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Lipid Storage Myopathy

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Abstract Lipid storage myopathy (LSM) is pathologically characterized by prominent lipid accumulation in muscle fibers due to lipid dysmetabolism. Although extensive molecular studies have been performed, there are only four types of genetically diagnosable LSMs: primary carnitine deficiency (PCD), multiple acyl-coenzyme A dehydrogenase deficiency (MADD), neutral lipid storage disease with ichthyosis, and neutral lipid storage disease with myopathy. Making an accurate diagnosis, by specific laboratory tests including genetic analyses, is important for LSM as some of the patients are treatable: individuals with PCD show dramatic improvement with high-dose oral L-carnitine supplementation and increasing evidence indicates that MADD due to *ETFDH* mutations is riboflavin responsive.

Keywords Lipid storage myopathy · Primary carnitine deficiency · Multiple acyl-coenzyme A dehydrogenase deficiency · Neutrolipid storage disease

Introduction

Lipid is defined as a hydrophobic biomolecule and consists of mainly two types of molecules: fatty acid and its derivatives including triglycerides, and sterol-containing metabolites such as cholesterol. To date, all known muscle lipid disorders are associated with dysmetabolism of fatty acid and its derivatives. Fatty acids are eventually catabolized through the β-oxidation cycle in the mitochondrial matrix and adenosine triphosphate (ATP) is produced. Short- and medium-chain fatty acids can enter cells and then mitochondria by diffusion. In contrast, long-chain fatty acids that are richer sources of ATP require specific transport mechanisms, namely fatty acid transporters at the plasma membrane and carnitine palmitoyltransferase system at the mitochondrial membranes. Not surprisingly, muscle lipid disorders are due to a defect in intracellular triglyceride (TG) catabolism, transport of long-chain fatty acids and carnitine, or fatty acid β-oxidation.

In infantile cases, clinical manifestations are often similar in all types of lipid dysmetabolism, including hypotonia, hypoketotic hypoglycemic encephalopathy, hepatomegaly, and cardiomyopathy. In late-onset cases, muscle lipid disorders usually show two different phenotypes [1]: recurrent rhabdomyolysis or progressive muscle weakness. Recurrent rhabdomyolysis has been associated with defects of intramitochondrial fatty acid transport and β-oxidation, such as deficiencies of carnitine palmitoyltransferase II, mitochondrial trifunctional protein and very-long-chain acyl-coenzyme A (CoA) dehydrogenase. Interestingly, muscle pathology usually shows only nonspecific findings with a variable degree of necrotic and regenerating changes in muscle fibers reflecting recent episodes of rhabdomyolysis and lipid droplets are not usually increased. In contrast, the muscle weakness phenotype is usually associated with increased lipid droplets in muscle fibers. Importantly, there are only four types of genetically diagnosable lipid storage myopathy (LSM): primary carnitine deficiency (PCD), multiple acyl-CoA dehydrogenase deficiency (MADD), neutral lipid storage disease with

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ichthyosis (NLSDI), and neutral lipid storage disease with myopathy (NLSDM) (Table 1). In this review, we will cover clinical, pathologic, and molecular aspects of these four LSMs.

Primary Carnitine Deficiency

PCD is an autosomal-recessive disorder, caused by the mutations in *SLC22A5*, which encodes a carnitine/organic cation transporter OCTN2 [2]. Many different mutations, including frameshift, nonsense, and missense mutations, have been reported in PCD patients. The mutations causing premature stop codons may lead to absent OCTN2 activity, whereas missense mutations result in different residual OCTN2 activity of carnitine transport. In addition, mutations in the promoter of *SLC22A5* have also been linked to Crohn's disease, an inflammatory bowel disease with autoimmune dysregulation [3].

As OCTN2 is a sodium-dependent carnitine transporter, which transfers carnitine across the plasma membrane using the sodium electrochemical gradient and because carnitine is essential for the transfer of long-chain fatty acids from the cytoplasm to the mitochondrial matrix for following oxidation (Fig. 1), defects in OCTN2 result in cytoplasmic accumulation of long-chain fatty acid, and consequently triglycerides as lipid droplets, as well as defective ATP synthesis by β-oxidation.

As mentioned earlier, infantile patients principally present with hypotonia, Reye-like syndrome, and cardiomyopathy. However, cardiomyopathy may develop in isolation or with a milder metabolic presentation during childhood or even older age [4]. Muscle weakness can also be seen. Conversely, some individuals can be asymptomatic for the whole life [5, 6], clearly demonstrating the wide variability of clinical severity in PCD. Other uncommon clinical symptoms of PCD include ventricular fibrillation and peripheral neuropathy [7, 8]. Interestingly, heterozygotes can develop cardiac hypertrophy but it is still controversial whether heterozygous mutations are directly responsible for the cardiomyopathy [9]. There is no clear correlation between genotype and phenotype, in either clinical or biochemical aspects, suggesting that the wide phenotypic variability may be related to epigenetic or exogenous factors that exacerbate carnitine deficiency [10]. Common blood tests may reveal increased levels of hepatic enzymes and creatine kinase (CK).

Muscle pathology is characterized by increased lipid droplets in both number and size in muscle fibers, especially in type 1 fibers, in addition to fiber size variation. On electron microscopy, lipid droplets are often present next to mitochondria.

