III　研究成果の刊行に関する一覧表

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IV　研究成果の刊行物・別刷

TMEM43 Mutations in Emery-Dreifuss Muscular Dystrophy-Related Myopathy

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Objective: Emery-Dreifuss muscular dystrophy (EDMD) is a genetically heterogeneous muscular disease that presents with muscular dystrophy, joint contractures, and cardiomyopathy with conduction defects. Mutations in several nuclear envelope protein genes have been associated with EDMD in less than half of patients, implying the existence of other causative and modifier genes. We therefore analyzed *TMEM43*, which encodes LUMA, a newly identified nuclear membrane protein and also a binding partner of emerin and lamins, to investigate whether LUMA may contribute to the pathomechanism of EDMD-related myopathy.
Methods: Forty-one patients with EDMD-related myopathy were enrolled. In vitro and in vivo transfection analyses were performed to assay the binding partners and oligomerization of mutant LUMA.
Results: We identified heterozygous missense mutations, p.Glu85Lys and p.Ile91Val in *TMEM43*, in 2 EDMD-related myopathy patients. Reduced nuclear staining of LUMA was observed in the muscle from the patient with p.Glu85Lys mutation. By in vitro transfection analysis, p.Glu85Lys mutant LUMA resulted to failure in oligomerization, a process that may be important for protein complex formation on nuclear membrane. Furthermore, we demonstrated for the first time that LUMA can interact with another nuclear membrane protein, SUN2, in addition to emerin. Cells expressing mutant LUMA revealed reduced nuclear staining with or without aggregates of emerin and SUN2 together with a higher proportion of abnormally shaped nuclei. In vivo expression of mutant LUMA by electroporation in mouse tibialis anterior muscles likewise demonstrated the decreased staining of emerin and SUN2 on myonuclei.
Interpretation: Our results suggest that mutant LUMAs may be associated with EDMD-related myopathy.
ANN NEUROL 2011;69:1005–1013

Emery-Dreifuss muscular dystrophy (EDMD) is a genetically heterogeneous neuromuscular disorder characterized by muscular dystrophy, joint contractures, and cardiomyopathy with conduction block.[1] In EDMD, 5 causative genes have been identified, including *EMD, LMNA, SYNE-1, SYNE-2,* and *FHL1,* that encode emerin, A-type lamins, nesprin-1, nesprin-2, and four and a half LIM domains 1 (FHL1), respectively.[2–5] All but FHL1 are nuclear envelope proteins. Emerin, an inner nuclear membrane (INM) protein, is essential for maintaining the structural integrity of nucleus and scaffolding a variety of gene-regulatory partners that might be involved in gene expression.[6–9] Mutations in *EMD* cause typical EDMD phenotype and albeit rarely limb girdle muscular dystrophy.[10] Nesprins, a family of spectrin-repeat proteins, link INM and cytoskeletal proteins to mediate nuclear envelope (NE) localization and integrity.[11,12] Patients with mutations in *SYNE-1* and *SYNE-2* also present EDMD-related phenotype.[4] A-type lamins, intermediate filament proteins, form nuclear lamina and anchor INM proteins, thus providing a mechanically resistant meshwork.[13–16] Mutations in *LMNA* cause not only EDMD, but a number of other diseases that affect skeletal muscle, cardiac muscle, peripheral nerve, or fat tissue,[17] among which limb-girdle muscular dystrophy type 1B displays proximal muscle weakness with later-onset cardiomyopathy but no marked joint contracture. Despite the identification of mutations in these genes, no genetic mutation was confirmed in >60% of patients with EDMD,[18] indicating the existence of other causative genes.

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Additional Supporting Information can be found in the online version of this article.

LUMA, an INM protein, was first identified in a proteomics-based approach to identify nuclear envelope proteins.[19] Recently, the encoding gene *TMEM43* was reported to cause arrhythmogenic right ventricular cardiomyopathy (ARVC) type 5, characterized by ventricular tachycardia, heart failure, sudden cardiac death, and fibrofatty replacement of cardiomyocytes.[20] In the meanwhile, the topological and functional characterization of LUMA showed that it contains a large hydrophilic and 4 transmembrane domains, and that the transmembrane spans are necessary for nuclear envelope targeting and homo-oligomerization.[21] In addition, it was demonstrated that LUMA interacts with lamins and emerin and is involved in the structural organization of nuclear membrane. As emerin is postulated to form complexes with different partners at NE to carry out distinct functions,[21,22] and mutation in *TMEM43* could cause cardiac conduction defect, a characteristic of EDMD, LUMA can logically be considered to contribute to the pathomechanism of EDMD-related myopathy.

Patients and Methods

Patients

All clinical materials used in this study were obtained for diagnostic purpose with informed consent. All experiments performed in this study were approved by the Ethical Committee of the National Center of Neurology and Psychiatry. In this study, we included the patients with at least 2 characteristic features of EDMD[1] but without mutations in *EMD*, *LMNA*, *SYNE-1*, *SYNE-2*, and *FHL-1*, who are described as having EDMD-related myopathy in this article. In all we had 41 unrelated patients. Twenty of the 41 were typical EDMD patients; 18 showed limb-girdle type muscular dystrophy with cardiac conduction defects, and 3 presented limb-girdle type muscular dystrophy with early joint contracture. Clinical information was assessed from the records provided by the physicians.

Histochemistry

Biopsied muscle specimens were frozen in liquid nitrogen-cooled isopentane and sectioned with a cryostat (10μm). A battery of histochemical stains was performed on biopsied muscle specimens.

Mutation Analysis

Genomic DNA was extracted from blood or muscle biopsy samples according to standard protocols. All 12 exons and their flanking intronic regions of *TMEM43* were amplified and sequenced using an automated 3100 DNA sequencer (Applied Biosystems, Foster, CA). Primer sequences are available upon request. DNA samples from 100 individuals without apparent muscular disorders were analyzed as control.

Plasmid Constructs, Site-Directed Mutagenesis, and Transfection

We produced plasmid constructs containing V5 or FLAG-tagged wild-type (WT) and mutant human LUMA cDNA, and transfected into HeLa or C2 cells as described in Supporting Information Methods.

Antibody to LUMA

Rabbits were immunized by mixed 2 synthetic polypeptides, peptide-1: LUMA 8-23 (TSTRREHVKVKTSSQPC) and peptide-2: LUMA 129-145 (ESREYTEDGQVKKE TRYC) (MBL, Nagoya, Japan). We used the affinity purified antiserum using peptide-2. Antibodies were prepared according to standard protocols.

Immunofluorescence Study

For immunocytochemistry, WT and mutant LUMA transfected cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton-100. Frozen sections of human muscle were fixed in 4% paraformaldehyde, and immunohistochemistry was performed according to standard protocol. Primary antibodies used in this study were as follows: antiemerin (Novocastra, Bannockburn, IL), anti-V5 (Abcam, Cambridge, MA), anti-laminA/C (1:200; Chemicon, Temecula, CA), antilamin B (1:200; AbD Serotec, Martinsried, Germany), anti-SUN1 and anti-SUN2 (1:200; ATLAS, Stockholm, Sweden), anti-nesprin (1;200; Abcam), and anti-LUMA antiserum (1:100).

Blue Native Polyacrylamide Gel Electrophoresis

Forty-eight hours after transfection, HeLa cells were homogenized in 20mmol/l HEPES with 0.25mol/l sucrose. Nuclear fraction was suspended in 1.5% digitonin lysis buffer and incubated for 30 minutes on ice, then centrifuged at 15,000 *g* for 30 minutes to remove insoluble materials. The supernatants were subjected to Blue Native polyacrylamide gel electrophoresis (PAGE) (NativePAGE Novex Bis-Tris Gel System, Invitrogen, Carlsbad, CA), then blotted onto Immobilon-P membranes (Millipore, Billerica, MA) and probed with anti-V5 antibody (1:5,000). Immunoreactive bands were detected using enhanced chemiluminescence plus detection reagent (GE Healthcare, Milwaukee, WI).

