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

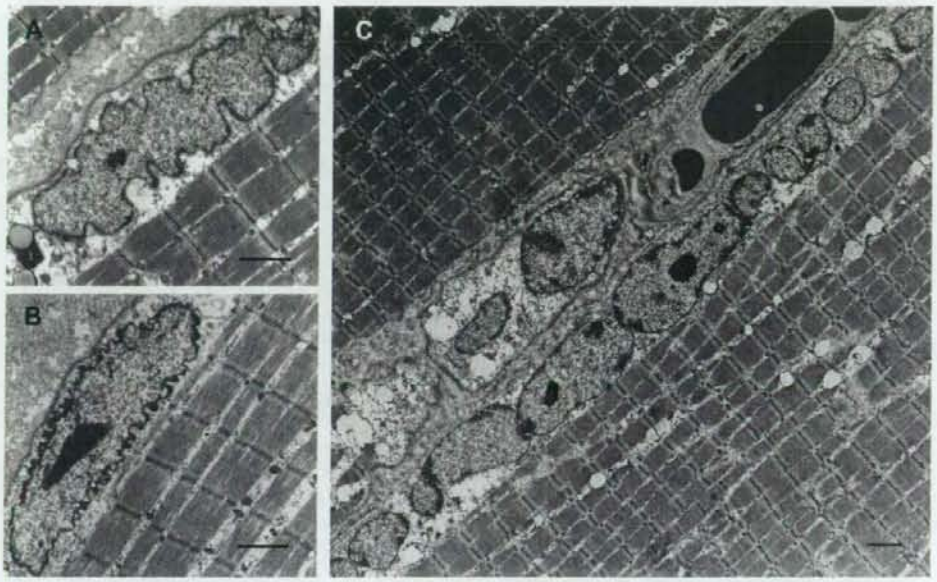

Fig. 3. Changes in the shape of myonucleus from patient 4 (A and B) and patient 1 (C). (A and B) Nuclear contours are irregular showing serpentine and sawtooth-like features. (C) A nuclear chain is identified in subsarcolemmal region, which is corresponded to those in the semi-thin section of Fig. 1C.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

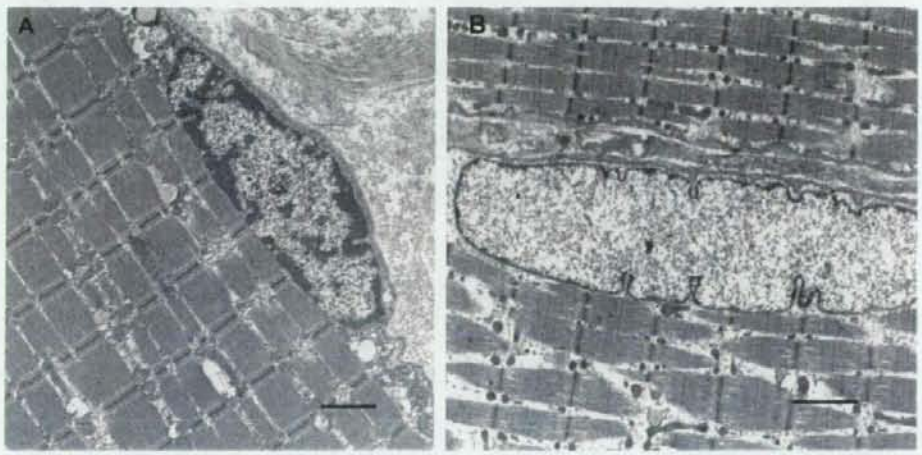

Fig. 4. Chromatin organization in myonuclei from one DMD control (A) and patient 4 (B). (A) Myonucleus of DMD has abundant heterochromatin and condensed euchromatin. (B) In myonucleus of the patient with AD-EDMD/LGMD1B, heterochromatin was scanty or nearly absent, and euchromatin reveals reduced density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

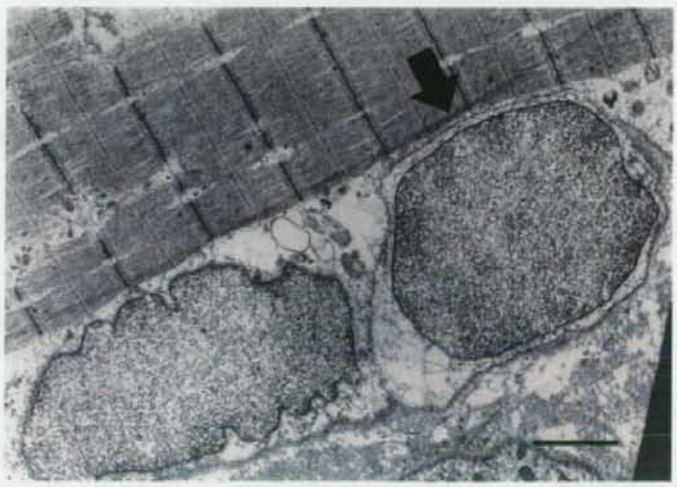

Fig. 5. Chromatin disorganization of satellite cell nuclei from patient 4. Arrow indicates the satellite cell having abnormal chromatin texture of nucleus, which is similar to that seen in myonucleus. Bar = 1 μm.

