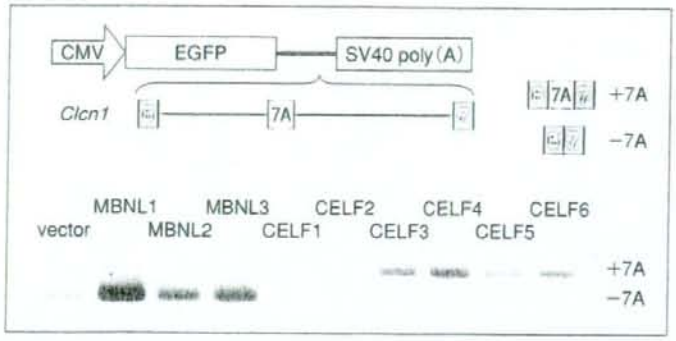

図 1　塩素チャネル遺伝子のスプライシング
MBNL ファミリーは−7A 型(成熟型)のスプライシングを促進するが，CELF ファミリーは逆に+7A 型(幼若型)スプライシングを促進する．

ころ，CUG リピートや CCUG リピートと特異的に結合することがわかった[5]．

DM の特徴的症状のひとつは，何といっても筋強直(ミオトニア)である．ミオトニアは活動電位の頻発と弛緩障害という興奮異常であり，塩素チャネルの機能低下によって起こる．DM 患者の筋では塩素チャネル CLCN1 遺伝子に異常スプライシングが起こり，エクソン 7A が入るようなスプライシングが起こって途中に停止コドンが入った幼若型 CLCN1 ができることがわかっている[6]．実はエクソン 7A をスキップさせる成熟型のスプライシングには MBNL1 が欠かせないのであるが，何らかの異常で MBNL1 が働かないために異常スプライシングが起こると推定されている．一般に，筋肉の分化過程で，機能のない幼若型から機能をもつ成熟型にスプライシングパターンが変わっていくのであるが，この変化にも MBNL1 がかかわっていると考えられている．

実は MBNL1 の発見の前に，CUG リピートと結合する蛋白質として CUG-BP(CUG 結合蛋白質)という蛋白質が同定されていた[7]．この蛋白質は核内にある長い CUG リピートに結合するといわれ，本症発症にかかわる重要な分子ではないかと推定されていた．しかし，著者らの結合特異性とスプライシング活性の研究によって，この CUG-BP は CUG リピートよりも UG リピートに強く結合することがわかり[8,9]，その関与は疑わしいともいわれるようになった．実際にヒトにはこのホモログが 6 個あり，CUG-BP を CELF1 として，CELF1-6 と再命名されている．

例として図 1 に，塩素チャネルミニ遺伝子を使った MBNL ファミリーと CELF ファミリーのスプライシング特異性を示す．この図で明らかなように，MBNL1-3 は成人型へのスプライシングを，CELF ファミリー，とくに CELF3-6，は胎児型へのスプライシングを促進することがわかる．興味深いことに，MBNL1 は CUG/CAG の二重鎖 RNA にはまったく結合せず，図 2 に示すようにミスマッチのある RNA 二重鎖に結合しやすいことが明らかになっている[5]．

このように，MBNL と CELF はたがいに逆方向の作用をしていることが多い．塩素チャネルだけでなく，インスリン受容体のスプライシングに際しても拮抗的に働くことが示されている．実際に DM 筋でどう働いているかについては次項で説明しよう．

DM筋でのスプライシング異常

著者らは西野の協力を得て，国立精神・神経センターに保管してある筋肉バンクの筋強直性ジストロフィー筋 21 例から RNA を抽出し，PCR によってスプライシングパターンを調べたところ，図 3 に示すように，塩素チャネルやインスリン受容体が幼若型優位なスプライシングを行っていることを発見した[10]．おそらくこれが DM 特有のミオトニアや耐糖能異常の原因と考えられた．

DM 筋で，これらスプライシング因子(MBNL と CELF)の発現がどう変化しているかについて

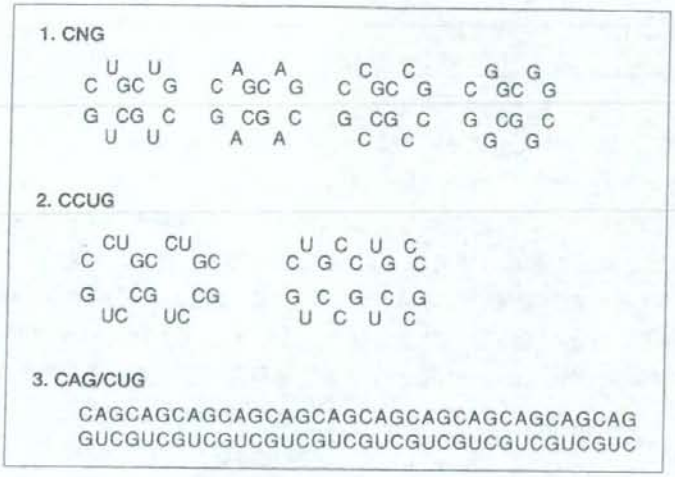

図 2　MBNL1が結合するリピート

MBNL1 は 1 と 2 のようなミスマッチのある二重鎖に結合し，3 のような完全な二重鎖には結合しない．

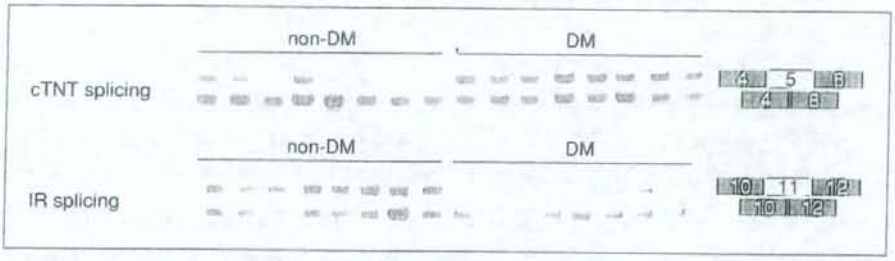

図 3　DM患者でのスプライシング異常

DM 筋では心筋トロポニン T(cTNT)の+5 型，インスリン受容体(IR)の−11 型スプライシングが促進していることが対照と比較して明らかである．

の詳細な検討はなかったので，著者らは患者筋での発現をリアルタイム PCR を用いて定量したところ，発現量が多い MBNL1，MBNL2，CELF1，CELF2 に関してはその発現はほとんど対照筋と差がないことを確認した[10]．この結果，DM 筋でのスプライシング異常は，スプライシング因子の発現量が変わっているせいではないことが明らかになった．

新しい治療法

このように，DM の原因と症状との関係は明らかになったが，発症メカニズムを利用した治療の試みはいぜんとして進んでいない．しかし，焦点が絞られてきた感がある．まず，ミオトニアの治療としては塩素チャネルのスプライシング正常化が第 1 の標的になる．図 3 で明らかなように，リピートに MBNL1 がトラップされて正常スプライシングが果たせないなら MBNL1 の発現を上げることが治療の第一歩と考えられる．また，逆方向のスプライシングに働く CELF ファミリーの遺伝子発現を低下させることも必要かもしれない．このような薬剤をスクリーニングすることは今後の治療にたいへん有用である．

その試みの一端をご紹介しよう．

1．スプライシング調節薬の探索

ヒト cDNA ライブラリーより MBNL と CELF 合わせて 9 種のリピート RNA 結合蛋白質(MBNL1, MBNL2, MBNL3, CUG-BP, CUG-BP2, CELF3, CELF4, CELF5, CELF6)をクローニングした[11]．スプライシングを調べるアッセイ系には塩素チャネル，インスリン受容体，αアクチニン，*c-src* などのミニ遺伝子を用い，HEK 細胞に

トランスフェクションした後，発現を確認した．筋強直に一番関係が深いと考えられている塩素チャネルのミニ遺伝子を用いて試験管内スプライシングアッセイを行った．マウス塩素チャネルのエクソン 6，7A，7 を使ったこのアッセイは，エクソン 7A を含む幼若型(6-7A-7)と 7A を含まない成熟型(6-7)の比を検出するものである．幼若型では停止コドンが入るため，機能のない遺伝子がつくられる．このミニジーンをトランスフェクトした COS 細胞に各種因子を添加し，時間を追って mRNA を抽出して，PCR 法によってスプライシング活性を検討した．