Mitochondria are usually enlarged but cristae are normally aligned and no inclusions are seen in contrast to so-called mitochondrial encephalomyopathy, which show abnormal mitochondrial cristae and inclusions [11].

Measurement of free carnitine and all acylcarnitine species is essential for the diagnosis. Typically, both are extremely low in PCD. Heterozygous individuals have half-normal carnitine transport activity in fibroblasts and borderline carnitine levels in plasma [12]. Secondary carnitine deficiency should be carefully excluded, which may show decreased free carnitine level but specific species of acylcarnitine are usually elevated. In rare occasions, plasma carnitine level can be normal in PCD. Under such conditions, assessment of carnitine transport in fibroblasts may be useful to confirm the diagnosis. However, because in vitro functional measurements of OCTN2 transport activity are not widely available, screening for mutations in *SLC22A5* is an alternative way to establish the diagnosis of PCD.

PCD patients usually respond very well to high-dose L-carnitine supplementation (100–400 mg/kg/d). Early carnitine therapy has been believed to prevent the occurrence of cardiomyopathy and other irreversible organ damage [10]. In recent years, activation of peroxisome proliferator-activated receptor-α (PPAR-α) has been demonstrated to cause an upregulation of OCTN2, leading to an increase of intracellular carnitine concentration in animal models [13, 14]. In addition, a study has shown that mutant OCTN2 is retained in the cytoplasm and not localized in the plasma membrane, probably resulting in the degradation in endoplasmic reticulum (ER) [15]. Accordingly, PPAR-α agonists and the drugs reducing the efficiency of protein degradation in the ER or capable of binding OCTN2 may be potential candidates for treating PCD patients in the future.

Multiple Acyl-CoA Dehydrogenase Deficiency

MADD, also known as glutaric aciduria type II, is caused by defects in electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH) (also called ETF-ubiquinone oxidoreductase). ETF, ETFDH, and most mitochondrial enzymes associated with electron transfer system as well are flavoproteins, which contain flavin adenine dinucleotide prosthetic groups. In the process of fatty acid β-oxidation in mitochondrial matrix, high-energy electrons, produced at several acyl-CoA dehydrogenases, are transferred to respiratory chain via ETF, which then transfers electrons to ETFDH located in the inner mitochondrial membrane. ETFDH subsequently passes the electrons to ubiquinone in the respiratory chain (Fig. 1). Therefore, a defect in ETF or ETFDH can theoretically affect all acyl-CoA dehydro-

Table 1 Summary of lipid storage myopathies

Disease	Gene	Protein/ function	Clinical manifestations	Pathologic features	Laboratory findings[b]	Treatment
Primary carnitine deficiency (PCD)	*SLC22A5*	OCTN2/carnitine transport	Infantile-onset: hypotonia, hepatomegaly, encephalopathy, cardiomyopathy Later-onset: Myopathy, cardiomyopathy	Lipid accumulation (muscle, liver)	Free carnitine ↓↓ Acylcarnitines ↓↓ CK ↑, could be normal	Carnitine
Multiple acyl-CoA dehydrogenase deficiency (MADD)	*ETFA, ETFB, ETFDH*	α subunit of ETF, β subunit of ETF, ETF-QO/ electron transfer	Neonatal-onset: Congenital anomalies, hypotonia, hepatomegaly, encephalopathy, cardiomyopathy Later-onset: Myopathy, hepatomegaly, encephalopathy, episodic metabolic crisis	Lipid accumulation (muscle, liver)	Free carnitine→or↑ acylcarnitines ↑↑ C5-10 dicarboxylic aciduria with acylglycine derivatives CK ↑ to↑↑, could be normal	Riboflavin
Neutral lipid storage disease with ichthyosis (NLSDI)	*ABHD5*	CGI-58/activator of ATGL	Multisystem involvement, including ichthyosis, mild myopathy, hepatomegaly, intestinal and ophthalmologic symptoms, hearing loss, mental retardation, short stature, microcephaly	Lipid accumulation (muscle and various tissues); Jordans' anomaly (leukocyte)	CK: usually normal	None
Neutral lipid storage disease with myopathy (NLSDM)	*PNPLA2*	ATGL/triglyceride lipase	Myopathy and cardiomyopathy predominantly	Lipid accumulation (muscle and various tissues); Rimmed vacuoles[a] (muscle); Jordans' anomaly (leukocyte)	CK ↑or↑↑	None

[a] This finding was seen in some patients, not all.

b↑: mild elevation; ↑↑: moderate to marked elevation; →: no change;↓↓: moderate to marked decrease.

ATGL adipose triglyceride lipase; *CGI-58* comparative gene identification-58; *CK* creatine kinase; *ETF* electron-transfer flavoprotein; *ETF-QO* ETF-coenzyme Q oxidoreductase; *OCTN2* plasma membrane sodium-dependent carnitine transporter.

