厚生労働科学研究費補助金（創薬基盤推進研究事業（政策創薬総合研究事業））
分担研究報告書

「ヒト組織・細胞の研究資源としての高度化と公共ヒト組織・細胞バンクシステムの利用促進に関する研究」

分担研究課題　効率の良い初代ヒト培養細胞の不死化法の開発

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研究要旨　　既に収集凍結保存されている先天性筋疾患筋生検由来の細胞から不死化ヒト筋芽細胞を樹立し細胞バンク化する方法論を確立するために、効率の良いヒト筋芽細胞不死化法ならびに細胞不死化における問題点の検討を進めた。変異CDK4、Cyclin D1とhTERTをレンチウイルスベクターにより初代培養細胞に導入することで効率よく不死化できるが、変異CDK4、Cyclin D1は細胞分化を抑制する危惧がある。しかし、これまでのところ分化誘導培地へ移すことで増殖は停止し細胞融合が認められている。その分子生物学的背景を検討した。また、昨年度までに高齢者由来の筋芽細胞もと若年者由来の筋芽細胞の同様遺伝子導入により倍加時間が短く容易に継代可能な不死化できることを示してきた。両者の間に質的な差があるかどうかを調べるため、さらに症例数を殖やして不死化細胞を樹立した。また、導入遺伝子の発現量は主にプロモーター活性と組み込まれたレンチウイルスのコピー数によって決まるが、発現量の差が表現系にあたえる影響は無視してきた。不死化細胞の表現型の差が個体差なのか、不死化遺伝子の導入効率によるものかの検討を始めた。

A. 研究目的

ヒトの正常体細胞にはテロメア短縮による分裂寿命が存在するが、筋芽細胞を含むほとんどの細胞種ではそれ以前にも pRB 経路の活性化による細胞老化（premature senescence）機構が存在する。そのためヒト正常体細胞を用いた研究において、十分量の細胞数を得、再現性あるデータを取るのは一般に困難である。特に稀少疾患である先天性筋疾患由来の生検試料は極めて貴重であり、本試料を出発点とした研究の進展のためには、筋芽細胞の不死化が極めて重要である。本研究では、筋芽細胞の簡便かつ安定した不死化法を確立することである。本研究の成果は、筋疾患由来不死化筋芽細胞バンクに有効であり先天性筋疾患の発症機構、病態、治療法の開発などの研究促進が期待される。

B.　研究方法（倫理面の配慮含む）

既に不死化した正常筋芽細胞ならびに神経精神センターで凍結保存されているDucchenne型筋ジストロフィーなど１０症例の筋生検試料由来の初代培養細胞および高齢者と若年者の正常筋芽細胞などの解析を共同研究者らが検討し、その有効性を示唆するデータが蓄積されている。初代培養細胞には筋芽細胞以外の細胞種や、分化能を失った細胞も混在している。これらの初代培養細胞中の筋芽細胞は短期間の内に増殖能を失う可能性があるため、筋芽細胞を純化せず遺伝

子導入を優先し延命・不死化を先行した。高齢者と若年者由来の筋芽細胞、あるいはDMD患者と正常者由来の筋芽細胞との間の質的な差を示唆するデータを橋本博士らが得たが、個体差や不死化時の遺伝子導入効率の差を見ている可能性もあるため、症例数を増やし検討することとした。新たに高齢者由来の筋芽細胞とDMD患者由来の筋芽細胞を不死化した。また、以前不死化した正常人由来の筋芽細胞と同一の初代筋芽細胞に対しレンチウイルスのMOIを厳密に計算し遺伝子導入した。

また、本研究で用いている不死化遺伝子を他の細胞腫にも導入しており、異なる細胞腫に対する有効性を検討した。

（倫理面への配慮）

ヒト細胞全般の不死化研究については、各施設の倫理委員会の承認ならびに国立がんセンター倫理審査委員会の承認（承認番号14-69）を得ている。

C.　研究結果

高齢者と若年者の検体間で筋芽細胞の延命（不死化）効率に差は見られなかった。現在、延命（不死化）細胞の性質に差がないかを橋本らが検討中である。筋疾患患者の筋生検より得られた細胞のうちDucchenne型筋ジストロフィー３例、福山型筋ジストロフィー１例由来の筋芽細胞では延命（不死化）後の筋芽細胞の含有率が大きく異なることが橋本らにより示された。延命不死化後には数%以下の筋芽細胞でもソーティングが可能な細胞表面マーカーを橋本らが同定し、不死化後に濃縮純化をすることで比較的簡便に不死化筋芽細胞を得られることが示された。D. 考察

