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

不死化ヒト筋疾患由来筋細胞の樹立とその有用性に関する研究

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研究要旨　筋疾患患者の骨格筋組織から分離した初代培養筋細胞を不死化することができれば、これまでに実施困難であった解析が可能になり、当該筋疾患研究にブレークスルーをもたらすと考えられる。私たちは、ヒト筋細胞を不死化する独自の技術を開発し、ディシェンヌ型および福山型筋ジストロフィーの患者由来初代培養筋細胞の不死化を試みた。その結果、いずれの筋ジストロフィー由来初代培養筋細胞についても、増殖・分化能を保持したまま不死化することに成功した。この技術を応用することによって、広範に分与可能な、安定した性質を有する「不死化筋細胞バンク」を構築することができれば、筋疾患の発症機序解明および治療法開発に大いに貢献することが期待できる。

A.　研究目的

ヒト初代培養筋細胞の増殖・分化能は、継代を繰り返すと著しく低下することが知られている。国立精神・神経センターには、様々な神経筋疾患患者由来の初代培養筋細胞が保存されている。1000例に達する初代培養ヒト筋細胞を不死化できれば、筋疾患由来細胞を広範に供給することが可能になり、筋疾患研究の進展に大いに貢献するものと考えられる。本研究は、先行研究において確立したヒト初代培養筋細胞の不死化法を、疾患由来筋細胞に応用し、不死化筋細胞バンクを構築するために必要な基盤技術の確立を目的とする。

B.　研究方法

細胞培養　国立精神・神経センターにおいて収集・保存されている初代培養ヒト筋細胞ライブラリーに含まれる、ディシェンヌ型筋ジストロフィーおよび福山型筋ジストロフィー患者由来筋細胞を、国立がんセンター研究清野　透博士による遺伝子導入実験に供した。細胞培養液としては Primary Myocytes Growth Medium (pmGM、Wada et al., 2002) を用いた。細胞培養ディッシュは I 型 コラーゲン・コート・ディッシュ（スミロン、c-1シャーレ）を用いた。筋分化は、Primary Myocytes Differentiation Medium (pmDM、Wada et al., 2002)中で培養することによって誘導した。不死化前および導入遺伝子の発現によって延命効果が認められた細胞に関して、増殖能力および筋分化能力を詳細に検討した。

免疫染色　細胞を4%パラフォルムアルデヒドで固定後、骨格筋最終分化マーカーであるmyosin heavy chain (MyHC) に対するモノクローナル抗体MF20と4℃で1-2晩反応させた。次に、Cy3標識抗マウスIgG抗体あるいはbiotin標識抗マウスIgG抗体と反応させた。後者を用いた場合は、さらにhorse radish peroxidase標識ストレプトアビジンと反応させた後、ジアミノベンチジンによる呈色反応により抗原を検出した。

組織化学

初代培養ヒト筋細胞を細胞を4%パラフォルムアルデヒドで固定した。アルカリ性フォスファターゼ (ALP) 活性は、Fast BICIP/NBT (SIGMA)を用いて、組織化学的に検出した。

C.　研究結果

不死化前の初代培養筋細胞の増殖分化特性の検討　国立精神神経センター・筋細胞バンクに保存されている、ディシェンヌ型筋ジストロフィー由来初代培養筋細胞３種（登録番号01-536M（略称DMD1），02-218M（DMD2），06-401M（DMD3））および福山型筋ジストロフィー患者由来筋細胞１種（登録番号07-111F（略称FCMD1））の不死化前における増殖・分化能力について検討した。継代数３代目の細胞をpmDM中で６日間培養し、筋管細胞の分化および筋分化マーカーMyHCの発現を検討した。DMD1では、ほとんど筋管細胞は形成されず、MyHC陽性細胞も数%以下であった。DMD2、DMD3細胞では筋管細胞の形成が認められ、fusion index（筋管細胞に含まれる核数の割合。細胞融合率を表す。）は、それぞれ20および30%で、MyHC陽性率も同程度だった。FCMD1細胞の筋管細胞の形成率は高く、fusion indexおよびMyHC陽性率は、約40%であった。

不死化後の初代培養筋細胞の増殖分化特性の検討

hTERT，cyclinD1，変異CDK4を導入し、延命効果が認められた筋細胞のうち、CMVプロモーターを用いて遺伝子を発現させた細胞（cmvシリーズと呼ぶ）の、増殖・分化能を検討した。DMD1,2，3およびFCMD1に由来する延命化細胞集団を、DMD1cmv、DMD2cmv、DMD3cmv、FCMD1cmvと名付けた。これらの細胞(継代数４代目)をpmDM中で４日間培養し、筋管細胞の分化および筋分化マーカーMyHCの発現を検討した。DMD1cmvは、核数２−３個の小さな筋管細胞を少数形成するのみで、MyHC陽性細胞も極めて少数（５%以下）であった。DMD3cmvおよびFCMD1cmvでは、筋管細胞の形成が顕著であり、MyHC陽性細胞の割合も高かった。DMD2cmvの筋管細胞形成率は、DMD1cmvよりも高いが、DMD3cmvおよびFCMD1cmvに比べると著しく低かった。

培養ヒト筋細胞の純度を図る指標の検討

骨格筋由来の初代培養においては、非筋細胞が混在する可能性があり、その除去が大きな課題の一つと考えられてきた。実際、私たちの先行研究においては、デスミン陽性細胞は初代培養全細胞の一部（10〜30%）にしか認められなかった。我々は先行研究において不死化した、ミトコンドリア関連筋疾患（Leigh脳症）患者由来筋細胞クローンを解析し、筋分化能力を保持した筋細胞クローンにおいても、デスミンの発現は一部の細胞にしか認められないことを明らかにした。すなわち、従来筋細胞系譜のマーカー(cell lineage marker)として用いられてきたデスミンが、ヒト筋細胞に関する限り、必ずしもlineage markerと成り得ない。私たちは、これまでの研究から、培養ヒト筋細胞が、例外なくbrain/liver/kidney-type ALP (BLK-ALP)を発現していることを見出した（Hashimoto et al., 2008)。今回、不死化前後の細胞におけるALP発現を組織化学的に検出したところ、DMD1,2,3およびFCMD1のいずれにおいても、不死化前後に関わらず、ほぼすべての細胞でALP活性が検出された。

（倫理面への配慮）

動物およびヒト材料を用いた実験に関しては、国立長寿医療センターの動物実験倫理委員会、倫理委員会の承認を得、規定にしたがって実施した。

D.　考察

私たちは、先行研究においてhTERT，cyclinD1，変異CDK4の導入により、ヒト筋細胞を効率良く不死化する方法を確立した。しかし、それらの研究では、正常筋組織および筋損傷の軽微なLeigh脳症筋組織から分