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-

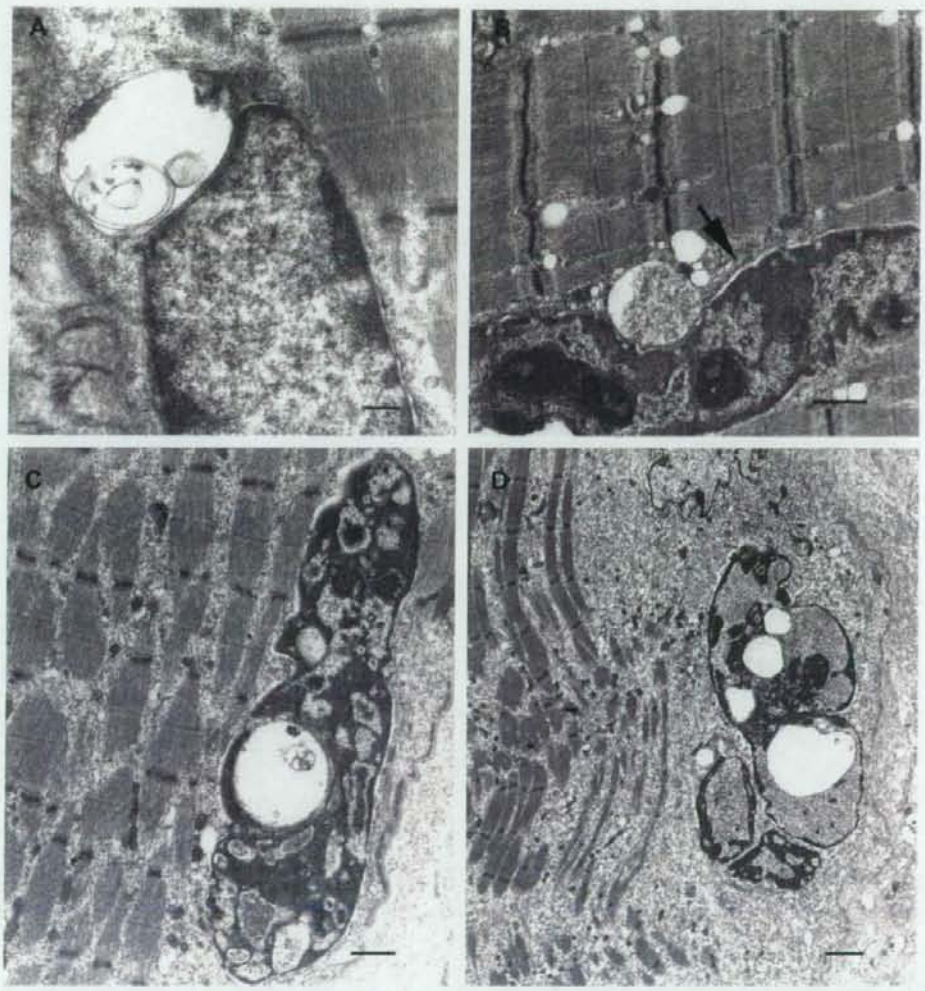

Fig. 6. Perinuclear and intranuclear vacuoles of myonuclei in patient 1 (A and B), patient 4 (C) and patient 3 (D). Vacuoles contain various materials (A, B and C), but larger intranuclear vacuoles are empty (D). (A) Nuclear membrane is difficult to be distinguished from the membrane of perinuclear vacuole. (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).

