表7-6　Leigh脳症の臨床像

発症年齢	多くは乳児（2〜24か月）
臨床症状	精神運動遅滞・筋緊張低下・痙攣などを認め，呼吸障害が早期にあらわれやすい
診断	CT/MRI上で，脳幹や大脳基底核に対称性病変 髄液中の高乳酸値
治療	有効なものなし
原因	mtDNA変異によるものが約20%

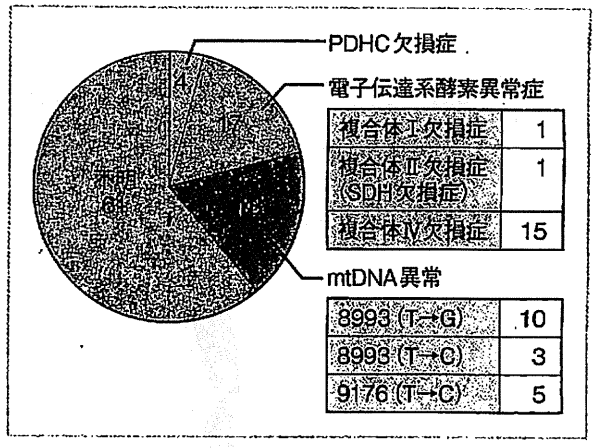

図7-11　Leigh脳症で認められるおもな酵素異常症の割合（n=100）
（国立精神・神経センター，1978〜2000年）

れた神経病理学的な疾患の概念で，当初は死亡後に解剖をして初めて診断がつくものでした。基本的には大脳基底核や脳幹に病変があって，組織学的には海綿状変性で神経細胞が脱落し，血管新生が多くみられるのが特徴です。非常に早く発症する病気で，乳児に多く，2歳くらいまでに発症して非常に重篤な経過をたどっていきます。特に脳幹部が障害されますので，呼吸不全が早晩あらわれてきて人工呼吸器を使うことになるという，非常に重い病気です（**表7-6**，**図7-11**）。

このLeigh脳症のなかの一部が，mtDNAの変異によるものであることがわかりました。ただし全体からみるとmtDNA変異は約20%で，残る80%は核DNA異常だろうといわれています。ただし，どちらであろうがこのような重篤な臨床経過をたどるということがあって，現在，小児科領域ではMELASと同じくらいの頻度で診断がされている病気です。Leigh脳症でみつかるmtDNAの変異を**図7-12**に示してあります。青地に書いてあるのが，ATPaseの8993や9176の変異です。この変異はホモプラスミーで認められる，要するに全部が変異型になっている状態で発見されることが多いです。先述したMELASの3243変異の場合はヘテロプラスミーで，細胞ごとの違いというのがあるわけですが，こちらの場合は全部の細胞が同じ変異をもっている点が異なります。3243変異の場合はヘテロプラスミーで起こって，ATPase変異の場合はホモプラスミーで起こる。ATPaseの領域で起こる病気の場合は筋肉をみてもRRFがみられない場合が多いです。

これは実は大きな違いでして，3243変異のようにヘテロプラスミーの場合は，組織や細胞での違いが大きいわけです。それは当然それぞれがもっている変異の比率が違うからであり，そうなると症状の多様性も大きいということになり，先述したように発症前・出生前診断ができないということになります。しかし8993変異はホモプラスミーですので，組織・細胞での違いが少なく，筋肉だろうが脳，血液だろうが，同じようにホモプラスミーであるということです。ということは羊水を取ってみたり，絨毛を調べてみることによって，変異が存在していれば予後がわかるということで，これに関しては実際に出生前診断が行われており，欧米では着床前診断も行われています。このように，同じmtDNAの変異であっても，ヘテロプラスミーで起こる病気と，ホモプラスミーで起こる病気とでは，生殖補助医療においても

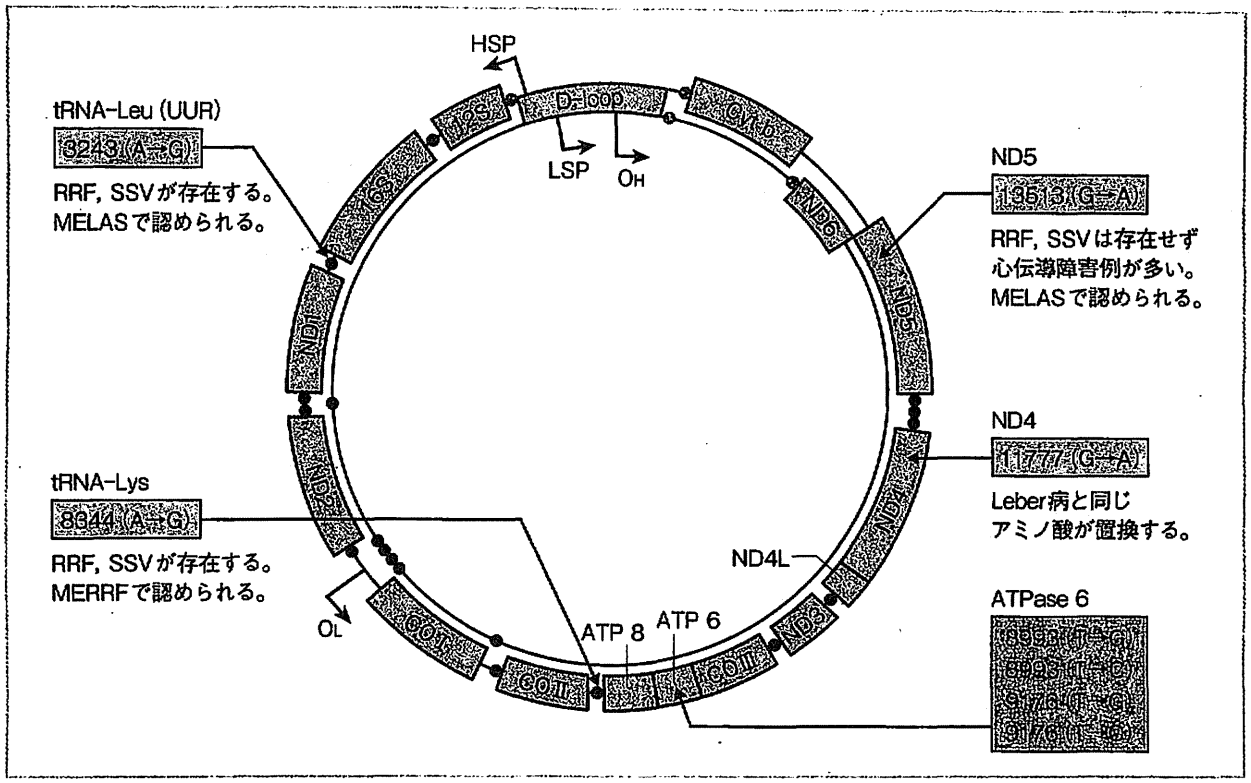

図7-12　Leigh脳症で認められるmtDNAの点変異

対応がまったく変わってくるといえます。

臨床の場において ミトコンドリア病がみつかったら

私たちが最も慎重を要しなければならないのは，検査をしてミトコンドリアに遺伝子変異がみつかった場合，それをどのように両親にお話しするかということです。母系遺伝という言葉から，母親が負担を感じるような話し方をしてはいけないわけで，加えて変異をもってるからといって即病気になるわけではないということを詳しく話す必要があります。説明したとおり，比率が低ければ，まったく病気にかかわらない可能性もあるわけです。それからもう1つは，mtDNAにも突然変異と考えられる変異がみつかってきているということです。患者には8993変異がほぼ100%存在しているのに，その母親や姉の血液をみたらまったく変異が存在していないという事例もあります。おそらくこれはたまたま母親の卵子のなかに起こった点変異が増えて，それがたまたま子に伝わったのではないかと考えられます。要するに *de novo*（新生）の突然変異ではないかということが推定されるわけです。こういう例は8993変異以外でも報告されています。何度もいいますが，診断がついたからといって，それが即母系遺伝であるとはいえない場合があるということに注意しましょう。

表7-7は最近のデータになりますが，新生児3168人の臍帯血を調べた例です。3168人のmtDNAのいろいろな病的点変異をチェックしたところ，15人で点変異がみつかったという結果になっています。たとえば3243変異をみていた

表7-7　臍帯血における主要なmtDNA点変異（新生児3168人の臍帯血）（文献1より）

種類	陽性例	母の血液で陽性の例
1555A→G	2	0
3243A→G*	4	2
3460G→A	3	0
7445A→G	0	0
8344A→G	0	0
8993T→G	0	0
11778G→A	3	1
13513G→A	0	0
14459G→A	0	0
14484T→C	3	0
合計	15（0.47%）	3

last-cycle fluorescent PCR：測定感度1.8%
*少なくとも出生200人に1人には点変異が存在。3243変異はその内の33%を占める。新生変異は10万人に107人の割合（1000人弱に1人）である。