まず，DM 患者で認められる酸化ストレスに対して防御的効果のあるビタミン E と N アセチルシステインの効果をみた．ビタミン E 添加については 5 μM で効果が認められたが，それ以上の濃度では有意差が認められなかった．N アセチルシステインでは 100 μM まで効果が認められなかった．このほかに抗生物質のネオマイシンも効果がなかった．つぎに，二糖類であるトレハロースの効果を調べた．その結果，100 mM 以上の濃度で塩素チャネルの正常型スプライシングを促進することが明らかになった．

2. 筋分化促進物質の探索

もうひとつのスクリーニングとして，筋芽細胞 C2C12 を用いて筋分化を促進する因子を探すことが考えられる．DM 筋は一般に未熟で，分化が遅れているといわれている．そのため，分化促進に働く薬剤は治療薬としても有用と考えられる．著者らはマイオチューブラリン関連蛋白質 1 (MTMR1)のアイソフォームが筋分化の指標になることを発見し，筋管細胞特異的なアイソフォーム C の出現を分化の指標として分化を促進させる因子の検討も行った．酸化ストレスを軽減するといわれる多くの化合物を C2C12 筋細胞培溶液に添加してみたが，はっきりと筋分化を促進させる因子は現在のところみつかっていない．とくにカテキン，アスタキサンチンなどの分子の効果は認められなかった．

3. エクソンスキップ

塩素チャネル遺伝子のところで説明したが，アンチセンスオリゴヌクレオチドを用いてこの遺伝子のエクソン 6B と 7A をスキップさせることができれば，成熟型塩素チャネルが優先的につくられ，ミオトニアの症状がよくなることが期待される．このエクソンスキップは Duchenne 型筋ジストロフィーでうまくいくのではないかと提唱されており，塩素チャネル遺伝子に応用することも可能である．今後，検討されていくであろう．

4. 新しいモデル動物の開発

どんなによい治療法があっても，ヒトに応用する前にモデルとなる動物で効果が得られなければならない．現在のところ，ヒトと同じほどの長さの CTG または CCTG リピートを組み込むことが困難なために，ヒトの DM と同じ症状となるマウスをつくることができていない．しかし，アクチンプロモーターの下流に CTG リピートのみをつないで発現させたり[12]，MBNL1 をノックアウトすることにより一部 DM と類似した症状が出るとの報告がある[13]．著者らはその障害を乗り越えるため，ヒトに近い動物でなければならないという発想を転換して線虫を使って DM のモデルをつくりだすことに成功した．線虫に GFP と融合させた CTG5, CTG130, CCTG100 をインジェクションし，筋肉細胞に発現させた(*myo3* プロモーター)．また，ヒト MBNL のホモログである K02H8.1 遺伝子をクローニングするとともに，K02H8.1 が欠損した線虫を作出した．結果的に，線虫の MBNL(CeMBL と命名，ヒト MBNL1 とのホモロジーは 37%)は，スプライシング活性がヒト MBNL1 と類似していることが証明された．

また，CTG リピートが伸びた線虫，CCTG リピートが伸びた線虫，MBNL が欠損している線虫(エクソン 1 を含む 511 塩基の欠失をもつ変異体 *Tm1563*)などの行動や世代交代の時期を検討したところ，MBNL が欠損すると寿命が短縮することを発見した．これはヒト DM における早老症を反映しているモデルになると考えられた．

おわりに

筋強直性ジストロフィーの示す全身症状のほとんどが塩素チャネル(ミオトニア)，インスリン受容体(耐糖能の異常)，トロポニン T(心筋異常)など種々の遺伝子のスプライシング異常に起因する

ことが明らかになってきた．そのため，正常スプライシングに変える薬剤があれば，DM の治療として有用である．

そのために本稿では薬剤を用いた治療法について議論した．スプライシングを正常化させるためにはまずスプライシングにかかわる因子を明らかにし，その生理作用をうまく利用することが必要である．系が確立できれば，あとはスクリーニングによって効率のよい化合物がみつかるであろう．現在，可能性のある物質としてトレハロースという候補がみつかったが，糖は大量摂取が難しい．今後はこれを突破口として薬物の探索を行い，モデル生物でのスプライシング調節を指標に，治療薬を検討していくという方法がとられるに違いない．

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Case report

Distal lipid storage myopathy due to *PNPLA2* mutation

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Abstract

Distal myopathy is a group of heterogeneous disorders affecting predominantly distal muscles usually appearing from young to late adulthood with very rare cardiac complications. We report a 27-year-old man characterized clinically by distal myopathy and dilated cardiomyopathy, pathologically by lipid storage, and genetically by a *PNPLA2* mutation. The patient developed weakness in his lower legs and fingers at age 20 years. Physical examination at age 27 years revealed muscle weakness and atrophy predominantly in lower legs and hands, and severe dilated cardiomyopathy. The patient had a homozygous four-base duplication (c.475_478dupCTCC) in exon 4 of *PNPLA2*.

Keywords: Distal myopathy; Lipid storage myopathy; Neutral lipid storage disease with myopathy; *PNPLA2*

1. Introduction

Lipid storage myopathy (LSM) is a pathologically defined entity with accumulation of triglycerides in the muscle fiber. Six causative genes for only four diseases have been identified: *SLC22A5* for primary carnitine deficiency (PCD); *ETFA*, *ETFB*, and *ETFDH* for multiple acyl-CoA dehydrogenase deficiency (MADD); *ABHD5* for neutral lipid storage disease with ichthyosis or Chanarin–Dorfman syndrome; and *PNPLA2* for neutral lipid storage disease with myopathy (NLSDM) [1–3].

PNPLA2 encodes an adipose triglyceride lipase; mutations in this gene were recently reported in three patients who presented with LSM and variable cardiac involvement [1]. Here, we report a Japanese patient with a *PNPLA2* mutation presenting with distal myopathy and severe dilated cardiomyopathy and showing numerous rimmed vacuoles on muscle pathology.

2. Case report

A 27-year-old man had slowly progressive muscle weakness. Despite being a slow runner since childhood, he belonged to a mountaineering club and had no difficultly climbing mountains. At 20 years, he noticed difficulty climbing down the stairs, and gradually developed distal dominant muscle weakness and atrophy. Family history was non-contributory.

Upon consultation with us at 27 years, he had marked muscle weakness and atrophy in the extremities predominantly in the lower legs (Fig. 1A) and fingers (Fig. 1B). Examination of the muscle strength showed 3–4/5 asymmetric weakness over the deltoid, biceps brachii, extensor digitorum, gastrocnemius, and tibialis anterior. Grasping power was 12 kg on right and 10 kg on left (normal

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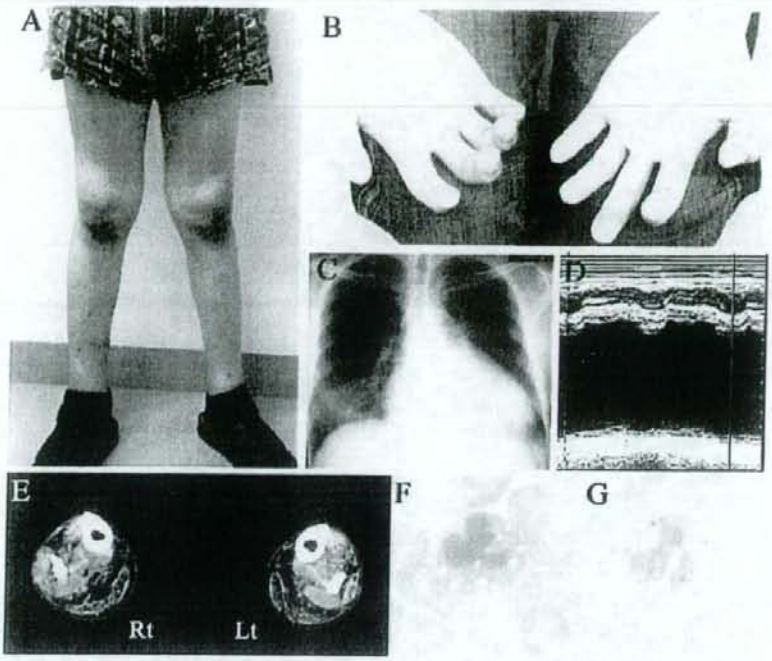