離した細胞を用いており、非筋細胞の混在はほとんど認められなかった。ディシェンヌ型および福山型筋ジストロフィーでは、筋損傷が著しく、線維化も起こりがちであるため、線維芽細胞などの非筋細胞の混入は避けられないと予想された。しかし、今回不死化した4種のヒト疾患由来筋細胞に関しては、非筋細胞の混在を示す結果は得られなかった。しかし、MyHC発現、筋管細胞形成などを指標とする限り、DMD1およびDMD2の筋分化率は著しく低く、筋細胞と非筋細胞が混在している可能は否定できない。一方、ヒト再生筋繊維は、ALPを発現することが知られており、ALPは筋再生過程においてヒト筋細胞系譜を特徴付けるマーカーのひとつなのではないかと考えられる。私たちは、これまでに解析した培養ヒト筋細胞37サンプル全てにおいて、ALPの発現を認めている。今回検討した4例の初代培養のいずれにおいても、ほぼすべての細胞でALP発現が認められたことから、非筋細胞は、ほとんど含まれていない可能性がある。私たちは、培養ヒト筋細胞におけるALP発現は、培養下におけるヒト筋細胞固有のストレス応答の一つであると推定している。「ALPが培養下における筋細胞系譜マーカーと成りうるか否か」を明らかにすることは、培養筋細胞の純度（非筋細胞の混入率）を示す指標を確立するために検討すべき重要な課題のひとつである。

E. 結論

ディシェンヌ型および福山型筋ジストロフィーの患者筋組織らから分離した初代培養細胞を不死化することに成功した。いずれの患者由来の細胞からも、筋分化能力を有する不死化筋細胞が得られた。形態的特徴およびALP発現から、非筋細胞の混在は著しくないと考えられた。しかし、今後、筋細胞と非筋細胞を識別する適切な指標を確立する必要がある。

F. 健康危険情報

G. 研究発表

1．論文発表

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H. 知的財産権の出願・登録状況

なし。

厚生労働科学研究費補助金（政策創薬総合研究事業）（総括・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・細胞バンクの利用促進に関する研究」
-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　　既に収集保存されている先天性筋疾患由来のヒト筋芽細胞をより多くの研究者が利用できるように、効率の良いヒト筋芽細胞不死化法の検討を進めた。これまでに、変異CDK4、Cyclin D1とhTERTの組み合わせを発現するレンチウイルスベクターを新たに筋ジストロフィーを含む種々の筋疾患１０症例の筋生検由来の初代培養細胞に導入し不死化法の至適化を検討した。初代培養細胞は継代後、著しい増殖能の低下が観察されたが、遺伝子導入細胞では倍加時間が短く培養維持が容易であった。各延命（不死化）細胞には筋芽細胞が豊富に含まれており、遺伝子導入前の細胞純化作業は省略できることが示唆された。Cyclin D1の強制発現が分化に与える影響を調べたが、分化能は維持されておりCyclin D1の強制発現は分化誘導に対する影響は少ないことが示唆された。

A. 研究目的

ヒトの正常体細胞を培養すると一定回数分裂した後増殖を停止する。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）

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Ducchenneg型筋ジストロフィー３例、福山型筋ジストロフィー１例、ミトコンドリア症３例の他、超稀少疾患３例の計１０症例の筋生検試料由来の初代培養細胞を用いて不死化法の検討を行った。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可能性があるが、今回は筋芽細胞を純化せずそのまま培養細胞に不死化遺伝子群を導入することで、延命・不死化を試みた。不死化遺伝子として、p16INK4aに結合しない変異CDK4, Cyclin D1, hTERTを発現するレンチウイルスベクターを作成し種々の組み合わせで筋芽細胞に導入した。

（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

１０種類の凍結細胞を解凍後、細胞数を数え培養皿に一定数を播種した。各細胞の生存率、生着率は50%を超えており、保存状態が良いことが示唆された。播種翌日にhTERT, 変異CDK4, Cyclin D1を発現するレンチウイルスベクターを導入した。Cyclin D1は高発現が期待されるCMV IEプロモーター制御のベクターと発現量の低いPGKプロモーター制御のベクターの２種類を用いた。変異CDK4＋（CMV-）Cyclin D1+hTERTの導入により全例で延命と倍加時間の短縮が観察された。変異CDK4＋（PGK-）Cyclin D1+hTERTを導入した細胞でも延命が観察されたが、CMV IEプロモーター制御のCyclin D1を導入した細胞に比べ増殖は緩やかであった。また、hTERT単独導入の細胞は延命効果が見られず、継代により著しく増殖能が低下し、２回目の継代が不可能なものも複数存在した。延命（不死化）した細胞の性質を解析中であるが、少なくとも分化能を有する筋芽細胞が存在することが確認されている。

D. 考察

筋芽細胞では、変異CDK4＋Cyclin D1+hTERTによる不死化が、増殖能、分化能の２点において優れており、増殖能はCyclin　D1の発現レベルに依存していることが示唆された。これらの細胞ではCyclin D1/CDK4によるpRbのリン酸化によりpRb経路が不活化された結果、p53発現が増加しているが、アポトーシスなどの像は見られず、正常なp53機能が維持され正常２倍体を保っている可能性が高い。先行して不死化した正常筋芽細胞では正常２倍体を維持していることが確認されている。これまでのところ、変異CDK4＋Cyclin D1導入により不死化した正常筋芽細胞は高い分化能を維持している。変異CDK4＋Cyclin D1導入によるpRbのリン酸化亢進は生理的なpRbの不活化機構であり、正常な細胞機能が維持された可能性が示唆されている。疾患由来の筋芽細胞では病態を反映した分化異常や細胞死が見られる可能性もあり、慎重に検討する必要がある。

E.　結論

Ducchenneg型筋ジストロフィー３例、福山型筋ジストロフィー１例、ミトコンドリア症３例の他、超稀少疾患３例の計１０症例の筋生検試料由来の初代培養細胞を用いて不死化法の検討を行った。筋芽細胞の増殖に問題があると考えられる疾患由来の細胞も効率よく延命(不死化)できることが示された。これらの初代培養細胞の中には、継代後急速に増殖能が低下するものが複数例見られた。疾患由来筋芽細胞の不死化には筋芽細胞の純化前に遺伝子導入による延命（不死化）を優先させる必要があると考えられた。

本研究で開発された筋芽細胞不死化法は、筋疾患由来不死化筋芽細胞バンク設立において必須の技術であり、本研究によりその基盤が確立された。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

本研究に用いる組換えレンチウイルスの使用にあたっては国立がんセンター遺伝子組み換え実験安全委員会の承認を受け適切に取り扱っている。

G．研究発表
1．　論文発表
該当なし（投稿準備中）

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

研究成果の刊行に関する一覧表

書籍

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発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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研究成果の刊行物・別刷（抜粋）

Biochimica et Biophysica Acta 1800 (2010) 313–315

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Pyruvate therapy for Leigh syndrome due to cytochrome *c* oxidase deficiency

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ARTICLE INFO

Article history:
Received 15 May 2009
Accepted 8 July 2009
Available online 17 July 2009

Keywords:
Pyruvate therapy
Leigh syndrome
Cytochrome *c* oxidase deficiency
Exercise intolerance
Lactate-to-pyruvate ratio

ABSTRACT

Background: Recently we proposed the therapeutic potential of pyruvate therapy for mitochondrial diseases. Leigh syndrome is a progressive neurodegenerative disorder ascribed to either mitochondrial or nuclear DNA mutations.
Methods: In an attempt to circumvent the mitochondrial dysfunction, we orally applied sodium pyruvate and analyzed its effect on an 11-year-old female with Leigh syndrome due to cytochrome *c* oxidase deficiency accompanied by cardiomyopathy. The patient was administered sodium pyruvate at a maintenance dose of 0.5 g/kg/day and followed up for 1 year.
Results: The exercise intolerance was remarkably improved so that she became capable of running. Echocardiography indicated improvements both in the left ventricle ejection fraction and in the fractional shortening. Electrocardiography demonstrated amelioration of the inverted T waves. When the pyruvate administration was interrupted because of a gastrointestinal infection, the serum lactate level became elevated and the serum pyruvate level, decreased, suggesting that the pyruvate administration was effective in decreasing the lactate-to-pyruvate ratio.
Conclusions: These data indicate that pyruvate therapy was effective in improving exercise intolerance at least in a patient with cytochrome *c* oxidase deficiency.
General significance: Administration of sodium pyruvate may prove effective for other patients with cytochrome *c* oxidase deficiency due to mitochondrial or nuclear DNA mutations.