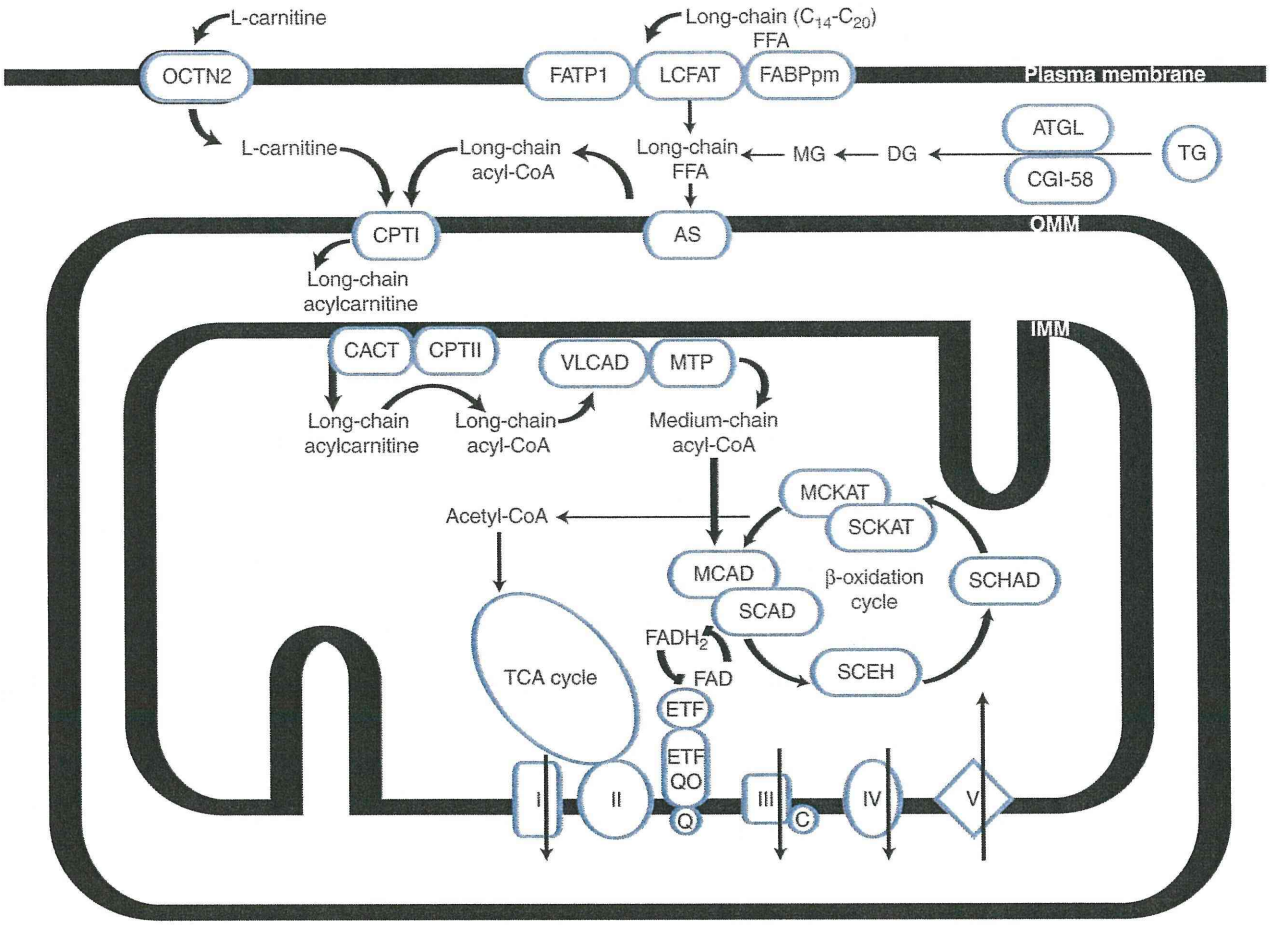

Fig. 1 Scheme of metabolic pathways of triglycerides and fatty acids. *AS* acyl-CoA synthetase; *ATGL* adipose triglyceride lipase; *C* cytochrome c; *CACT* carnitine-acylcarnitine translocase; *CGI-58* comparative gene identification-58; *CoA* coenzyme A; *CPTI* carnitine palmitoyltransferase I; *CPTII* carnitine palmitoyltransferase II; *DG* diglycerides; *ETF* electron-transfer flavoprotein; *ETF-QO* ETF-coenzyme Q oxidoreductase; *FABPpm* plasma membrane-associated fatty acid-binding protein; *FAD* flavin adenine dinucleotide; $FADH_2$ flavin adenine dinucleotide [reduced form]; *FATP1* fatty acid transporter protein 1; *FFA* free fatty acid; *IMM* inner mitochondrial membrane; *I~V* respiratory chain complex I~V; *LCFAT* long-chain fatty acid transporter; *MG* monoglyceride; *MTP* mitochondrial trifunctional protein; *OCTN2* plasma membrane sodium-dependent carnitine transporter; *OMM* outer mitochondrial membrane; *Q* coenzyme Q; *SCAD/MCAD* acyl-CoA dehydrogenases; *SCEH* enoyl-CoA hydratase; *SCHAD* 3-hydroxyacyl-CoA dehydrogenase; *SCKAT/MCKAT* 3-ketoacyl-CoA thiolase; *TCA* tricarboxylic acid; *TG* triglycerides; *VLCAD* very long chain acyl-CoA dehydrogenase

genases in fatty acid β-oxidation and, consequently, the disease is called MADD.