本研究で不死化に用いている遺伝子は転写因子ではなくpRB蛋白質をリン酸化し不活化するキナーゼ複合体を形成するCDK4/Cyclin D1とテロメア伸長酵素であるTERTの３つである。さらにCDK4はp16INK4aと結合しない変異CDK4(CDK4R24C)を用いている。従って、基本的にはG1期からS期への侵入を促進しG0への停止を防ぐことで細胞周期を回転させると共に繰り返される細胞分裂に伴うテロメアの短縮をTERTの導入により回復させることで不死化されている。細胞周期回転が良くG1期は短くなるが細胞自体の形質には比較的影響が少ないことが期待できる。しかし、筋芽細胞の分化時には細胞周期停止と細胞融合がカップルしているためCDK4R24C/Cyclin D1の高発現は分化を抑制する可能性がある。しかし、実際には分化誘導培地に移すと不死化細胞は速やかに増殖を停止し細胞融合し最終分化マーカーの発現も見られる。同じ遺伝子の組み合わせで不死化したヒト角化細胞も3次元培養により最終分化することを確認しておりDK4R24C/Cyclin D1の高発現は異なる細胞種でも最終分化にほとんど影響を与えないことが示唆された。現在分化誘導時の細胞周期停止の分子機構と分化後のS期への再侵入がどのように阻害されているかについて解析を進めている。

Cyclin D1はS期に入るとCDKによりリン酸化され核外移行しCDK4/6の活性は低下することが知られている。そのリン酸化サイトに変異を導入したCyclin D1を替わりに導入した場合、分化にどのような影響があるかを解析中である。

これまでに不死化してきた疾患由来の筋芽細胞、正常高齢者と若年者由来の筋芽細胞などの性質を比較検討している。いくつかの興味深い差異が見つかってきているが、これ

が疾患や年齢に依存しているのか、不死化の過程に影響しているのかを慎重に検討するため新たな検体から不死化筋芽細胞を樹立した。残りの研究期間で差異が再現されるか否かを検討する。

筋芽細胞の含有率の低い初代培養細胞の場合、不死化遺伝子を導入し種々の細胞種をまとめて不死化した後、細胞表面マーカーで比較的簡単に筋芽細胞のみを濃縮できる方法を橋本らが開発した。本研究の不死化技術と不死化後の筋芽細胞純化技術を合わせることにより、既存の希少筋疾患患者由来の筋生検初代培養細胞から不死化細胞バンクを構築する基本技術は確立できたものと考える。

E. 結論

これまで試した正常高齢者、若年者由来の筋芽細胞、Duchenne型筋ジストロフィー、福山型筋ジストロフィー、ミトコンドリア症、超稀少疾患などの筋手術時検体あるいは筋生検試料由来の初代培養細胞全例に対して変異CDK4＋Cyclin D1+hTERTによる延命（不死化）の有効性が示された。延命（不死化）された細胞中の筋芽細胞の含有率は様々で、純化の必要のないものから、数%以下のものまであることが分かった。しかし、橋本らが細胞表面マーカーによる筋芽細胞の濃縮法を見いだしており、数%以下のものでも濃縮と培養を繰り返すことで比較的簡単に純化できることが示されている。本研究で実証された効率の良い不死化技術は筋芽細胞の分化にはほとんど影響が見られないことから、筋芽細胞の形質を保持したまま不死化する技術はほぼ完成したと言える。また、橋本らの開発した筋芽細胞の濃縮（純化）技術と組み合わせることで、既存の希少筋疾患由来の初代培養細胞から筋疾患由来不死化筋芽細胞バンク設立のための基盤が確立された。

今後、不死化法、純化法をプロトコール化し順次、既存の希少筋疾患由来の初代培養細胞から不死化筋芽細胞を樹立し、筋疾患由来不死化筋芽細胞バンクが設立されれば未知の先天性筋疾患の発症機構、病態、治療法の開発などの研究促進が期待される。

F．健康危険情報

なし

G．研究発表

1. 論文発表

Shiomi, K., T. Kiyono, K. Okamura, M. Uezumi, Y. Goto, S. Yasumoto, S. Shimizu, and N. Hashimoto.CDK4 and cyclin D1 allow human myogenic cells to recapture growth property without compromising differentiation potential. Gene Ther 18:857-66, 2011.

H．知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得

該当なし

2. 実用新案登録

該当なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果一覧

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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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