1. Introduction

Mitochondrial diseases are intractable disorders, including encephalomyopathy, cardiomyopathy, hearing or visual loss, and diabetes; and they are caused by either mitochondrial or nuclear DNA mutations. In spite of the research efforts for gene therapy aiming at removal of a specific mitochondrial DNA mutation by use of restriction enzymes, *e.g.*, SmaI or XmaI for the m.8993T>G mutation [1], definite therapies have not been established for mitochondrial diseases. The supplementation of vitamins and cofactors are not satisfactory except for a limited number of patients, such as those with thiamine-responsive pyruvate dehydrogenase complex deficiency [2] or those with defects in the biosynthetic pathway of coenzyme Q [3, 4]. Earlier we proposed that pyruvate has a therapeutic potential for mitochondrial diseases, because: (a) pyruvate can stimulate the glycolytic pathway by reducing the NADH/NAD ratio in the cytoplasm, (b) pyruvate can activate the pyruvate dehydrogenase complex (PDHC) by inhibiting pyruvate dehydrogenase kinase, and (c) pyruvate can scavenge hydrogen peroxide by a non-enzymatic reaction [5].

Leigh syndrome (LS) is an early-onset progressive neurodegenerative disorder characterized by developmental delay or regression, lactic acidosis, and bilateral symmetrical lesions in the basal ganglia, thalamus, and brainstem [6, 7]. The disease is caused by mutations in both nuclear and mitochondrial genes involved in energy metabolism; however, the underlying gene defects remain unidentified in nearly half of the patients [8, 9]. Because of the clinical and genetic heterogeneity of the disorder, there is no established treatment for patients with LS.

Our recent trial showed that sodium pyruvate produced a slightly favorable change in the plasma lactate and pyruvate levels for the treatment of mitochondrial disease [5]. This preliminary result prompted us to apply sodium pyruvate to a patient with LS due to cytochrome *c* oxidase. In the present report, we describe our clinical experience with pyruvate therapy in an adolescent with cytochrome *c* oxidase deficiency.

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doi:10.1016/j.bbagen.2009.07.008

2. Administration of pyruvate to a patient with cytochrome *c* oxidase deficiency

An 11-year-old female complained of frequent falls during walking and slowness in running. This patient was born with a weight of 3590 g after a normal pregnancy. At the age of 6 years, she complained of double and blurred vision. Neurological examinations revealed gaze nystagmus and bilateral paresis of the abducens nerve. Cranial magnetic resonance imaging (MRI) demonstrated bilateral lesions in the putamen (Fig. 1). The lactate level was elevated in the cerebrospinal fluid (31 mg/dL, normal 10–20 mg/dL). Histopathological study of the skeletal muscle revealed the presence of diffuse cytochrome *c* oxidase-negative fibers (Fig. 2) without ragged-red fibers (RRF) or strongly succinate dehydrogenase-reactive blood vessels (SSV). Biochemical analysis of the mitochondria isolated from the skeletal muscle indicated a marked deficiency of cytochrome *c* oxidase activity (17% of the normal control value). The sequencing of the entire mtDNA identified no pathogenic mutations either in the protein-coding regions or in the ribosomal and transfer RNA genes. From these findings she was diagnosed as having LS due to cytochrome *c* oxidase deficiency. From the age of 8 years oral administration of coenzyme Q was started, but her motor dysfunction became gradually aggravated and her easy fatigability, enhanced. Neurological examination revealed dystonia and an ataxic gait. She sometimes needed assistance in walking, and her speech became gradually slurred. At the age of 10 years, echocardiography revealed mild cardiac dysfunction: her left ventricular ejection fraction was 52% (normal 55%–80%), and the fractional shortening was 26% (normal>28%). An electrocardiogram revealed inverted T waves in leads V3 and V4, suggesting cardiac muscle involvement. The blood lactate and pyruvate levels were 20.5 mg/dL and 1.13 mg/dL, respectively, with a lactate-to-pyruvate ratio of 18.1. The levels of lactate and pyruvate in the cerebrospinal fluid were 32.4 mg/dL and 1.21 mg/dL, respectively, giving a lactate-to-pyruvate ratio of 26.8.

At the age of 11 years, administration of sodium pyruvate (0.5 g/kg/day) was started. After the administration both the blood lactate and pyruvate levels decreased to 10.3 mg/dL and 0.88 mg/dL, respectively, with a reduction in the lactate-to-pyruvate ratio to 11.7.

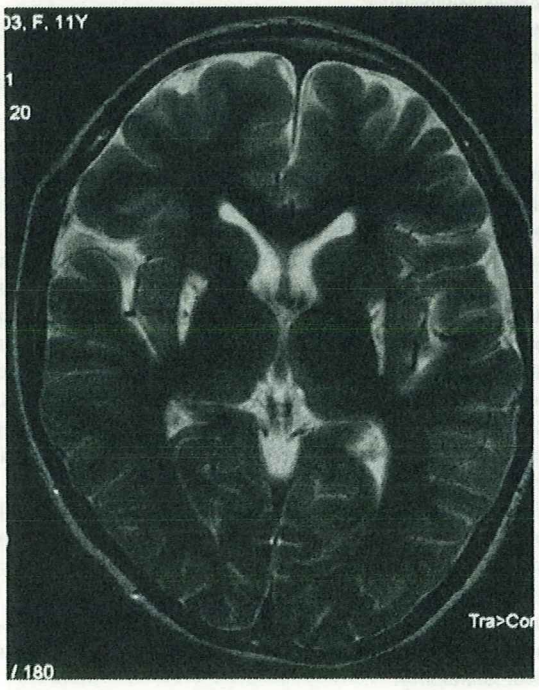

Fig. 1. T2-weighed magnetic resonance imaging (MRI) of the brain of the patient at 11 years of age.

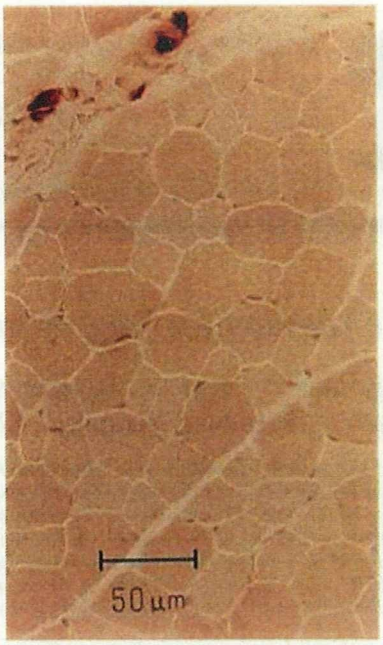

Fig. 2. Histochemical staining for cytochrome *c* oxidase in the biopsied skeletal muscle of the patient. Diffuse deficiency of cytochrome *c* oxidase is to be noted.