Homozygous or compound heterozygous mutations in *ETFA*, *ETFB*, or *ETFDH*, which encode α- and β-subunits of ETF and ETFDH, respectively [16], are responsible for MADD. Intriguingly, MADD seem to be common in the southern Chinese population due to a probable founder mutation, c.250G> A (p.A84T) in *ETFDH*, with an estimated carrier frequency of about 0.8% in Taiwanese [17, 18]. To date, all 20 reported patients from the southern Chinese population harbor this mutation, with 16 from Taiwan [19, 20]. In contrast, according to PubMed-listed reports, only 15 MADD patients with *ETFDH* mutations have been identified in Japan, which has a 5.5 times larger population than Taiwan. This finding indicates that the incidence of MADD is likely to be much higher than previously estimated and many MADD patients may actually be underdiagnosed at least among Southern Chinese, including Taiwanese people.

The clinical phenotype of MADD is quite heterogeneous and has been classified as neonatal-onset forms with or without congenital anomalies, and mild- and/or later-onset form. The congenital anomalies include facial dysmorphism, cystic renal dysplasia, and other features. Patients with neonatal-onset forms usually present with hypotonia, hepatomegaly, nonketotic hypoglycemia, and metabolic acidosis and usually die early in infancy. Later-onset patients manifest proximal myopathy often with hepatomegaly, encephalopathy, and episodic lethargy, vomiting and hypoglycemia; these episodes can be lethal [21]. Cardiomyopathy has also been

reported in both neonatal- and later-onset MADD patients [22]. Routine biochemical tests often show mildly to moderately elevated CK levels, especially during the episodes of metabolic decompensation.

Mutations in *ETFA* and *ETFB* tend to cause neonatal-onset forms, whereas *ETFDH* mutations often present with the later-onset form [23, 24], but disease severity may not solely depend upon the primary gene defect but also upon other factors [25•]. It has been long known that a group of MADD patients are riboflavin-responsive but others are not. Recent report suggests that all riboflavin-responsive MADD (RR-MADD) are associated with *ETFDH* mutations [26•]. We also confirmed this in our own series [11, 17]. *ETFDH* mutations have also been associated with the myopathic form of coenzyme Q_{10} (CoQ_{10}) deficiency [27]. However, this association is not consistent because CoQ_{10} levels are not decreased in some MADD patients due to *ETFDH* mutations [17].

Muscle pathology is characterized by increased lipid droplets in muscle fibers as in PCD. Regenerating fibers may also be seen if muscle biopsy is performed after metabolic crisis episode, suggesting mild rhabdomyolytic events can occur during metabolic decompensation in MADD. On electron microscopy, lipid droplets are often present next to mitochondria.

Measurement of plasma carnitine, acylcarnitines, and urinary organic acid profiles is useful to make a diagnosis. Blood acylcarnitine analysis usually displays elevated concentrations of all-chain-length, but mainly medium- and long-chain acylcarnitines. Plasma free carnitine level is usually decreased but can sometimes be normal. Urine organic acid analysis typically shows C5 to C10 dicarboxylic aciduria and acylglycine derivatives. In addition, reduced biochemical activities of other mitochondrial enzymes, including flavin-dependent and respiratory chain enzymes, have also been reported in MADD [26•, 28, 29], although it is still unknown if the mitochondrial dysfunction is directly associated with *ETFDH* mutations or caused by other factors. Of note, biochemical assays occasionally show normal results between episodes of metabolic decompensation; thus, mutation analyses of *ETFA*, *ETFB*, and *ETFDH* may be the most reliable diagnostic method for MADD at this moment.

Although the molecular mechanism of MADD is still unclear, riboflavin supplementation (100–400 mg/d) has been known to strikingly improve the clinical symptoms and metabolic profiles in a group of MADD patients, particularly those with the late-onset form. As mentioned earlier, the RR-MADD patients seem to have *ETFDH* mutations. Accordingly, riboflavin should be tried for all MADD patients. There is still a controversy about the combination therapy with carnitine. We think that L-carnitine supplementation helps when secondary carnitine deficiency is present. CoQ_{10} supplementation has also been reported to improve muscle weakness in patients with secondary CoQ_{10} deficiency, together with riboflavin use. However, this is still in dispute. Probably, CoQ_{10} supplementation should be considered only when secondary CoQ_{10} deficiency is confirmed.

Neutral Lipid Storage Diseases with Ichthyosis and Myopathy

Neutral lipid storage disease (NLSD) is a rare lipid storage disorder caused by a defect in either an adipose triglyceride lipase (ATGL; also called patatin-like phospholipase domain-containing 2 [PNPLA2]) or alpha/beta-hydrolase domain-containing protein 5 (ABHD5; also called comparative gene identification-58 [CGI-58]). ATGL catabolizes TG and releases the first fatty acid from the glycerol backbone and produces diglyceride (DG). The enzyme exhibits high substrate specificity for TG, but not DG or other lipids [30•]. ABHD5 activates ATGL and acylates lysophosphatidic acid. Activation of ATGL initiates the hydrolytic catabolism of cellular TG stores to glycerol and nonesterified fatty acids (Fig. 1). Naturally, dysfunction of these two proteins prevents the degradation of TG, resulting in the accumulation of TG in the cytoplasm of various organs including skeletal muscle in which TG accumulation is recognized as increased lipid droplets in muscle fibers.