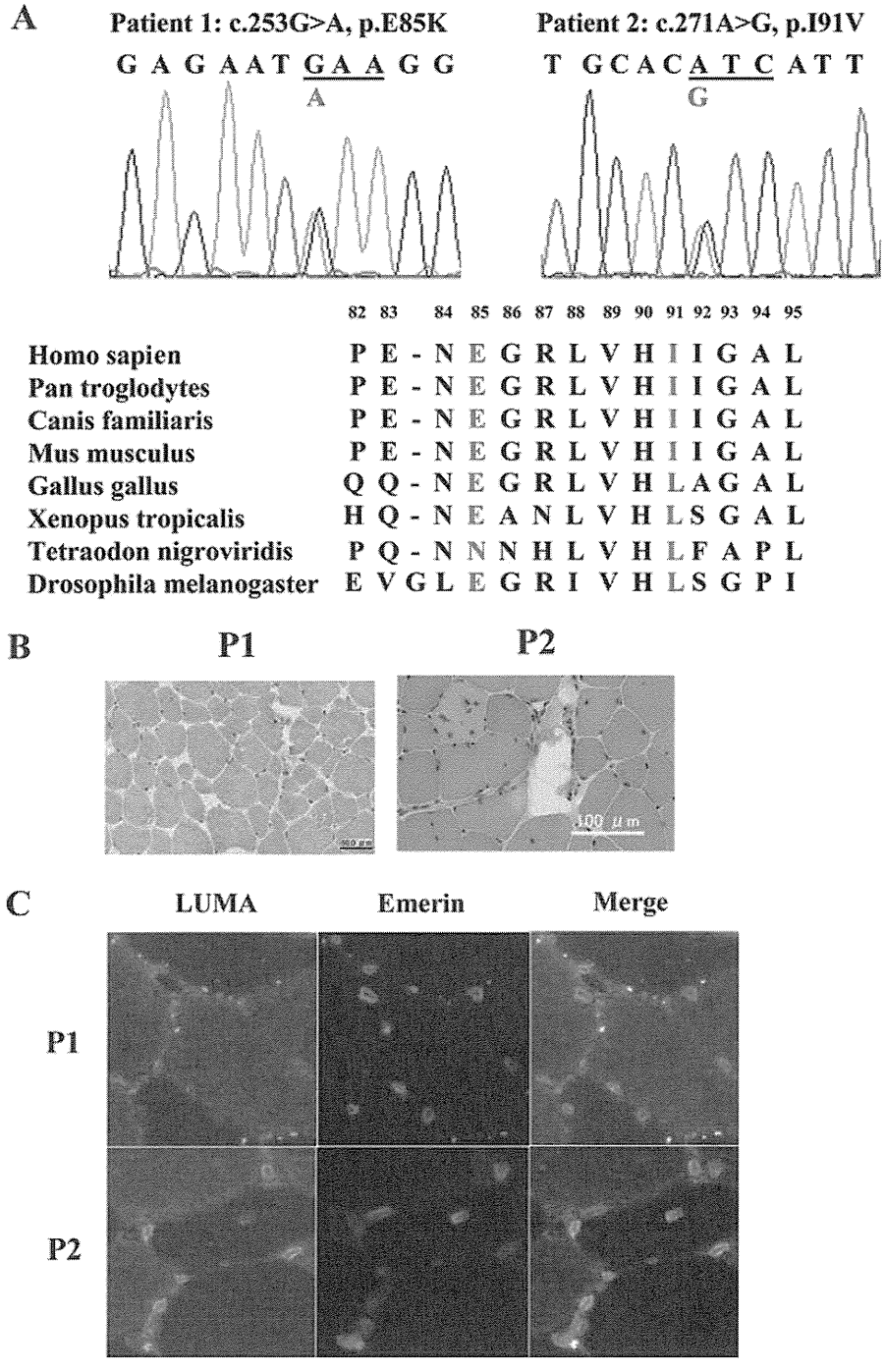

FIGURE 1: (A) DNA sequence illustration of patients shows *TMEM43* mutations c.253G>A and c.271A>G; multiple alignment of amino acid for part of *TMEM43* exon 3 shows conservation of glutamate at position 85 for p.Glu85Lys and isoleucine at position 91 for p.Ile91Val. (B) Hematoxylin and eosin staining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