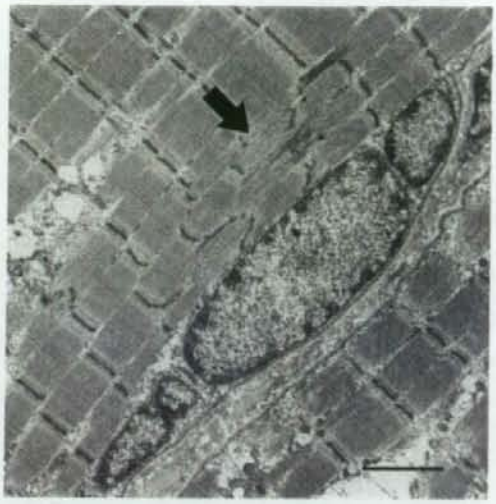

Fig. 7. In a few fibers in patient 4, myofibrils were disorganized in the region close to the altered myonucleus showing Z-line streaming as indicated by an arrow. Bar = 1 μm.

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.

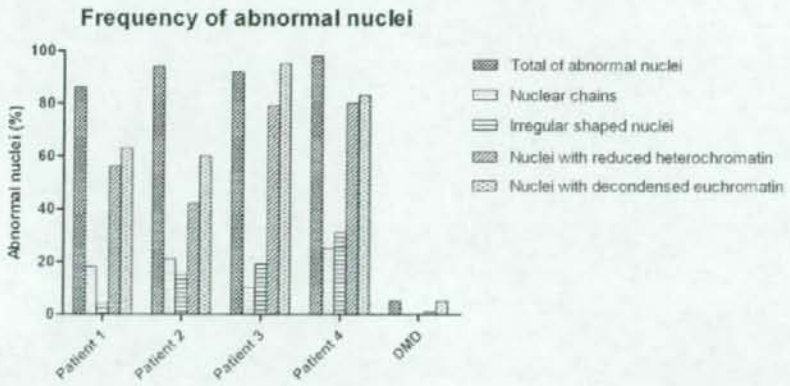

Fig. 8. The frequency of myonuclear abnormalities in four patients with AD-EDMD/LGMD1B and one DMD patient. Various kinds of nuclear changes are observed in high frequency in muscles from all four patients with AD-EDMD/LGMD1B. These changes are not correlated with the patients' age at biopsy, clinical 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.

Acknowledgements

We thank Dr. May Christine V. Malicdan (National Institute of Neuroscience, NCNP) for reviewing the manuscript. This study was supported by "Research on Psychiatric and Neurological Diseases and Mental Health" of "Health Labour Sciences Research Grant" and the "Research Grant for Nervous and Mental Disorders" from the Ministry of Health, Labor, and Welfare; by grants from the Human Frontier Science Program; by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science; by Research on Publicly Essential Drugs and Medical Devices from the Japanese Health Sciences Foundation; and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

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,[2,5] 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

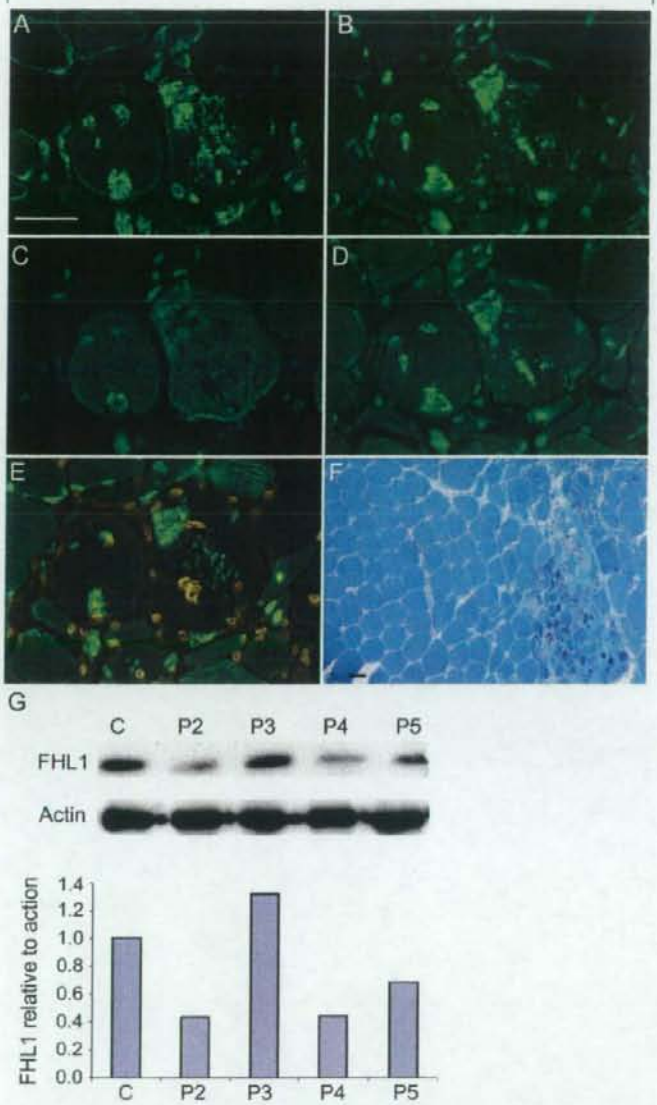
Discussion. All our RBM patients, with a wide range of clinical phenotypes, fatal infantile (patient 1 and 2), benign childhood (patient 4), and adult-onset (patients 3 and 5), had novel *FHL1* mutations, confirming the recent report that *FHL1* is the causative gene for RBM.[1] All the mutations identified 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.[5] 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 α5β1-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-

