar = 50 μm. (F) Modified Gomori-trichrome staining from patient 3 shows focal involvement in the muscle section. Bar = 50 μm. (G) Immunoblotting analysis of FHL1 in muscle specimens from patients 2, 3, 4, and 5 show variable amount of FHL1. Patients 2, 4, and 5 show significant reduction in FHL1 amount. Patient 4 (son) shows more reduction in FHL1 amount than patient 5 (his mother). Patient 3 shows slight increase in FHL1. Relative amount of FHL1 was calculated and normalized to actin (Nichirei).

NBT staining without substrate is not performed unless RBM is suspected. *FHL1* mutations have also been reported as the cause of X-linked scapuloperoneal myopathy (SPM)[6] and X-linked myopathy with postural atrophy (XMPMA).[7] Certainly, RBM, SPM, and XPMPA share common clinicopathologic features such as scapuloperoneal dominant muscle involvement, asymmetric muscle weakness, rigid spine, myofibers with core-like appearance on NADH, and rimmed vacuoles, and this finding raises a possibility that they may be a single entity. In addition, reducing bodies detected in a SPM patient strengthens this idea (unpublished data).

Further studies together with the identification of more RBM patients may help refine the diagnostic criteria for RBM and may explain the pathomechanism underlying the formation of reducing bodies which is unclear.

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Case report

A novel *POMT2* mutation causes mild congenital muscular dystrophy with normal brain MRI

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Abstract

We report a patient harboring a novel homozygous mutation of c.604T > G (p.F202V) in *POMT2*. He showed delayed psychomotor development but acquired the ability to walk at the age of 3 years and 10 months. His brain MRI was normal. No ocular abnormalities were seen. Biopsied skeletal muscle revealed markedly decreased but still detectable glycosylated forms of alpha-dystroglycan (α-DG). Our results indicate that mutations in *POMT2* can cause a wide spectrum of clinical phenotypes as observed in other genes associated with α-dystroglycanopathy. Presence of small amounts of partly glycosylated α-DG may have a role in reducing the clinical symptoms of α-dystroglycanopathy.

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Keywords: POMT2; α-Dystroglycan; α-Dystroglycanopathy; Congenital muscular dystrophy; Limb girdle muscular dystrophy; Brain MRI

1. Introduction

Alpha-dystroglycan (α-DG) is a surface membrane protein that links extracellular basal lamina and intracellular cytoskeleton. α-DG is a highly glycosylated protein mainly composed of unique *O*-mannosyl glycans. Reduced/altered glycosylation of α-DG causes a wide variety of muscular dystrophies including Walker–Warburg syndrome (WWS), muscle-eye-brain disease (MEB), Fukuyama-type congenital muscular dystrophy (FCMD), congenital muscular dystrophies type 1C and type 1D, and limb girdle muscular dystrophies (LGMD) type 2I, 2K to 2N. They are collectively called alpha-dystroglycanopathies (α-DGP). So far, six causative genes for α-DGP have been identified including *protein-O-mannosyl transferase 1* and *2* (*POMT1 and POMT2*), *protein O-mannose β-1,2-N-acetylglucosaminyltransferase* (*POMGnT1*), *fukutin* (*FKTN*), *fukutin-related protein* (*FKRP*), and *acetylglucosaminyl transferase-like protein* (*LARGE*). Here we report a mild congenital muscular dystrophy patient associated with a novel homozygous mutation in *POMT2*.

2. Case report

A 4-year-old Japanese boy, the only child from healthy consanguineous parents, was delivered uneventfully at full term. During few days after birth, he was