Binding Assay and Immunoprecipitation

For oligomerization analysis, HeLa cells were cotransfected with 5μg of both V5-tagged and FLAG-tagged LUMA constructs, and the nuclear fraction was lysed in 1% Triton X-100 lysis buffer for 1-hour incubation at 4°C. After centrifugation, the supernatants were then precleared with protein-A agarose beads (Roche, Basel, Switzerland). Immunoprecipitations (IPs) with anti-V5 (1:500), and antiemerin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) were performed for 2 hours at 4°C, followed by incubation with protein-A agarose beads for 1 hour at 4°C. After washing with lysis buffer, the proteins were eluted from beads by boiling for 5 minutes at 95°C in sodium dodecyl sulfate sample buffer. For IP with anti-FLAG (1:200), anti-FLAG M2 affinity gel (Sigma-Aldrich, St Louis, MO) was used.

In Vivo Electroporation

The tibialis anterior muscles of 8-week-old WT mice were injected with 80μg of purified FLAG-tagged LUMA constructs. In vivo transfection was performed using a square electroporator (CUY-21SC; NEPA GENE, Ichikawa, Japan). A pair of

electrode needles was inserted into the muscle to a depth of 5mm to encompass the DNA injection sites. Each injected site was administered with three 50-millisecond-long pulses of the required voltage (50–90V) yielding about 150mA current with 1-second interval and following 3 more pulses of the opposite polarity. Seven days after electroporation, mice were sacrificed, and tibialis anterior muscles were analyzed.

Statistics

The percentage of cells with abnormal-shaped nuclei between WT, and M1 or M2 LUMA transfected groups was analyzed by unpaired samples *t* test.

Results

TMEM43 Sequence Variants in Patients with EDMD-Related Myopathy

MUTATION ANALYSIS. We sequenced all coding exons of *TMEM43* in 41 unrelated patients. Seven sequence variants were identified. Among these, c.504A>C (p.Lys168Asn) and c.536T>C (p.Met179Thr) were reported as polymorphisms (Ensembl database: http://www.ensembl.org/index.html), whereas c.1111T>C (p.Tyr371His) was identified in 2 of 100 Japanese control individuals. c.265G>A (p.Val89-Met) and c.896G>C (p.Arg299Thr), identified in 1 of 41 patients, were probably polymorphisms, as both of them were also found in 1 control individual, in addition to the finding that the healthy mother of this patient harbored both sequence variants. The remaining variants, c.235G>A (p.Glu85Lys) (M1) in Patient 1 and c.271A>G (p.Ile91Val) (M2) in Patient 2, we considered possibly pathogenic, because they were not found in control individuals and are involved in amino acids that are well conserved among mammals (Fig 1A).

CLINICAL AND PATHOLOGICAL FEATURES. Patient 1, a 40-year-old man, was diagnosed as EDMD with typical clinical manifestations when muscle biopsy was performed in 1996. Muscle pathology revealed marked fiber size variation with scattered internalized nuclei (see Fig 1B). The patient died a few years after muscle biopsy; thus, the detailed medical record was unavailable. According to the limited clinical information obtained at the time of biopsy, his son had similar symptoms, indicating autosomal-dominant inheritance. However, his son was lost to follow-up; thus, mutation analysis and segregation study were not done for this family.

Patient 2 was a 68-year-old woman with muscle atrophy involving paraspinal, neck, upper arm, and thigh muscles. Slowly progressive proximal muscle weakness was noticed from the age of 64 years when pacemaker implantation was performed due to atrial fibrillation with bradycardia. Her parents died without mention of any specific causes, and she does not have any children. Muscle pathology demonstrated a necrotic and regenerating process (see Fig 1B). Immunohistochemistry for dystrophin, dystroglycans, sarcoglycans, dysferlin, caveolin-3, and emerin all showed normal positive staining pattern.

LUMA Expression in Human Skeletal Muscle

In the previous report, LUMA mRNA was barely detectable in skeletal muscle.[21] However, as our patients have muscular dystrophy, we examined the expression of LUMA in this tissue. We performed reverse transcription polymerase chain reaction using human mRNA from total 11 different tissues and detected LUMA cDNA in all examined tissues, including cardiac and skeletal muscles (Supporting Information Fig 1A).

Immunocytochemical analysis using C2 cells showed clear nuclear staining by our affinity-purified anti-LUMA antiserum that became barely detectable after adsorption by antigen peptide-2 (see Supporting Information Fig 1B). Consistently, immunoblotting analysis using this antibody detected a ~43kDa band corresponding to the expected molecular weight of LUMA in total cell lysates and nuclear fractions of HEK, HeLa, and C2 cells (see Supporting Information Fig 1C). LUMA transfected cells could show both endogenous and larger-sized tagged LUMA. Adsorbed antiserum recognized only week bands corresponding to transfected LUMA (see Supporting Information Fig 1D).

On muscle cryosections, LUMA staining was observed around nuclei in human skeletal muscles from controls and both Patient 1 and Patient 2. The staining intensity of LUMA looked fainter in Patient 1 compared to Patient 2 (see Fig 1C). We also performed immunofluorescence study in the skeletal muscles from EDMD patients with *EMD* and *LMNA* mutations, and interestingly, laminopathy muscles also contained many faintly stained myonuclei (Supporting Information Fig 2).

Disrupted Oligomerization of LUMA in HeLa Cells Overexpressing Mutant LUMA (p.Glu85Lys; M1)

As LUMA can form homo-oligomers,[21] we performed blue native PAGE and binding assay to examine the oligomerization of LUMA. Using nuclear fraction from WT LUMA transfected HeLa cells, monomer, dimers, trimers, tetramers, and even oligomers of LUMA were clearly visible. Similar result was seen in the cells expressing M2 LUMA, as well as V89M, 1 of the possible polymorphisms. Conversely, M1 LUMA can only form predominantly monomers with very few dimers (Fig 2A). To confirm these findings, we checked binding assay by IP and found that FLAG-tagged WT LUMA could coimmunoprecipitate V5-tagged WT LUMA, but the binding to M1 LUMA was markedly reduced. Consistently, FLAG-tagged M1 LUMA could bind a reduced