Figure Immunohistochemical and immunoblotting analyses



(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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Supported by a Grant-in-Aid for Scientific Research and a Grant-in-Aid for Exploratory Research from Japan Society for the Promotion of Science; by Research on Psychiatric and Neurological Diseases and Mental Health of Health Labor Sciences Research Grants and the Research Grant for Nervous and Mental Disorders from the Ministry of Health, Labor, and Welfare; by Research on Publicly Essential Drug and Medical Devices from the Japanese Health Sciences Foundation; and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

Disclosure: The authors report no disclosures.

Received May 21, 2008. Accepted in final form August 19, 2008.

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Brain & Development xxx (2008) xxx–xxx

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Case report

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

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Received 12 June 2008; received in revised form 18 July 2008; accepted 6 August 2008

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.

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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doi:10.1016/j.braindev.2008.08.005

Please cite this article in press as: Murakami T et al., A novel *POMT2* mutation causes mild congenital ..., Brain Dev (2008), doi:10.1016/j.braindev.2008.08.005

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.

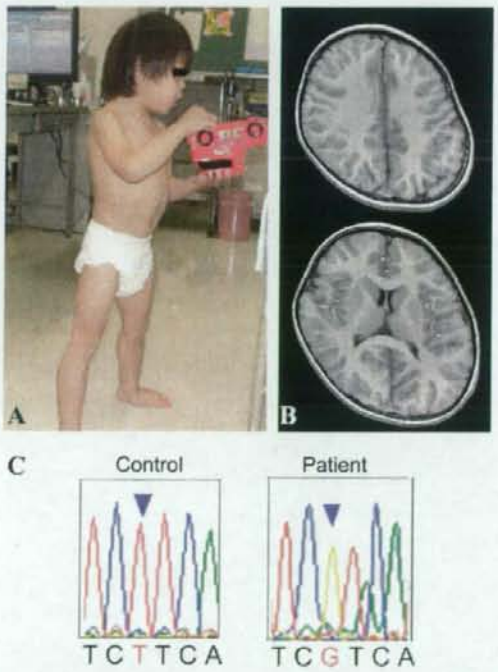

Fig. 1. (A) The patient can stand and walk with no support. Minimal calf hypertrophy is seen. (B) T2 weighted brain magnetic resonance imaging shows no obvious brain anomaly, cortical dysplasia, or white matter changes. (C) Sequence analysis of *POMT2* revealed a homozygous mutation at c.604T > G in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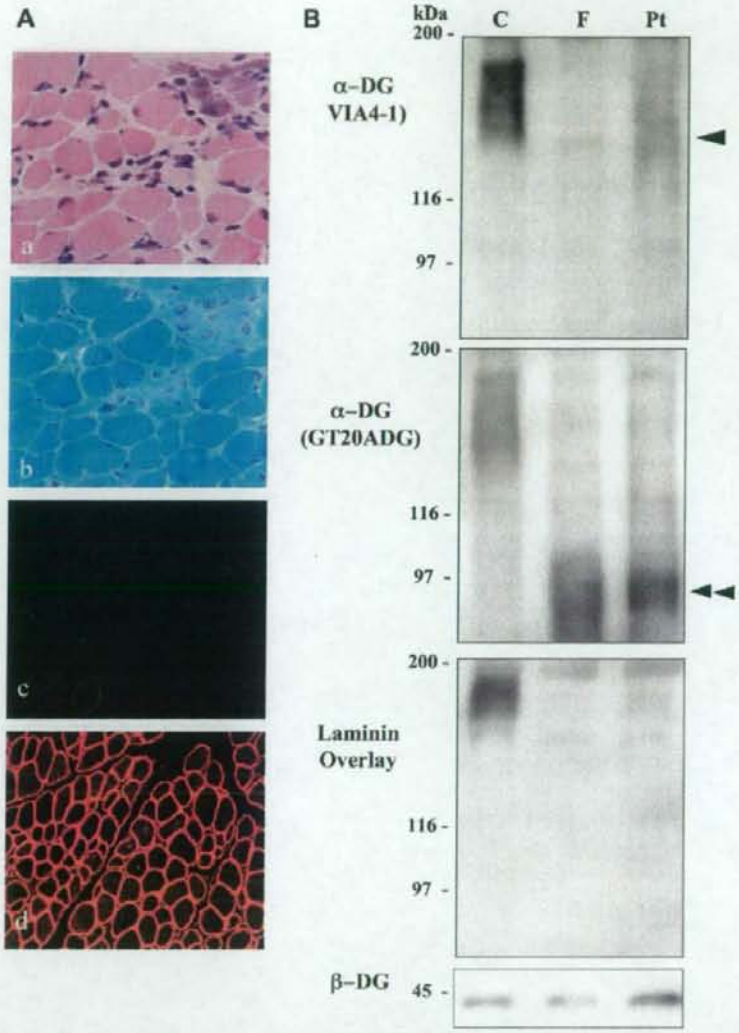