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low spirited and showed sucking weakness. Floppiness was not prominent but serum CK levels were markedly elevated up to 33,000 IU/l (normal < 70). His condition was improved within 2 weeks, but serum CK levels were persistently higher than 1000 IU/l. His motor milestones were delayed and he could control his head at 5 months of age. At 6-month-old, he could not sit without support, and muscle weakness and atrophy were noticed in lower limbs. Deep tendon reflexes were normal. No high arched palate or macroglossia were seen. Enjoji Scale of Infant Analytical Development (ESID) at his age of 7 months revealed mild delay in body movement (developmental age was 4 months, expression of language: 5 months), and his DQ was 83. Brain computed tomography (CT) revealed no definite abnormalities. Nerve conduction study was normal. His motor functions developed gradually and he was able to walk without support at 3 years and 10 months old. Gowers' sign was positive. Mild calf hypertrophy was seen with no joint contractures (Fig. 1A). Deep tendon reflexes were normal except for diminished Achilles tendon reflexes. ESID performed at his age of 3 years and 11 months showed general developmental delay (body movement: 15 months, hand movement: 24 months, activity of daily living: 27 months, personal relations: 24 months, expression of language: 18 months, and comprehension of language: 24 months), and his DQ was 47. Brain magnetic resonance imaging at 4 years and 1-month-old revealed no notable anomaly or cortical dysplasia (Fig. 1B). Detailed ophthalmological examinations revealed no abnormalities. No cardiac involvement was detected by chest X-ray, electrocardiogram, and echocardiography.

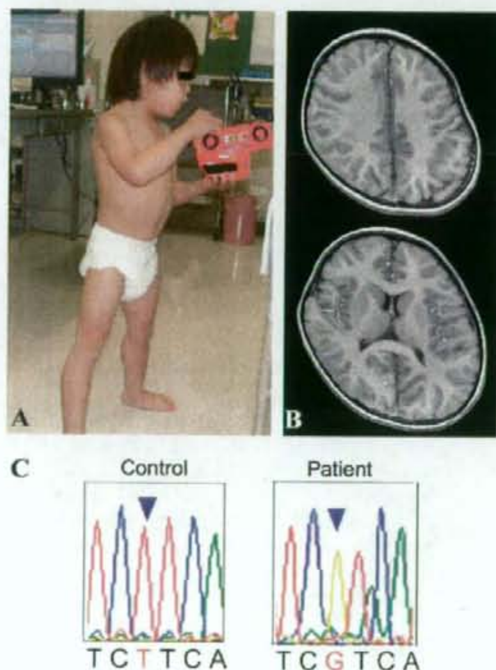

Fig. 1. (A) The patient can stand and walk with no support. Minimal calf hypertrophy is seen. (B) T2 weighted brain magnetic resonance imaging shows no obvious brain anomaly, cortical dysplasia, or white matter changes. (C) Sequence analysis of *POMT2* revealed a homozygous mutation at c.604T > G in exon 5.

Muscle biopsy taken at 7 months of age with informed consent showed dystrophic changes with scattered necrotic and regenerating fibers and mild endomysial fibrosis (Fig. 2A). No inflammatory changes were seen. On immunohistochemistry, glycosylated forms of α-DG detected by VIA4-1 antibody (Upstate Biotechnology, NY) was markedly reduced in the sarcolemma, while immunoreactions for the core region of α-DG using GT20ADG antibody [1] (data not shown) and for β-DG (43DAG1/8D5; Novocastra Laboratories, UK) was well preserved (Fig. 2A). On immunoblotting analysis, faint, broad band of around 140 kDa in size was detected by VIA4-1, whereas GT20ADG recognized a band of around 90 kDa in size. Laminin overlay assay showed barely detectable binding product (Fig. 2B). These results suggested altered glycosylation of α-DG in the muscle.

We performed mutation screening in all six causative genes for α-DGP. Genomic DNA was extracted from peripheral lymphocytes using standard technique after informed consent. Primer sequences we used are available on request. All exons and their franking intronic regions were directly sequenced by ABI PRISM 3100 (PE Applied Biosystems, CA). We identified a homozygous missense mutation of c.604T > G (p.F202V) in exon 5 of *POMT2* (Fig. 1C), which is not described in previous publications [3–8] and the mutation database (http://www.dmd.nl/).

The protein *O*-mannosyltransferase (POMT) activity was measured as previously described [2]. Mutant POMT2 (F202V) co-expressed with POMT1 in COS cells showed barely detectable POMT activity (data not shown).