Interestingly, the exercise tolerance of the patient improved after the start of pyruvate administration; and she became capable of participating in athletic games in school. One year after the start of pyruvate administration, although none of the neurological symptoms or signs had significantly improved, her cardiac function returned to within the normal ranges: left ventricular ejection fraction of 58% and fractional shortening of 30%. Inverted T waves in leads V3 and V4 of the electrocardiogram were diminished. These findings suggest that pyruvate administration might have beneficial effects on mitochondrial cardiomyopathy.

When pyruvate administration was interrupted because of a gastrointestinal infection, the serum lactate level of the patient increased from 11.3 mg/dL to 14.3 mg/dL; and her serum pyruvate level decreased from 0.96 mg/dL to 0.94 mg/dL, suggesting that the pyruvate administration was effective in decreasing the lactate-to-pyruvate ratio.

The present observations suggest that oral administration of sodium pyruvate at a dose of 0.5 g/kg/day had no harmful effects, although diarrhea was sometimes observed when the pyruvate was administered at a high concentration. We therefore recommend administering sodium pyruvate at 16.5 g/L (150 mM) diluted in either water, milk or fruit juice.

3. Discussion

In the present study, we reported a patient with LS who responded to pyruvate administration. The histochemical finding of diffuse cytochrome *c* oxidase deficiency indicated that this condition was distinct from the benign infantile mitochondrial myopathy due to reversible cytochrome *c* oxidase deficiency [10]. The sustained levels of blood lactate and pyruvate suggested that the enzyme defect itself was persisting in the present patient.

We also administered sodium pyruvate to several patients with mitochondrial encephalomyopathies in advanced stages. In such patients having respiratory disturbance necessitating artificial ventilation, dysphagia requiring tube feeding or a gastric fistula, severe psychomotor developmental delay, and/or multiple organ failure, we were unable to assess the efficacy of pyruvate administration. Considering the progressive nature of LS, and given that the pyruvate

administration is efficacious in preventing neurodegeneration, therapeutic intervention should be started in the early stage of disease progression.

There are some limitations in the present study. First, despite vigorous analysis of mitochondrial DNA mutations, we were unable to identify the causative etiology of LS in the present patient. Further survey for nuclear DNA mutations is needed. Second, this was a clinical study on one patient, in which the results must be interpreted with caution. For validation of our findings, multi-institutional research including the present case should be conducted.

In a patient with LS associated with cardiomyopathy examined previous to the present one (Wakamoto et al., unpublished observation), MRI conducted 1 year after the start of pyruvate therapy demonstrated remarkable improvement with distinct decreases in the size and intensity of the lesions located in the basal ganglia. Echocardiography also demonstrated marked improvements in the values of left ventricular end-diastolic diameter, left ventricular end-systolic diameter, fractional shortening, and left ventricular ejection fraction; although the degree of hypertrophy of the heart muscle was not influenced by the pyruvate administration. These observations indicated improved cardiac function after the treatment of this LS patient.

In another patient with LS, a marked improvement in the electroencephalographic findings was noticed after administration of sodium pyruvate (Koga et al., unpublished). Because LS is caused by a wide variety of the molecular and genetic defects, we need to identify the specific subtypes that are responsive to pyruvate therapy. For this purpose, we have started constructing a rapid and comprehensive detection system for pathogenic mutations of mitochondrial DNA by use of the Luminex suspension array technology (Nishigaki et al., in preparation). Efficient and systematic screening for nuclear DNA mutations should be also established.

Hermann et al. investigated the effect of intracoronary pyruvate in 8 patients with congestive heart failure, and concluded that pyruvate had a favorable inotropic effect [11]. Pyruvate affects energy metabolism by its input into the tricarboxylic-acid (TCA) cycle in 2 ways. First, pyruvate enters the TCA cycle as acetyl-CoA after decarboxylation via pyruvate dehydrogenase. Second, pyruvate enriches the TCA cycle after carboxylation to oxaloacetate and/or malate via pyruvate carboxylase and/or malic enzyme. Actually the ^{13}C NMR spectroscopic study by Weiss et al. demonstrated that the addition of 0.8 mM pyruvate significantly increased in the levels of citrate in the rat heart perfused with 5 mM [2-^{13}C]acetate [12]. This anaplerotic effect of pyruvate would increase the flux through the TCA cycle, supplementing oxidative phosphorylation. The exact mechanisms by which pyruvate improved the exercise intolerance in the present patient with cytochrome *c* oxidase deficiency should be further investigated.

We previously demonstrated that pyruvate infusion lowered the lactate-to-pyruvate ratio and corrected the deficit in ureogenesis in the liver of citrin-knockout ($Ctrn^{-/-}$) mice, a model of adult-onset type II citrullinaemia [13]. Recently, Mutoh et al. reported the use of arginine and sodium pyruvate for the treatment of a citrin-deficient patient at the early stage of adult-onset type II citrullinaemia [14]. Oral administration of arginine and sodium pyruvate for over 3 years improved the clinical symptoms and almost completely normalized the laboratory findings of the patient. The authors concluded that the administration of arginine and sodium pyruvate with low-carbohydrate meals may be an effective therapy for patients with citrin deficiency in order either to prolong metabolic normalcy or to provide a safer and more affordable alternative to liver transplantation [14]. Thus, extended studies are needed to confirm the therapeutic potential of pyruvate for both citrin deficiency and mitochondrial respiratory defects.

In conclusion, although the pathogenic mutation causing the mitochondrial dysfunction was not determined, our results suggest that exercise intolerance, mild cardiac dysfunction, and lactic acidosis were ameliorated by the pyruvate administration. Administration of sodium pyruvate may prove effective for other patients with cytochrome *c* oxidase deficiency due to mitochondrial or nuclear DNA mutations. Validation of our findings will require their replication with additional patients having different mitochondrial abnormalities confirmed by genetic or biochemical analysis.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research [C-18590317 to Y.N., and C2-10832009 and A (2)-15200051 to M.T.], a Grant-in-Aid for Young Scientists (B-18700541 to N.F.), and a Grant-in-Aid for Exploratory Research (20650113 to N.F.) from the Ministry of Education, Culture, Sports, Science and Technology; by a grant from the Third-Term Comprehensive 10-year Strategy for Cancer Control (to M.T.); by grant 20B-13 from the program Research Grants for Nervous and Mental Disorders of the Ministry of Health, Labour, and Welfare (to M.T.); and by grants for scientific research from the Kao Research Council for the Study of Healthcare Science (to Y.N.), the Takeda Science Foundation (to Y.N. and to M.T.), and the Sankyo Foundation of Life Science (to Y.N.).