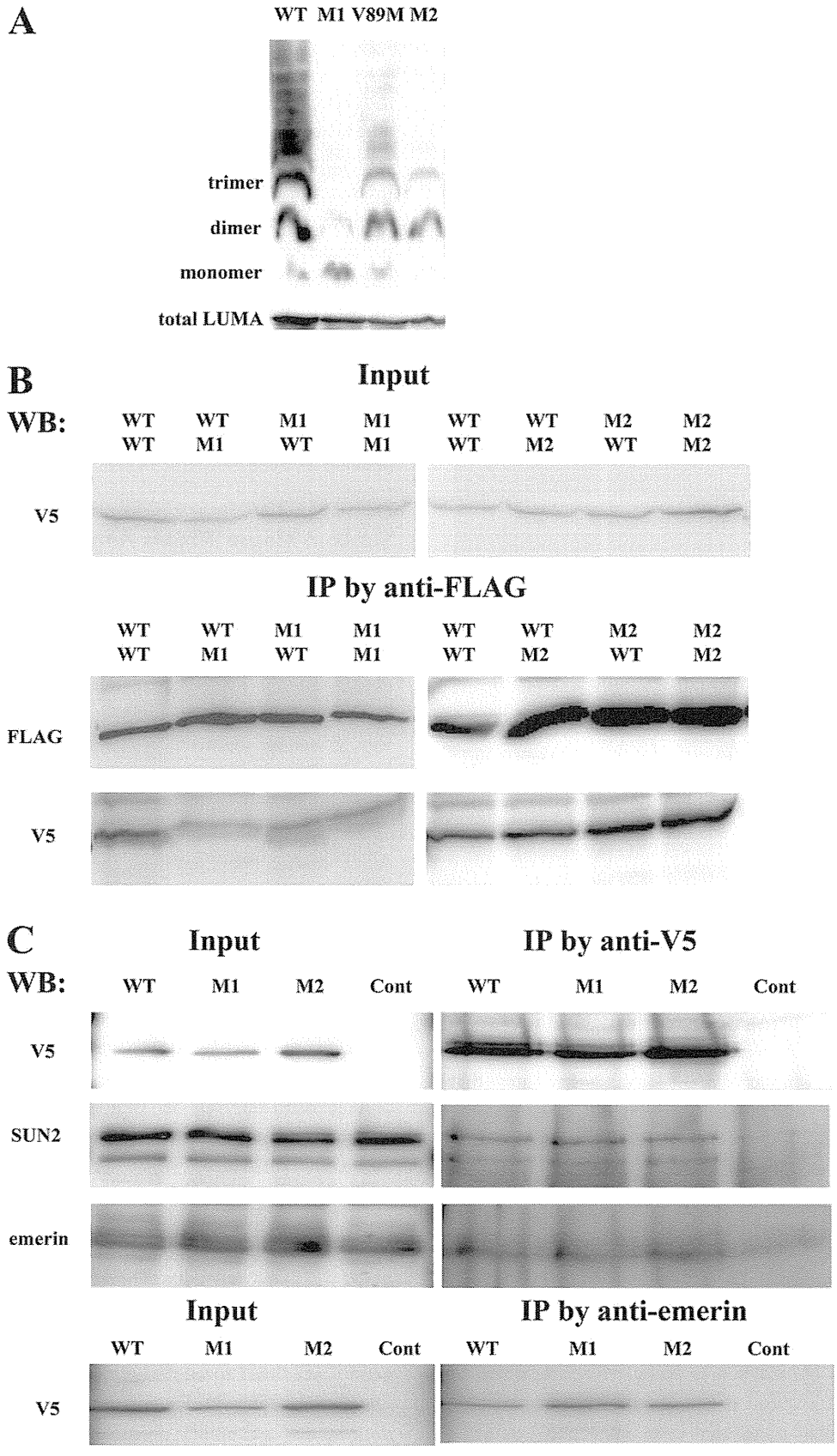

FIGURE 2: (A) On Blue Native polyacrylamide gel electrophoresis, mainly 1 band corresponding to LUMA monomers was detected in HeLa cells overexpressing M1 LUMA, whereas multiple bands corresponding to LUMA monomers, dimers, trimers, and oligomers were identified in the cells overexpressing WT, V89M,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

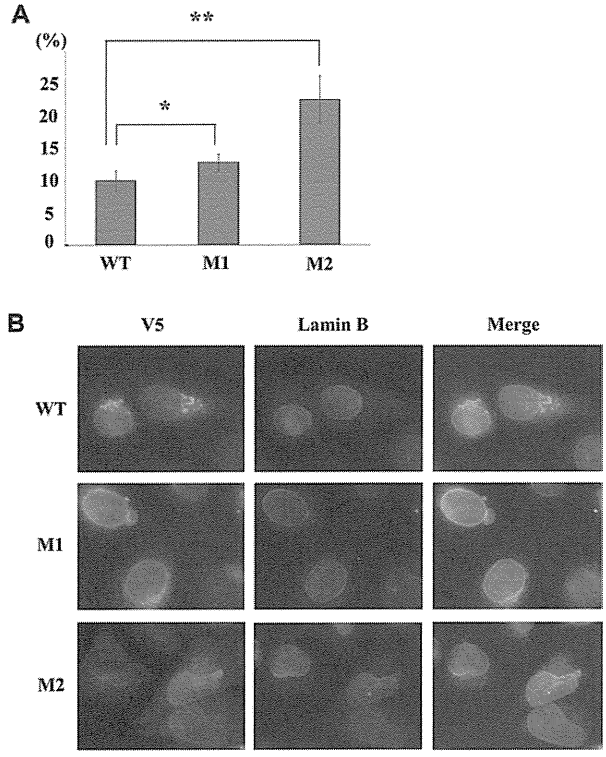

FIGURE 3: (A) Compared to WT LUMA transfected HeLa cells (10.0%), abnormal nuclear shape was observed in 12.8% and 22.6% of the cells overexpressing M1 and M2 LUMA, respectively. (B) Immunocytochemistry showed abnormally shaped nuclei of the cells overexpressing mutant LUMAs. Nuclear localization of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