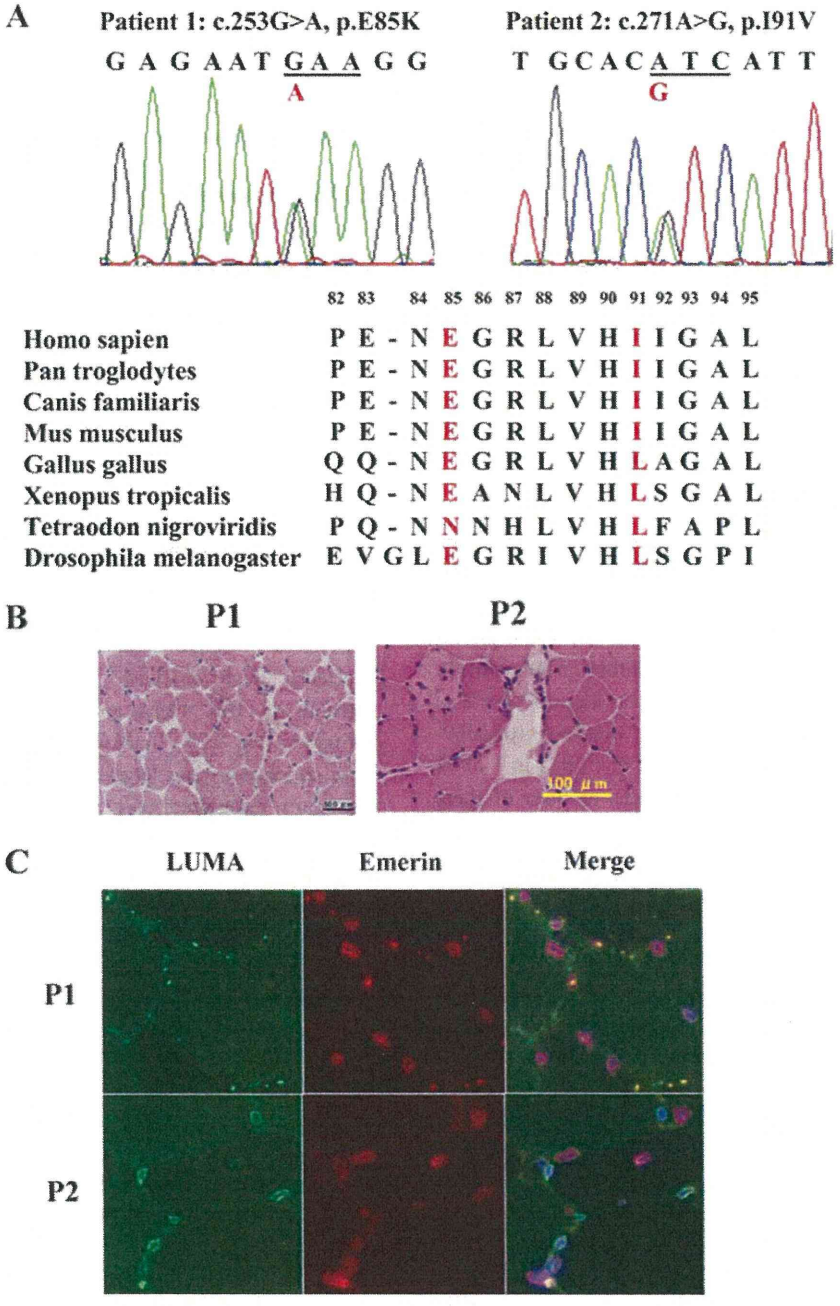

FIGURE 1: (A) DNA sequence illustration of patients shows *TMEM43* mutations c.253G>A and c.271A>G; multiple alignment of amino acid for part of *TMEM43* exon 3 shows conservation of glutamate at position 85 for p.Glu85Lys and isoleucine at position 91 for p.Ile91Val. (B) Hematoxylin and eosin staining in biceps brachii of Patient 1 (P1) showed marked fiber size variation with internalized nuclei. In Patient 2 (P2), necrotic and regenerating changes were observed. (C) Immunohistochemistry of biopsied muscle from biceps brachii showed fainter staining intensity of LUMA (green) in Patient 1 compared to Patient 2. Normal emerin staining (red) is seen in all muscles.

amount of V5-tagged WT LUMA and also M1 LUMA. Conversely, FLAG-tagged M2 LUMA could bind to both V5-tagged WT and M2 LUMA similarly (see Fig 2B). These results suggest that WT and M2 LUMA can form oligomers, but the oligomerization of M1 LUMA was impaired.

Interaction of LUMA with Emerin and SUN2

To know whether mutant LUMA could interact with emerin and other INM proteins, we performed IP. We found that WT and both M1 and M2 LUMA could coimmunoprecipitate with emerin and SUN2 (see Fig 2C), but

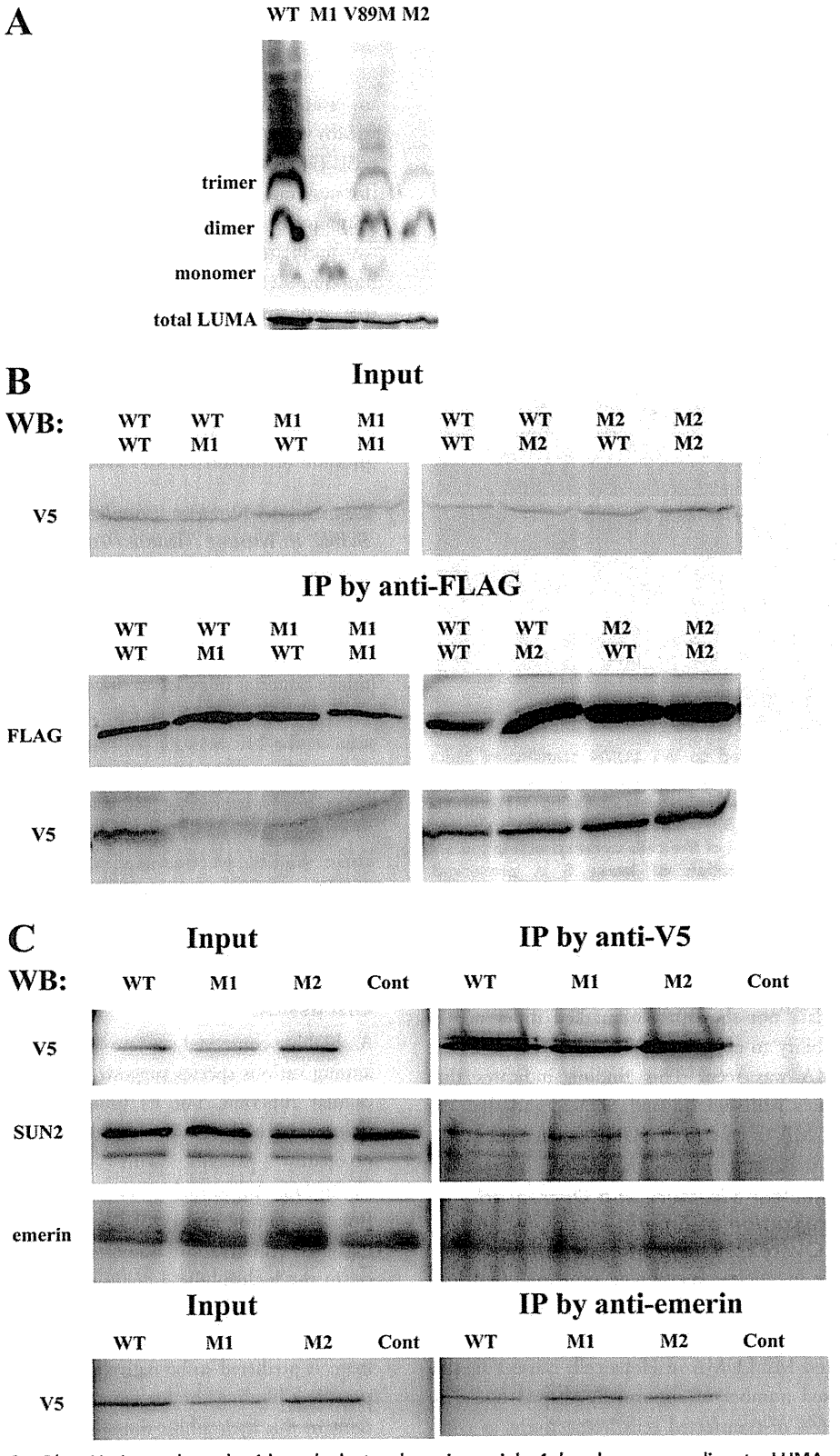

FIGURE 2: (A) On Blue Native polyacrylamide gel electrophoresis, mainly 1 band corresponding to LUMA monomers was detected in HeLa cells overexpressing M1 LUMA, whereas multiple bands corresponding to LUMA monomers, dimers, trimers, and oligomers were identified in the cells overexpressing WT, V89M, and M2 LUMA. (B) On binding assay, FLAG-tagged M1 LUMA coimmunoprecipitated reduced amount of V5-tagged WT and M1 LUMA, whereas no marked difference of binding ability was seen between WT and M2 LUMA, indicating impaired oligomerization of M1 LUMA. (C) Immunoprecipitation (IP) showed that both emerin and SUN2 were coimmunoprecipitated by anti-V5 antibody. V5-tagged LUMA was also coimmunoprecipitated by antiemerin antibody. No noticeable difference of binding ability to emerin and SUN2 was seen among WT, M1, and M2 LUMAs. V89M is a possible polymorphism, used as control. WB = Western blotting; WT = cells transfected with wild-type LUMA construct; M1 = cells transfected with E85K mutant LUMA construct; M2 = cells transfected with I91V mutant LUMA construct; V5 = anti-V5 antibody, recognizing V5-tagged LUMA.