だくと，陽性例が4人，その母親の血液では陰性が2例となっています。これは母親は変異をもっておらず，卵子のなかで起こった突然変異でお子さんが変異をもったということです。3460変異では3例とも母親では変異がみつかっていません。このように，おそらく相当程度，新生突然変異が起こっているだろうということが，このデータから推定されます。診断してお話しする際には，比率が低い場合には何も起こらないし，突然変異の場合もあることをきちんと説明することが重要かと思います。

最後になりますが，これが最も本講でお話ししたかったことになります。mtDNAの変異が，実際の臨床症状としてあらわれるまでにはいくつもの段階があります。mtDNAに変異がみつかったというだけではまったく意味がなくて，それがどの臓器，どの細胞でどれくらい存在しているかということまでわからないと臨床症状の説明ができませんし，ヘテロプラスミーで起こる病気の場合は，その比率がわかったからといって1つの細胞からでは全身のことは到底わかりません。そういうことをきちんと理解したうえで，病理検査，生化学検査などを総合的に組み合わせながら，ミトコンドリアの遺伝子検査を行っていくべきだろうと思います。

◉文献

1) Elliott HR, Samuels DC, et al: Pathogenic mitochondrial DNA mutations are common in the general population. Am J Hum Genet 2008; 83: 254-60.

出ずに生直後から人工呼吸管理が必要になる．良性型でも進行すると呼吸筋が障害され，人工呼吸管理が必要となることが多い．

ミオチュブラーミオパチーや先天性筋線維型不均等症などでは，知的発達障害やてんかんが合併することがある．デスミン関連ミオパチーでは，骨格筋以外に心筋も侵される．

経過・予後

筋症状は非進行性であったり，緩徐進行性であったりするが，ときに呼吸障害が急速に進むことがあるので注意を要する．重症型は，中枢神経症状がなければ，呼吸管理とその合併症である呼吸器感染症への対処の善し悪しによってその予後が決まるといってよい．

良性型は，粗大運動発達の遅れが主訴で診断に至ることがふつうであり，その後も発達が遅れ，ついには次第に運動機能が低下してくる．

治療・予防・リハビリテーション

原病の治療として有効なものはなく，合併症の予防やその早期治療が目標となる．患者がもつ運動機能に対応した，適切な補助器具の使用や関節拘縮の防止のためのリハビリテーションが必要になる．〔後藤雄一〕

■文献

埜中征哉：先天性ミオパチー．臨床のための筋病理 第4版，pp110-131，日本医事新報社，東京，2011.

9）ミトコンドリア病
mitochondrial disease

定義・概念

ミトコンドリアは成熟赤血球以外のあらゆる細胞に存在しており，ミトコンドリア病は，ミトコンドリアの機能低下によって起こる疾患の総称である．その機能低下は細胞機能の低下や細胞死を引き起こす．したがって，症状は多彩であるが，比較的エネルギー依存度の高い細胞が障害を受けやすく，中枢神経や骨格筋の症状が前景に出ることが多いためにミトコンドリア脳筋症とも称される．しかし，単にミトコンドリア病という病名が一般的になりつつある．

分類

ミトコンドリア病の分類は，その診断に用いた方法により，遺伝子変異による分類，生化学的異常による分類，臨床症状による分類がある（表 15-21-7）．これらの分類は，互いに1対1に対応せず，同じ臨床病型でありながら，遺伝子変異が異なったり，生化学異常が異なったりする．

原因・病因

ミトコンドリア内には，エネルギー代謝にかかわる多くの酵素が存在しているが，そのなかでも電子伝達系の機能低下を示す症例が最も多い．電子伝達系酵素複合体は5種類あり，それぞれが複数のサブユニットでできており，その一部はミトコンドリア内に存在するミトコンドリア DNA（mtDNA）上にコードされている．よって病因としては，核DNA 上の遺伝子変異の場合と mtDNA 異常の場合とがある．mtDNA は核DNA と異なり，1細胞に数千コピー存在することから，単にその数が減っても病的状態になる（mtDNA 欠乏症候群）．また，質的には mtDNA の欠失と点変異が知られているが，変異型と野生型が細胞内で任意の比率で存在する（ヘテロプラスミー）場合があり，核DNA 上の遺伝子変異で認めるホモとヘテロという概念とは基本的に異なっている．また精子に存在する mtDNA は受精の際に卵のなかに侵入できないか，侵入しても消失することが知られており，受精卵の mtDNA はすべて卵由来になる．したがってもともと卵のなかに変異 mtDNA が存在する場合にだけ，それが子に伝わってゆく（母系遺伝）．しかし，すべてが母系遺伝ではなく，欠失の場

表 15-21-7 ミトコンドリア病の分類

- Ⅰ.生化学的分類
  - a.複合体Ⅰ欠損症
  - b.複合体Ⅱ欠損症
  - c.複合体Ⅲ欠損症
  - d.複合体Ⅳ欠損症
  - e.複合体Ⅴ欠損症
  - f.複数の複合体欠損症
- Ⅱ.DNA 異常による分類
  - 1.核DNA 変異
  - 2.ミトコンドリアDNA 異常
    - a.欠失，重複
    - b.点変異
    - c.欠乏状態
  - 3.核DNA 変異とmtDNA異常が同時に存在する場合
- Ⅲ.臨床症状による分類
  - 1.3大病型
    - a.慢性進行性外眼筋麻痺（Kearns-Sayre 症候群を含む）
    - b.ミオクローヌスを伴うミトコンドリア病：MERRF
    - c.卒中様症状を伴うミトコンドリア病：MELAS
  - 2.その他の病型
    - a.Leber 遺伝性視神経萎縮症
    - b.Leigh 脳症
    - c.Pearson 病
    - d.NARP
    - e.MNGIE
    - f.その他（Wolfram 症候群，Alzheimer 病など）

MERRF：myoclonus epilepsy associated with raggedred fibers, MELAS: mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episode, NARP：neuropathy, ataxia and retinitis pigmentosa, MNGIE: mitochondrial neurogastrointestinal encephalomyopathy.

合はほとんどが散発例であり遺伝性を認めない．一方，点変異の場合は，母系遺伝であることがほとんどである．注意すべきことは，変異率が高くないと病気を発症しないことであり，母から子に変異mtDNAが伝わったからといって，必ずしも病気になるとは限らないということを十分銘記することが重要である．

電子伝達系以外の酵素欠損は核DNA上に存在する遺伝子変異であり，通常は常染色体劣性遺伝形式である．最近になって，mtDNAの複製にかかわる酵素をコードする遺伝子の変異をもつ例が報告され，これらは二次的にmtDNAの欠乏状態や多重欠失を起こす．この場合の遺伝形式は，母系遺伝ではなく核DNA上の遺伝子変異の遺伝形式に依存する．

臨床症状

代表的な臨床病型としては，慢性進行性外眼筋麻痺症候群（chronic progressive external ophthalmoplegia：CPEO），赤色ぼろ線維・ミオクローヌスてんかん症候群（myoclonus epilepsy associated with ragged-red fibers：MERRF），ミトコンドリア脳筋症・乳酸アシドーシス・脳卒中様発作症候群（mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episode：MELAS）がある．これら3病型は主症状である中枢神経症状によって分類されている．

1）慢性進行性外眼筋麻痺症候群： 慢性進行性外眼筋麻痺症候群は眼瞼下垂・眼球運動制限（もしくは麻痺）を特徴とする．眼筋症状のみの症例は少なく，骨格筋症状（筋力低下，筋萎縮），中枢神経症状（網膜色素変性，知能低下，感音性難聴，下垂体障害など），心症状（伝導障害など），腎症状（Bartter症候群やFanconi症候群など），内分泌症状（低身長，糖尿病，副甲状腺障害など），皮膚症状（多毛症，無汗症など）などを合併し，全身の多臓器が障害されることが多い．特に若年者で網膜色素変性と心伝導障害を伴う慢性進行性外眼筋麻痺症候群をKearns-Sayre症候群（KSS）とよんでいる．KSSは慢性進行性外眼筋麻痺症候群のなかでも，症状が多臓器に及ぶ傾向があり，若年発症が多いことなどから慢性進行性外眼筋麻痺症候群の重症型と考えられる．慢性進行性外眼筋麻痺症候群は，大きな欠失をもつ変異mtDNAを正常mtDNAと合わせもつ例が多く，このような変異DNAと正常DNAが共存する状態をヘテロプラスミーとよんでいる．このヘテロプラスミーはKSSを除く慢性進行性外眼筋麻痺症候群では40～65%，KSSでは90%以上の症例で認められ，両者の病因は同一のものと予想されている．