Fig. 1. The patient had distal muscle atrophy especially in the lower legs (A) and thenar muscles (B). Chest X-ray showed cardiomegaly with cardiothoratic ratio of 63% (normal cardiothoratic ratio <50%) (C). Echocardiogram showed left ventricular enlargement with decreased ejection fraction of 18% (normal >60%) (D). Calf muscles were involved relatively sparing tibialis anterior on CT (E). Note many vacuoles of leukocyte by Wright–Giemsa (F), which are positively stained by oil red O (G).

values = 43–56 kg). Deep tendon reflexes were absent. No skin abnormality was seen. Chest X-ray revealed cardiomegaly (Fig. 1C). Echocardiogram showed left ventricular enlargement with decreased left ventricular ejection fraction of 18% (normal >60%), left ventricular end-diastolic dimension of 78 mm, left ventricular end-systolic dimension of 70 mm, interventricular septum thickness of 8 mm and posterior wall thickness of 8 mm (Fig. 1D). ECG showed negative Q wave in lead I, negative P wave in V_1 and occasional ventricular extra-systoles. EMG showed myopathic changes. His respiratory function was normal. Serum creatine kinase was elevated (412–1697 IU/L; normal value <170). Serum cholesterol, TG, LDL-cholesterol and glucose were within normal ranges. In leukocytes, Jordans anomaly [4], multiple tiny vacuoles due to lipid accumulation, was seen (Fig. 1F and G). Muscle CT showed decreased densities in both soleus, both gastrocnemius, and right tibialis anterior muscles (Fig. 1E).

Muscle biopsy from the left biceps brachii muscle revealed marked variation in fiber size. Numerous lipid droplets were seen in virtually all type one fibers (Fig. 2A). In addition, rimmed vacuoles were observed in scattered fibers (Fig. 2B). Dystrophin, caveolin-3, and dysferlin immunohistochemistry were normal. On electron microscopy, markedly increased lipid droplets were seen between myofibrils where mitochondria appeared pyknotic (Fig. 3A). Numerous autophagic vacuoles were also observed (Fig. 3B). Total and free muscle carnitine levels were 13.2 and 3.9 nmol/mg non-collagen protein, respectively (reference: total, 15.7 ± 2.8; free, 12.9 ± 3.7).

We sequenced all exons and the flanking intronic regions of all six known causative genes for LSM in genomic DNA. In the patient, we identified a homozygous four-base duplication (c.475_478dupCTCC) in exon 4 of *PNPLA2* (Gene ID: 57104), predicted to result in a premature stop codon at amino acid position 178. Heterozygous c.475_478dupCTCC mutation was confirmed in both healthy parents. We did not find any sequence variant in other candidate genes, including *GNE* gene.

3. Discussion

The patient presented has been followed up with a tentative diagnosis of distal myopathy. In fact, one patient in the first report of *PNPLA2* mutations had distal dominant muscle weakness although the other two had proximal muscle involvement [1]. Therefore, distal myopathy may not be uncommon in LSM associated with *PNPLA2* mutations.

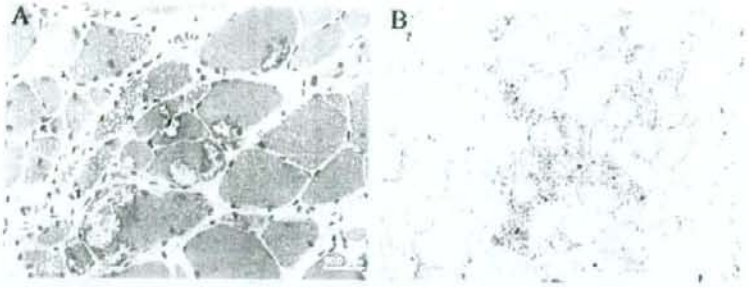

Fig. 2. In addition to variation in fiber size, numerous small vacuoles and rimmed vacuoles were seen with H&E staining (A). Numerous lipid droplets were seen with oil red O (B).

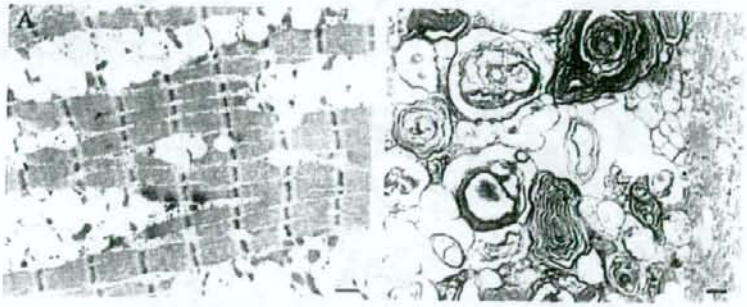

Fig. 3. Onelectron microscopy, markedly increased lipid droplets were seen intermyofibrillar spaces in most of fibers (A). In areas with the rimmed vacuoles, the lipid droplets were not actively scavenged by autophagosome (K). Bar = 1 μm.

Miyoshi myopathy and distal myopathy with rimmed vacuoles are the two most common distal myopathies in Japan, but these were excluded by immunohistochemistry for dysferlin and sequence analysis of *GNE* gene; moreover, finger muscle atrophy and weakness are not usually seen in these distal myopathies. There is a peculiar distal myopathy due to caveolin-3 gene mutation that selectively affected small muscles in hands and feet [5]. However, caveolin-3 immunohistochemistry was normal (data not shown).

Rimmed vacuoles can also be seen in myofibrillar myopathy and inclusion body myopathy with Paget's disease of bone and frontotemporal dementia (IBMPFD) [6,7]. Myofibrillar myopathy is pathologically characterized by disorganization of myofibrillar alignment and protein aggregations, such as cytoplasmic body and spheroid body, which were absent in our patient. IBMPFD is caused by mutations in the gene encoding valosin-containing protein and is clinically characterized by variable extent of dementia and polyostotic skeletal disorganization. IBMPFD is unlikely as our patient had neither intellectual deficit nor bone abnormality although Kimonis et al. recently postulated that IBMPFD is underdiagnosed and reported that 86% of patients had muscle disease while frontotemporal dementia and Paget disease of bone was diagnosed in 27% and 57%, respectively [8]. On top of it, lipid droplets are not a feature of any of the above-mentioned disorders.

In our patient, free carnitine was low in the muscle while total amount was normal. Two patients in the first report of *PNPLA2* mutations showed normal serum carnitine levels [1]. However, muscle carnitine levels were not measured in these patients. Further studies are necessary to determine a relationship between NLSDM and carnitine levels.

The increased amount of lipid droplets in muscle fibers led us to make a diagnosis of LSM. In PCD and MADD, lipid droplets are seen next to mitochondria that are structurally normal. In contrast, mitochondria are pyknotic in our case. Furthermore, autophagic vacuoles have never been reported in other LSM. These observations suggest a possibility that NLSDM may have a myodegenerative process different from other LSM.

We have 47 muscle biopsies diagnosed as LSM collected from 1978–2006. Interestingly, all other 46 patients had proximal dominant muscle weakness except for the present case, suggesting a possibility that distal muscle involvement may be unique to *PNPLA2* mutations although further studies are necessary to draw any conclusion.

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Case report

Rigid spine syndrome caused by a novel mutation in four-and-a-half LIM domain 1 gene (*FHL1*)

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ABSTRACT

Four-and-a-half LIM domain 1 gene (*FHL1*) has recently been identified as the causative gene for reducing body myopathy (RBM), X-linked scapuloperoneal myopathy (SPM) and X-linked myopathy with postural muscle atrophy (XMPMA). Rigid spine is a common clinical feature of the three diseases. We searched for *FHL1* mutations in eighteen patients clinically diagnosed as rigid spine syndrome (RSS). We identified one RSS patient with *FHL1* mutation. Reducing bodies were observed in few fibers of the patient's muscle sample. Amount of FHL1 protein was decreased on immunoblotting. In conclusion, *FHL1* can be one of the causative genes for RSS.