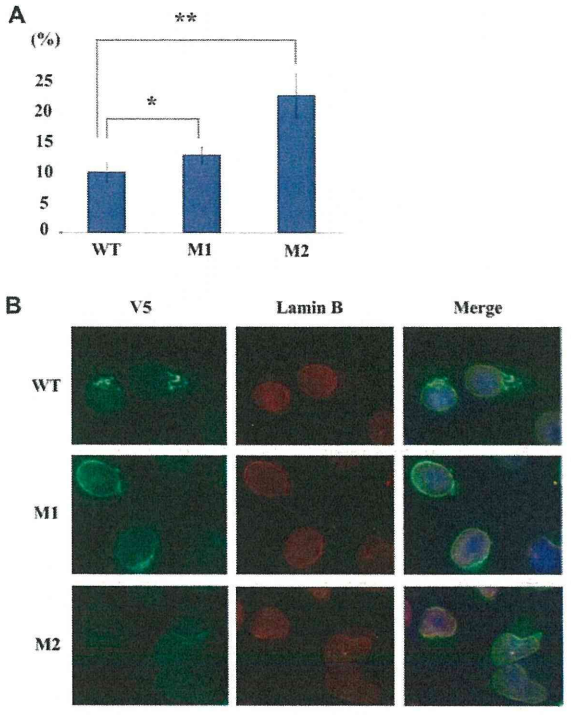

FIGURE 3: (A) Compared to WT LUMA transfected HeLa cells (10.0%), abnormal nuclear shape was observed in 12.8% and 22.6% of the cells overexpressing M1 and M2 LUMA, respectively. (B) Immunocytochemistry showed abnormally shaped nuclei of the cells overexpressing mutant LUMAs. Nuclear localization of lamin B is preserved. *$p < 0.05$, **$p < 0.01$. WT = cells transfected with wild-type LUMA construct; M1 = cells transfected with E85K mutant LUMA construct; M2 = cells transfected with I91V mutant LUMA construct; V5 = anti-V5 antibody.

not SUN1 (data not shown). No marked difference in the binding ability to emerin or SUN2 between WT and mutant LUMA was seen. This finding indicates that LUMA interacts with emerin and SUN2, and that the binding of LUMA to emerin or SUN2 was not affected.

Abnormal Nuclear Structure and Decreased Nuclear Localization of Emerin and SUN2 in HeLa Cells Overexpressing Mutant LUMA

As the defects in nuclear membrane proteins often lead to altered nuclear shape, we examined nuclear morphology by staining lamin B. We observed that overexpression of M1 and M2 LUMA in HeLa cells cause a significantly increased number of abnormally shaped nuclei in 12.8 and 22.6% of transfected cells, respectively, as compared to WT (10.0%) (Fig 3A, B; $p < 0.05$). We further checked the localization of mutant LUMA and its binding proteins. Overexpressed V5-tagged mutant LUMA could localize to the NE similar to WT LUMA, but some extranuclear aggregates were seen. Interestingly, emerin staining was reduced at the NE and mislocalized to endoplasmic reticulum (ER) with mutant LUMA, which was more prominent in M2 LUMA transfected cells (Fig 4A). In contrast, the nuclear localization of lamins was not affected by overexpression of mutant LUMA (Supporting Information Fig 3A). In addition to emerin, we found that the nuclear membrane staining of SUN2, but not SUN1 (data not shown), was also decreased in the cells overexpressing mutant LUMA, especially M2 LUMA (see Fig 4B). Intriguingly, no aggregate of SUN2 was seen in these cells. Nuclear localization of other nuclear membrane proteins, including nesprins and LAP2, another LEM-domain protein, was not affected by overexpression of mutant LUMA (data not shown).

Decreased Nuclear Localization of Emerin and SUN2 in Mouse Tibialis Anterior Muscle Overexpressing Mutant LUMA

To confirm whether overexpression of mutant LUMA could also cause mislocalization of emerin and SUN2 in vivo, we electroporated FLAG-tagged LUMA constructs to tibialis anterior muscles of 8-week-old WT mice. No remarkable difference of emerin and SUN2 expression was seen at the NE between WT and M1 LUMA transfected muscle fibers (data not shown). However, in the muscle fibers highly expressing M2 LUMA, the nuclear staining of emerin and SUN2 was reduced (Fig 5). Based on the above studies, we examined the localization of emerin, SUN2, and A-type lamins in patients' muscles, but no noticeable abnormality was seen, compared to normal control muscle (see Fig 1C, Supporting Information Fig 3B).

Discussion

A highly conserved amino-acid sequence of LUMA among various species suggests its important fundamental cellular function, but its biological role is still largely unknown. In this study, we identified 2 EDMD-related myopathy patients with missense mutations in *TMEM43*. Autosomal dominant inheritance is suggested from the family history of Patient 1. In addition to having good conservation among species, M1 and M2 localize in the hydrophilic domain of LUMA, which has been shown to be crucial for the maintenance of nuclear structure.[21] Moreover, the sequence of a large part of this domain is predicted to be natively unfolded and proposed to provide a binding site for signal proteins.[21,23] Thus, mutations in this hydrophilic domain might affect both the integrity of the NE and protein–protein interaction. Accordingly, these 2 mutations could be associated with EDMD-related myopathy, involving both skeletal and cardiac muscles. Interestingly, previously reported *TMEM43* mutation in the transmembrane domain, p.Ser358Leu, causes ARVC type 5 without skeletal muscle symptoms,