2）MERRF： MERRFは通常10歳前後に発症し，ミオクローヌスもしくはミオクローヌスてんかんと小脳失調を特徴とし，多くの例で精神運動発達障害を伴う．病名に含まれる赤色ぼろ線維（ragged-red fiber：RRF）とは，ミトコンドリア病患者の骨格筋で特徴的に出現するミトコンドリアの形態異常（図15-21-13A）のことである．この病理変化は3大病型のいずれにも認められるものであり，MERRFだけの特異的所見ではない．生化学的に検出される異常のほとんどは，複合体Ⅳ活性低下である．mtDNAのリジンtRNA内の8344変異がMERRF患者の80%に存在する．

3）MELAS： MELASは脳卒中様症状を主徴とするミトコンドリア病であり，比較的若年で発症する（80%が20歳以前）．臨床症状はきわめて多彩である（表15-21-8）．卒中様症状を示すときの脳画像検査では血管の支配領域とは必ずしも一致しない梗塞に似た像を認め，また症状の回復とともに画像所見も正常化する

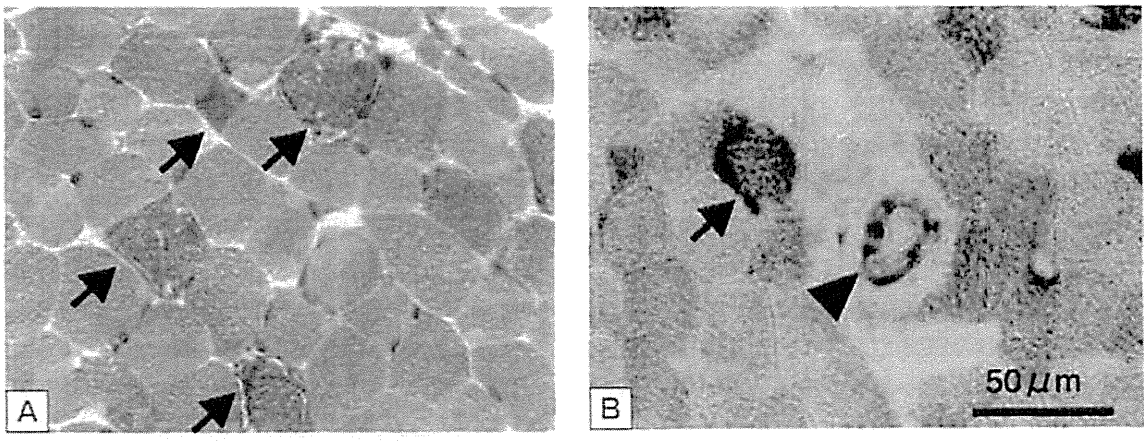

図15-21-13 ミトコンドリア病で認める病理所見

A：Gomori-トリクローム染色でミトコンドリアは赤く染まる．赤色ぼろ線維（ragged-red fiber：RRF）は特に筋鞘膜直下にミトコンドリアの集積が著明で赤みが強い（矢印）．

B：コハク酸脱水素酵素（succinate dehydrogenase：SDH）染色でも，RRFは高い活性を示す（矢印）．また，MELASでは小動脈の血管平滑筋細胞内のミトコンドリアが増加した高SDH反応性血管（strongly SDH-reactive blood vessel：SSV）を認めることが多い（矢頭）．

のが普通である．生化学的には複合体Ⅰ欠損が最も多く，複合体ⅣやⅠ＋Ⅳ欠損も認められ一定していない．

この疾患の病理所見として特徴的なことは，全身の小動脈，特に血管平滑筋細胞が強く侵されていることである．この所見は，生検筋のコハク酸脱水素酵素（succinate dehydrogenase：SDH）染色で容易に検出できることから，高SDH反応性血管（strongly SDH-reactive blood vessel：SSV）とよばれ，RRFとともにミトコンドリア形態異常を示す重要な所見である（図15-21-13B）．またMELASにおいては，ミトコンドリア転移RNAの1つ，tRNA-Leu（UUR）内の1塩基置換が次々に明らかにされた．そのなかでも塩基番号3243のAがGに変異している症例が80%の患者で認められる．

4）Leber遺伝性視神経萎縮症（Leber病）：Leber病は，思春期から成人期にかけて急性あるいは亜急性に視力低下で発症する遺伝性の視神経萎縮症である．視神経以外でもWPW症候群などの心症状やジストニアなどの神経症状を伴うことがある．日本人のLeber病患者の90%に11778変異を認める．

5）糖尿病/難聴：中枢神経症状はなく糖尿病と難聴だけが存在している患者が報告された．それらには，MELASと同じ3243変異が認められた．糖尿病患者の約1%がミトコンドリア異常によることが，日本を含め諸外国から報告されている．

6）アミノグリコシド感受性難聴：ストレプトマイシンやゲンタマイシンなどのアミノグリコシド系抗菌薬の投与により難聴をきたす家系で，mtDNAの1555変異が発見された．1回の投与で遅発性進行性に難聴を起こす例も報告されており，臨床の現場でもアミノグリコシド系抗菌薬の投与前にこの変異を調べておくことが必要になるであろう．平成24年度から保険収載された難聴遺伝子検査のなかに，3243変異とともに含まれている．

表15-21-8 MELASの臨床症状

症状	%
脳卒中様症状	100
痙攣	87
意識障害（一過性）	82
視野・視力障害	62
運動麻痺（一過性）	33
頭痛・嘔吐発作	79
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検査成績・診断

検査は，障害がどの臓器に，どの程度及んでいるかを調べる検査と，ミトコンドリア異常の有無を確認する検査とに分けられる．前者の検査は，各臓器特有の検査法に従うことになり，状況に応じて各専門医の協力を得る必要がある．後者の検査は，ミトコンドリア病の確定診断に不可欠な検査であり，血液のpHや乳酸・ピルビン酸値，中枢神経症状がある場合は髄液の乳酸・ピルビン酸値の測定が重要である．確定診断に至るうえで最も情報量が多いのが筋生検である．Gomori-トリクローム染色でのRRF，コハク酸脱水素酵素染色でのRRFやSSV，チトクロームc酸化酵素染色での欠損像が診断に有用である．生検筋や線維芽細胞で酵素活性を測定することができる．

診断は生化学的検査，形態学的検査（筋生検），分子遺伝学的検査（mtDNAや核DNA）などを行い，総合的に診断する．ミトコンドリア病はミオパチーばかりでなく，中枢神経や心臓の症状など多彩な症状を示すことが特徴であり，多彩な臓器症状をもつ患者を診たときは本症を疑うことが診断の端緒になる．

経過・予後

臨床経過は進行性のことが多いものの，症状の進行度やほかの臓器症状の出現の予想はまったく不可能である．定期的な検診による経過観察が重要である．

治療・予防・リハビリテーション

ミトコンドリア異常そのものに対する治療に，現在のところ特効薬はない．エネルギー代謝に影響を与える薬物，たとえば，コエンザイムQ10，補酵素である各種ビタミン類などを使用するが，その効果は明らかではない．しかし，MELASに対するアルギニン治療が発作症状を軽減させ，発作を予防する場合がある．またミトコンドリア内の代謝に悪影響を与える薬剤などを避けるべきであり，抗痙攣薬のバルプロ酸は，カルニチン代謝を通じてミトコンドリア内エネルギー産生を阻害する．また，乳酸の入った輸液を大量に行うことは避ける．アルコールはそもそもエネルギー代謝を抑制するので，患者では禁忌である．〔後藤雄一〕

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10）グリコーゲン病（糖原病）
glycogen storage disease, glycogenosis

グリコーゲン病はグリコーゲン代謝に関与する酵素の先天的異常のため，組織内に異常な量または質のグリコーゲンが蓄積する疾患群であり（表 15-21-9，図 15-21-14）大部分が常染色体劣性遺伝である．骨格筋障害を呈する代表的なものは，Ⅱ，Ⅲ，Ⅴ型であり，この 3 型で筋型糖原病の 70 ～ 80％を占める．症候はグリコーゲンやその中間代謝産物の蓄積による筋障害，あるいはグリコーゲン分解の最終産物であるグルコースの欠乏によるエネルギー危機によって出現する．筋症状としては運動時の筋痛やクランプ，進行性の筋力低下および筋萎縮などがみられる．ライソゾーム以外の解糖代謝異常の場合には，前腕阻血運動負荷試験（forearm ischemic exercise test）で乳酸増加を認めない．確定診断は酵素欠損証明によるが遺伝子診断も併用されている．【⇨ 13-2-6）】