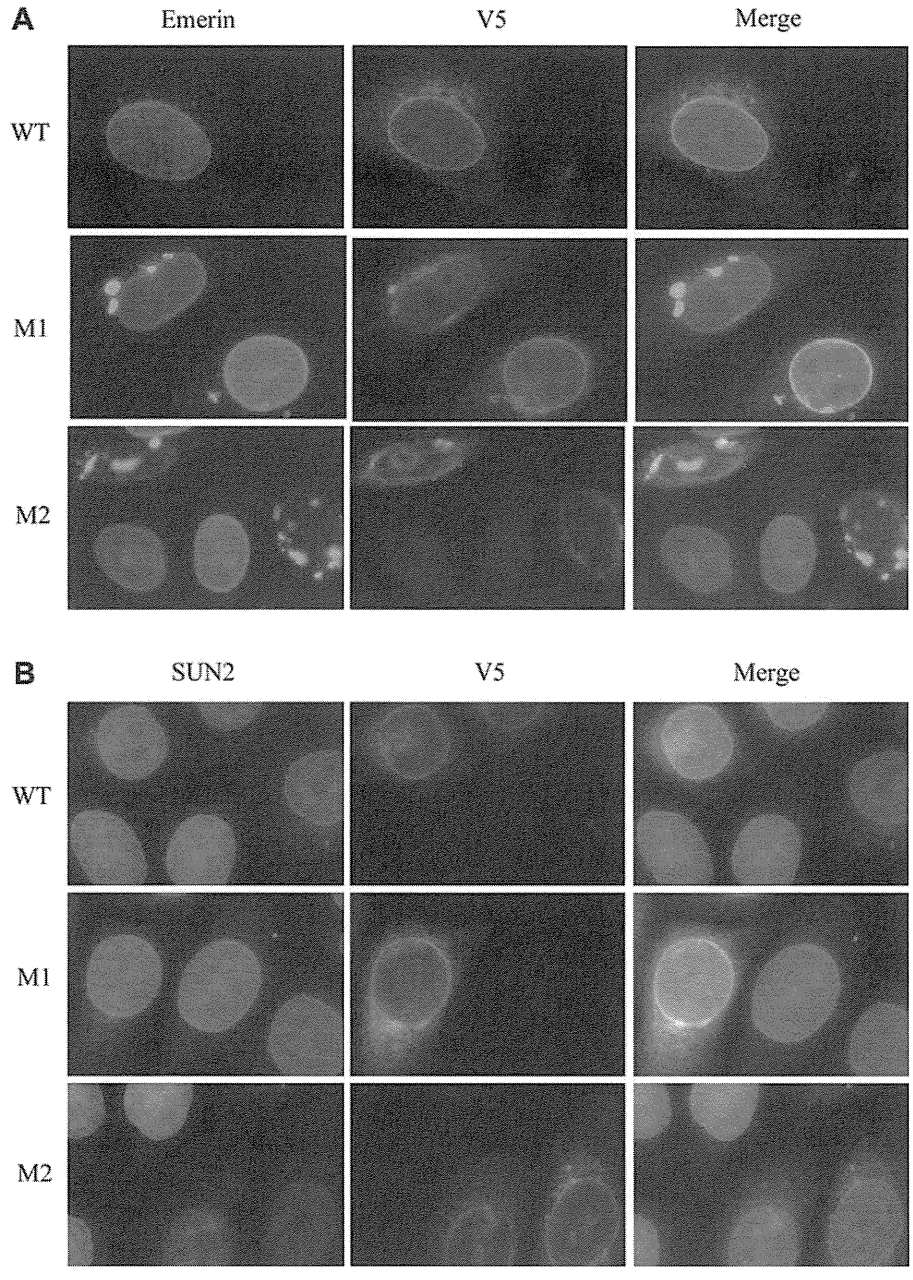

FIGURE 4: . (A) Aggregation and loss of emerin from nuclear envelope were seen in HeLa cells overexpressing mutant LUMA, especially in M2 LUMA transfected cells. (B) SUN2 staining was diminished in HeLa cells overexpressing mutant LUMA, especially in M2 LUMA transfected cells. WT = cells transfected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

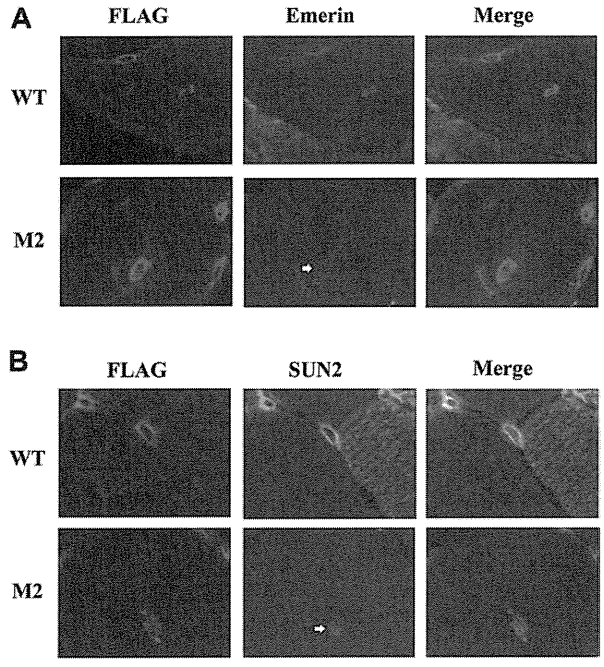

FIGURE 5: The expressions of emerin and SUN2 were much reduced in mouse tibialis anterior muscles overexpressing M2 LUMA. Arrows indicate the myonuclei highly expressing M2 LUMA. WT = cells transfected with wild-type LUMA construct; M2 = cells transfected with I91V mutant LUMA construct; FLAG: anti-FLAG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

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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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A Congenital Muscular Dystrophy with Mitochondrial Structural Abnormalities Caused by Defective De Novo Phosphatidylcholine Biosynthesis