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Brain & Development 31 (2009) 465–468

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

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Case report

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

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

Abstract

We report a patient harboring a novel homozygous mutation of c.604T > G (p.F202V) in *POMT2*. He showed delayed psychomotor development but acquired the ability to walk at the age of 3 years and 10 months. His brain MRI was normal. No ocular abnormalities were seen. Biopsied skeletal muscle revealed markedly decreased but still detectable glycosylated forms of alpha-dystroglycan (α-DG). Our results indicate that mutations in *POMT2* can cause a wide spectrum of clinical phenotypes as observed in other genes associated with α-dystroglycanopathy. Presence of small amounts of partly glycosylated α-DG may have a role in reducing the clinical symptoms of α-dystroglycanopathy.

Keywords: POMT2; α-Dystroglycan; α-Dystroglycanopathy; Congenital muscular dystrophy; Limb girdle muscular dystrophy; Brain MRI

1. Introduction

Alpha-dystroglycan (α-DG) is a surface membrane protein that links extracellular basal lamina and intracellular cytoskeleton. α-DG is a highly glycosylated protein mainly composed of unique *O*-mannosyl glycans. Reduced/altered glycosylation of α-DG causes a wide variety of muscular dystrophies including Walker–Warburg syndrome (WWS), muscle-eye-brain disease (MEB), Fukuyama-type congenital muscular dystrophy (FCMD), congenital muscular dystrophies type 1C and type 1D, and limb girdle muscular dystrophies (LGMD) type 2I, 2K to 2N. They are collectively called alpha-dystroglycanopathies (α-DGP). So far, six causative genes for α-DGP have been identified including *protein-O-mannosyl transferase 1* and *2* (*POMT1 and POMT2*), *protein O-mannose β-1,2-N-acetylglucosaminyltransferase* (*POMGnT1*), *fukutin* (*FKTN*), *fukutin-related protein* (*FKRP*), and *acetylglucosaminyl transferase-like protein* (*LARGE*). Here we report a mild congenital muscular dystrophy patient associated with a novel homozygous mutation in *POMT2*.

2. Case report

A 4-year-old Japanese boy, the only child from healthy consanguineous parents, was delivered uneventfully at full term. During few days after birth, he was

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0387-7604/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.braindev.2008.08.005

low spirited and showed sucking weakness. Floppiness was not prominent but serum CK levels were markedly elevated up to 33,000 IU/l (normal < 70). His condition was improved within 2 weeks, but serum CK levels were persistently higher than 1000 IU/l. His motor milestones were delayed and he could control his head at 5 months of age. At 6-month-old, he could not sit without support, and muscle weakness and atrophy were noticed in lower limbs. Deep tendon reflexes were normal. No high arched palate or macroglossia were seen. Enjoji Scale of Infant Analytical Development (ESID) at his age of 7 months revealed mild delay in body movement (developmental age was 4 months, expression of language: 5 months), and his DQ was 83. Brain computed tomography (CT) revealed no definite abnormalities. Nerve conduction study was normal. His motor functions developed gradually and he was able to walk without support at 3 years and 10 months old. Gowers' sign was positive. Mild calf hypertrophy was seen with no joint contractures (Fig. 1A). Deep tendon reflexes were normal except for diminished Achilles tendon reflexes. ESID performed at his age of 3 years and 11 months showed general developmental delay (body movement: 15 months, hand movement: 24 months, activity of daily living: 27 months, personal relations: 24 months, expression of language: 18 months, and comprehension of language: 24 months), and his DQ was 47. Brain magnetic resonance imaging at 4 years and 1-month-old revealed no notable anomaly or cortical dysplasia (Fig. 1B). Detailed ophthalmological examinations revealed no abnormalities. No cardiac involvement was detected by chest X-ray, electrocardiogram, and echocardiography.

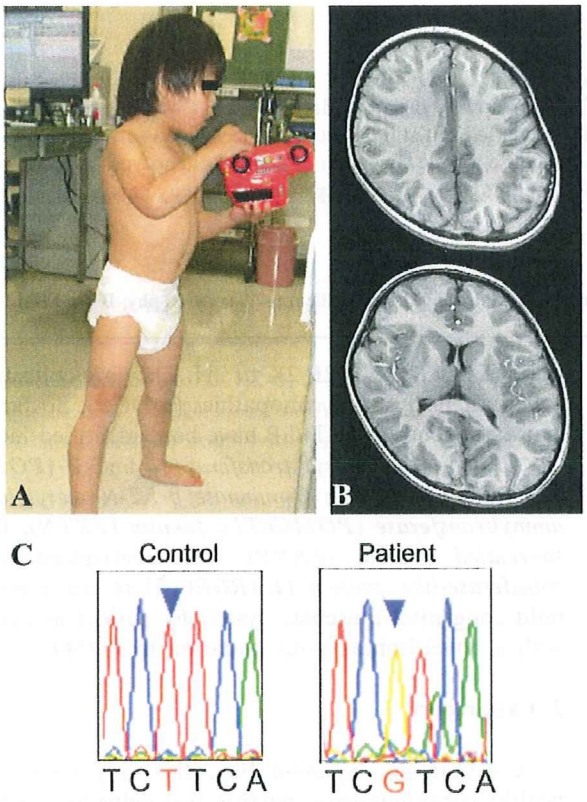

Fig. 1. (A) The patient can stand and walk with no support. Minimal calf hypertrophy is seen. (B) T2 weighted brain magnetic resonance imaging shows no obvious brain anomaly, cortical dysplasia, or white matter changes. (C) Sequence analysis of *POMT2* revealed a homozygous mutation at c.604T > G in exon 5.

Muscle biopsy taken at 7 months of age with informed consent showed dystrophic changes with scattered necrotic and regenerating fibers and mild endomysial fibrosis (Fig. 2A). No inflammatory changes were seen. On immunohistochemistry, glycosylated forms of α-DG detected by VIA4-1 antibody (Upstate Biotechnology, NY) was markedly reduced in the sarcolemma, while immunoreactions for the core region of α-DG using GT20ADG antibody [1] (data not shown) and for β-DG (43DAG1/8D5; Novocastra Laboratories, UK) was well preserved (Fig. 2A). On immunoblotting analysis, faint, broad band of around 140 kDa in size was detected by VIA4-1, whereas GT20ADG recognized a band of around 90 kDa in size. Laminin overlay assay showed barely detectable binding product (Fig. 2B). These results suggested altered glycosylation of α-DG in the muscle.

We performed mutation screening in all six causative genes for α-DGP. Genomic DNA was extracted from peripheral lymphocytes using standard technique after informed consent. Primer sequences we used are available on request. All exons and their franking intronic regions were directly sequenced by ABI PRISM 3100 (PE Applied Biosystems, CA). We identified a homozygous missense mutation of c.604T > G (p.F202V) in exon 5 of *POMT2* (Fig. 1C), which is not described in previous publications [3–8] and the mutation database (http://www.dmd.nl/).

The protein *O*-mannosyltransferase (POMT) activity was measured as previously described [2]. Mutant POMT2 (F202V) co-expressed with POMT1 in COS cells showed barely detectable POMT activity (data not shown).