（1）Pompe 病（グリコーゲン病Ⅱ型，ライソゾーム α-グルコシダーゼ欠損症）
lysosomal acid α-glucosidase（α-1,4-グルコシダーゼ，酸性マルターゼ）欠損による常染色体劣性遺伝疾患である．本酵素はライソゾーム酵素でマルトースやグリコーゲンからグルコースを遊離する．本症は発症年齢，障害臓器，予後などの違いから 3 病型に分類される．

1）乳児型：生後数カ月以内に筋力低下（筋緊張低下児，floppy infant）が明らかになり，病変は全身に及び心臓，肝臓，舌などの臓器腫大が進行する．呼吸困難や哺乳困難が著明で無治療の場合半年から 2 年で呼吸不全や心不全で死亡する．先天性筋ジストロフィーなどとの鑑別が必要である．

2）小児型：小児期早期より筋力低下や筋萎縮が進行し，無治療の場合呼吸不全で成人するまでに死亡する．臓器腫大はまれである．男子例では Duchenne 筋ジストロフィーなどとの鑑別を要する．

3）成人型：20～30歳以降に緩徐進行性のミオパチーとして発症する．約 30％の症例で呼吸不全が出現する．多発性筋炎や肢帯型筋ジストロフィーとの鑑別が必要である．

診断
血清 CK，LDH，AST などの筋原性酵素が上昇する．前腕阻血運動負荷試験は正常である．筋電図では筋原性変化のほかに筋線維の異常興奮がありミオトニア放電（myotonic discharge）がみられるが，臨床的にはミオトニアは認められない．筋病理所見は，酸性ホスファターゼの活性亢進を伴う空胞変性，PAS 陽性物質の蓄積がみられ，電顕的には限界膜で囲まれたグリコーゲン顆粒の増加が認められる．確定診断は骨格筋やリンパ球における酵素欠損あるいは遺伝子異常を証明することによる．

表 15-21-9 筋症状を呈するグリコーゲン病

欠損症	染色体位置	通称	重度呼吸不全	セカンドウインド現象
α-グルコシダーゼ欠損症（Ⅱ型）	17q25	Pompe 病	＋	
脱分枝酵素欠損症（Ⅲ型）	1p21	Forbes 病	±	
筋ホスホリラーゼ欠損症（Ⅴ型）	11q13	McArdle 病		＋
ホスホフルクトキナーゼ欠損症（Ⅶ型）	12q13	垂井病		±
ホスホリラーゼbキナーゼ欠損症（Ⅷ型） X染色体性 常染色体性	 Xq12-q13 16q12-q13			
ホスホグリセリン酸キナーゼ欠損症	Xq13			
ホスホグリセリン酸ムターゼ欠損症	7p12-p13			
ホスホグルコムターゼ欠損症	1p31（PGM 1）			
M型乳酸脱水素酵素欠損症	11p15	菅野・西村病		
A型アルドラーゼ欠損症	16p11			

JNNP Online First, published on September 11, 2013 as 10.1136/jnnp-2013-305587

RESEARCH PAPER

Mutation profile of the *GNE* gene in Japanese patients with distal myopathy with rimmed vacuoles (GNE myopathy)

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▸ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jnnp-2013-305587).

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Received 4 June 2013
Revised 21 August 2013
Accepted 22 August 2013

To cite: Cho A, Hayashi YK, Monma K, *et al. J Neurol Neurosurg Psychiatry* Published Online First: [*please include* Day Month Year] doi:10.1136/jnnp-2013-305587

ABSTRACT

Background GNE myopathy (also called distal myopathy with rimmed vacuoles or hereditary inclusion body myopathy) is an autosomal recessive myopathy characterised by skeletal muscle atrophy and weakness that preferentially involve the distal muscles. It is caused by mutations in the gene encoding a key enzyme in sialic acid biosynthesis, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE).

Methods We analysed the *GNE* gene in 212 Japanese GNE myopathy patients. A retrospective medical record review was carried out to explore genotype–phenotype correlation.

Results Sixty-three different mutations including 25 novel mutations were identified: 50 missense mutations, 2 nonsense mutations, 1 insertion, 4 deletions, 5 intronic mutations and 1 single exon deletion. The most frequent mutation in the Japanese population is c.1714G>C (p.Val572Leu), which accounts for 48.3% of total alleles. Homozygosity for this mutation results in more severe phenotypes with earlier onset and faster progression of the disease. In contrast, the second most common mutation, c.527A>T (p.Asp176Val), seems to be a mild mutation as the onset of the disease is much later in the compound heterozygotes with this mutation and c.1714G>C than the patients homozygous for c.1714G>C. Although the allele frequency is 22.4%, there are only three homozygotes for c.527A>T, raising a possibility that a significant number of c.527A>T homozygotes may not develop an apparent disease.

Conclusions Here, we report the mutation profile of the *GNE* gene in 212 Japanese GNE myopathy patients, which is the largest single-ethnic cohort for this ultra-orphan disease. We confirmed the clinical difference between mutation groups. However, we should note that the statistical summary cannot predict clinical course of every patient.

INTRODUCTION

GNE myopathy, which is also known as distal myopathy with rimmed vacuoles,[1] quadriceps sparing myopathy[2] or hereditary inclusion body myopathy (hIBM),[3] is an autosomal recessive myopathy characterised by skeletal muscle atrophy and weakness that preferentially involve the distal muscles such as the tibialis anterior. It is a progressive disease, whereby the symptoms of muscle weakness start to affect the patient from the second or third decade of life, and most of the patients become wheelchair-bound between twenties and sixties.[4] The characteristic histopathological features in muscle biopsy include muscle fibre atrophy with the presence of rimmed vacuoles and intracellular congophilic deposits.[4 5] GNE myopathy is caused by mutations in the gene encoding a key enzyme in sialic acid biosynthesis, UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE).[6–8] Genetically confirmed GNE myopathy was initially recognised in Iranian Jews and Japanese,[7 9] but later appeared to be widely distributed throughout the world. More than 100 mutations in the *GNE* gene have been described up to date.

During the last decade, there has been extensive experimental work to elucidate the pathogenesis and to develop therapeutic strategies of GNE myopathy.[6 10–12] Better knowledge on the basis of those research achievements have currently enabled us to enter the era of clinical trial for human patients. At this moment, the identification of new GNE myopathy patients with precise genetic diagnosis and the expansion of global spectrum of *GNE* mutations are timely and important. Here, we report the molecular profile of Japanese GNE myopathy patients with a brief discussion of genotype–phenotype correlations.

METHODS

Patients

Two hundred and twelve patients from 201 unrelated Japanese families were included in this study. There were 117 female and 95 male patients. All cases were genetically confirmed as GNE myopathy. A retrospective medical record review was carried out to explore genotype–phenotype correlation. Informed consent was obtained for the collection of clinical data and extraction of DNA to perform mutation analysis.

Genetic analysis

DNA was extracted from peripheral blood leukocytes or skeletal muscle tissue. We used the previously described sequencing method to describe mutations at cDNA level.[7] All exons and splice regions of the *GNE* gene were sequenced. NM_005476.5 was used as a reference sequence. We screened 100 alleles from normal Japanese individuals to determine the significance of novel variations.

Pathological analysis

To evaluate histopathological phenotype according to genotype, we analysed muscle biopsies from two

most common genotype groups in Japanese population. Each of the three age-matched and biopsy site-matched samples from c.1714G>C homozygous group and c.1714G>C/c.527A>T compound heterozygous group was compared. Muscle samples were taken from biceps brachii and frozen with isopentane cooled in liquid nitrogen. Serial frozen sections of 10 μm were stained using a set of histochemical methods including haematoxylin-eosin and modified Gomori trichrome.

Statistical analysis

Statistics were calculated using GraphPad Prism 5 software (GraphPad Software, La Jolla, California, USA). Between-group comparison for clinical data was performed using one-way analysis of variance with Dunnett's post-test. All values are expressed as means±SD. We performed two-sided tests with a $p<0.05$ level of significance.

Table 1 Allele frequency for GNE mutations in 212 Japanese GNE myopathy patients

	Allele frequency
Mutation type	
Missense	402 (94.8%)
Nonsense	3 (0.7%)
Insertion	1 (0.2%)
Small deletion	4 (0.9%)
Single exon deletion	2 (0.5%)
Intron	12 (2.8%)
Three most common mutations	
c.1765G>C (p.Val572Leu)	205 (48.3%)
c.578A>T (p.Asp176Val)	95 (22.4%)
c.38G>C (p.Cys13Ser)	15 (3.5%)
Total alleles	424

RESULTS

Mutation profile

We identified homozygous or compound heterozygous *GNE* mutations in all 212 patients (see online supplement 1). In total, 63 different mutations were found including 50 missense mutations, 2 nonsense mutations, 1 insertion, 4 deletions, 5 intronic mutations and 1 single exon deletion (figure 1). Twenty-five novel mutations were identified including 17 missense mutations, 4 small deletions, 3 intronic mutations and 1 single exon deletion (figure 1, see online supplement).