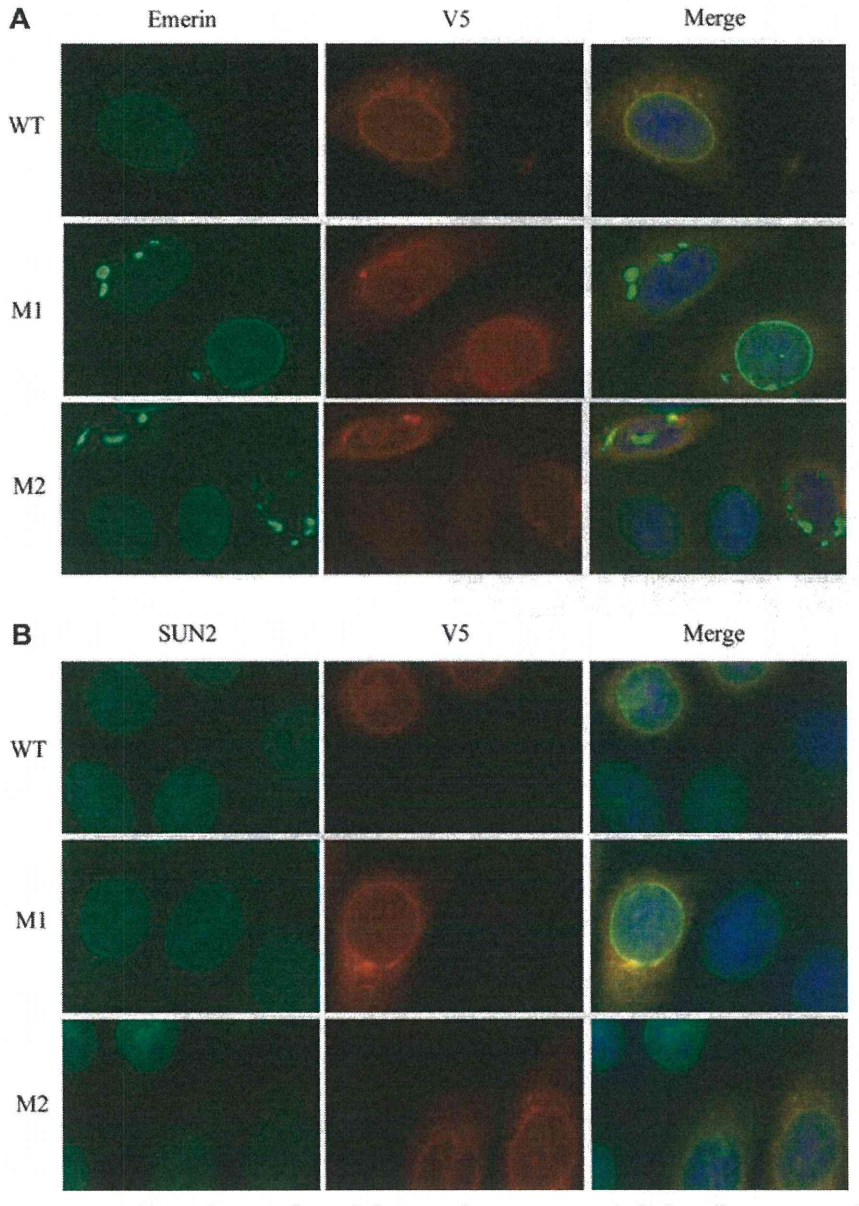

FIGURE 4: . (A) Aggregation and loss of emerin from nuclear envelope were seen in HeLa cells overexpressing mutant LUMA, especially in M2 LUMA transfected cells. (B) SUN2 staining was diminished in HeLa cells overexpressing mutant LUMA, especially in M2 LUMA transfected cells. WT = cells transfected with wild-type LUMA construct; M1 = cells transfected with E85K mutant LUMA construct; M2 = cells transfected with I91V mutant LUMA construct; V5 = anti-V5 antibody.

which indicates that different domains of LUMA may be involved in different biological functions, eventually leading to distinct phenotypes.

Our study confirmed that LUMA can form homo-oligomers.[21] Although LUMA oligomers were proposed to provide a platform for formation or organization of protein complexes at the NE,[21] its precise role is unclear. The defect of oligomerization in M1 LUMA might be related to the reduced LUMA staining observed in the muscle from Patient 1, although the precise mechanism still awaits further elucidation. The reduced NE expression of emerin, together with its mislocalization into the ER and formation of aggregates with mutant LUMA, indicates that M2 LUMA oligomers might prevent emerin from localizing to NE properly. So far, the intracellular trafficking and nuclear localization of emerin is still not clarified,[24–26] although it might be affected by the deficiency of A-type lamins and nesprins.[4,27] Our findings suggest that LUMA may also be a determinant for the proper nuclear localization of emerin. Further investigations are necessary

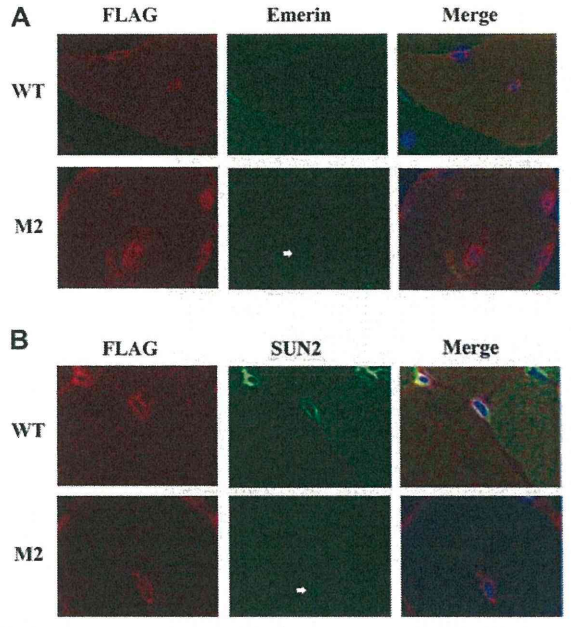

FIGURE 5: The expressions of emerin and SUN2 were much reduced in mouse tibialis anterior muscles overexpressing M2 LUMA. Arrows indicate the myonuclei highly expressing M2 LUMA. WT = cells transfected with wild-type LUMA construct; M2 = cells transfected with I91V mutant LUMA construct; FLAG: anti-FLAG antibody.

to uncover other binding partners of LUMA and the precise function of LUMA oligomerization, which would increase understanding of the whole pathomechanism.

We also demonstrated that LUMA interacts with SUN2, and overexpression of mutant LUMAs induces decreased nuclear staining of SUN2. Reduced nuclear staining of SUN2 without aggregate formation suggests that mutant LUMAs may bind SUN2 to prevent its nuclear localization, and then accelerate its degradation. SUN proteins have been known to anchor the nesprins to nuclear lamina and contribute to nuclear positioning and cellular rigidity.[28–30] However, as the interaction with SUN proteins for the normal nuclear localization of nesprin-1 is dispensable,[31] and there is a redundant role between SUN1 and SUN2,[30] the preserved nesprin localization observed in our study (data not shown) is understandable because of the normal distribution of SUN1.

The cells overexpressing mutant LUMA showed an increased number of abnormal-shaped nuclei in our study. As emerin deficiency can also lead to nuclear changes,[7] it may be argued that the abnormal nuclear shape resulted from the effect of mutant LUMA or was brought about by the mislocalization of emerin. Nevertheless, in the cells overexpressing mutant LUMA, abnormally shaped nuclei could be recognized, regardless of emerin expression at the NE, which indicates that the altered nuclear shape may not be primarily caused by the change of emerin distribution, but most likely be secondary to the effect of mutant LUMA. In addition, knockdown of SUN proteins in vitro and in vivo did not show abnormal nuclear shape, except for aberrant nuclear positioning,[30,32] suggesting that the loss of SUN2 at the NE might not be responsible for the alternation of nuclear shape. Hence, LUMA is primarily involved in the structural organization of nuclear membrane, as previously reported.[21]

In nuclear envelopathy, the mutant or deficient nuclear proteins often cause altered localization of their binding partners at the NE. For example, in the cells lacking A-type lamins or overexpressing mutant A-type lamins, the nuclear localization of LAP2, Nup153, and lamin B was altered; in addition, mislocalization of emerin and lamins in cells with nesprin deficiency was also reported.[4,27,33] In this study, we showed that overexpression of mutant LUMA can disturb the nuclear localization of emerin and SUN2 not only in vitro but in vivo. The effect of mutant LUMA for the correct nuclear localization of its binding partners, including emerin and SUN2, suggests its possible role in the pathomechanism of nuclear envelopathy. As emerin is an important factor to maintain nuclear integrity and regulate gene expression, mislocalization of emerin from NE in the cells expressing mutant LUMAs may lead to similar dysfunction observed in emerin deficiency. Likewise, SUN proteins have been shown to interact with lamins, emerin, and nesprins and be involved in various cellular functions.[34] Although mutations of *SUN1* and *SUN2* have not been reported to cause human muscular diseases so far, the mislocalization of SUN2 are surmised to perturb the functions of SUN2 and also its binding partners at the NE. Accordingly, mutations in *TMEM43* may generate similar cellular dysfunctions present in cardiac and skeletal muscles in EDMD-related myopathy.