3. Discussion

POMT2 is the gene encoding an enzyme for protein *O*-mannosylation, and it is required to form a complex with POMT1 for the enzyme activity [2]. Recently, some patients with mutations in *POMT2* have been reported [3–8]. Most patients showed floppiness at birth, delayed psychomotor development, congenital muscular dystrophy, and severe mental retardation with or without ocular involvement. Brain anomalies are prominent including hydrocephalus, lissencephaly, agenesis of the corpus callosum, fusion of the hemispheres, and cerebel-

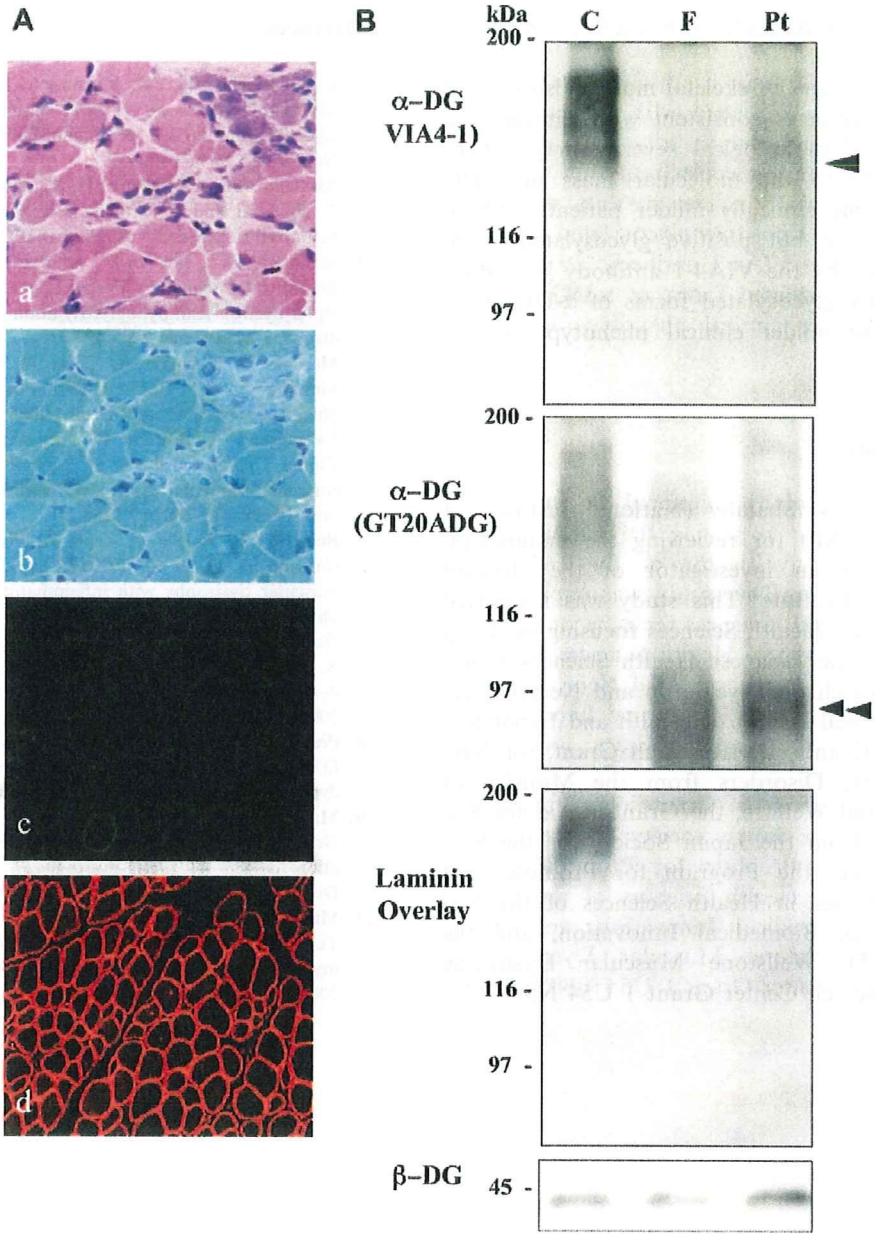

Fig. 2. (A) Histological analysis. On Hematoxylin and eosin (a) and modified Gomori-trichrime (b) staining, variation in fiber size and scattered necrotic and regenerating fibers are seen. Immunohistochemical analysis using antibodies VIA4-1 (c), which recognize heavily glycosylated form of α-dystroglycan (α-DG), showed greatly reduced sarcolemmal staining in patient, but well preserved immunoreactivities of β-DG (d) is seen. Bar = 50 m. (B) Immunoblotting analysis. Immunoblotting analysis using antibodies of VIA4-1, GT20ADG for α-dystroglycan (α-DG) and laminin overlay assay are performed using skeletal muscle from control (C), Fukuyama-type congenital muscular dystrophy (FCMD; F), and the patient (Pt). VIA4-1 recognizes a broad band about 156 kDa in size in control, and approximately 90 kDa in FCMD. In the patient muscle, reduced in size and amount compared with control was observed. GT20ADG revealed bands at approximately 90 kDa in both the patient and FCMD muscles. Laminin overlay assay shows barely detectable band in both the patient and FCMD.

lar hypoplasia [3–5]. In contrast, the patient reported here shows milder clinical features. Although his psychomotor milestones were delayed, he achieved independent ambulation with no marked brain malformation and ocular involvement. His clinical phenotype was intermediate between congenital muscular dystrophy and limb girdle muscular dystrophy. Milder clinical features with mutations in *POMT2* have been recently reported and designated as limb girdle muscular dystrophy type 2N [6,7]. Mutations in *POMT2* can cause wide spectrum of clinical phenotypes from Walker–Warburg syndrome to limb girdle muscular dystrophy (LGMD),

as demonstrated in patients with *FKRP*, *FKTN*, or *POMT1* mutations.

Pathological changes of skeletal muscle also showed mild dystrophic changes consistent with clinical findings. Clinical and pathological severity may not be always correlated to the molecular mass of α-DG [9]. However, some clinically milder patients with α-DGP show reduced but positive glycosylated forms of α-DG detected by the VIA4-1 antibody [10]. Preservation of partly glycosylated forms of α-DG could contribute to the milder clinical phenotype of this patient.

Acknowledgements

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

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J Neuropathol Exp Neurol

Vol. 68, No. 6
June 2009
pp. 701–707

ORIGINAL ARTICLE

Defective Myotilin Homodimerization Caused by a Novel Mutation in *MYOT* Exon 9 in the First Japanese Limb Girdle Muscular Dystrophy 1A Patient

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Abstract

Myotilin is a muscle-specific Z disk protein. Several missense mutations in the myotilin gene (*MYOT*) have been identified in limb girdle muscular dystrophy (LGMD), myofibrillar myopathy, and distal myopathy patients. All previously reported pathogenic *MYOT* mutations have been identified only in Exon 2. We sequenced *MYOT* in 138 patients diagnosed as having LGMD, myofibrillar myopathy, or distal myopathy, and identified a novel *MYOT* mutation in Exon 9 encoding the second immunoglobulin-like domain in 1 patient with clinically typical LGMD. By light microscopy, there were scattered fibers with rimmed vacuoles and myofibrillary disorganization in the patient's muscle biopsy; accumulation of Z disk proteins was observed by immunohistochemistry. Immunoblot analysis demonstrated that the amount of myotilin monomer was increased in the patient muscle, but that the myotilin homodimeric band was decreased. Functional analysis of the myotilin mutation using a yeast 2-hybrid system revealed defective homodimerization of the mutant myotilin and decreased interaction between mutant myotilin and α-actinin. The homodimerization defect was further demonstrated by immunoprecipitation. This is the first *MYOT* mutation outside of Exon 2 in an LGMD type 1A patient and the first *MYOT* mutation identified in the Japanese population. This mutation in the second immunoglobulin-like domain impairs myotilin dimerization and alters the binding between myotilin and α-actinin, which is known to be important for actin bundling.