Twenty-one mutations were found to be shared between two or more unrelated families. The three mutations occurring most frequently in the Japanese population were c.1714G>C (p.Val572Leu), c.527A>T (p.Asp176Val) and c.38G>C (p.Cys13Ser); these comprised 48.3%, 22.4% and 3.5%, respectively, of the total number of alleles examined (table 1).

Genotype–phenotype correlations

The mean age of genetic analysis was 41.6±14.1 years (n=212), and the mean age of symptom onset based on the data available was 28.4±10.2 years (n=195). The earliest onset age was 10 and the latest was 61 years old in our cohort. Thirty-six among 154 patients (23.4%) were full-time wheelchair users at the point of genetic diagnosis with the average age at loss of ambulation being 36.8±11.3 years (n=36). The youngest wheelchair-bound age was 19, and the oldest ambulant age was 78. To investigate genotype–phenotype correlations in the major *GNE* mutations of Japanese population, we compared the age at symptom onset and loss of ambulation between the patients groups carrying either of the two most frequent mutations, c.1714G>C and c.527A>T (table 2). As with a previous report,[13] homozygous c.1714G>C mutations resulted in earlier symptom onset (23.9±7.1 years, $p<0.01$) and the majority of full-time wheelchair users were in this group. On the other hand, c.1714G>C/c.527A>T compound heterozygous patients first developed symptoms at a later age (37.6±12.6 years, $p<0.01$), and there were no wheelchair-bound patients at the time of genetic analysis in this group. Only three homozygous c.527A>T mutation patients were identified, and their average onset age (32.3±5.7 years) was also higher among total patients (28.4±10.2 years). All three patients were ambulant until the last follow-up visits (29, 40 and 44 years).

Among 212 cases, 80 patients underwent muscle biopsies. Overall pathological findings in our series were compatible with GNE myopathy. The characteristic rimmed vacuoles were observed in the majority (76/80, 95.0%) of the cases. Through the analysis of muscle biopsies from age-matched and biopsy site-matched samples, we found that the histopathological phenotypes were in line with these genotype–phenotype correlations (figure 2). Homozygous c.1714G>C mutations have led to much more advanced pathological changes with severe myofibre atrophy and increased numbers of rimmed vacuoles. Marked adipose tissue replacement was appreciated in a case with reflecting very advanced stage of muscle degeneration.

DISCUSSION

As shown in figure 1, mutations were located throughout the whole open reading frame of the *GNE* gene. The majority (94.8%, 402/424 alleles) of the mutations in our series were missense mutations (table 1), and there were no homozygous null mutations. These results are in accordance with previous reports[7 9] signifying that total loss of GNE function might be

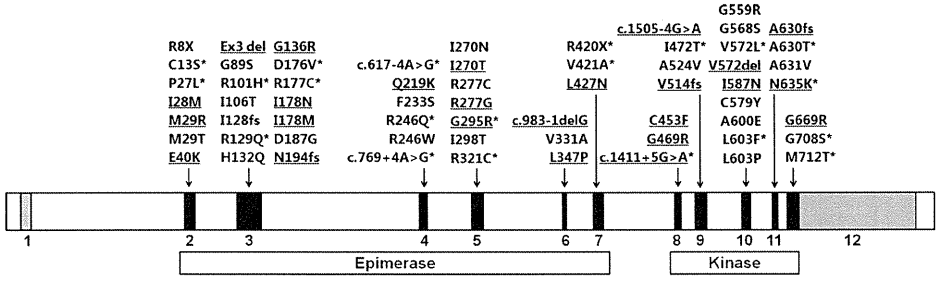

Figure 1 Mutation spectrum of *GNE* in the Japanese population. The mutations are located throughout the whole open reading frame. Twenty-five novel mutations are underlined, and 21 shared mutations are indicated with asterisks.

Table 2 Comparison of clinical course between two most frequent GNE mutations in Japanese population

Mutations	Age at exam (years)		Age at onset (years)		Age at WB (years)		Ambulant
c.1714G>C/c.1714G>C	38.6±13.4	(n=71)	23.9±7.1	(n=65)**	35.4±10.6	(n=28)	n=22
c.1714G>C/other	32.3±13.2	(n=25)	21.9±6.8	(n=22)*	37.0±8.6	(n=4)	n=16
c.1714G>C/c.527A>T	48.9±14.1	(n=38)	37.6±12.6	(n=35)**		(n=0)	n=29
c.527A>T/c.527A>T	37.7±7.7	(n=3)	32.3±5.7	(n=3)		(n=0)	n=3
c.527A>T/other	41.3±11.1	(n=51)	30.6±8.0	(n=46)		(n=2)	n=33
other/other	49.8±14.7	(n=24)	28.8±9.5	(n=24)		(n=2)	n=16
Total	41.6±14.1	(n=212)	28.4±10.2	(n=195)	36.8±11.3	(n=36)	n=118

Dunnett's multiple comparison test (control: total patients) *p<0.05, **p<0.01.
Other: a mutation other than c.1714G>C and c.527A>T; WB, wheelchair-bound.

lethal in human beings. The embryonic lethality of null mutation in *GNE* had also been proved in the mouse model.[14] Only three of total 212 patients carried a nonsense mutation; clinical data were available for two of them. Interestingly, one patient with compound heterozygous c.22C>T (p.Arg8X)/c.1714G>C (p.Val572Leu) mutations developed his first symptoms at the age of 15, while the other patient with c.1258C>T (p.Arg420X)/c.527A>T (p.Asp176Val) mutations developed her symptoms much later, at the age of 45. The similar difference was also observed in the phenotypes of patients with frame-shift mutations. A patient carrying c.383insT (p.I128fs) and c.1714G>C (p.Val572Leu) mutations developed his first symptom at the age of 13, whereas another two patients with c.1541-4del4 (p.Val514fs)/c.527A>T (p.Asp176Val) and c.581delA (p.N194fs)/c.527A>T (p.Asp176Val) mutations had later symptom onset, at the age of 30 and 32 years, respectively. This clinical variation can be explained as it reflects alternative missense mutations, because the two patients with very early onset shared the same missense mutation c.1714G>C, while the patients with the milder phenotype shared c.527A>T.

Among five intronic mutations identified in our series, c.617 −4A>G and c.769 + 4A>G were previously reported as pathological mutations.[7 15] Three novel variants were located at splice junction of exon 6 (c.983–1delG), exon 8 (c.1411 +5G>A) and exon 9 (c.1505–4G>A), raising the high possibility of relevant exons skipping. These variants were not detected in 200 alleles from normal Japanese individuals and also in the single nucleotide polymorphism (SNP) database.