NLSD is characterized by systemic TG deposition in multiple tissues, including skin, muscle, liver, central nervous system, and blood leukocytes. Not surprisingly, NLSD patients present with a wide variety of clinical manifestations, including myopathy, hepatomegaly, variable ophthalmologic symptoms (cataract, nystagmus, strabismus), neurosensory hearing loss, mental retardation, short stature, microcephaly, and intestinal involvement [31–33].

There are two well-characterized NLSDs: NLSDI and NLSDM. In NLSDI, which is also known as Chanarin-Dorfman syndrome, patients typically have rather extensive nonbullous congenital ichthyosiform erythroderma and thus it is called NLSDI, whereas no ichthyosis is seen in NLSDM. Of note, in NLSDI, myopathy can be seen but the weakness is usually mild. In NLSDM, patients develop slowly progressive myopathy, which can be either proximal- or distal-dominant. Importantly, cardiomyopathy is exclusively found in almost half of the patients with NLSDM [33], but not NLSDI, whereas neurosensory defects and mental retardation are commonly seen in NLSDI but not NLSDM. The CK level is usually mildly to moderately elevated. In both NLSDs, lipid accumulation is observed in leukocytes, which is called Jordans' anomaly. This intracytoplasmic lipid storage is visible on peripheral blood smear. In skeletal muscles, increased lipid droplets can be easily recognized even during the presymptomatic period.

On muscle pathology, lipid droplets are increased in both size and number. Interestingly, rimmed vacuoles are shown in the muscle fibers in a significant number of patients with NLSDM, unlike PCD and MADD [11, 34]. In addition, fiber size variation seems to be more significant than PCD and MADD. These features suggest a different pathomechanism of the disease. Because DG is a source for phospholipid, these changes might be associated with membrane phospholipid abnormalities caused by decreased DG availability, although no solid evidence is available at this moment.

NLSDI is caused by mutations in *ABHD5* [35] and NLSDM by mutations in *PNPLA2* [36•]. Null mutations in *ABHD5* have never been reported, but several mutations removing the functional domains have been identified, suggesting that the mutant ABHD5 is not completely deficient but functionally impaired [35]. Interestingly, almost all mutations in *PNPLA2* are located on the C-terminal region, leaving the probable active site of the enzyme intact but impairing the binding ability to cellular lipid droplets in vitro [37].

Up to now, there is still no effective treatment for NLSD. Although *ATGL* and ABHD5 have been known to play a crucial role in lipid metabolism, many aspects of their functions are incompletely understood. However, the appearance of ichthyosis indicates an ATGL-independent function of ABHD5 in skin and/or other organs. Mice lacking ATGL have shown defective lipolysis and altered energy metabolism, which mimics human phenotype [38], providing an opportunity to enhance understanding of NLSDM. The phenotype of ABHD5-deficient mice has not been reported yet. More detailed genetic and clinical characterization of NLSDI patients may be helpful to elucidate the biological role of CGI-58 in lipid dysmetabolism and to develop promising therapeutic strategy.

Conclusions

LSM is pathologically defined by excessive lipid deposition in muscles. However, to identify the underlying disease, detailed characterization of clinical features combined with distinctive results of biochemical assays is required. In addition, mutation analyses are usually helpful for making the final diagnosis, especially when clinical phenotype and laboratory tests show indistinguishable and nonspecific findings. Prompt diagnosis is important for treatment of patients because carnitine for PCD and riboflavin for RR-MADD have demonstrated excellent efficacy in eliminating the clinical symptoms. Although no specific therapeutic management is available for NLSD so far, accurate diagnosis is necessary to predict disease course, to provide genetic counseling, and to advance further research.

To date, except for PCD, MADD, NLSDI, and NLSDM discussed in this review, the causative genes remain unknown in the majority of patients with LSM [11]. Although secondary change due to variable metabolic alterations can also generate significant lipid accumulation in the muscles, it may be mainly caused by other undetermined primary defects in lipid metabolism. Thus, further studies for the proteins involved in lipid metabolism are crucial for discovering the novel causative genes, probing molecular mechanisms, and developing useful therapeutic strategies.

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Disclosure Conflicts of interest: W.-C. Liang: none; I. Nishino: is an employee of the National Institute of Neuroscience, NCNP; is a Guest Professor at Waseda University Faculty of Science and Engineering, Tokyo, Japan; is an Executive Board member for the World Muscle Society; is a Secretary, Executive Board member, and Founding Board member for the Asian Oceanian Myology Center; is an Executive Board member for the Japanese Society of Neurology; is an Editorial Board member for the Neuromuscular Conference in Japan; is a Founding member and Executive Board member for the Myositis Workshop in Japan; is a Scientific Advisory Board member for the Patients Association for Distal Myopathies in Japan; he reads muscle biopsies sent from all over Japan and abroad as part of his job at the National Institute of Neuroscience, NCNP; and his travel expenses were paid by the Neuromuscular Foundation when he was a speaker for the HIBM Workshop held in Los Angeles, CA, in 2009. He also has patents pending for the following: 1) The method to develop a model mouse for distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy. 2) The development of the therapy for distal myopathy with rimmed vacuoles/hereditary inclusion body myopathy. 3) The method to diagnose congenital muscular dystrophy with mitochondrial structural abnormalities.