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Congenital muscular dystrophy is a heterogeneous group of inherited muscle diseases characterized clinically by muscle weakness and hypotonia in early infancy. A number of genes harboring causative mutations have been identified, but several cases of congenital muscular dystrophy remain molecularly unresolved. We examined 15 individuals with a congenital muscular dystrophy characterized by early-onset muscle wasting, mental retardation, and peculiar enlarged mitochondria that are prevalent toward the periphery of the fibers but are sparse in the center on muscle biopsy, and we have identified homozygous or compound heterozygous mutations in the gene encoding choline kinase beta (*CHKB*). This is the first enzymatic step in a biosynthetic pathway for phosphatidylcholine, the most abundant phospholipid in eukaryotes. In muscle of three affected individuals with nonsense mutations, choline kinase activities were undetectable, and phosphatidylcholine levels were decreased. We identified the human disease caused by disruption of a phospholipid de novo biosynthetic pathway, demonstrating the pivotal role of phosphatidylcholine in muscle and brain.

A spontaneous mutant mouse with a neonatal-onset autosomal-recessive rostral-to-caudal muscular dystrophy (*rmd* mouse) due to a loss-of-function mutation in choline kinase beta (*Chkb*) was identified in 2006.[1] Interestingly, *rmd* mice exhibit a unique mitochondrial morphology in muscle fibers, which show enlarged mitochondria at the periphery of the fiber but none at the center (Figure S1). These features are similar to those seen in a congenital muscular dystrophy (CMD) that we previously reported in four Japanese individuals.[2] We therefore screened 15 genetically undiagnosed cases of CMD with fairly homogenous clinical features (Table 1) for mutations in choline kinase beta (*CHKB*); we included the four cases from in our previous study in these 15 cases. Features included peculiar mitochondrial changes in muscle as well as motor delay followed by the appearance of severe mental retardation and microcephaly without structural brain abnormalities (Figure 1 and Table 1).

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent. The study was approved by the Ethical Committee of the National Center of Neurology and Psychiatry. All mouse protocols were approved by the Ethical Review Committee on the Care and Use of Rodents in the National Institute of Neuroscience, National Center of Neurology and Psychiatry. For muscle pathology, samples of skeletal muscle were obtained from biceps brachii or quadriceps femoris in humans and from quadriceps femoris muscle in 8-week-old *rmd* mice. Muscles were frozen and sectioned at a thickness of 10 μm according to standard procedures, and a battery of routine histochemical stains, including hematoxylin and eosin (H&E), modified Gomori trichrome (mGT), NADH-tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), cytochrome c oxidase (COX), and Oil Red O, were analyzed. For electron microscopic analysis, muscles were fixed as previously described,[3] and ultra-thin sections were observed at 120kV or 80kV. All affected individuals exhibited nonspecific dystrophic features (Figure 1A). However, in mGT, NADH-TR, SDH, and COX staining, prominent mitochondria at the periphery as well as central areas devoid of mitochondria were seen (Figures 1B and 1C). Oil Red O staining was unremarkable (data not shown). Electron microscopy confirmed enlarged mitochondria (Figure 1D).

We directly sequenced all exons and their flanking intronic regions in *CHKB* (MIM 612395, NM_005198.4, GenBank Gene ID 1120) in genomic DNA extracted from individuals' peripheral lymphocytes. All 15 individuals in three different populations (Japanese, Turkish, and British) had homozygous or compound heterozygous mutations in

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Table 1. Summary of Clinical and Laboratory Features

			Phenotypic Findings									Muscle Pathology					Mutations				
Individual	Sex	Origin	Age at Last Follow-Up	Floppy at Birth	Walk Alone	Serum Creatine Kinase (IU/liter)	Head Circumference (percentile)	Mental Retardation	Seizure	Cardiomyopathy	Skin Change	Age at Muscle Biopsy	Necrotic Fiber	Regenerative Fiber	Endomysial Fibrosis	Mitochondrial Enlargement	Status	cDNA	Consequence	Exon	Literature ref. on phenotype
1	F	Japanese	died at 13 yr	+	2 yr 6 mo	370	ND	+	-	+	-	7 yr3 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
2	M	Japanese	died at 23 yr	+	1 yr 9 mo	190–2676	25–50	+	+	+	-	1 yr 2 mo	+	+	+	+	homo	c.810T>A	p.Tyr270X	7	2
3	F	Japanese	28 yr	+	1 yr 6 mo	502	ND	+	+	+	-	8 yr	+	+	+	+	het	c.116C>A	p.Ser39X	1	2
																	het	c.458dup	p.Leu153PhefsX57	3	2
4	M	Japanese	22 yr	+	2 yr 6 mo	230	3–10	+	+	-	-	4 yr 11 mo	+	+	+	+	het	c.116C>A	p.Ser39X	1	
																	het	c.458dup	p.Leu153PhefsX57	3	
5	M	Turkish	7 yr	-	2 yr 6 mo	843	<3	+	-	-	+	6 yr	±	+	+	+	homo	c.611_612insC	p.Thr205AsnfsX5	5	
6[a]	M	Turkish	died at 2 yr 6 mo	+	no	258	<3	+	-	+	-	1 yr 3 mo	±	±	+	+	homo	c.922C>T	p.Gln308X	8	
7	F	Turkish	2 yr	-	no	368	3-10	+	-	-[b]	-	9 mo	-	±	+	+	homo	c.847G>A	p.Glu283Lys	8	
8	M	Turkish	13 yr	ND	2 yr	1122	ND	+	-	-	-	12 yr 10 mo	±	±	+	+	homo	c.1130 G>T	p.Arg377Leu	11	
9	F	Turkish	17 yr	+	3 yr	2669	<3	+	-	ND	-	17 yr	±	±	+	+	homo	c.554_562del	p.Pro185_Trp187del	4	
10	F	Turkish	16 yr	+	3 yr	1103	<3	+	-	-[c]	+	3 yr	-	±	+	+	homo	c.677+1G>A	ND	5	
11	F	Turkish	3 yr 3 mo	+	no	497	10-25	+	-	ND	-	3 yr	±	-	+	+	homo	c.677+1G>A	ND	5	
12	F	Turkish	5 yr	-	3 yr 6 mo	467	25–50	+	-	-[d]	+	4 yr 6 mo	±	+	+	+	homo	c.677+1G>A	ND	5	
13	M	Turkish	3 yr 6 mo	+	no	428	<3	+	-	+	+	3 yr	+	+	+	+	homo	c.1031+1G>A	aberrant splicing	9	
14	F	Turkish	6 yr 4 mo	-	1 yr 3 mo	1606	3–10	+	-	+	-	4 yr	+	+	+	+	homo	c.1031+1G>A	ND	9	
15	M	British	died at 8 yr	-	3 yr 4 mo	607–1715	<3	+	-	+	+	2 yr 2 mo	+	-	+	+	homo	c.852_859del	p.Trp284X	8	