Fig. 2. (A) Histological analysis. On Hematoxylin and eosin (a) and modified Gomori-trichrime (b) staining, variation in fiber size and scattered necrotic and regenerating fibers are seen. Immunohistochemical analysis using antibodies VIA4-1 (c), which recognize heavily glycosylated form of α-dystroglycan (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.

Acknowledgements

We thank Dr. S. Shalaby (National Institute of Neuroscience, NCNP) for reviewing the manuscript. K.P. Campbell is an investigator of the Howard Hughes Medical Institute. This study was supported by the Research on Health Sciences focusing on Drug Innovation from the Japanese Health Sciences Foundation, the Research on Psychiatric and Neurological Diseases and Mental Health of Health and Labor Sciences Research Grants, the Research Grant, for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare, the Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and the Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center Grant 1 U54 NS053672.

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Please cite this article in press as: Murakami T et al., A novel *POMT2* mutation causes mild congenital ..., Brain Dev (2008), doi:10.1016/j.braindev.2008.08.005

Acta Myologica • 2007; XXVI; p. XX-XX

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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of the mutation can also be identified by immunohistochemistry showing mosaic expression (mixed with immunopositive and negative nuclei) of emerin. From the clinical point of view, it is important to identify the female carrier of *EMD* mutations because of the risk of developing lethal cardiac conduction defects (12, 13).

Following the identification of *EMD*, mutations in *LMNA* were reported both in AD-EDMD and AR-EDMD (14, 15). *LMNA* mapped in chromosome 1q21.2-q21.3 encodes A-type lamins, including lamins A and C through alternative mRNA splicing (16-19). Lamins are type V intermediate filament proteins consisting of a N-terminal head domain, a central rod domain, and a C-terminal globular tail. A-type lamins and B-type lamins (lamin B1 and B2) are major components of the nuclear lamina underlying the inner nuclear membrane. *LMNA* mutations are subsequently identified in patients with autosomal dominant LGMD with atrioventricular conduction disturbances (LGMD1B) (20). To date, the clinical spectrum caused by mutations in *LMNA* has expanded to at least 10 heterogeneous diseases listed under laminopathies including EDMD, LGMD, dilated cardiomyopathy with conduction defects (DCM-CD), lipodystrophy, neuropathy, and premature senescence (21, 22). In contrast to emerinopathy, definitive diagnosis of laminopathy is solely undertaken by mutation analysis, since protein analysis would show nearly normal expression and localization of lamin A/C. *LMNA* contains 12 exons. To date, more than 200 different mutations have been reported (http://www.umd.be:2000/, http://www.dmd.nl/). Most of the mutations in *LMNA* are heterozygous missense mutations and there is no hot spot identified throughout the gene.

In our experience of 13 years inclusion, a total of 47 muscular dystrophy patients were identified associated with nuclear envelopathy. Here, we thoroughly reviewed the clinical, pathological and molecular features of these patients.

Mutation screening

All human samples used in this study were obtained for diagnostic and research purposes with informed consent. All exons and their franking intronic regions of *EMD* and *LMNA* were amplified using genomic DNA extracted from peripheral lymphocytes or skeletal muscle. Direct sequence analysis was performed using the standard method. We identified 20 patients in 17 families with hemizygous mutation in *EMD*, and 27 patients in 24 families with heterozygous mutation in *LMNA*. Mutations identified in *EMD* and *LMNA* are listed in Table 1.

In *EMD*, 13 types of mutations were identified including 4 novel mutations. Twelve mutations were nonsense or frame-shift mutations that created premature termination. One patient had a total deletion of the coding region of the gene. All patients showed negative immunostaining for emerin on biopsied muscles.