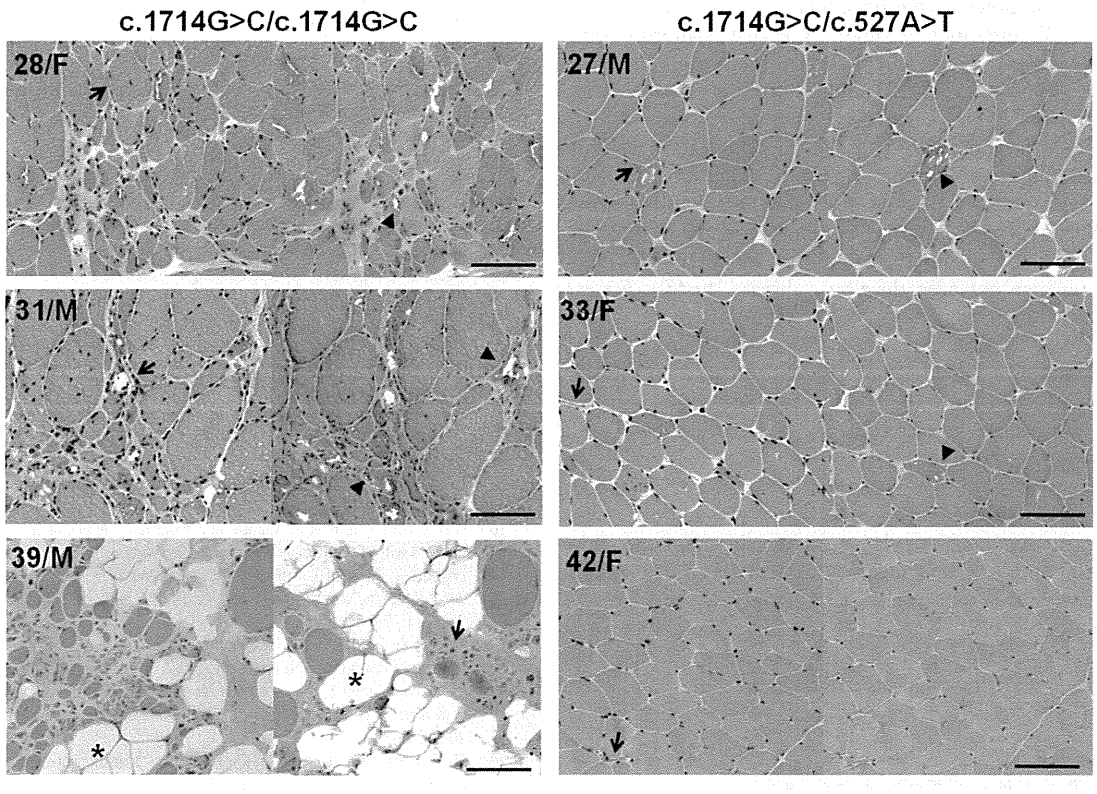

Figure 2 Comparison of muscle pathology between patients with homozygous c.1714G>C (p.Val572Leu) and with compound heterozygous c.1714G>C (p.Val572Leu)/c.527A>T (p.Asp176Val) mutations. Homozygous c.1714G>C (p.Val572Leu) mutations have led to much more advanced histopathological changes compared with compound heterozygous c.1714G>C (p.Val572Leu)/c.527A>T (p.Asp176Val) mutations. Haematoxylin-eosin (left) and modified Gomori trichrome (right) stains of muscle sections from age (c.1714G>C/c.1714G>C: 28, 31 and 39 years, c.1714G>C/c.527A>T: 27, 33 and 42 years) and biopsy site (biceps brachii muscles) matched samples. Bar=100μm; triangles: rimmed vacuoles; arrows: atrophic fibres; asterisks: adipose tissue.

As there are ethnic differences in *GNE* mutation frequencies,[9 16–19] establishing the mutation spectrum and defining predominant mutations in a certain population may be helpful for the diagnosis. Three most common mutations in the Japanese population and their allele frequencies (table 1) were in agreement with previous data.[7 13] The allele frequencies of top two mutations (c.1714G>C and c.527A>T) comprise more than two-third of the total number of alleles suggesting that founder effects are involved in the relatively higher incidence of GNE myopathy in Japan.

Although most of patients showed characteristic pathological features, the existence of exceptional cases with atypical biopsy findings implies that GNE myopathy cannot be totally excluded from the absence of rimmed vacuoles in muscle biopsies. On the other hand, we found 94 patients who were pathologically or clinically suspected but not had mutations in *GNE*. Several cases of VCP myopathy mutations in (*VCP*), myofibrillar myopathy mutations in (*DES*) and reducing body myopathy (*FHL1*) were later identified in this group, suggesting these diseases should be included as differential diagnosis of GNE myopathy.[20]

In terms of genotype–phenotype correlations, we confirmed that homozygosity for c.1714G>C (p.Val572Leu) mutation resulted in more severe phenotypes in clinical and histopathological aspects. In contrast, the second most common mutation, c.527A>T (p.Asp176Val), seems to be a mild mutation as the onset of the disease is much later in the compound heterozygotes with this mutation and c.1714G>C. Several evidences further strengthened the link between the more severe phenotype and c.1714G>C, and between the milder phenotype and c.527A>T. Compound heterozygosity for c.1714G>C and non-c.527A>T mutations resulted in earlier symptom onset (22.9 ± 6.8 years, $p<0.05$) compared with the average onset age of the total group, whereas c.527A>T, both presented as homozygous and as compound heterozygous mutations, lead to slower disease progression (table 2). In addition, only three patients carrying this second most common mutation c.527A>T in homozygous mode were identified, which is much fewer than the number expected from high allele frequency (22.4%), raising a possibility that considerable number of c.527A>T homozygotes may not even develop a disease. In fact, we ever identified an asymptomatic c.527A>T homozygote at age 60 years.[7] Now he is at age 71 years and still healthy. Overall, these results indicate that different mutations lead to different spectra of severity. However, this is a result of a statistical summary that cannot predict clinical course of each individual patient.

Here, we presented the molecular bases of 212 Japanese GNE myopathy patients with 25 novel *GNE* mutations. Based on the current status of knowledge, sialic acid supplementation may lead to considerable changes in the natural course of GNE myopathy within near future. The ongoing identification of *GNE* mutations and further studies regarding the clinicopathological features of each mutation will provide better understanding of GNE myopathy and lead to accelerated development of treatment for this disease.

Acknowledgements The authors thank Kanako Goto and Yuriko Kure for their invaluable technical support and assistant in genetic analysis.

Contributors AC had full access to all of the data in the study and wrote the manuscript; YKH supervised all aspects of this study including study design, data interpretation and manuscript preparation; KM and YO participated in collecting and analysing all the clinical and genetic data; SN, I Nonaka and I Nishino were involved in data analysis and interpretation and also supervised manuscript preparation.

Funding This study was supported partly by Intramural Research Grant 23-4, 23-5, 22-5 for Neurological and Psychiatric Disorders of NCNP; partly by Research on Intractable Diseases, Comprehensive Research on Disability Health and Welfare, and Applying Health Technology from the Ministry of Health Labour and Welfare; and partly by JSPS KAKENHI Grant Number of 23390236.

Competing interests None.

Ethics approval This study was approved by the ethics committee of National Center of Neurology and Psychiatry.

Provenance and peer review Not commissioned; externally peer reviewed.

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Mutation profile of the *GNE* gene in Japanese patients with distal myopathy with rimmed vacuoles (GNE myopathy)

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J Neurol Neurosurg Psychiatry published online September 11, 2013
doi: 10.1136/jnnp-2013-305587

Notes

PLOS | CURRENTS MUSCULAR DYSTROPHY

The C2A domain in dysferlin is important for association with MG53 (TRIM72)

November 5, 2012 · Advanced Diagnostics and Biomarkers

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Matsuda C, Miyake K, Kameyama K, Keduka E, Takeshima H, Imamura T, Araki N, Nishino I, Hayashi Y. The C2A domain in dysferlin is important for association with MG53 (TRIM72). PLOS Currents Muscular Dystrophy. 2012 Nov 5 [last modified: 2012 Nov 5]. Edition 1. doi: 10.1371/5035add8caff4.

Abstract

In skeletal muscle, Mitsugumin 53 (MG53), also known as muscle-specific tripartite motif 72, reportedly interacts with dysferlin to regulate membrane repair. To better understand the interactions between dysferlin and MG53, we conducted immunoprecipitation (IP) and pull-down assays. Based on IP assays, the C2A domain in dysferlin associated with MG53. MG53 reportedly exists as a monomer, a homodimer, or an oligomer, depending on the redox state. Based on pull-down assays, wild-type dysferlin associated with MG53 dimers in a Ca2+-dependent manner, but MG53 oligomers associated with both wild-type and C2A-mutant dysferlin in a Ca2+-independent manner. In pull-down assays, a pathogenic missense mutation in the C2A domain (W52R-C2A) inhibited the association between dysferlin and MG53 dimers, but another missense mutation (V67D-C2A) altered the calcium sensitivity of the association between the C2A domain and MG53 dimers. In contrast to the multimers, the MG53 monomers did not interact with wild-type or C2A mutant dysferlin in pull-down assays. These results indicated that the C2A domain in dysferlin is important for the Ca2+-dependent association with MG53 dimers and that dysferlin may associate with MG53 dimers in response to the influx of Ca2+ that occurs during membrane injury.

To examine the biological role of the association between dysferlin and MG53, we co-expressed EGFP-dysferlin with RFP-tagged wild-type MG53 or RFP-tagged mutant MG53 (RFP-C242A-MG53) in mouse skeletal muscle, and observed molecular behavior during sarcolemmal repair; it has been reported that the C242A-MG53 mutant forms dimers, but not oligomers. In response to membrane wounding, dysferlin accumulated at the injury site within 1 second; this dysferlin accumulation was followed by the accumulation of wild-type MG53. However, accumulation of RFP-C242A MG53 at the wounded site was impaired relative to that of RFP-wild-type MG53. Co-transfection of RFP-C242A MG53 inhibited the recruitment of dysferlin to the sarcolemmal injury site. We also examined the molecular behavior of GFP-wild-type MG53 during sarcolemmal repair in dysferlin-deficient mice which show progressive muscular dystrophy, and found that GFP-MG53 accumulated at the wound similar to

wild-type mice. Our data indicate that the coordination between dysferlin and MG53 plays an important role in efficient sarcolemmal repair.