1. Introduction

FHL1, four-and-a-half LIM domain 1 is a 32 kDa protein which is highly expressed in skeletal muscle with intermediate expression in the heart [1]. LIM domains are a cysteine-rich double zinc finger protein-binding motif denoted by the sequence (CX2–CX17–19HX2C)X2(CX2CX16–20CX2(H/D/C)) and mediate interactions with transcription factors and cytoskeletal proteins. LIM domain proteins play critical roles in tissue differentiation and cytoskeletal integrity, respectively. FHL1 was implicated in many cellular functions; (1) α5β1-integrin-dependent myocyte elongation [2], (2) regulation of myosin filament formation and sarcomere assembly by binding to myosin-binding protein C [3], and (3) modulation of Notch signalling pathway through interaction of FHL1C (one of the splicing isoforms of FHL1) with transcription factor RBP-J and RING1 [4].

Recently, mutations in *FHL1* have been identified in patients with RBM [5], SPM [6] and XMPMA [7]. We have also identified mutations in *FHL1* in all RBM patients we reported previously, and confirmed that *FHL1* is the causative gene for RBM (unpublished data). Clinical picture of RBM patients varies from congenital lethal form to benign childhood and adult forms. However, four out of the six RBM families reported to date show rigid spine [5,8]. In addition rigid spine was reported in SPM families [9] and was also seen in the British and Italian–American families reported as XMPMA [7]. This finding suggests that rigid spine is a common clinical feature of patients with *FHL1* mutations.

Here we found a patient with rigid spine syndrome (RSS) harboring a mutation in *FHL1* among 18 patients clinically diagnosed as RSS.

2. Case report

The patient is a 16-year-old male who was a good runner during his childhood. He was first noted to have scoliosis on a routine medical examination when he was 13 years old. Gradually, his walking and running speed became slower, and hip muscle atrophy was noted. Two years late he started experiencing difficulty in bending his body and difficulty in neck flexion. He could not stand on one foot. By the age of 16 years, bilateral hip and thigh muscle atrophy was prominent. On examination, he showed muscle weakness and atrophy in the sternomastoid, trapezius, paravertebral, pelvic girdle and proximal lower limb muscles. Winging of scapula and Gowers' sign were observed. Funnel chest and joint contractures in neck, spine, hip and ankle joints were seen. He walked slouchingly and his left leg was slightly lagged and outward rotated. Serum creatine kinase level was mildly elevated and respiratory functions were mildly impaired. His elder brother showed mild scoliosis but not rigid spine or muscle weakness. His father had IRBBB while his mother was healthy.

Genomic DNA was isolated from peripheral lymphocytes using a standard technique after obtaining informed consent. Seven sets of primers were used to amplify genomic fragments of *FHL1*. All

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exons and their flanking intronic regions of *FHL1* were directly sequenced using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems). We identified a hemizygous in-frame nine base-pair (bp) deletion mutation at c.451–459delGTGACTTGC (p.151–153delVTC) of *FHL1* in this patient. A total 250 controls and the other 17 RSS patients did not carry the mutation in *FHL1*. Genetic analysis of other family members including the elder brother was not allowed.

Biopsied muscle specimen was frozen in isopentane cooled in liquid nitrogen. Serial 10 μm cryostat sections were stained with haematoxylin and eosin (HE), modified Gomori trichrome (mGt) and a battery of histochemical methods. Menadione–nitroblue tetrazolium (NBT) staining in the absence of the substrate α-glycerophosphate was also performed to detect reducing bodies (RBs). Histological analyses of muscle showed marked variation in fiber size and fibers with rimmed vacuoles. Only a limited number of fibers contained RBs. These abnormal fibers detected were localized in focal areas of the muscle specimen (Fig. 1A and B).

Immunohistochemical analysis revealed diffusely increased FHL1 staining in some muscle fibers. The strong FHL1 staining was observed in both types of fibers as seen in serial sections stained by slow type of myosin heavy chain (MHC-slow) (Fig. 1C and D). Protein amount of FHL1 by immunoblotting analysis was significantly reduced in the patient muscle when compared to normal control after normalization to actin amount (Fig. 2).

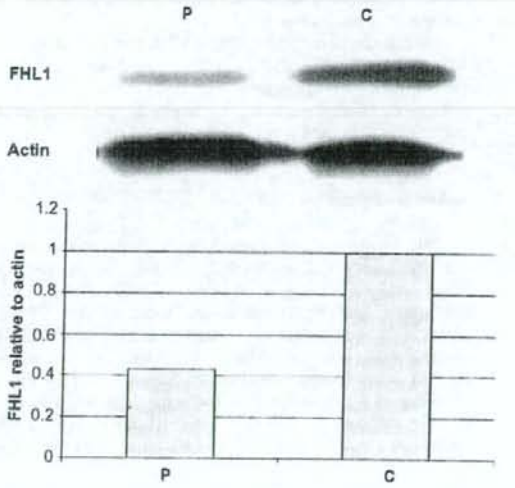

Fig. 2. Immunoblotting analysis of FHL1. Amount of FHL1 in biopsied muscle from the RSS patient show significant reduction compared to actin.

3. Discussion

The term *rigid spine syndrome* was first proposed by Dubowitz to highlight the essential clinical problem seen in myopathy with prominent spinal rigidity [10]. Nevertheless, spinal rigidity is not a specific finding as it is a characteristic feature in Emery–Dreifuss muscular dystrophy, Bethlem myopathy, and in selenoprotein related myopathies. In addition it has also been reported in other congenital myopathies and muscular dystrophies. Patients with *FHL1* mutations also show spinal rigidity [5,7,9].

Here we identified a RSS patient with a novel mutation in *FHL1*. The mutation affects a cysteine residue in the second LIM domain of FHL1 similar to all mutations causing RBM [5].

The most important feature to differentiate RSS from other muscular diseases associated with spinal rigidity is the limitation of flexion of the cervical and dorsolumbar spine in absence of

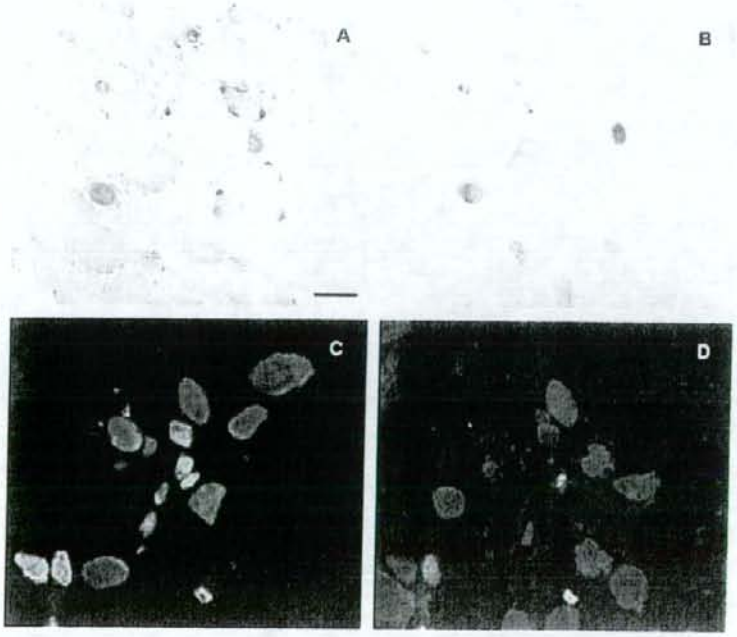

Fig. 1. Muscle pathology. (A) Intracytoplasmic inclusions and rimmed vacuoles are seen on mGT staining. (B) Reducing bodies are positive on melanodine–NBT staining. (C) Diffuse strong immunoreactivity to FHL1 is seen in both MyHC-slow positive and negative fibers (D). Bar = 20 μm.