Key Words: Distal myopathy, Homodimer, Immunoglobulin-like domain, Limb girdle muscular dystrophy, Myofibrillar myopathy, Myotilin, Myotilinopathy.

INTRODUCTION

Myotilin (myofibrillar protein with titin-like immunoglobulin domains) is a sarcomeric Z disk protein encoded by the myotilin gene (*MYOT* or *TTID*) on chromosome 5q31 (1). Myotilin is composed of a unique serine-rich N-terminus and 2 immunoglobulin-like domains at the C-terminus (1–4). Myotilin forms a homodimer and interacts with α-actinin (1), actin (5), and filamin C (FLNC) (6, 7) at the Z disk through these immunoglobulin-like domains (Fig. 1A). Myotilin is highly expressed in skeletal muscle, cardiac muscle, and peripheral nerves (1). Myotilin plays a significant role in sarcomere assembly by acting together with α-actinin and FLNC to cross-link actin filaments into tightly packed bundles (1, 5, 8). The resulting structures support the integrity of the contracting muscle cell (6).

Missense mutations in *MYOT* cause myofibrillar myopathy (MFM) (9–11), limb girdle muscular dystrophy type 1A (LGMD1A) (11–13) and late-onset distal myopathy (14, 15). These myotilinopathies are usually associated with cardiac involvement and peripheral neuropathy and rarely with respiratory involvement. Currently, there are only 8 known *MYOT* mutations associated with myopathy (i.e. K36E, S39F, S55F, T57I, S60C, S60F, Q74K, and S95I) (8–11); all are located in Exon 2 encoding the serine-rich amino-terminal (Fig. 1A).

Here, we report the first Japanese LGMD1A patient associated with a novel mutation in the second immunoglobulin-like domain of myotilin and provide data suggesting the pathobiologic significance of this mutation.

MATERIALS AND METHODS

Clinical Materials

All clinical materials used in this study were obtained for diagnostic purposes with informed consent. We searched

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This study was supported by a grant-in-aid for scientific research and a grant-in-aid for exploratory research from the Japan Society for the Promotion of Science; by the Research on Psychiatric and Neurological Diseases and Mental Health of the Health Labour Sciences Research Grant and research grants (20B-12, 20B-13, 19A-4, and 19A-7) for Nervous and Mental Disorders from the Ministry of Health, Labour, and Welfare; by the Research on Health Sciences focusing on Drug Innovation from the Japanese Health Sciences Foundation; and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation.

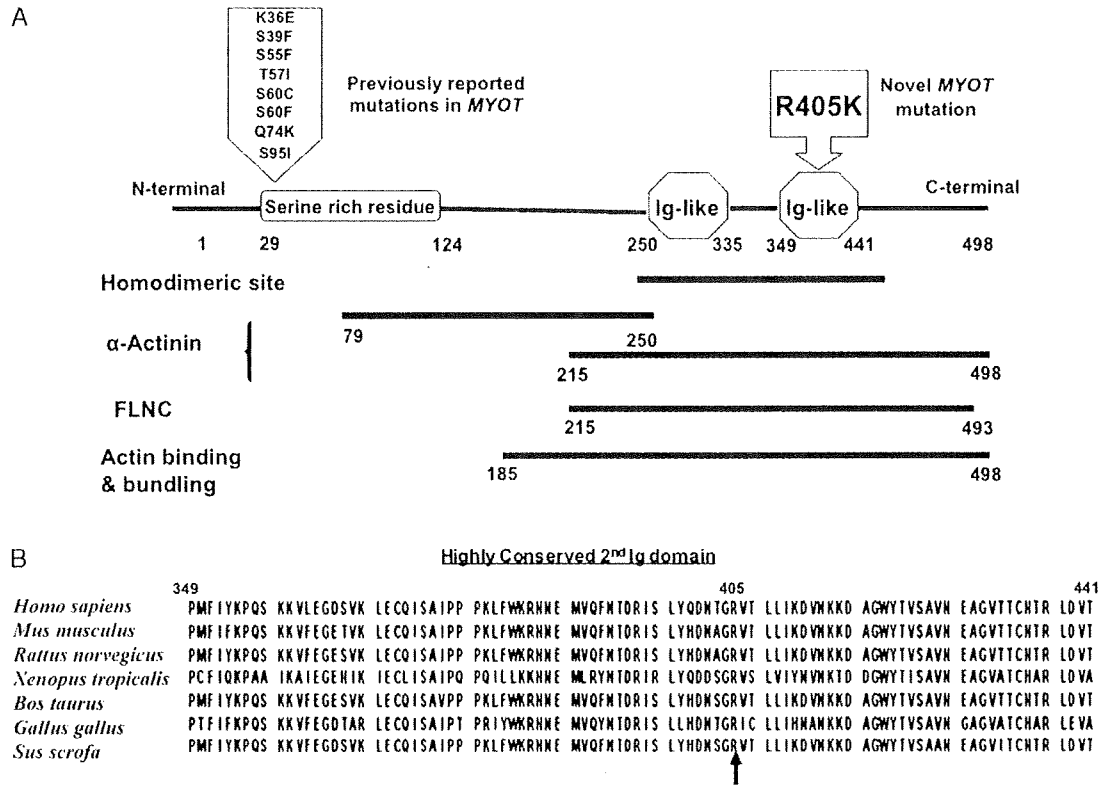

FIGURE 1. Myotilin structure, interacting partners, and reported mutations. **(A)** All previously reported mutations are located in the serine-rich domain; the novel mutation p.R405K identified in this study is located in the second immunoglobulin (Ig)-like domain of myotilin. Immunoglobulin domains of myotilin are regions of most myotilin interactions with other proteins. **(B)** The second Ig domain is highly conserved through species including the mutated residue p.R405 in the patient (arrow) (6–8). FLNC, filamin C.

for *MYOT* mutations in a total of 138 Japanese patients who had been diagnosed pathologically or clinically as MFM (n = 48), LGMD (n = 40), or distal myopathy (n = 50).

Genetic Analysis

Genomic DNA was isolated from peripheral lymphocytes or muscle specimens using standard techniques. Nine sets of primers were used to amplify genomic fragments of *MYOT*. All exons and their flanking intronic regions of *MYOT* were sequenced directly in all 138 patients using an ABI PRISM 3100 automated sequencer (PE Applied Biosystems, Foster City, CA). Nearly all of the patients with the different diagnoses were screened for *ZASP*, *DES*, and *CRYAB* mutations. Distal myopathy patients were also screened for *GNE* mutations. Primer sequences are available on request.