Funding Statement

This study was partly supported by intramural Research Grant 23-4 (YKH) and 23-5 (CM, IN) for Neurologicaland Psychiatric Disorders of NCNP; partly by Research on Intractable Diseases, Comprehensive Research on Disability Health and Welfare (YKH, IN), and Applying Health Technology from the Ministry of Health Labour and Welfare (IN); and partly by JSPS KAKENHI Grant Numbers of 18590966 (CM), 24390227 (YKH), and 24659437 (YKH).

Introduction

Dysferlin is a sarcolemmal protein, and dysferlin deficiency causes Miyoshi myopathy (MM) and limb girdle muscular dystrophy type 2B (LGMD2B) [1,2]. Based on the observation that dysferlin accumulates at wound sites in myofibers in a Ca^{2+}-dependent manner, dysferlin is thought to mediate Ca^{2+}-dependent sarcolemmal repair [3].

Mitsugumin 53 (MG53), also known as muscle-specific tripartite motif 72, is a recently identified protein involved in membrane repair in skeletal muscle [4]. Mice lacking MG53 suffer progressive myopathy [4], similar to dysferlin-null mice [3]. MG53 is localized in intracellular vesicles and plasma membranes in skeletal muscle, and it accumulates at injury sites in an oxidation-dependent, but not Ca^{2+}-dependent, manner [4].

MG53 interacts with dysferlin and caveolin-3 to regulate sarcolemmal repair [5]. When expressed in C2C12 myoblasts that lack endogenous MG53, damaged membrane sites cannot be repaired in the presence of GFP-dysferlin, however, co-transfection of MG53 and GFP-dysferlin in these myoblasts results in GFP-dysferlin accumulation at injury sites [5]. These findings indicated that recruitment of dysferlin to the injury site of the membrane depends on MG53. However, it remains unclear whether the absence of dysferlin perturbs recruitment of MG53 to the injury site for membrane repair. A previous report has demonstrated the association of dysferlin with MG53 with co-immunoprecipitation (IP) assays using mouse skeletal muscle and C2C12 myoblasts transfected with dysferlin and MG53 [5]. However, which protein domains participate in this interaction between dysferlin and MG53 and whether this interaction is dependent on Ca^{2+} remain unclear. MG53 oligomerizes via disulfide bonds [4] and forms homodimers via a leucine-zipper motif in the coiled-coil domain [6]. The interaction between dysferlin proteins and MG53 monomers or oligomers has not been characterized in detail. To understand the precise role of dysferlin and MG53 in sarcolemmal repair, it would be helpful to determine whether dysferlin associates with MG53 monomers, oligomers, or both in a Ca^{2+}-dependent manner.

Thus, to examine the biological role of the association between dysferlin and MG53, we used the following strategy to examine the effect of the absence of MG53 oligomers on dysferlin. We co-transfected mouse skeletal muscle with wild-type dysferlin-EGFP and RFP-tagged wild-type MG53 or a RFP-tagged MG53 mutant (RFP-C242A –MG53), and conducted a membrane-repair assay using a two-photon laser microscope. The C242A–MG53 mutant has been reported to form dimers, but not oligomers [6]. There is no report of simultaneous observation of dysferlin and MG53 during sarcolemmal repair; however, we have successfully performed real-time imaging of dysferlin-GFP and MG53-RFP after membrane injury in mouse skeletal muscle.

Dysferlin protein is absent or severely reduced in the skeletal muscle of patients with dysferlinopathy [7] and of SJL and A/J mice with mutations in the dysferlin genes [8]. To examine whether the absence of dysferlin affects the recruitment of MG53 to injury sites, we transfected skeletal muscle from dysferlin-deficient SJL and A/J mice with EGFP-MG53 and conducted membrane repair assays. These experiments are helpful in elucidating the

molecular pathology of dysferlinopathy and revealed that MG53 accumulated in the skeletal muscles of dysferlin-deficient mice, which develop progressive muscular dystrophy.

We present evidence indicating that efficient sarcolemmal repair requires both dysferlin and MG53.

Methods

Immunoprecipitation. To examine the interaction between MG53 and dysferlin, mouse gastrocnemius muscles were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, and Complete mini EDTA-free protease inhibitor cocktail (Roche) [9] supplemented with 1 mM $CaCl_2$ or 2 mM EGTA. Lysates pre-cleared with Protein A/G agarose (Pierce) were incubated with polyclonal antibodies against mouse MG53 [4] or mouse dysferlin; the anti-dysferlin antibody was made in rabbit by injecting bacterial recombinant protein containing residues 1669 to 1790. The immunoprecipitated proteins were separated by SDS-PAGE and detected on immunoblots using the same antibodies used for IP or the anti-human dysferlin monoclonal antibody, NCL-Hamlet (Novocastra Laboratories).

A human MG53 cDNA was amplified by PCR and subcloned into pFLAG-CMV-4 (Sigma). Wild-type and truncated human dysferlin that were each tagged with c-myc were generated previously [10]. We also created five truncated human dysferlin constructs with the C2A domain (aa 1-149, 1-349, and 1-1080) and without the C2A domain (aa 130-2080 and 1081-2080). The sequence of each construct was verified by DNA sequencing. FuGENE 6 or E-xtremeGENE 9 (Roche) was used to transiently transfect COS-7 cells with MG53 and wild-type or mutant dysferlin constructs. Transfectants were cultured for 48 h and subsequently lysed in the same lysis buffer used to lyse mouse muscle, except that this buffer lacked $CaCl_2$ and EGTA. Lysates pre-cleared with Protein G-Sepharose (GE Healthcare) were incubated with anti-FLAG (M2, Sigma) or anti-c-myc (9E10, Santa Cruz Biotechnology) monoclonal antibodies; Protein G-Sepharose was then added. Immunoprecipitated proteins were analyzed by immunoblotting using M2 and anti-c-myc polyclonal (A14, Santa Cruz Biotechnology) antibodies.

Pull-down assay. Fragments of the dysferlin C2A domain (corresponding to aa 1-129 of human dysferlin) were amplified as cDNA by PCR and subcloned into pGEX-5X-3 (GE Healthcare). Dysferlin p.W52R (TGG to CGG at c.527-529) and p.V67D (GTG to GAT at c.572-574) mutations were introduced by PCR using appropriate primers. GST fusion proteins expressed in BL21 *E. coli* were purified using sarkosyl [11] and bound to glutathione Sepharose 4B (GE Healthcare). COS-7 cells overexpressing FLAG-tagged human MG53 were lysed in lysis buffer containing 10 mM Na_2HPO_4, 1.8 mM KH_2HPO_4, 1% NP-40 (pH 7.4), 2 mM EGTA, various concentration of $CaCl_2$, and Complete mini EDTA-free protease inhibitor cocktail. EGTA was used to chelate the free Ca^{2+} in solution and $CaCl_2$ at various concentrations. The free calcium concentration was calculated using the free software CALCON3.6. Lysates were centrifuged to remove cellular debris, supplemented with 5 mM N-methylmaleimide (NEM) or 5 mM dithiothreitol (DTT), and finally subjected to protein cross-linking by treating with 2 mM glutaraldehyde (GA) for 5 min at room temperature, which was quenched with 100 mM Tris-HCl (pH 7.5) [6]. The cross-linked lysates were diluted with 75 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 2 mM EGTA, various concentrations of $CaCl_2$, and Complete mini EDTA-free protease inhibitor cocktail. Lysates pre-cleared with GST bound to glutathione Sepharose 4B were divided into aliquots and incubated with wild-type, p.W52R, and p.V67D dysferlin C2A-GST fusion protein bound to beads for 2 hr at 4°C. After three washes in lysis buffer containing 75 mM Tris-HCl (pH 7.5), 2× sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% (v/v) glycerol, and 0.004% bromophenol blue) was added to the beads, and the mixtures were incubated for 10 min at 85°C. Bound proteins were separated by SDS-PAGE and subjected to immunoblotting with the anti-FLAG antibody M2.