Acknowledgment

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Potential Conflicts of Interest

I. Nishino: grants/grants pending, Ministry of Education, Culture, Sports, Science, and Technology (Japan); Ministry of Health, Labor, and Welfare (Japan); Neuromuscular Foundation (USA); Japan Foundation for Neuroscience and Mental Health (indirectly from Genzyme); patents pending or planned, method to develop model mouse for distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy; development of therapy for distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy; method to diagnose congenital muscular dystrophy with mitochondrial structural abnormalities; travel expenses, Neuromuscular Foundation.

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サルコメア配列異常を主病変とする筋ジストロフィーの

病因・病態の解明と治療法の開発

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Inflammatory changes in infantile-onset *LMNA*-associated myopathy

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Abstract

Mutations in *LMNA* cause wide variety of disorders including Emery–Dreifuss muscular dystrophy, limb girdle muscular dystrophy, and congenital muscular dystrophy. We recently found a *LMNA* mutation in a patient who was previously diagnosed as infantile onset inflammatory myopathy. In this study, we screened for *LMNA* mutations in 20 patients suspected to have inflammatory myopathy with onset at 2 years or younger. The diagnosis of inflammatory myopathy was based on muscle pathology with presence of perivascular cuffing and/or endomysial/perimysial lymphocyte infiltration. We identified heterozygous *LMNA* mutations in 11 patients (55%), who eventually developed joint contractures and/or cardiac involvement after the infantile period. Our findings suggest that *LMNA* mutation should be considered in myopathy patients with inflammatory changes during infancy, and that this may help avoid life-threatening events associated with laminopathy.

Keywords: Inflammatory myopathy; Laminopathy; Emery–Dreifuss muscular dystrophy; Limb girdle muscular dystrophy; Congenital muscular dystrophy; *LMNA*; Infantile; Pathology; Steroid therapy; Muscle image

1. Introduction

Laminopathy is a group of disorders caused by mutations in the *LMNA* gene encoding A-type lamins that includes autosomal forms of Emery–Dreifuss muscular dystrophy (AD- and AR-EDMD) and limb girdle muscular dystrophy type 1B (LGMD1B). EDMD is characterized by the triad of: (1) early contractures of the elbows, Achilles tendons, and posterior cervical muscles; (2) slowly progressive muscle weakness and atrophy that begins in a humeroperoneal distribution; and (3) cardiomyopathy with conduction defects which culminates in complete heart block and atrial paralysis [1]. LGMD1B patients show progressive proximal dominant muscle involvement and

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cardiomyopathy with conduction defects, but joint contracture is not prominent. The onset of these diseases is usually 2 years or later. Recently, *LMNA*-related congenital muscular dystrophy (L-CMD) was reported as a novel and severe form of laminopathy [2]. L-CMD has variable severity and can be divided in two main groups: a severe group with absent motor development and patients with dropped-head syndrome.

We recently came across an infantile-onset laminopathy patient with marked mononuclear cell infiltrations in his muscle mimicking inflammatory myopathy (Patient 1 in Table 1, Fig. 1A). This patient showed hypotonia and delayed motor milestones with elevation of serum CK levels from 3 months of age. Although, he became ambulant at 15 months of age, he presented proximal dominant muscle weakness and atrophy with no dropped-head at 2 years of age. Corticosteroid therapy was started based on the muscle pathological findings that had beneficial effects on his motor development. *LMNA* gene analysis was done at 6 years of age when his ankle and elbow joint contractures appeared and a heterozygous p.Glu358Lys mutation was identified.

From this result, we screened *LMNA* mutation in the 20 patients with the onset at 2 years or younger who were pathologically suspected as inflammatory myopathy.

2. Patients and methods

2.1. Patients

All clinical materials used in this study were obtained for diagnostic purposes and written informed consent was obtained from guardians of all patients. This work was approved by the Ethical Committee of National Center of Neurology and Psychiatry (NCNP). We retrospectively recruited patients with onset at 2 years or younger who were pathologically suspected to have inflammatory myopathy from a total of 10,874 muscle biopsies stored in the

Table 1
Clinical, radiological, and genetic findings of patients with *LMNA* mutations and inflammatory changes.

Patient #/gender/ *LMNA* mutations	Age at onset /age at biopsy/ age at last consultation	Initial signs/ CK at biopsy	Muscle pathology	Steroid treatment: responsiveness/ age at start of administration/ duration of administration	Age at acquired ambulation/ maximum motor ability	Cardiac involvement	Joint contracture	Respiratory dysfunction	CT/MRI (age)/imaging at thigh	CT/MRI (age)/imaging at calf
1/M/E358K*	3 m/2 y/11 y	Motor delay/900	IC: marked, diffuse; NR: moderate; Fib: mild	Effective/2 y/9 y	15 m/Ambulant	No	6 y: Ankles, elbows, 8 y: rigid spine	No	MRI (8 y)/ selective involvement of VL, VI, VM	MRI (8 y)/ selective involvement of SO, mGC
2/M/R249W*	10 m/10 m/12 y (Died by respiratory failure)	Motor delay/1000	IC: marked, pathy; NR: mild; Fib: mild	Effective/10 m/11 y	Unknown/ambulant	9 y: Heart failure	4 y: Ankles, knees	9 y: Nocturnal NPPV	ND	ND
3/M/N39D	11 m/1 y/16 y	Motor delay/1100	IC: marked, pathy; NR: marked; Fib: mild	Effective/1 y/15 y	18 m/Ambulant	13 y: 200B0 A–V block, 15 y 3° A–V block, pacemaker implantation	1 y: Ankles, knees, hips, Rigid spine from childhood	No	CT (13 y)/DI with relative sparing of RF, GR, SA	CT (13 y)/DI
4/F/R249Q*	2 y/2 y/15 y	High CK/2000	IC: moderate, focal; NR: moderate; Fib: moderate	Effective/3 y/6 m	14 m/Ambulant	12 y: 1° A–V block	3 y: Ankles, 8 y: elbows	No	CT (6 y)/DI with relative sparing of RF, GR	CT (6 y)/ selective involvement of SO, mGC
5/M/R28Q	5 m/1 y/11 y	Motor delay/800	IC: marked, pathy; NR: moderate; Fib: moderate	Ineffective/1 y/2 y	18 m/9 y: Inability to walk	Atrial fibrillation, A–V block, PAC, PVC	No	No	CT (11 y)/DI with relative sparing of RF, GR, SA	ND
6/M/R41S	9 m/1 y/13 y	Motor delay/900	IC: moderate, diffuse, NR: moderate Fib: moderate	Ineffective/1 y/8 y	16 m/9 y: Inability to walk	11 y: PSVT attack	6 y: Ankles, elbows	11 y: Nocturnal NPPV	MRI (10 y)/ DI/DI	MRI (10 y)/ DI/DI
7/F/K32del*	1 y/2 y/6 y	Unsteady gait/800	IC: mild, focal; NR: mild; Fib: mild	Ineffective/2 y/8 m	15 m/5 y: Inability to walk	No	2 y: Ankles	No	CT (4 y)/DI with relative sparing of RF, GR/Selective involvement of SO, mGC	CT (4 y)/DI with relative sparing of RF, GR/Selective involvement of SO, mGC
8/M/R249W*	11 m/1 y/24 y (Died by arrhythmia)	Motor delay/600	IC: marked, pathy; NR: mild; Fib: moderate	Ineffective/1 y/unknown	2 y/12 y: Inability to walk	17 y: 2° A–V block, 23 y complete A–V block	17 y: Ankles, knees	No	ND	ND
9/F/L292P	1 y/8 y/10 y	Motor delay/300	IC: mild, focal; NR: moderate; Fib: marked	Unadministered	16 m/4 y: Inability to walk	6 y: LV dysfunction, 8 y: PAC, PVC	No	No	MRI (8 y)/DI with relative sparing of RF, GR, SA	MRI (8 y)/DI
10/F/R377C*	2 y/4 y/7 y (Died by heart failure)	Unsteady gait/1000	IC: moderate, focal; NR: moderate; Fib: moderate	Unadministered	10 m/ambulant	7 y: DCM (EF:32%)	5 y: Ankles	No	ND	ND
11/F/N456H	2 y/5 y/10 y	Unsteady gait/3000	IC: moderate, focal; NR: moderate; Fib: marked	Unadministered	12 m/ambulant	No	6 y: Ankle, knee, neck, 8 y: rigid spine	No	MRI (10 y)/ DI with relative sparing of RF, GR, SA	MRI (10 y)/ DI