Detailed clinical information for individual 1 to 4 was previously described (2). Eleven CHKB mutations were identified in 15 affected individuals. All exhibited generalized muscle hypotonia and weakness from early infancy. Ambulation was delayed, and gait in those who achieved walking was limited. In addition, all displayed marked mental retardation, and most never acquired meaningful language. Microcephaly with head circumferences at or below the 3rd to 10th percentile was observed in most cases. Cranial magnetic resonance imaging showed no developmental brain defects. Six individuals had dilated cardiomyopathy, and two had cardiac anomaly. Individuals 1, 2, 6, and 15 died from cardiomyopathy at ages 13 yr, 23 yr, 2 yr 6 mo, and 8 yr, respectively. No one had respiratory insufficiency. Ichthyosiform skin changes were frequent. All showed mildly to moderately elevated serum creatine kinase (CK) levels. Individuals 7 and 9 also had homozygous single-nucleotide substitutions, c.902C>T (p.Thr301Ile) and c.983A>G (p.Gln328Arg), respectively. CHK activities of recombinant CHK-β proteins with p.Thr301Ile and p.Gln328Arg were only mildly decreased (Figure S2), suggesting these are likely to be neutral polymorphisms or only mildly hypomorphic mutations. Individuals 10, 11, and 12, who have same c.677+1G>A mutation, and individuals 13 and 14, who have same c.1031+1G>A mutation, are not siblings. Abbreviations are as follows: ND, not determined; p, percentile; F, female; and M, male.

[a] An affected sibling had ichthyosis and died at age 6 years with cardiomyopathy.
[b] Patent ductus arteriosus.
[c] Atrial septal defect.
[d] Mitral valve prolapse.