3. Discussion

POMT2 is the gene encoding an enzyme for protein *O*-mannosylation, and it is required to form a complex with POMT1 for the enzyme activity [2]. Recently, some patients with mutations in *POMT2* have been reported [3–8]. Most patients showed floppiness at birth, delayed psychomotor development, congenital muscular dystrophy, and severe mental retardation with or without ocular involvement. Brain anomalies are prominent including hydrocephalus, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, and cerebel-

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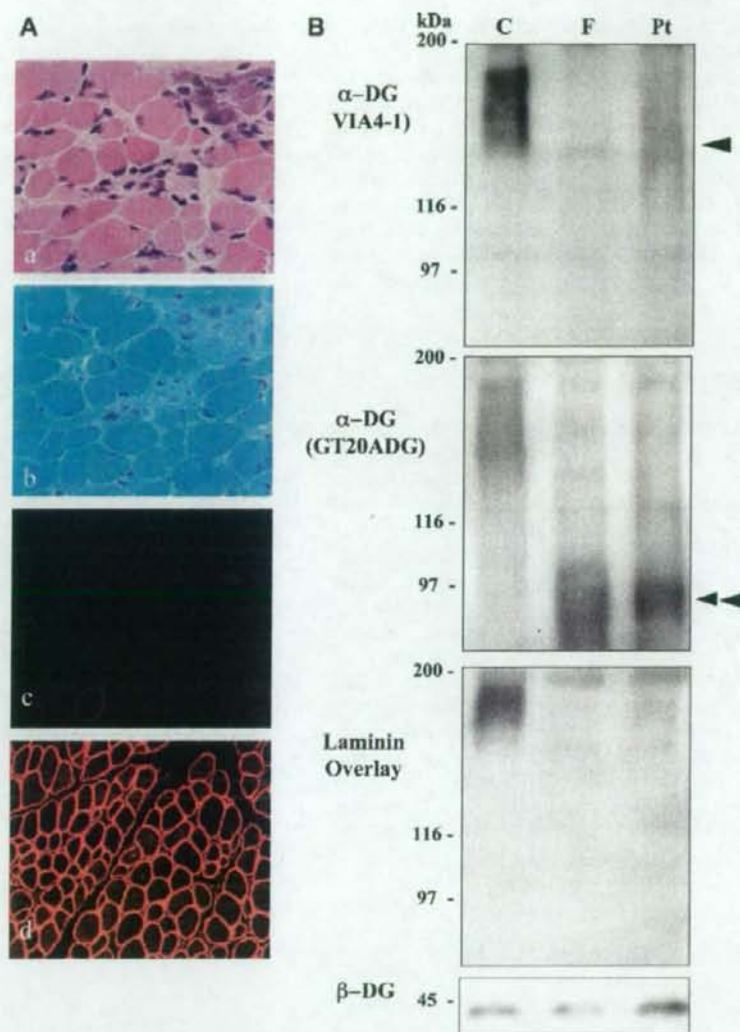

Fig. 2. (A) Histological analysis. On Hematoxylin and eosin (a) and modified Gomori-trichrime (b) staining, variation in fiber size and scattered necrotic and regenerating fibers are seen. Immunohistochemical analysis using antibodies VIA4-1 (c), which recognize heavily glycosylated form of α-dystroglycan (α-DG), showed greatly reduced sarcolemmal staining in patient, but well preserved immunoreactivities of β-DG (d) is seen. Bar = 50 m. (B) Immunoblotting analysis. Immunoblotting analysis using antibodies of VIA4-1, GT20ADG for α-dystroglycan (α-DG) and laminin overlay assay are performed using skeletal muscle from control (C), Fukuyama-type congenital muscular dystrophy (FCMD; F), and the patient (Pt). VIA4-1 recognizes a broad band about 156 kDa in size in control, and approximately 90 kDa in FCMD. In the patient muscle, reduced in size and amount compared with control was observed. GT20ADG revealed bands at approximately 90 kDa in both the patient and FCMD muscles. Laminin overlay assay shows barely detectable band in both the patient and FCMD.

lar hypoplasia [3–5]. In contrast, the patient reported here shows milder clinical features. Although his psychomotor milestones were delayed, he achieved independent ambulation with no marked brain malformation and ocular involvement. His clinical phenotype was intermediate between congenital muscular dystrophy and limb girdle muscular dystrophy. Milder clinical features with mutations in *POMT2* have been recently reported and designated as limb girdle muscular dystrophy type 2N [6,7]. Mutations in *POMT2* can cause wide spectrum of clinical phenotypes from Walker–Warburg syndrome to limb girdle muscular dystrophy (LGMD),

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as demonstrated in patients with *FKRP*, *FKTN*, or *POMT1* mutations.