On the other hand, 17 types of mutations in *LMNA* were identified including 15 missense and 2 nonsense mutations. Six of these were novel mutations. *LMNA* p.R453W, found in 6 families (25%), is the most common mutation in our series.

Clinical features of emerinopathy

Clinical attributes of 20 emerinopathy in our series were reviewed. All the patients were male and the age at examination ranged from 6 to 56 years old (mean ± SD = 29.0 ± 16.1). The age at onset of the disease was varied considerably from 2.5 to 37 years old (mean ± SD = 10.1 ± 9.5). Of the 20 patients, 16 (80%) were diagnosed with X-EDMD, whereas 4 patients (20%) had proximal dominant muscle involvement with no or minimal joint problems. They are diagnosed with X-LGMD (23).

Mean age at onset of these 4 X-LGMD patients was 15.5 ± 13.5 years, and all the patients noticed lower limb muscle weakness as the initial symptom. Three adult patients had severe conduction defects that required pacemaker implantation at 40.0 ± 8.5 years of age, on average. Two of them also had dilated cardiomyopathy, and one had valvular heart disease. The youngest LGMD patient (6-year-old male) did not show any cardiac involvement (23). This result suggests that cardiac involvement is likewise common in patients with X-LGMD as in LGMD1B, caused by *LMNA* mutations.

Clinical findings of 16 X-EDMD patients in our series were rather variable. Mean age at onset was 8.8 ± 9.5 years which is younger than X-LGMD. Of 16 patients, 12 had all the cardinal triad of EDMD; i.e., joint, muscle, and cardiac involvements. The initial symptoms of X-EDMD patients were variable. Early joint contracture before appearance of any significant muscle weakness is a characteristic feature of EDMD. Patients starting from joint contractures were most frequent (37.5%) in our series, and their mean age at onset was 6.3 ± 2.1 years. One patient was clinically diagnosed to have rigid spine syndrome (24). The patients starting from muscle symptoms reached 31.25%, and mean age at onset was 4.5 ± 2.7 years old. Muscle involvement was usually noticed from slow running or gait disturbance. Humeroperoneal muscles are affected from an early stage, with subsequent diffuse limb muscle involvement in a later stage. Only one patient noticed transient mild calf hypertrophy. Conduction block was the initial symptom for 5 patients (31.25%) with X-EDMD, and mean age at onset was 16.0 ± 12.1 years old, which is older than those starting with muscle/joint problems. Half of the X-EDMD patients received pacemaker implantation at 26.0 ± 11.6 years old, on average, because of severe conduction defects. Cardiomyopathy and/or valvular heart disease were seen in 43.8% of X-EDMD patients. The youngest, a 7-year-old patient with entire deletion of the gene, has not shown any cardiac symptoms yet. Interestingly, 3 patients (19,

Table 1. Mutations of EMD and LMNA identified in our series (*: novel mutations).

	EMD			LMNA	
Exon	cDNA	Protein	Exon	cDNA	Protein
Ex.1	c.31del G	p.E11SfsX2	Ex.1	c.73G > C	p.R25G
Ex.1	c.82 + 5G > C	c.54_82del	Ex.1	c.306T > A *	p.L102P
Ex.2	c.83-2A > G *	fs?	Ex.2	c.374G > C *	p.G125A
Ex.2	c.123C > G	Y 41 X	Ex.4	c.746G > A	p.R249Q
Ex.2	c.144dupC *	p.S49LfsX11	Ex.5	p.907T > C	p.S303P
Ex.3	c.197delC *	p.S66X	Ex.5	c.931A > G *	p.K311R
Ex.3	c.251-255delTCTAC	p.L84_Y85 > PfsX7	Ex.6	c.1058A > G *	p.Q353R
Ex.3	c.359-362delCAGT	p.S120X	Ex.6	c.1063 C > T	p.Q355X
Ex.5	c.400-2A > G *	fs?	Ex.6	c.1129C > T	p.R377C
Ex.6	c.677G > A	W226X	Ex.7	c.1357 C > T	p.R453W
Ex.6	c.619delC	p.R207GfsX30	Ex.7	c.1366A > C *	N456H
Ex.6	c.650_654dup	p.G218_Q219insWAfsX20	Ex.8	c.1412G > A	p.R471H
	entire deletion		Ex.9	c.1540T > C	p.W514R
			Ex.9	c.1580G > C	p.R527P
			Ex.9	c.1583C > A	T528K
			Ex.9	c.1527-1529 TAC > AA *	p.T509Tfs39X
			Ex.10	c.1622G > A	p.R541H

22 and 37 years old) had severe conduction defects and mild joint contractures with no muscle weakness. Previously, a patient, likewise harboring *EMD* mutation presenting as severe conduction cardiomyopathy with mild muscle involvement, has been reported (25). These results suggest that cardiac symptoms can be a major symptom for some emerinopathy patients despite minor joint and muscle involvements.