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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

ORIGINAL ARTICLE

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.
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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

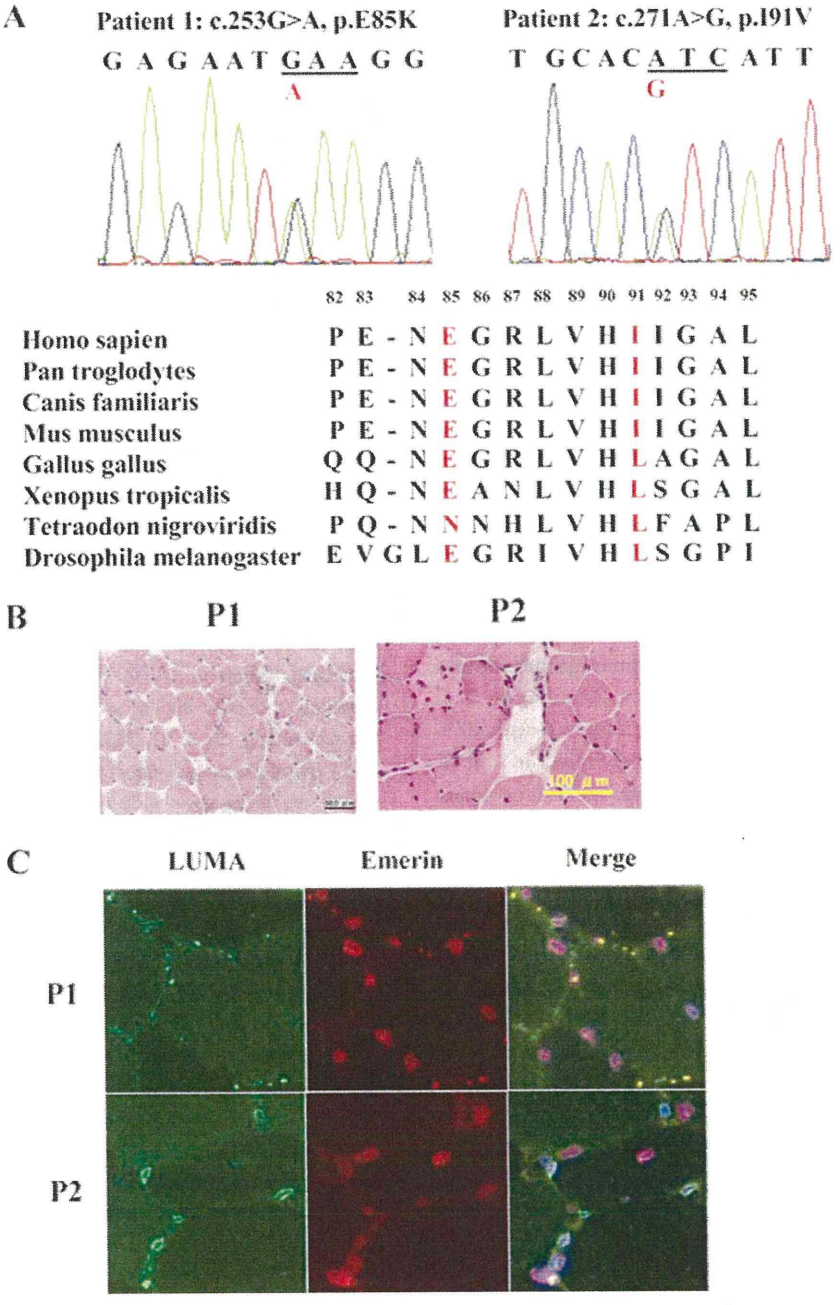

FIGURE 1: (A) DNA sequence illustration of patients shows *TMEM43* mutations c.253G>A and c.271A>G; multiple alignment of amino acid for part of *TMEM43* exon 3 shows conservation of glutamate at position 85 for p.Glu85Lys and isoleucine at position 91 for p.Ile91Val. (B) Hematoxylin and eosin staining 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