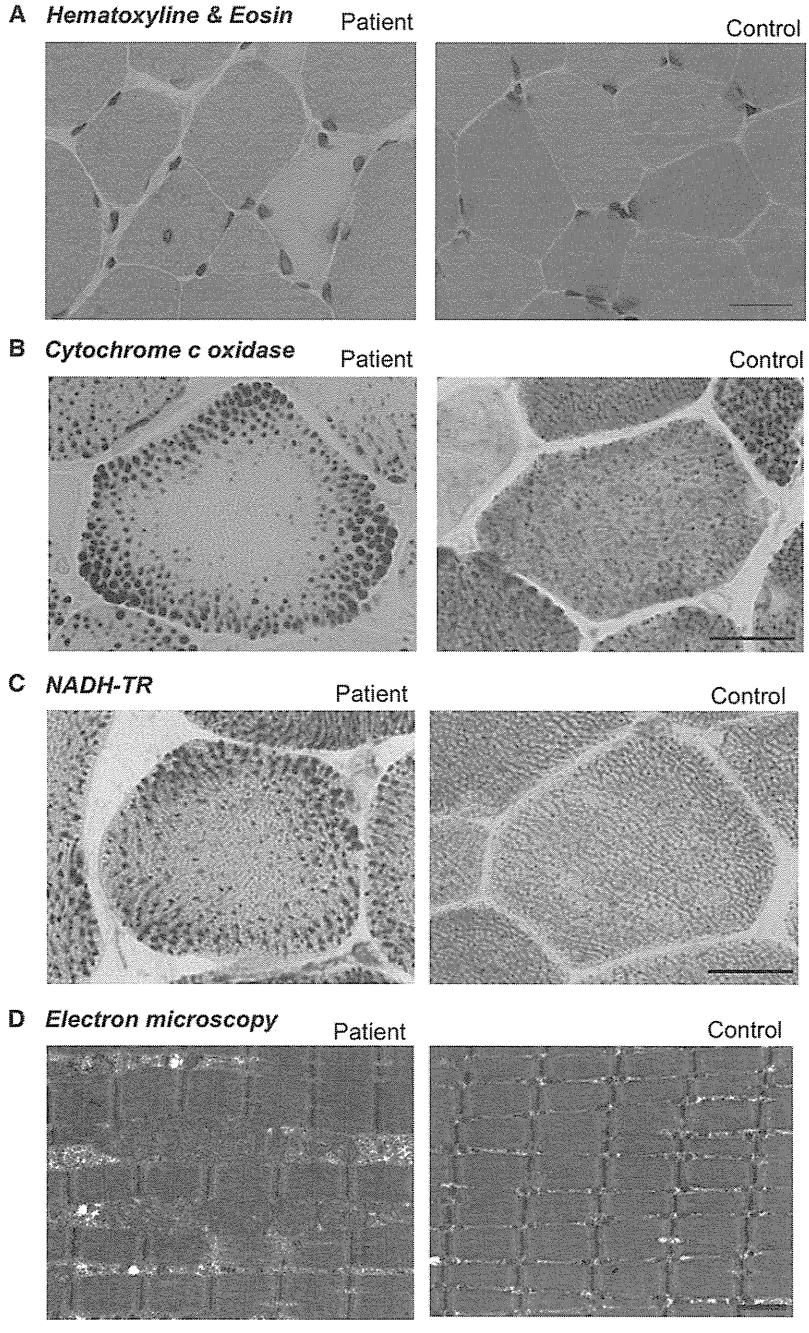

Figure 1. Muscle Pathology of the Affected Individuals

Cross-sections of muscle fiber from a human control and individual 4.

(A) On H&E staining, nonspecific dystrophic features with necrotic and regenerating fibers, internalized nuclei, and endomysial fibrosis are seen. The scale bar represents 25 μm.

(B) On cytochrome c oxidase staining, enlarged mitochondria at the periphery and central areas devoid of mitochondria were seen. The scale bar represents 20 μm.

(C) On NADH-TR staining, the intermyofibrillar network was preserved even in the central areas that are devoid of mitochondria, suggesting the presence of myofibrils and only absence of mitochondria. The scale bar represents 20 μm.

(D) Electron microscopy confirmed enlarged mitochondria. The scale bar represents 1 μm.

CHKB (Table 1). Among a total of 11 mutations identified, six were nonsense, two were missense, one was a 3 amino acid deletion, and two were splice-site mutations. The six nonsense mutations, c.116C>A (p.Ser39X), c.458dup (p.Leu153PhefsX57), c.611_612insC (p.Thr205AsnfsX5), c.810T>A (p.Tyr270X), c.852_859del (p.Trp284X), and c.922C>T (p.Gln308X), were predicted to truncate the protein and eliminate highly conserved domains of CHK.[4,5] Individuals 1 and 2 (unrelated, Japanese) had the same homozygous nonsense mutation of c.810T>A (p.Tyr270X). Individual 2's mother, who was healthy, had the heterozygous c.810T>A (p.Tyr270X) mutation. Unfortunately, a DNA sample from the father of individual 2 was not available. DNA samples from other family members of individual 1 and 2 were not available. Individuals 3 and 4 (siblings, Japanese) had the same compound heterozygous mutation c.116C>A (p.Ser39X) and c.458dup (p.Leu153PhefsX57). Both parents were healthy, and the father was heterozygous for mutation c.116C>A (p.Ser39X), whereas the mother was heterozygous for mutation c. 458dup (p.Leu153-PhefsX57), thus confirming a recessive inheritance pattern. These mutations cosegregated with the disease phenotype in all family members tested.

We therefore measured CHK activity in biopsied muscle. For all biochemical analyses, because of the limiting amounts of remaining tissue, biopsied muscle samples were available only from individuals 2, 3, and 4. Biopsied muscle samples from these three individuals were homogenized in 3 volumes of 20 mM Tris-HCl (pH 7.5), 154 mM KCl, and 1 mM phenylmethanesulfonyl fluoride with a sonicator (MISONIX), and supernatant fractions (105,000 × g, 60 min) were prepared and analyzed for CHK activity as previously described.[6] Similar to muscles of *rmd* mice,[1] muscles from individuals 2, 3, and 4, who carried homozygous or compound heterozygous nonsense mutations, did not have any detectable CHK activity (Figure 2A). Individuals 7, 8, and 9 had homozygous missense mutations c.847G>A (p.Glu283Lys) and c.1130 G>T (p.Arg377Leu) and a homozygous 3 amino acid deletion, c.554_562 del (p.Pro185_Trp187del), respectively.We screened 210 control chromosomes for the identified missense mutations and small in-frame deletion by direct sequencing or single-strand conformation polymorphism (SSCP) analysis. SSCP was performed with Gene Gel Excel (GE Healthcare) as previously described.[7] These missense mutations and this small in-frame deletion were not identified in control