A–V block = atrioventricular conduction block, CK = creatine kinase, CT = computed tomography, DI = diffuse involvement, EF = ejection fraction, Fib = endomysial fibrosis, GR = gracilis, IC = inflammatory cellular infiltration, LV = left ventricle, mGC = medial head of gastrocnemius, MRI = magnetic resonance imaging, NPPV = noninvasive positive-pressure ventilation, NR = necrotic and regenerating process, PAC = premature atrial contraction, PSVT = paroxysmal supraventricular tachycardia, PVC = premature ventricular contraction, RF = rectus femoris, SA = Sartorius, SO = soleus, VI = vastus intermedius, VL = vastus lateralis, VM = vastus medialis.

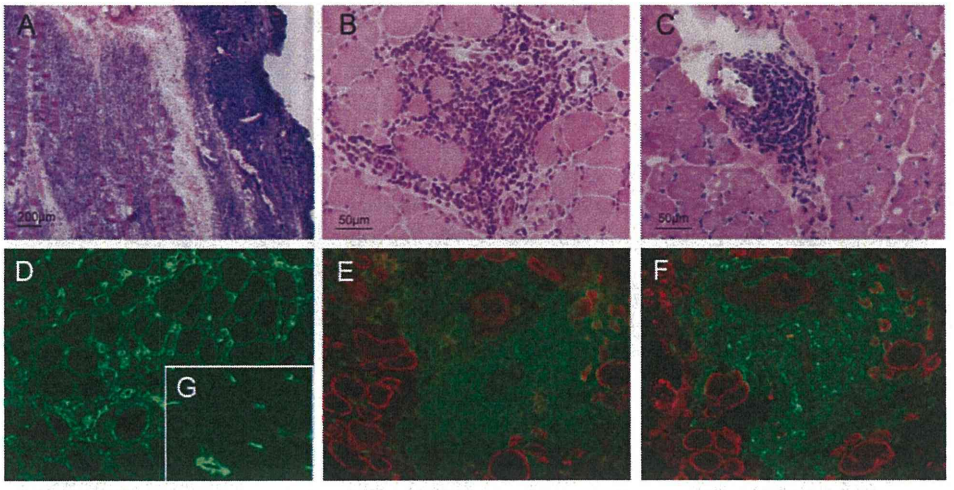

Fig. 1. Inflammatory cellular infiltration observed in the patients with *LMNA* mutations on hematoxilin and eosin staining (A: Patient 1, B: Patient 3, C: Patient 9). Serial frozen sections of muscle from Patient 5 were immunostained with HLA-ABC (D), double immunostained with CD4 (green) and dystrophin (red) (E), and CD20 (green) and dysrophin (red) (F). HLA-ABC stain in control muscle is shown in (G).

National Center of Neurology and Psychiatry. The diagnosis of inflammatory myopathy was based upon the mononuclear cell infiltrations at perimysial, endomysial, and perivascular sites [3]. Patients suspected to have dermatomyositis with skin rash and/or perifascicular atrophy on muscle pathology were excluded in this study. Then we gathered a total of 20 patients including one patient (Patient 2) who had previously been reported as infantile polymyositis [4].

2.2. Histopathological studies

All biopsied samples were taken from biceps brachii. Muscle specimens were frozen in isopentane chilled in liquid nitrogen. Serial frozen sections were stained with hematoxylin and eosin, modified Gomori trichrome, and a battery of histochemical methods. Immunohistochemical analysis was performed as described previously [5]. Antibodies used in this study are: dystrophin (DMDP-II [6], DYS1, DYS2, and DYS3 from Novocastra, Newcastle upon Tyne, UK); sarcoglycans (SGCA, SGCB, SGCG, and SGCD: Novocastra); laminin-α2 chain (ALEXIS, Farmingdale, NY); α-dystroglycan (Upstate Biotech, Lake Placid, NY); caveolin-3 (BD Transduction Laboratories, Franklin Lakes, NJ); dysferlin (Novocastra); emerin (Novocastra); collagen VI (Novocastra); CD4 and CD8 (Nichirei, Tokyo, Japan); CD20, and HLA-ABC (DAKO, Glostrup, Denmark).

2.3. Mutational analysis of LMNA

Genomic DNA was extracted from either frozen muscles or peripheral lymphocytes using standard protocols [7]. All exons and their flanking intronic regions of *LMNA* were amplified by PCR and directly sequenced using automated 3130 sequencer (PE Applied Biosystem, Foster City, CA). Primer sequences are available upon request.

2.4. Clinical information

Clinical characteristics collected from attending physicians were demographic data, age of onset, initial signs, motor functions, presence of cardiac involvement, presence of joint contractures, respiratory function, effectiveness of steroid, and pertinent laboratory examinations including serum creatine kinase (CK), electrocardiogram, Holter electrocardiogram, and echocardiogram.

2.5. Muscle imaging

Muscle computed tomography (CT) or magnetic resonance imaging (MRI) was done with some modifications depending on the facilities in each hospital. Scans were performed at thigh (the largest diameter of thigh) and calf (the largest diameter of lower leg) levels. Involvement of each muscle was evaluated at both scan levels.

3. Results

Ten types of heterozygous single nucleotide substitutions in *LMNA* were identified in 11 of 20 patients. Four (p.Arg249Gln, p.Leu292Pro, p.Asn456His and p.Arg377Cys) mutations were previously reported in patients with AD-EDMD or LGMD1B, one (p.Arg249Trp) was found only in L-CMD patients, and two (p.Lys32del and p.Glu358Lys) were identified in AD-EDMD, LGMD1B, or L-CMD patients [2,8–10]. Another three (p.Arg28Gln, p.Asn39Asp, p.Arg41Ser,) were novel mutations and not detected in 300 control chromosomes. All 11 patients had neither consanguinity nor family history of myopathy or