Histochemical Analysis

Biopsied muscle specimens were frozen in isopentane cooled in liquid nitrogen. Serial 10-μm cryosections were stained with hematoxylin and eosin, modified Gomori trichrome, nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase, myosin ATPase phosphatase, and a battery of histochemical methods.

Immunohistochemistry and Immunoblotting

Immunohistochemistry and immunoblotting were performed as previously described (13, 16, 17). Antibodies used in this study are to: myotilin (kindly provided by Dr Carpén, University of Helsinki) (13), desmin (Abcam, Tokyo, Japan), Z-band alternatively spliced PDZ motif protein (ZASP) (Abcam), skeletal muscle actin (Nichirei, Tokyo, Japan), and αB-crystallin (Stressgen, Victoria, British Columbia, Canada).

Antibodies to dystrophin (Novocastra Laboratories, Newcastle upon Tyne, UK), sarcoglycans (Novocastra), α-dystroglycan (Upstate Biotechnology, Lake Placid, NY), dysferlin (Novocastra), caveolin-3 (BD Transduction Laboratories, Lexington, KY), calpain-3 (Novocastra), merosin (Chemicon International, Temecula, CA), collagen VI (ICN Biomedicals, Inc, Cleveland, OH), and emerin (Novocastra) were also used in the LGMD patients to exclude other diagnosable causes of muscular dystrophy.

Plasmid Construction

Total RNA was extracted from control human skeletal muscle using standard techniques; cDNA was synthesized by reverse transcription polymerase chain reaction with random hexamers. Full-length myotilin (wtMYOT), skeletal muscle

actin (ACTA1), α-actinin 2 (ACTN2), and the C-terminal portion (i.e. amino acids from 1967 to 2699) of FLNC were amplified using the following primers: MYOT, 5′-GGAATT CAGTAATAATTTGCCTTCA TCTTCCA-3′ and 5′-CGGGATCCACAAATCCATATACCCAGATTTCCT-3′; ACTA1, 5′-GGAATTCCAGAAACTAGACACAATGTG CGA-3′ and 5′-CGGGATCCAGTTGTTACAAAGAAAGT GACTGCG-3′; ACTN2, 5′-CCGGAATTCGCGGCCAC CATGGATTACAAGGATGACGACGACGATAAGAAC CAGATAGAGCCCGGCGT-3′ and 5′-CCGCTCGAGTCA CAGATCGCTCTCCCCGTA-3′; and FLNC, 5′-GGAATT CAAGATCACCGAGAGTGATCTGAGC-3′ and 5′-GTCGACCTCCTTGACAGTGTAGGTGACATTG-3′. The polymerase chain reaction products were cloned into the pGEM-T-easy vector (Promega, Madison, WI), and their sequences were confirmed. For expression in yeast, the cDNAs inserted in pGEM-T-easy were digested and ligated into the vectors pGBKT7 containing *GAL4* DNA-binding domain and pGADT7 containing *GAL4* activating domain (Takara Bio, Shiga, Japan). Mutant myotilin (mMYOT) (c.1214G>A) was generated by site-directed mutagenesis using the primers 5′-GATAACACTGGAAAAGTTACTTTACTG-3′ and 5′-CAGTAAAGTAACTTTTCCAGTGTTATC-3′.

Yeast 2-Hybrid Experiment

Yeast 2-hybrid (Y2H) assays aimed at testing specific interaction pairs were carried out as previously described (18–20). *Saccharomyces cerevisiae AH109* was double transformed with pGBKT7 constructs and pGADT7 constructs and selected on minimal medium lacking leucine and tryptophan and containing histidine (SD/-Leu/-Trp/+His) plates (low-stringency plate). The transformants were picked up and spotted onto selective medium lacking histidine (SD/-Leu/-Trp/-His) plates with 0.2, 0.5, 1.0, 2.0, or 5.0 mmol/L 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the HIS3 reporter gene (high-stringency plate) and plates without 3-AT (medium-stringency plate). The plates were then incubated at 30°C for 4 days, and the growth of the transformants was analyzed.

Immunoprecipitation and Immunoblot Analysis

For expression in mammalian cells, wtMYOT or mMYOT inserted in pGADT7 were digested by *Eco*RI and *Bam*HI, and ligated into FLAG-tag-inserted pcDNA3.1/V5-HisA (kindly gifted by Dr Ishiura, University of Tokyo). The cDNAs inserted in pGADT7 were also digested by *Sfi*I and *Xho*I and ligated into pCMV-Myc vector (Takara Bio).

COS-7 cells were cultured in Dulbecco modified Eagle medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% carbon dioxide. The cells were transiently transfected using FuGENE HD transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Mouse horseradish peroxidase–conjugated anti-Myc antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-FLAG polyclonal antibody was purchased from Sigma.

COS-7 cells were cotransfected with 5 μg of each plasmid. Forty-eight hours after transfection, the cells were then lysed in 1.0 mL of lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA (pH 8.0), 10% glycerol, 1% Nonidet P-40, and Complete Protease Inhibitor Cocktail (Roche Diagnostics). The lysates were incubated at 4°C for 30 minutes with gentle rotation and then centrifuged at 15,000 × *g* at 4°C for 30 minutes. The supernatants were collected, and their protein concentrations were determined using the protein assay kit (Bio-Rad, Hercules, CA).

For immunoprecipitation, the protein concentration of the cleared lysates was adjusted to 1.5 μg/μL, and anti-FLAG M2 affinity gel (Sigma) was added. The mixtures were incubated at 4°C overnight. The resulting immune complexes were washed once with lysis buffer and 3 times with Tris buffered saline. The proteins were eluted by boiling at 95°C for 5 minutes in the sample buffer without reducing agent (50 mmol/L Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 1% glycerol [vol/vol], 0.1% bromophenol blue), and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The polyacrylamide gel electrophoresis–separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and the membranes were blocked with blocking buffer (5% nonfat dry milk in PBS containing 0.05% Tween-20) at room temperature for 1 hour. The blocked membranes were incubated with a primary antibody at room temperature for 1 hour. Anti-Myc-horseradish peroxidase antibody was diluted in Can Get Signal solution 2 (Toyobo, Osaka, Japan) at 1:1000. Anti-FLAG polyclonal antibody was diluted in blocking buffer at 1:4000) Anti-FLAG was followed with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G antibody at 1:4000 at room temperature for 30 minutes. Immunoreactive complexes on the membranes were visualized using enhanced chemiluminescence or enhanced chemiluminescence–plus detection reagent (GE Healthcare UK Ltd, Buckinghamshire, UK).

RESULTS

Genetic Analysis

We identified a novel heterozygous missense mutation c.1214G>A (p.R405K) in Exon 9 of *MYOT* in 1 LGMD patient (Fig. 1B). This mutation was not identified in a panel of 100 healthy Japanese controls.

Clinical Data

The 57-year-old female patient presented with gait disturbance. She started experiencing difficulty in standing up and climbing the stairs by age 41 years. Her condition gradually progressed, and by age 50 years, she could not walk long distances and could not stand up or climb stairs without support. Her deceased father and elder sister had a similar condition. Her sister was previously diagnosed as having sporadic inclusion body myositis, but further information could not be obtained. On examination, the patient had proximal dominant muscle weakness, especially in neck flexors, iliopsoas, hamstring, and quadriceps muscles (3/5 by