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

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

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Clinical/Scientific Notes

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

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

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

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

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

Supplemental data at www.neurology.org

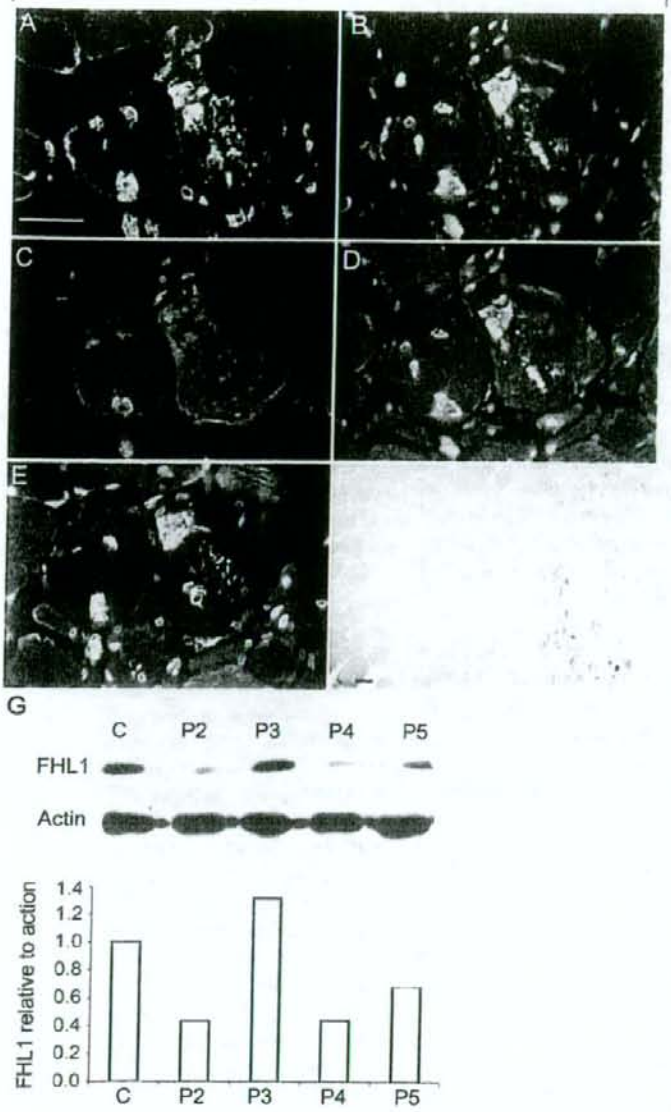
Discussion. All our RBM patients, with a wide range of clinical phenotypes, fatal infantile (patient 1 and 2), benign childhood (patient 4), and adult-onset (patients 3 and 5), had novel *FHL1* mutations, confirming the recent report that *FHL1* is the causative gene for RBM.[1] All the mutations identified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

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

Here we showed that MyHC-slow is aggregated in patient muscles. It was reported that both overexpression and underexpression of FHL1 were associated with the failure of myosin to assemble into thick filaments. Aggregation of myosin was also noted in FHL1 knockdown cells. In RBM muscles, mislocalization of myosin filaments and the sarcomeric disassembly may be caused by FHL1 dysfunction. Surprisingly, α5-integrin was also highly aggregated in RBM patients although normally α5-integrin is expressed in myoblasts and during primary myogenesis, and is downregulated in mature muscle. FHL1 was reported to induce α5β1-integrin-dependent myocyte elongation. Whether or not there is a correlation between α5-integrin aggregation and the suggested role of FHL1 in integrin signaling and regulation of cytoskeletal dynamics during muscle differentiation is not clear.

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

Figure **Immunohistochemical and immunoblotting analyses**



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

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

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

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ABSTRACT: Causative genes have been identified only in four types of lipid storage myopathies (LSMs): *SLC22A5* for primary carnitine deficiency (PCD); *ETFA*, *ETFB*, and *ETFDH* for multiple acyl-coenzyme A dehydrogenation deficiency (MADD); *PNPLA2* for neutral lipid storage disease with myopathy (NLSDM); and *ABHD5* for neutral lipid storage disease with ichthyosis. However, the frequency of these LSMs has not been determined. We found mutations in only 9 of 37 LSM patients (24%): 3 in *SLC22A5*; 4 in MADD-associated genes; and 2 in *PNPLA2*. This low frequency suggests the existence of other causative genes. Muscle coenzyme Q_{10} levels were normal or only mildly reduced in two MADD patients, indicating that ETFDH mutations may not always be associated with CoQ_{10} deficiency. The 2 patients with *PNPLA2* mutations had progressive, non-episodic muscle disease with rimmed vacuoles. This suggests there is a different pathomechanism from other LSMs.

Muscle Nerve **39:** 333–342, 2009

CLINICAL AND GENETIC ANALYSIS OF LIPID STORAGE MYOPATHIES

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Defects in muscle lipid metabolism are due to a heterogeneous group of metabolic conditions. They are caused by problems in transport of fatty acids and carnitine, mitochondrial matrix β-oxidation enzymes, or endogenous triglyceride synthesis. The clinical spectrum of these disorders is variable. Patients often present with hypotonia, muscle weakness, recurrent rhabdomyolysis, and peripheral neuropathy.[21] Lipid storage myopathies (LSMs), which are categorized under the broad category of disorders of lipid metabolism, are invariably characterized by accumulation of lipid droplets in muscle fibers. Among LSMs, genetic causes have been identified in only four disorders: primary carnitine deficiency (PCD); multiple acyl-coenzyme A (acyl-CoA) dehydrogenation deficiency (MADD); neutral lipid storage disease with myopathy (NLDSM); and neutral lipid storage disease with ichthyosis (NLDSI).[4,10,21]

PCD is an autosomal-recessive disorder caused by mutations of the *SLC22A5* gene, which encodes an integral plasma membrane protein, organic cation transporter 2 (OCTN2). It functions to transport extracellular carnitine into cells.[17,22] OCTN2 mutations lead to defective renal reabsorption and reduced tissue storage of carnitine and impairment of long fatty acid metabolism, as carnitine is necessary to incorporate long-chain fatty acids into the mitochondrial matrix for β-oxidation. Clinical features of PCD include severe hypoglycemia and dilated cardiomyopathy in addition to skeletal muscle involvement.[21]

Abbreviations: ABHD5, abhydrolase domain- containing 5; ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification 58; CPT II, carnitine palmitoyltransferase type II; Cho, cholesterol; CoA, coenzyme A; CoQ_{10}, coenzyme Q_{10}; ECG, electrocardiogram; ETF, electron transfer flavoprotein; ETFDH, electron transfer flavoprotein dehydrogenase; FFA, free fatty acids; HSL, hormone-sensitive lipase; LSM, lipid storage myopathies; MADD, multiple acyl-coenzyme A dehydrogenation deficiency; NLSDI, neutral lipid storage with ichthyosis; NLSDM, neutral lipid storage disease with myopathy; OCTN2, organic cation transporter 2; PCD, primary carnitine deficiency; PL, phospholipids; PNPLA2, patatin-like phospholipase domain–containing protein 2; RT-PCR, reverse transcript-polymerase chain reaction; SDH, succinate dehydrogenase; TG, triglyceride; VLCAD, very-long-chain acyl-CoA dehydrogenase

Key words: *ABHD5*; *ETF*; *ETFDH*; lipid storage myopathy; *PNPLA2*; *SLC22A5*

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MADD, also known as glutaric aciduria type II, is an autosomal-recessive disorder of fatty and amino acid metabolism[6] caused by defects in electron transfer flavoprotein (ETF) or ETF dehydrogenase (ETFDH). ETF is a heterodimeric protein consisting of two subunits, α and β, that are encoded by different genes, *ETFA* and *ETFB*. ETF receives electrons from mitochondrial flavin-containing dehydrogenases to ETFDH in the inner mitochondrial membrane. ETDFH, in turn, transfers electrons to coenzyme Q. The MADD phenotype varies widely from a fatal neonatal-onset form[19,20] to a much milder late-onset form, which is often associated with a lipid storage myopathy that manifests with muscle weakness and pain. Recently, patients with ETDFH mutations were shown to have secondary coenzyme Q_{10} (CoQ_{10}) deficiency.[7]

Neutral lipid storage disease is characterized by systemic accumulation of triglycerides (TG) in the cytoplasm and includes two distinct diseases: NLSDM and NLSDI (also called Chanarin–Dorfman syndrome). NLSDM is caused by mutations in a gene that encodes adipose triglyceride lipase (ATGL), which is also referred to as patatin-like phospholipase domain-containing protein 2 (PNPLA2).[4,9,23] This protein catalyzes the initial step in TG hydrolysis. On the other hand, NLSDI is due to defects in the gene that encodes the coactivator of ATGL, comparative gene identification-58 (CGI-58), which is also known as abhydrolase domain–containing 5 (ABHD5).[10]

Although the pathological characteristics of LSM are rather uniform, the phenotypic manifestations are remarkably heterogeneous, possibly due to different genetic backgrounds. Thus, genetic analysis has always posed a challenge. In this study, we analyzed all known causative genes for LSM (*SLC22A5*, *ABHD5*, *PNPLA2*, *ETFA*, *ETFB*, and *ETFDH*), as well as *LIPE*, which encodes hormone-sensitive lipase (HSL),[8] among patients who had pathological confirmation of LSM. Our aim was to determine the actual frequency of identifiable mutations and to look for genotype–phenotype correlations that could be helpful for diagnosis.