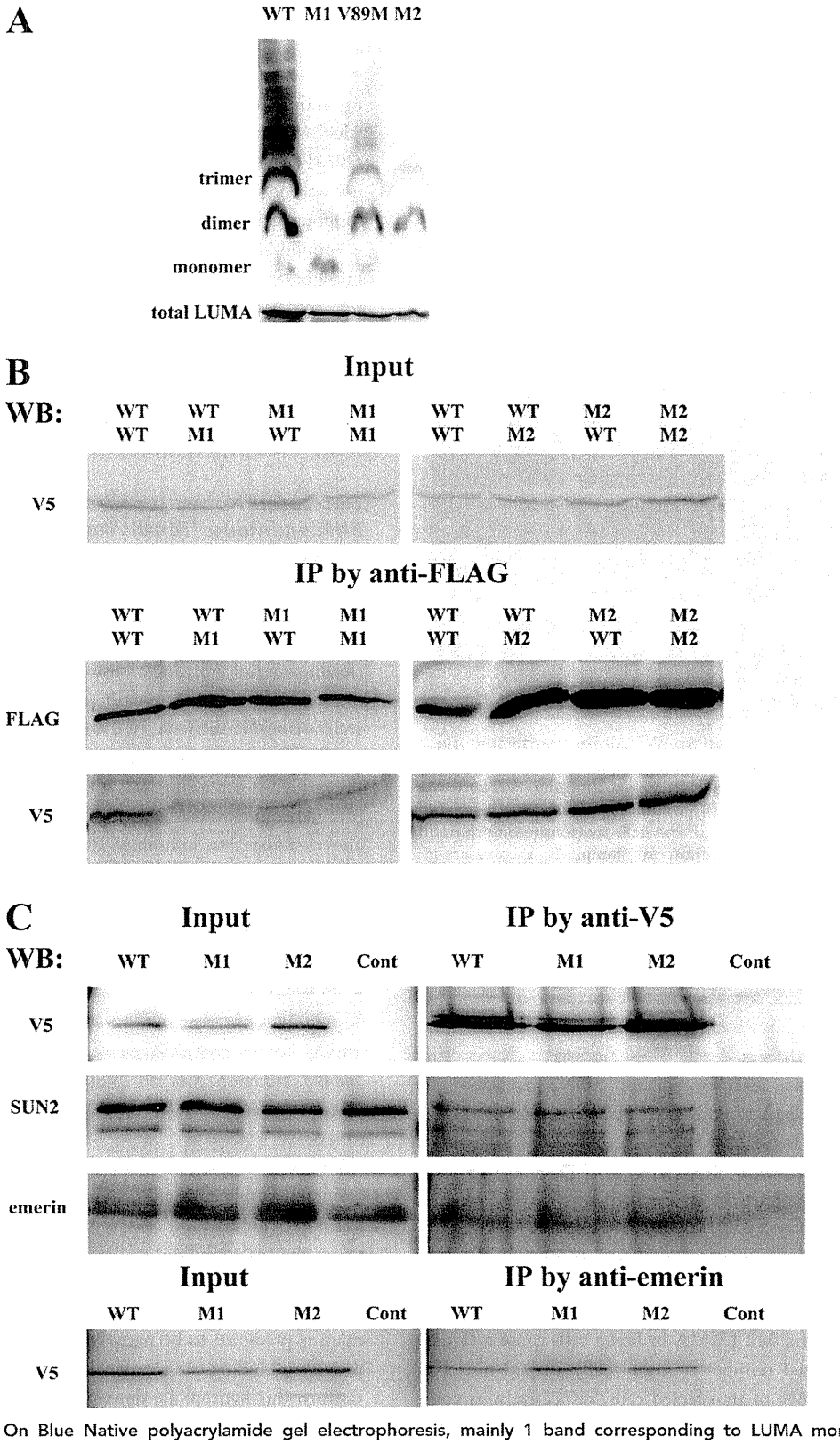

FIGURE 2: (A) On Blue Native polyacrylamide gel electrophoresis, mainly 1 band corresponding to LUMA monomers was detected in HeLa cells overexpressing M1 LUMA, whereas multiple bands corresponding to LUMA monomers, dimers, trimers, and oligomers were identified in the cells overexpressing WT, V89M, 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.

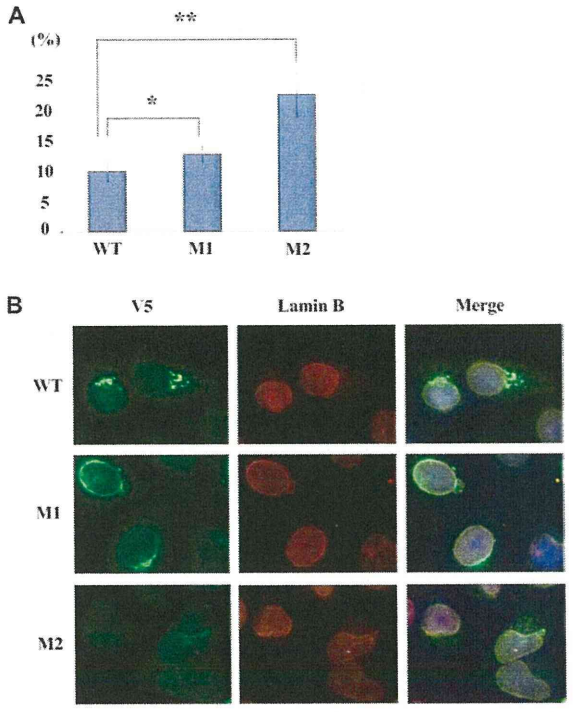

FIGURE 3: (A) Compared to WT LUMA transfected HeLa cells (10.0%), abnormal nuclear shape was observed in 12.8% and 22.6% of the cells overexpressing M1 and M2 LUMA, respectively. (B) Immunocytochemistry showed abnormally shaped nuclei of the cells overexpressing mutant LUMAs. Nuclear localization of 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,