Pathological changes of skeletal muscle also showed mild dystrophic changes consistent with clinical findings. Clinical and pathological severity may not be always correlated to the molecular mass of α-DG [9]. However, some clinically milder patients with α-DGP show reduced but positive glycosylated forms of α-DG detected by the VIA4-1 antibody [10]. Preservation of partly glycosylated forms of α-DG could contribute to the milder clinical phenotype of this patient.

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Emerinopathy and Laminopathy Clinical, pathological and molecular features of muscular dystrophy with nuclear envelopathy in Japan

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Mutations in the genes for nuclear envelope proteins of emerin (*EMD*) and lamin A/C (*LMNA*) are known to cause Emery-Dreifuss muscular dystrophy (EDMD) and limb girdle muscular dystrophy (LGMD). We compared clinical features of the muscular dystrophy patients associated with mutations in *EMD* (emerinopathy) and *LMNA* (laminopathy) in our series. The incidence of laminopathy was slightly higher than that of emerinopathy. The age at onset of the disease in emerinopathy was variable and significantly older than in laminopathy. The initial symptom of emerinopathy was also variable, whereas nearly all laminopathy patients presented initially with muscle weakness. Calf hypertrophy was often seen in laminopathy, underscoring the importance of mutation screening for *LMNA* in childhood muscular dystrophy with calf hypertrophy. The clinical spectrum of emerinopathy is actually wider than previously known including EDMD, LGMD, conduction defects with minimal muscle/joint involvement, and their intermittent forms. Pathologically, no marked difference was observed between emerinopathy and laminopathy. Increased number and variation in size of myonuclei were detected. More precise observations using electron microscopy is warranted to characterize the detailed nuclear changes in nuclear envelopathy.

Key words: Emerin, lamin A/C, muscular dystrophy

Introduction

In eukaryotic cells, nucleus is delineated from cytoplasm by nuclear envelope which comprise the outer and inner nuclear membranes, perinuclear space, nuclear pore complexes and the nuclear lamina (1). The functions of nuclear envelope encompass preserving the structural integrity of the nucleus, controlling molecular passage between the nucleus and cytoplasm, DNA replication and gene transcription (2, 3). Mutations in the genes encoding nuclear envelope proteins are known to cause a wide variety of disorders, the so-called nuclear envelopathy. The number of genes related to nuclear envelopathy and their associated diseases are rapidly increasing. Among these, mutations in the emerin gene (*EMD*) and the lamin A/C gene (*LMNA*) are known to cause Emery-Dreifuss muscular dystrophy (EDMD) and limb girdle muscular dystrophy (LGMD).

EDMD is clinically characterized by the triad of:

1) early joint contractures of the elbows, Achilles tendons, and postcervical area;
2) slowly progressive muscle wasting and weakness with a humeroperoneal distribution in the early stages;
3) cardiomyopathy with conduction defects that require pacemaker implantation to avoid sudden death (4).

X-linked recessive (X-EDMD; OMIM 310300), autosomal dominant (AD-EDMD; OMIM 181350) and rare autosomal recessive (AR-EDMD; OMIM 604929) forms are known.

In 1994, the *STA* (or *EMD*) gene was identified as the causative gene for X-EDMD (5). *EMD* is located on chromosome Xq28 and composed of 6 exons encoding a 254-amino acid proteinm known as emerin. Emerin is a 34-kDa integral inner nuclear membrane protein (6, 7), which is involved both in tissue-specific gene regulation and mechanical integrity of the nucleus. At present, more than 100 mutations distributed homogeneously along the *EMD* gene have been reported (http://www.dmd.nl/). Most mutations create premature termination in the coding region or frame-shift mutations, and only a few missense mutations have been reported. For the screening of emerinopathy, protein analysis is quite useful. Emerin is a ubiquitously expressed nuclear membrane protein and several kinds of tissues/cells can be used for the protein analysis including biopsied skeletal and cardiac muscles, skin biopsy or fibroblasts, peripheral lymphocytes, and oral exfoliative buccal cells (6, 8-10). Almost all patients with *EMD* mutations show absence of emerin by immunohistochemistry and western blotting. Only rare patients have been reported to show reduction of the protein (11). Since *EMD* is located on X chromosome, female carrier

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