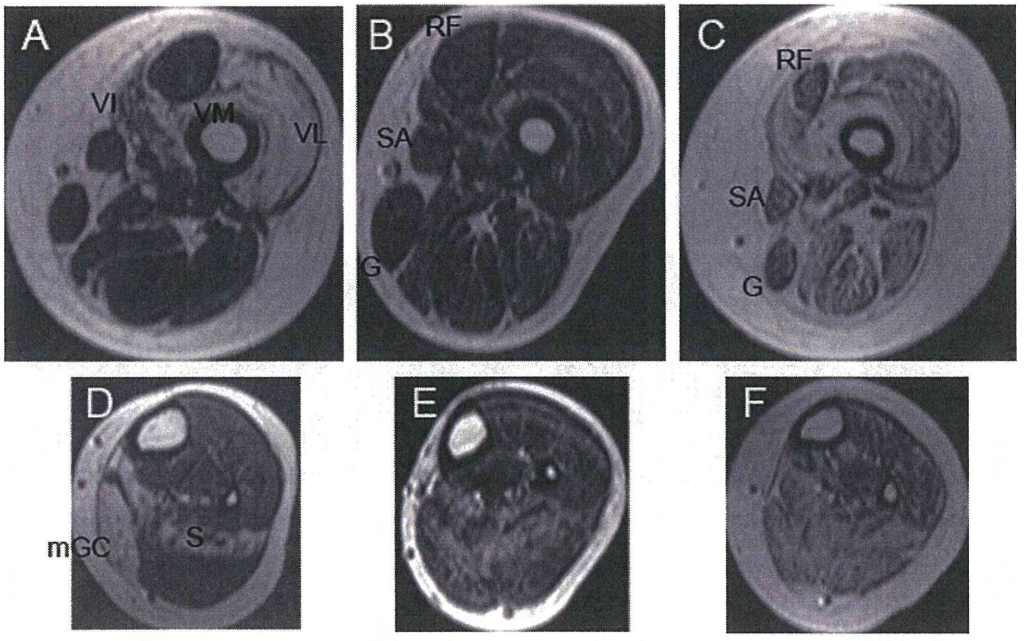

Fig. 2. Selective muscle involvement of thigh and calf muscles. Transverse sections of T1 weighted magnetic resonance imaging of thigh (A–C) and calf (D–F) in patients with *LMNA* mutations. Selective involvements of vastus lateralis (VL), vastus intermedius (VI), vastus medialis (VM), soleus (S), and medial head of gastrocnemius (mGC, A, D: Patient 1), relatively mild and diffuse involvements with relative sparing of rectus femoris (RF), gracilis (G), sartorius (SA, B, E: patient 11), and diffuse and severe involvement with relative sparing of rectus femoris, gracilis, sartorius (C, F: patient 9) are observed.

cardiomyopathy. DNA samples from the parents of 11 patients were not available.

Table 1 shows clinical summary of the 11 patients with *LMNA* mutations. Initial clinical signs were motor developmental delay or progressive muscle weakness. Head drop was not observed in any patient. Serum CK levels were mildly to moderately elevated in all patients. Joint contractures, spinal rigidity, and cardiac involvement were not observed at the time of the biopsy but became prominent in some patients in later age. Importantly, Patient 6 had an episodic paroxysmal supraventricular tachycardia during general anesthesia at age 11 years, and Patient 3 received pacemaker implantation due to complete atrioventricular conduction block at age 15 years. Patient 8 succumbed to sudden death due to arrhythmia at age 24 years and Patient 10 died by cardiac failure at age 7 years. Two patients developed chronic respiratory failure requiring non-invasive positive-pressure ventilation. Patient 2 died by respiratory failure at age 12 years. Steroid was used in eight patients but beneficial effects such as improvement of muscle power and reduction of serum CK levels were seen only in four.

On muscle biopsy, the most striking inflammatory change was observed in Patient 1 showing numerous inflammatory cells predominantly located in the perimysial connective tissue (Fig. 1A). This finding was diffusely seen in the whole muscle specimen. The other 10 patients also showed variable degrees of mononuclear cellular infiltration with active necrosis and regenerating process (Fig. 1B, C, Table 1). Fiber size variation and endomysial fibrosis were also seen. Fiber type grouping, groups of atrophic fibers, and abnormal oxidative stains were not observed. Immunohistochemically, sarcolemmal HLA staining was increased in many fibers in all patients examined (Fig. 1D). Infiltrated mononuclear cells were positive for lymphocyte markers of CD4 (Fig. 1E), CD8 (data not shown), or CD20 (Fig. 1F). No abnormal immunostaining was seen for the antibodies associated with muscular dystrophy (data not shown).

Muscle imaging was performed in relatively later stages of the disease in eight out of 11 patients with *LMNA* mutations (Fig. 2). At the level of thigh, Patient 1 showed selective involvement of vastus lateralis, vastus intermedius and vastus medialis. Patient 6 showed diffuse involvement of all thigh muscles. The remaining six patients showed diffuse involvement of thigh muscles with relative sparing of sartorius, gracilis and rectus femoris. At lower leg levels, three patients (Patients 1, 4, and 7) showed selective involvement of soleus and medial head of gastrocnemius. The remaining four patients showed diffuse involvement of calf muscles.

4. Discussion

In our series, surprisingly, more than half of the infantile patients showing inflammatory changes are due to *LMNA* mutations. Prominent mononuclear cell infiltrations can sometimes be evident in biopsies from muscular dystrophy patients including CMD, LGMD, and facioscapulohumeral muscular dystrophy, leading to misdiagnosis of inflammatory myopathy [11–16]. Apparently, however, frequency of inflammatory changes is much higher in infantile striated muscle laminopathy patients, suggesting a possibil-

ity that *LMNA* mutations may cause active inflammation in skeletal muscle during infancy by a certain mechanism. In support of this notion, three of 15 L-CMD patients report by Quijano-Roy et al. had inflammatory cell infiltration [2]. In Patients 4, 7, 9, 10 and 11, muscle biopsies were done at the age of 2 years or later and inflammatory changes were relatively milder compared to the other earlier biopsies. These findings suggest that severities of inflammation may be related to the age of biopsies.

Inflammatory myopathy manifesting with muscle weakness starting during infancy is a poorly defined muscle disorder and limited number of patients were described in the literature [4,17–20]. Thompson emphasized that responsiveness to corticosteroid is one of the crucial findings that define the infantile myositis [17]. However, this is unlikely to be always the case as some of our laminopathy patients, who were initially diagnosed as infantile-onset inflammatory myopathy also showed some clinical improvement by corticosteroid therapy. Good response to steroids is not only a feature of myositis but can also be seen in other muscular dystrophies including Duchenne muscular dystrophy. Therefore, the possibility of laminopathy should not be excluded solely based upon steroid responsiveness. Interestingly, all steroid-responsive patients were ambulant whereas non-responsive patients could not walk, which might imply some genotype–phenotype correlation. Nonetheless, the correlation between genotype and steroid responsiveness cannot be discussed at this moment as all patients for whom steroid was used had distinct mutations. In any case, corticosteroid therapy could be considered for infantile striated muscle laminopathy patients as some patients respond, although its long-term efficacy is still unknown.

The p.Arg249Trp mutation found in this study was previously reported in L-CMD patients [2], but not in AD-EDMD or LGMD1B. In contrast, p.Glu358Lys mutation has also been reported with extremely variability of phenotypes, including AD-EDMD, LGMD1B, or L-CMD [10]. Thus, the same mutation can result in different phenotypes and severities. These findings raise a possibility that other unknown factor(s) may play a role in the development of laminopathy phenotype.

Muscle imaging demonstrated selective muscle involvement in all eight patients examined. Vastus lateralis and intermedius were markedly affected, while involvement of adductor magnus was minimal. In addition, medial head of the gastrocnemius was remarkably involved while lateral head was relatively spared in most patients. This selective muscle involvement is basically identical to that observed in AD-EDMD/LGMD1B patients [21] and may be helpful for the diagnosis of laminopathy in children.

Cardiomyopathy with conduction defects is a common serious clinical problem in patients with EDMD and LGMD1B [1]. In the present study, 8 of 11 patients developed cardiac complications such as arrhythmia and heart failure in their childhood and two died due to arrhythmia and heart failure, respectively. These findings clearly demonstrate that accurate diagnosis followed by periodic examination of cardiac function including electrocardiogram, holter electrocardiogram and echocardiogram, and appropriate implantation of defibrillators is necessary to avoid unexpected sudden death [22,23].

Our results expand clinical and pathological variation of striated muscle laminopathy and the inflammatory histology is an important diagnostic clue to the *LMNA* related myopathy patients. Further analysis is needed to elucidate the role of mutant A-type lamins in inducing inflammatory process during infancy.

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