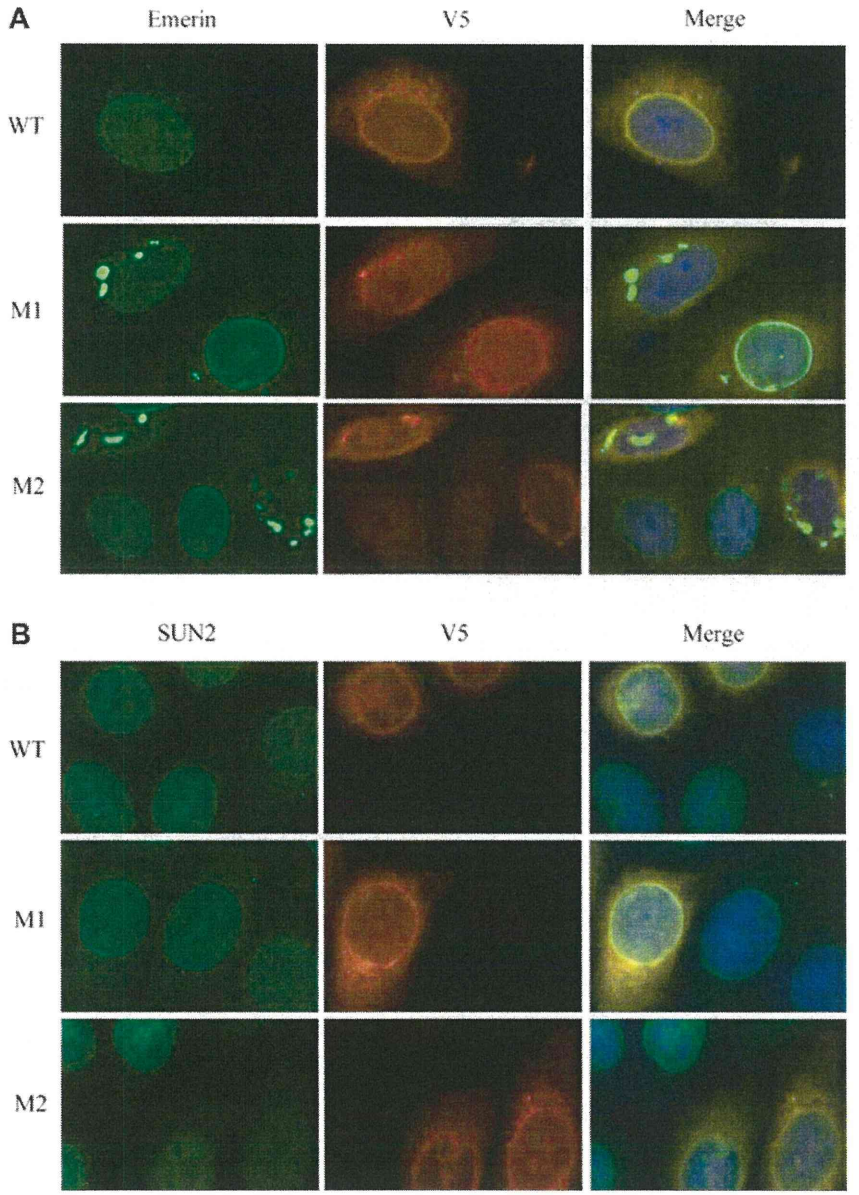

FIGURE 4: . (A) Aggregation and loss of emerin from nuclear envelope were seen in HeLa cells overexpressing mutant LUMA, especially in M2 LUMA transfected cells. (B) SUN2 staining was diminished in HeLa cells overexpressing mutant LUMA, especially in M2 LUMA transfected cells. WT = cells transfected 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

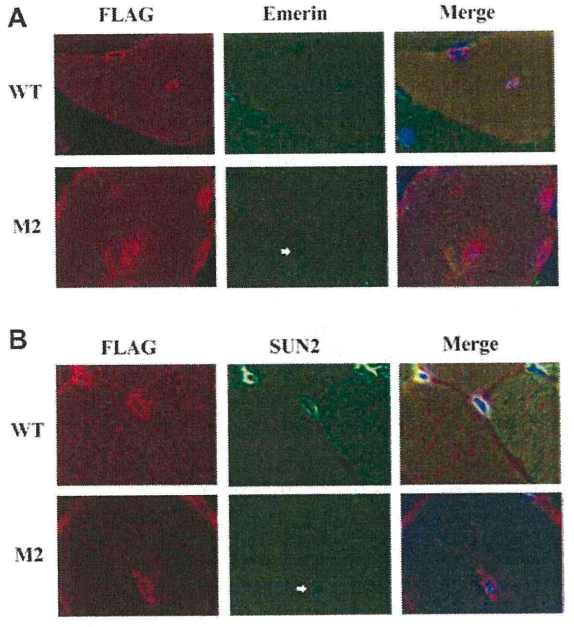

FIGURE 5: The expressions of emerin and SUN2 were much reduced in mouse tibialis anterior muscles overexpressing M2 LUMA. Arrows indicate the myonuclei highly expressing M2 LUMA. WT = cells transfected with wild-type LUMA construct; M2 = cells transfected with I91V mutant LUMA construct; FLAG: anti-FLAG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

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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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