METHODS

Patients. We retrospectively recruited cases diagnosed with LSM at the National Center of Neurology and Psychiatry (NCNP) from a total of 9639 muscle biopsies obtained between 1978 and 2006. The diagnosis of LSM was made based on characteristic muscle pathology findings: small clear vacuoles on hematoxylin and eosin staining and intramyofiber accumulation of lipid droplets on oil-red-O staining. We excluded cases with obvious mitochondrial abnormalities such as ragged-red fibers, strongly succinate dehydrogenase (SDH)-reactive vessels, and cytochrome *c* oxidase deficiency. Detailed retrospective review of the clinical and pathological findings was performed. Informed consent was obtained from the patients using a form approved by the NCNP ethics board committee.

Mutation Analysis. We sequenced all exons and their flanking regions of all the known causative genes for LSM: *SLC22A5*, *ABHD5*, *PNPLA2*, *ETFA*, *ETFB*, and *ETFDH* in genomic DNA of patients with LSM.

Genomic DNA was extracted from the muscle biopsies using a standard method.[16] We sequenced all exons and their flanking regions of *SLC22A5*, *ABHD5*, *PNPLA2*, *ETFA*, *ETFB*, *ETFDH*, and *LIPE*. Primers were designed from the genomic sequences reported in GenBank (Gene IDs: 6584 for *SLC22A5*, 51099 for *ABHD5*, 57104 for *PNPLA2*, 2108 for *ETFA*, 2109 for *ETFB*, 2110 for *ETFDH*, and 3991 for *LIPE*). We performed direct sequencing of amplified fragments using an automated 3100 DNA sequencer (Applied Biosystems, Foster City, California) with the BigDye Terminator cycle sequencing system, and analyzed DNA sequences with the SeqScape program (Applied Biosystems).

We performed quantitative reverse transcript–polymerase chain reaction (RT-PCR) in RNA obtained from muscle using the QuantiTect SYBR-Green PCR Kit (Qiagen GmbH, Hilden, Germany) and iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, California). We analyzed the amount of transcript for *ETFDH* relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA.

Biochemical Analyses. We measured CoQ_{10} in frozen muscles from patients with *ETF* mutations using a high-performance liquid chromatography (HPLC) method described previously[7] in 2 cases with enough sample size for analysis (patients 6 and 7). For muscle lipid analysis, total lipid was extracted from muscles according to the methods of Folch et al.[5] Extracted lipids were adopted to TLC with petroleum ether/diethyl ether/acetic acid (60:40:1) as a developing solvent to separate TG, cholesterol (Cho), and free fatty acids (FFA) from phospholipid (PL). The lipids were visualized with 50% sulfuric acids/methanol vapor. Band intensities were measured with Quantity One software (Bio-Rad Laboratories). We measured the levels of TG, PL, and FFA relative to Cho amount (TG/Cho, PL/Cho, FFA/Cho). Muscle

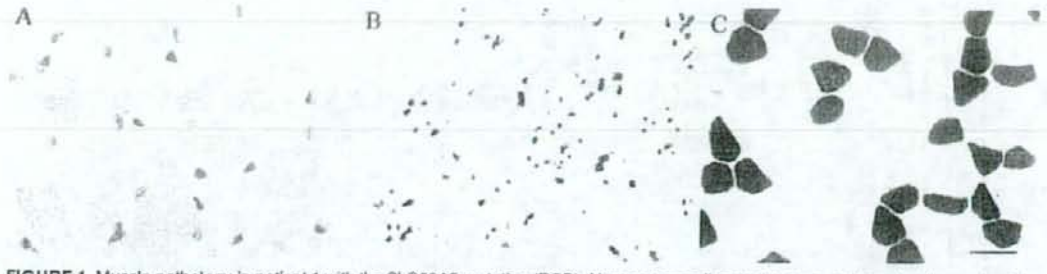

FIGURE 1. Muscle pathology in patient 1 with the SLC22A5 mutation (PCD). Numerous small vacuoles seen on hematoxylin–eosin stain **(A)** are actually lipid droplets, as shown on oil-red-O **(B)**. These vacuoles are seen predominantly in type 1 fibers **(C)**. Bar = 20 μm.

carnitine palmitoyltransferase type II (CPT II) activity was measured using a method described previously.[2]

RESULTS

Pathological and Clinical Features of LSM. Of 9639 frozen muscle biopsies that we had examined pathologically, 47 (0.5%) had LSM. In all 47 patients, there were numerous small vacuoles that were filled with lipid droplets in scattered type 1 and 2 muscle fibers (Fig. 1A, B). Measurement of the width of these vacuoles, indirectly representing the amount of lipid, did not reveal any significant differences among patients (data not shown). In addition, these lipid droplets were found predominantly in type 1 fibers (Table 1 and Fig. 1C), except in patients 10 and 11, who exhibited lipid droplets predominantly in type 2 fibers (Fig. 2).

The clinical features of the 47 patients (23 males and 24 females) are summarized in Table 1. Age at onset varied from 37 days to 75 years. Eight patients had a positive family history. The majority of the patients (55%) had muscle weakness, and all except 1 had generalized or proximal dominant muscle weakness. No correlation was found between the clinical/pathological phenotype and genotype of patients (data not shown).

Genetic Analysis of LSM. DNA was available for only 37 patients. We identified mutations in 9 (24%) patients: 3 in *SLC22A5*; 3 in *ETFDH*; 1 in *ETFA*; and 2 in *PNPLA2* (Table 2). In patient 4, we identified a heterozygous c.1519T>G *ETFDH* mutation in genomic DNA; however, by RT-PCR, only the transcript with this mutation was detected, indicating absence of transcript from the other allele. All mutations were novel except in patients 2 and 3.[11,12] We did not find similar mutations in 100 control chromosomes of Japanese individuals. In addition, we did not find any mutations in *ABHD5* or *LIPE*.

Biochemical Analysis. CoQ_{10} levels were normal in patient 7 and mildly decreased in patient 6, who had *ETFDH* and *ETFA* mutations, respectively (Table 3). The size of the samples permitted lipid analysis in only 14 patients, including 2 patients with mutations: patient 1 with PCD, and patient 6 with MADD. The amount of TG was significantly elevated in all LSM patients (TG/Cho: 12.5 ± 2.26 [mean ± standard error of mean]) when compared with control individuals (5.95 ± 1.72). In contrast, FFA were not increased, and PL were not significantly different (data not shown). In all 10 patients tested, CPT II activity was normal.

PCD Patients. Patients 1, 2, and 3 harbored mutations in *SLC22A5*. Patient 1 exhibited normal early motor development and appeared healthy until age 8 months when she developed hepatomegaly, coma, hyperammonemia, and non-ketotic dicarboxylic aciduria. On liver biopsy, numerous lipid droplets were seen. Clinical improvement was seen with L-carnitine supplementation, but she eventually succumbed to heart failure when she had an infection. Patients 2 and 3, who are siblings, have been reported previously.[2,12] Briefly, they had slowly progressive muscle weakness and hypertrophic cardiomyopathy, and their developmental milestones were normal until 3 years of age, when mild weakness in the lower limbs became evident. Laboratory examination showed transient high creatine kinase (CK) levels and hyperammonemia. Carnitine levels were decreased in skeletal muscles of these 3 patients (data not shown). Serum carnitine was likewise reduced in patients 2 and 3. Total and free carnitine levels (in μmol/L), respectively, were: 36.1 (normal: 67.6 ± 11.3) and 12.3 (normal: 52.2 ± 10.4) in patient 2; and 35.7 and 11.4 in patient 3. L-carnitine treatment in both cases resulted in marked clinical improvement.