From these results and previous reports, mutations in *EMD* could cause a wider variety of clinical features than previously considered, including EDMD, LGMD, cardiac conduction defects, and their intermediate phenotypes (23, 25).

Clinical features of laminopathy

We found 27 patients (12 male, 15 female) associated with *LMNA* mutations in our series. Age at examination varied from 6 months to 54 years old (mean = 25.0 ± 17.5). All laminopathy patients revealed their symptoms before 14 years of age, and mean age at onset of the disease was 3.3 ± 2.9 years old, which was significantly younger than those with emerinopathy. In contrast to emerinopathy, the initial symptom was fairly homogeneous. All except one noted lower limb muscle weakness as the initial symptom presenting unsteady gait, easy to fall down, or slow runner. Only one patient noticed rigidity of hind neck before muscle symptoms. Cardiac symptoms appeared later than muscle/joint problems in all the patients.

Joint contractures of Achilles tendons, elbows and/or hind neck were observed in 21 out of 27 patients (77.8%), however, only 6 patients showed humeroperoneal distribution of muscle involvement, as observed in typical EDMD patients. Twelve patients showed proximal dominant limb muscle weakness with joint contractures, which suggested the existence of an intermediate form between AD-EDMD and LGMD1B. Five patients had proximal dominant limb muscle weakness with no joint contractures. They were diagnosed LGMD1B. It is worthwhile mentioning that calf hypertrophy was frequently seen in patients showing proximal dominant muscle involvement with no/minimal joint contractures. Therefore, mutation screening of *LMNA* should be considered for childhood muscular dystrophy with calf hypertrophy.

Cardiac involvement was seen in 17 of the 27 patients (63.0%) with laminopathy, and only 5 patients were identified to have dilated cardiomyopathy. Six patients received pacemaker implantation at the age of 34.5 ± 10.7 years (average). In a mouse model of laminopathy carrying homozygous *LMNA* H222P mutation, the male mice showed more severe cardiomyopathy and shorter life span than the female (26). However, in our human series, no marked gender difference was seen.

Clinical manifestations of the patients are heterogenous even though they carry the same mutation in *LMNA*. In our series, we found 6 patients with p.R453W substitution in *LMNA*. One patient showed proximal limb muscle weakness with no joint contractures, and was diagnosed

as having LGMD1B. On the other hand, the other five patients had joint contractures and 2 were clinically diagnosed to have rigid spine syndrome. One patient manifested as humeroperoneal muscle involvement with joint contractures of Achilles tendons, elbows and hind neck, and was diagnosed as AD-EDMD. Among 6 patients with p.R453W mutation in *LMNA*, cardiomyopathy with conduction defects was seen only in one oldest patient from the age of 34 years.

Recently, Benedetti, et al. reported that premature termination mutations in *LMNA* cause rather late onset cardiac disorders or limb girdle muscular dystrophy (27). In our series, three laminopathy patients, in 2 families, had a nonsense mutation of p.Q355X (c.1063C > T) or p.T509Tfs.39X (c.1527-1529 TAC > AA) in *LMNA*. The mutation of p.Q355X had been previously reported in a patient with DCM-CD, but one patient with the same Q355X mutation in our series appeared EDMD phenotype at 6 years of age. His son, carrying the same mutation, was seen to have unsteadiness of sitting at 6-month-old. A LGMD patient with *LMNA* p.T509Tfs39X showed slow running from 3 years old. In our series, however, no marked difference in disease onset was seen between patients with missense and nonsense mutation in *LMNA*.

Pathological findings of skeletal muscles

Biopsied skeletal muscles from 11 emerinopthy and 12 laminopathy cases were examined in detail. Serial frozen sections were stained with hematoxylin and eosin (H&E), modified Gomori-trichrome, and a battery of histochemical staining. Immunohistochemical analysis was also performed using anti-emerin (Novocastra Lab.) and anti-lamin A and C antibodies (28).