On muscle pathology, both number and size of mitochondria were mildly increased (Fig. 4A). Lipid-

Table 1. Clinical summary of 47 patients with LSM

Pt	Age	Sex	Clinical feature	Weakness	Hypotonia	Muscle pain and cramp	Prodrome	Seizure	Coma	Respiratory failure	Cardiac symptom	Liver disease	CK	Familial history	Consanguinity	Lipid droplets distribution by fiber type
Mutations																
1	8m	F	vomiting, diarrhea	NA	NA	NA	+	−	+	−	−	+	243–1006	−	−	type 1>
2	4y	M	gait disturbance	+	+		−	−	−	−	+	−	150	+	−	type 1>
3	5y	M	gait disturbance	+	+	−	−	−	−	−	+	−	69	+	−	type 1>
4	5m	F		+	+	NA	NA	−	−	−	−	+	55	−	−	type 1>
5	6m	M		+	NA	NA	+			−	−	+	2000–4000	−	−	type 1>
6	11m	M	diarrhea	+	+	NA	+	+	+	−	+	+	128–618	−	−	type 1>
7	13y4m	F	muscle weakness	+	+		+	−		−	−	+	127	−		type 1>
8	27y	M	muscle weakness	+	−	−	−	−	−	−	+	−	757–1697	−	−	type 1>
9	35y	F	gait disturbance	+	+		−	−	−	−	+	−	654	+		type 1>
No Mutations																
10	15y	M	muscle cramp		−					−	−		587	−	−	< type2
11	67y	M	gait disturbance	+	−	−	−	−	−	−	−	−	4904	−	−	< type2
12	37d	F	metabolic acidosis	+	+	NA	−	−	−	+	+	−	44–200	−	−	type1 = type2
13	4m	F		NA	NA	NA	NA	NA	NA	NA	+	NA	NA	NA	NA	type1 = type2
14	4m	M	dyspnea	+	−	NA			−	+	−		112	+	−	type 1>
15	7m	M	developmental delay	+	−	NA	+	+	−				67	−	+	type 1>
16	1y	M	status epilepticus		−	NA		+	+	+	−	−	1593	−	−	type 1>
17	1y6m	F	metabolic acidosis							−			330	+		type 1>
18	1y1m	M		+	−	NA	+	−		+	+	−	559	−		type 1>
19	1y2m	F	developmental delay	+	−	NA	+	+	+	+	−	−	NA	−	−	type 1>
20	1y6m	M	albinism	+	+	NA							200–300			type 1>
21	2y2m	M	diarrhea	NA	+	−	+	−	+	−	−	−	163900	−	−	type 1>
22	2y7m	M	developmental delay	−	+	−					−		603	−		type 1>
23	3y	M	status epilepticus	NA	NA	NA	+	+	+		−		2034	−		type 1>
24	3y	F	developmental delay	+	−		−				−	−	63		−	type 1>
25	3y	M	developmental delay	NA	+	NA	−	−	NA	+	−	−	NA	−	−	type 1>
26	4y	F	periodic paralysis	+	+	−		+		−	−		162	−	−	type 1>
27	5y7m	M	developmental delay	+	+		−			−	−		47	−	−	type 1>
28	6y	M	dyspnea, abdominal pain	+	+	−	+	−	+	+	−	−	15	+	−	type 1>
29	13y8m	F	muscle weakness	+	NA				−		−		normal	+	+	type 1>
30	30y	F	lumbago										NA			type1 = type2
31	40y	F	diplopia, muscle cramp			−			−	−			NA	−		type 1>
32	49y	F	hypokalemic myopathy	+				−					3480			type 1>
33	54y	F	weakness	+						−			623	−	−	type 1>
34	59y	F	dyspnea, weakness	+				−		+	−		878	−		type 1>
35	66y	F	gait disturbance	+	−	−		−		−	−	−	49	−	−	type 1>
36	69y	M	gait disturbance			−		−			−	−	400			type 1>
37	75y	F	gait disturbance			−		−			−		5418	−	−	type 1>

Table 1. Continued

Pt	Age	Sex	Clinical feature	Weakness	Hypotonia	Muscle pain and cramp	Prodrome	Seizure	Coma	Respiratory failure	Cardiac symptom	Liver disease	CK	Familial history	Consanguinity	Lipid droplets distribution by fiber type
No Available DNA																
38	4m	F		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
39	7m	M	dyspnea	NA	NA	NA	NA	NA	NA	+	NA	NA	125	NA	NA	type1 = type2
40	9m	F	developmental delay	NA	+	NA	−	+	−	+	−	−	NA	−	−	NC*
41	9m	M	ketoacidosis		−	−	+	−	−	−	−	+	214	−	−	type 1>
42	1y	M	vomiting, diarrhea	NA	NA	NA	+	+	+	+	−	−	394	−	−	type 1>
43	1y	F	dyspnea, weakness	+	−	−			NA	+	−	−	12310	−	−	type 1>
44	5y	M	fever, arthralgia	+	NA	+	−	−	−	+	−	−	39	+	−	type1 = type2
45	21y	F	vomiting, diarrhea	−	−	−	+	−	+	−	−	−	NA	−	−	type 1>
46	23y	F	muscle pain	−	−	+	−	−	−	−	−	−	62180	−	−	type1 = type2
47	24y	F	lumbago	−	−	+	−	−	−	−	−	−	47-4797	−	−	type 1>

NC = not counted
NA = not available

containing vacuoles in skeletal muscle were predominantly observed in type 1 fibers (Fig. 4B). Patient 1 had type 2 fiber atrophy, whereas patients 2 and 3 showed type 2A fiber atrophy and type 2B fiber deficiency. On electron microscopy, there was an increase in number of lipid droplets and mitochondria. Incidentally, lipid droplets were often next to mitochondria (Fig. 5A, B).

MADD Patients. The clinical features of the 4 patients genetically confirmed to have MADD are summarized in Table 3. The diagnosis of MADD in patients 6 and 7 was initially made based on the results of urinary organic acid analysis by gas chromatography/mass spectroscopy. All 4 patients had the infantile form. They all had generalized muscle weakness and hypotonia. Serum CK levels varied from normal to 4000 IU/L. Hepatomegaly was documented in patients 4 and 5. Patient 5, who received L-carnitine and riboflavin treatment, had normal growth and development, except for some mild metabolic episodes, and is now 20 years old. Patient 6 had hypertrophic cardiomyopathy. He was treated with L-carnitine, but he died of pulmonary alveolar bleeding at the age of 1 year and 11 months. Patient 7 was always a slow runner and poor athlete with easy fatigability since her preschool years. She developed nausea and vomiting at age 13 years and started experiencing difficulty climbing stairs. She had proximal dominant muscle weakness and atrophy on examination at age 13 years and 4 months. After treatment with L-carnitine and riboflavin, muscle weakness was ameliorated.

In skeletal muscle, lipids were observed predominantly in type 1 fibers. Mitochondria were not as prominent as in PCD (Fig. 4C, D). Type 2 fiber atrophy was seen in patient 5. Electron-microscopic findings were similar to those seen in PCD patients: intracytoplasmic lipid droplets were markedly increased both in number and size, and lipid droplets were often present next to mitochondria (Fig. 5C, D).