Histologically, non-specific dystrophic changes were commonly seen including variation in fiber size, necrotic and regenerating process, increased interstitial fibrosis, increased number of fibers with internal nuclei and fiber splitting. Intermyofibrillar networks are often disorganized. Both type 1 and type 2 fibers are affected and no fiber type grouping was seen. There is no difference between EDMD and LGMD, regardless of the type of causative genes. Interestingly, one AD-EDMD patient showed active necrosis and a regenerating process associated with marked lymphocytic infiltration in endomysium and around blood vessels that was indistinguishable from inflammatory myopathy.

Interestingly, an increased number of myonuclei was often observed in muscles, especially from both older emerinopathy and laminopathy patients. Together with enlarged nuclei, smaller sized nuclei are scattered in the periphery of muscle fibers. Chained nuclei were also frequently seen. The total number of myonuclei was counted in 100 fibers and the mean number of myonuclei per muscle fiber with 100 μm diameter was calculated. We used skeletal muscles from 11 emerinopathy (mean age at biopsy 26.2 years), 12 laminopathy patients (mean age at biopsy 13.8 years), and 15 controls (mean age at biopsy 34.3 years) including dystrophinopathy, dysferlinopathy, calpainopathy, mitochondrial myopathy, inflammatory myopathy, congenital myopathy, neuropathy, and nearly normal muscles. Average number of myonuclei per fiber in emerinopathy, laminopathy, and controls was 13.8 ± 3.4, 9.2 ± 3.6, and 6.4 ± 1.7, respectively. This result suggests an increased number of myonuclei per muscle fiber in nuclear envelopathy. Together with variation in nuclear size, a few vacuoles were observed close to the myonuclei in some muscles from both emerinopathy and laminopathy cases. Similar perinuclear vacuoles were observed in emerin knockout mouse (29). These nuclear changes

Table 2. Clinical difference between emerinopathy and laminopathy

	Emerinopathy (n = 20)	Laminopathy (n = 27)
Mean age at exam. (years) ± SD	29.0 ± 16.1	25.0 ± 17.5
Mean age at onset (years) ± SD	10.1 ± 9.5	3.3 ± 2.9
Initial symptoms		
Muscle involvement	45.0%	96.3%
Joint contractures	30.0%	3.7%
Conduction block	25.0%	0.0%
Clinical symptoms		
Muscle involvement	85.0%	100.0%
Joint contractues	90.0%	77.8%
Cardiac involvement	90.0%	63.0%
Mean age at PMI (years) ± SD	28.9 ± 12.1	34.5 ± 10.7

PMI: pacemaker implantation

may be closely associated with fragile nuclear envelope, however, detailed electron microscopic examination is still warranted.

Immunohistochemically, lamins A and C were nearly normal in all the patients examined including laminopathy. Immunoreactions of emerin were negative in all muscles from emerinopathy. Interestingly, one patient with *LMNA* p.Q311R mutation showed reduced nuclear staining of emerin. No mutation was identified in *EMD*. This result suggests that instability of emerin could be induced in the presence of mutant lamin A/C.

Conclusions

The clinical difference between emerinopathy and laminopathy is outlined in Table 2. In our series, the incidence of laminopathy was similar, but slightly higher, than emerinopathy, although X-EDMD was previously thought to be much more frequent (4). In both emerinopathy and laminopathy, the distribution and severity of symptoms are variable and different in each patient despite harboring the same gene mutation. Classification into the disease category of EDMD, LGMD, or DCM-CD is sometimes difficult. The intermediate form is more frequently seen in laminopathy. Furthermore, LGMD, caused by mutations in *EMD*, is not rare. Mean age at onset of the disease was significantly younger in laminopathy than in that of emerinopathy. The initial clinical symptom was variable in emerinopathy, while earlier muscle involvement is common in laminopathy. Cardiac involvement is more notably observed in emerinopathy with younger mean age at onset of symptoms (21.9 ± 13.1) than in laminopathy (28.0 ± 15.3). Calf hypertrophy is often seen in laminopathy. Childhood onset muscular dystrophy with calf hypertrophy is quite similar to that in dystrophinopathy patients. Considering the lethal cardiac conduction defects, early diagnosis is important for patients with nuclear envelopathy.

Acknowledgements

Authors thank attending physicians, patients, and their families for participation in this study. Study was supported by grants from the Human Frontier Science Program; by "Research on Psychiatric and Neurological Diseases and Mental Health" of "Health Labour Sciences Research Grant" and "Research Grant for Nervous and Mental Disorders" from Ministry of Health, Labor, and Welfare; by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science; by Research on Health Sciences focusing on Drug Innovation from Japanese Health Sciences Foundation; and by Program for Promotion of Fundamental Studies in Health Sciences of National Institute of Biomedical Innovation (NIBIO).

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