NLSDM Patients. Patients 8 and 9 had mutations in *PNPLA2*. Patient 8 developed progressive weakness in the lower legs and fingers at age 20 years (article in submission); at age 27 years, echocardiogram revealed dilated cardiomyopathy with left ventricular enlargement. Serum CK was elevated from 757 to 1697 IU/L.[14] Patient 9 was a slow runner since childhood.[1] At age 33 years, she noticed weakness of all extremities and developed marked generalized muscle weakness at 35 years. Electrocardiogram (ECG) showed left ventricular hypertrophy, but echocardio-

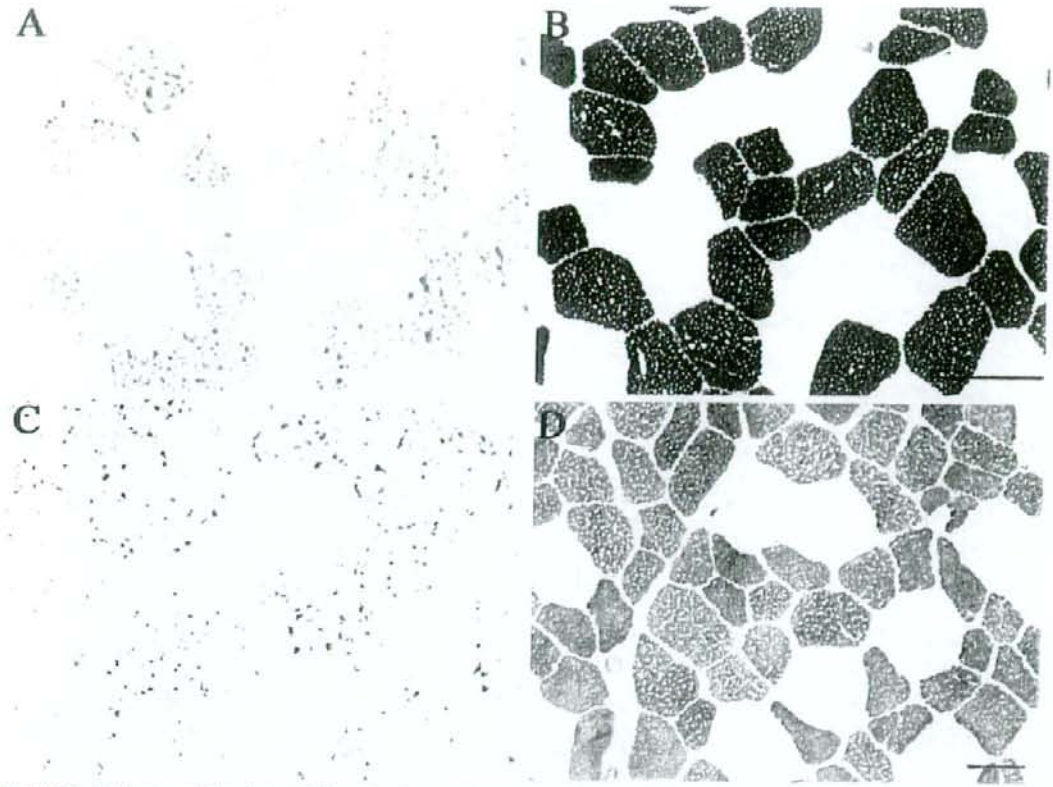

FIGURE 2. Lipid accumulation in type 2 fibers of patients with no mutations in known genes associated with LSM: patient 10 **(A, B)** and patient 11 **(C, D)**. Lipid droplets stained with oil-red-O **(A, C)** are only seen in type 2 fibers (routine adenosine triphosphatase stain) **(B, D)**. Bar 50 μm.

gram was normal. Serum CK was elevated to 654 IU/L. In both patients, peripheral blood smear revealed lipid-containing vacuoles in leukocytes, namely Jordan's anomaly. Both patients had numerous lipid droplets mainly in type 1 fibers in addition to variation in fiber size. Surprisingly, there were scattered rimmed vacuoles within the myofibers (Fig. 4E, F), which were demonstrated to be autophagic vacuoles on electron microscopy (Fig. 5F). Interestingly, increased lipid droplets were seen between myofibrils where mitochondria appeared pyknotic (Fig. 5E).

DISCUSSION

Among all LSM cases, we identified mutations in known causative genes in only 24% of the cases. This brings to our attention two possibilities: the existence of yet-unknown causative genes, and secondary increase of lipid in muscle under a variety of metabolic alterations without inheritance.

Analysis of muscle lipids demonstrated an increase in the amount of TG, but not FFA. The accumulated lipid droplets in the cytoplasm of skeletal myofibers are therefore likely to be mainly composed of TG. Although, theoretically, triglyceride accumulation should occur in NLSDM and NLSDI, in which genes encoding TG hydrolase or its activator are mutated, it is accumulated in virtually all patients analyzed regardless of the causative gene. Reduction of mitochondrial fatty acid metabolism may negatively regulate the hydrolysis of TG in cytosol.

We identified 3 PCD patients with mutations in *SLC22A5*. Their clinical characteristics were consistent with the typical PCD symptoms with severe hypoglycemia, dilated cardiomyopathy, and progres-

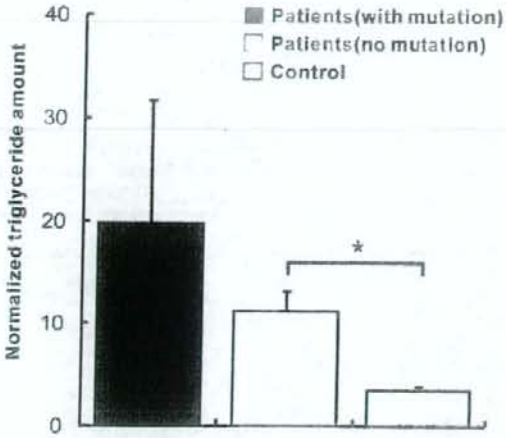

FIGURE 3. TLC analysis of lipid composition of skeletal muscle with LSM. The bars represent the mean triglyceride (TG) amount which is normalized with cholesterol (Cho) content. Values are shown for patients with mutation (black bar; $N = 2$), patients with no mutation (gray bar; $N = 12$), and in controls (white bar; N 4). Error bars represent standard error of means. Note the remarkable increase of TG in patients with mutations. $^*P < 0.05$ (Student's *t*-test).

sive muscle weakness, as reported elsewhere.[11,12] A positive response to L-carnitine treatment was seen in all 3 patients, a feature that has been shown to be characteristic of PCD.[11]

Among the patients with MADD, 2 had a good response to riboflavin. Olsen et al. noted that riboflavin-responsive MADD may result from defects in ETFDH combined with general mitochondrial dysfunction.[15] In support of this notion, both of our patients who responded to riboflavin had mutations in *ETFDH*. With regard to CoQ_{10} levels, however, our case contradicts the recent report.[7] Although we measured CoQ_{10} levels in only 2 patients due to sample size limitation, the finding of a normal CoQ_{10} level in a patient with the *ETDFH* mutation is still relevant for clinicians, because it indicates that *ETFDH* mutations may not always be associated with CoQ_{10} deficiency. Further studies are necessary to determine whether there is a detailed relationship between the *ETFDH* mutation and CoQ_{10} deficiency.

The first step of the mitochondrial β-oxidation cycle is catalyzed by four fatty acyl-CoA dehydrogenases (very long, long, medium, and short chain), all of which are affected in MADD. We previously reported that very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency does not show increased lipid droplets in muscle.[13] In contrast, MADD is characterized pathologically by lipid storage, raising the possibility that lipid droplets may not accumulate when one of the four acyl-CoA dehydrogenases, such as VLCAD, is defective.

Our patients with NLSDM presented with distal myopathy and cardiac symptoms, accompanied by lipid accumulation in muscle and peripheral leukocytes, suggesting multisystemic lipid accumulation. Notably, in the patient with NLSDM, mitochondria on electron microscopy were pyknotic, in stark contrast to those in PCD and MADD. This morphological difference is contrary to that expected from function of each causative gene, because PCD and MADD have defects in the mitochondrial β-oxidation cycle, whereas NLSDM is due to a defect in cytoplasmic TG hydrolysis. In addition, rimmed vacuoles were observed in the 2 NLSDM patients and not in the other LSM patients. Together with the fact that both patients had progressive, rather than episodic, muscle disease, these clinicopathological peculiarities should reflect a distinct pathomechanism that is yet to be elucidated. Clearly, further studies

Table 2. Identified mutations.

Patient	Age	Gender	Gene name	Nucleotide change	Amino acid change
1	8 mo	F	*SLC22A5*	c.396G>A* c.844C>T	p.W126X p.A282X
2[11,12]	4 y	M	*SLC22A5*	-91_22del†	
3[11,12]	5 y	M	*SLC22A5*	-91_22del†	
4	5 mo	F	*ETFDH*	c.1519T>G*	p.Y507D
5	6 mo	M	*ETFDH*	c.1208C>T†	p.A403V
6	11 mo	M	*ETFA*	c.284T>G†	p.L95W
7	13 y	F	*ETFDH*	c.524G>A*	p.R175H
				c.1774T>G	p.C592R
8	27 y	M	*PNPLA2*	c.477_478insCCTC*	Frameshift 178X
9	35 y	F	*PNPLA2*	c.477_478insCCTC*	Frameshift 178X

*Compound heterozygous.
†Homozygous.