In vivo transfection and membrane repair assay. Twenty micrograms of N-terminal RFP-tagged human MG53 cDNA/pcDNA3.1 and/or C-terminal GFP-tagged human dysferlin cDNA/pcDNA3.1 plasmid DNA were injected into

the flexor digitorum brevis of anesthetized, 4-week-old male C57BL6J and dysferlin-deficient SJL and A/J mice. Electroporation of plasmid DNA was performed using an electric pulse generator (CUY21SC, NEPAGENE) as described previously [12]. Seven days after electroporation, skeletal muscle myocytes (for whole-mount viewing) or individual myofibers were isolated and subjected to plasma membrane injury created by a two-photon laser microscope, LSM 710NLO with GaAsp Detectors (Zeiss) and Chameleon Vision II System (Coherent)[3]. Myofiber wounding using the 820-nm infrared laser and resealing analysis based on the kinetics and extent of FM1-43 or 4-46 dye (Molecular Probes) entry through open disruptions was carried out as previously described [3,13,14].

Ethics Statement. All experiments involving animals were performed according to the Procedure for Handling Experiments involving Animals of AIST (National Institute of Advanced Industrial Science and Technology) and approved by the Institutional Animal Care and Use Committee of AIST.

Results

Association of MG53 and dysferlin in mouse skeletal muscle

We used an IP assay with protein from mouse muscle to confirm that endogenous MG53 associates with dysferlin *in vivo*. MG53 and dysferlin associated only in the absence of EGTA and $CaCl_2$ (Fig. 1). The same result was obtained using C2C12 myotubes (data not shown). MG53 was specifically co-immunoprecipitated by the anti-dysferlin antibody, and conversely dysferlin was specifically co-immunoprecipitated by the anti-MG53 antibody. Thus, we confirmed that endogenous MG53 and endogenous dysferlin form a protein complex in mouse skeletal muscle without EGTA or $CaCl_2$ supplementation.

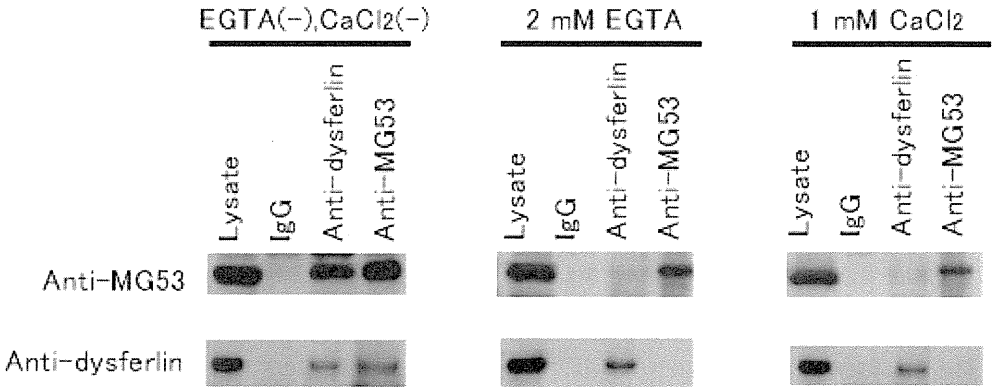

Fig. 1: IP assay of dysferlin and MG53.

MG53interacts with dysferlin in mouse skeletal muscle. Extracts from wild-type mouse skeletal muscle were subjected to IP with polyclonal anti-MG53 antibodies or polyclonal anti-dysferlin antibodies. Immunoprecipitated proteins were subjected to SDS-PAGE and visualized on immunoblots treated with the same antibodies that were used for IP.

Identification of the MG53-associating domain of dysferlin

Next, we used IP to define the region of dysferlin that associates with MG53. Specifically, we used transient co-transfection to introduce a construct encoding full-length human MG53 tagged with FLAG and a construct encoding human dysferlin tagged with c-myc into COS-7 cells; for each co-transfection, full-length dysferlin or one of five deletion mutant forms of tagged dysferlin was used (Fig. 2). For deletion mutants that lacked the C-terminal domain of dysferlin, the transmembrane domain of dysferlin was retained to increase protein stability [10]. Transfectants were lysed in the same buffer that was used for IP assays of mouse skeletal muscle extract, except that this buffer lacked EGTA and $CaCl_2$. Full-length dysferlin and deletion mutants that retained the N-terminal C2 (C2A) domain of dysferlin were co-immunoprecipitated by anti-MG53 antibody. In contrast, dysferlin mutants that lacked this N-terminal domain, Δ2-1080 and Δ2-129, failed to interact with MG53. These results indicated that the C2A domain of dysferlin was necessary for association with MG53.

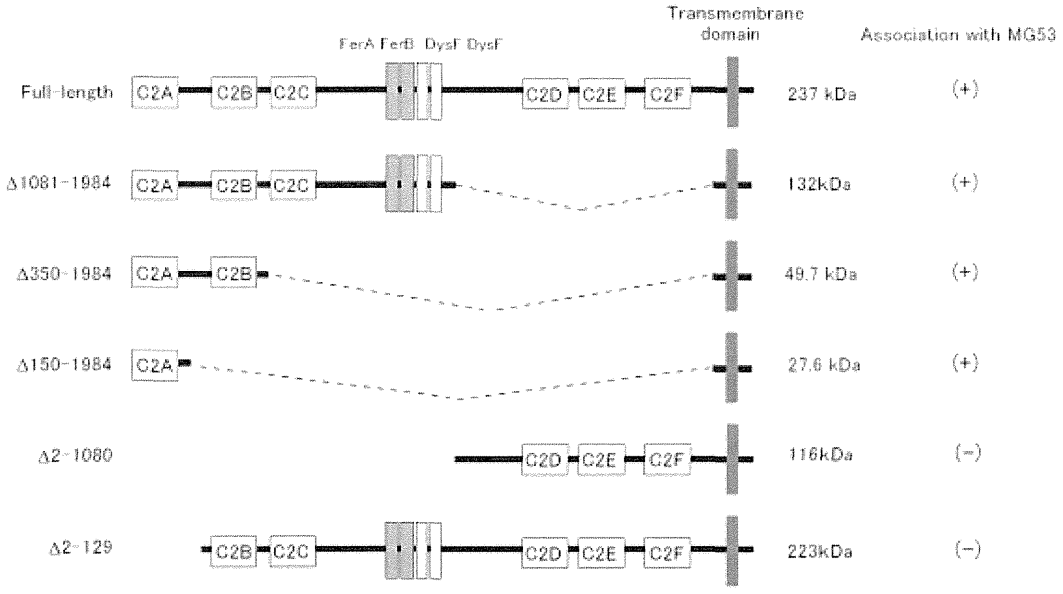

Fig. 2: Identification of MG53-binding region of dysferlin.

The dysferlin C2A domain associates with MG53. Constructs encoding dysferlin deletion mutants were used for co-IP assays, and the results of these experiments are shown on the right. Deletion mutants encoding c-myc-tagged dysferlin mutants and FLAG-tagged full-length MG53 were co-expressed in COS-7 cells. IP and immunoblotting were performed using antibodies against the c-myc and FLAG tags. MG53 was co-immunoprecipitated with full-length dysferlin and the dysferlin mutants that lacked the C-terminus, but not with the dysferlin mutants that lacked the N-terminus.

Characterization of the association of dysferlin C2A domain with MG53 monomers and MG53 oligomer

C2 domains are known to bind to phospholipids and/or proteins in a Ca^{2+}-dependent or Ca^{2+}-independent manner [15]. Therefore, we used a pull-down assay to examine whether the Ca^{2+} concentration affected the association between MG53 and the dysferlin C2A domain. We used lysis buffer containing 75 mM Tris to reduce the change in pH that can result from the addition of $CaCl_2$, to examine the calcium-dependency of the association between dysferlin and MG53. Reportedly, MG53 can exist as a monomer or an oligomer, depending on the redox state [4]. We used DTT for monomerization of MG53 by reducing sulfhydryl groups. Addition of 5 mM DTT resulted in complete dissociation of all MG53 oligomers (Fig. 3). To conduct a pull-down assay for MG53 oligomers, we treated cell lysates with an alkylating reagent, NEM, which reacts with sulfhydryl groups to form stable thioether bonds [6]. Multimers of MG53 were stabilized by chemical cross-linking with GA. Addition of 5 mM NEM to cell lysates resulted in oligomerization of MG53 (Fig. 3). In the presence or absence of Ca^{2+}, MG53 oligomers associated with wild-type C2A-GST, whereas MG53 monomers did not associate with wild-type C2A-GST. In the absence of DTT or NEM, MG53 existed as oligomers including dimers, which associated with WT C2A-GST only in 10 mM free Ca^{2+} (Fig. 3, top).

Next, we generated two mutant versions of C2A-GST (W52R or V67D) to further characterize the association between MG53 and the C2A domain. A V67D missense mutation in the human dysferlin gene has been found in patients with MM and patients with LGMD2B [16]; similarly, the W52R dysferlin missense mutation has been found in patients with LGMD2B [17]. Each mutant C2A-GST, like the wild-type C2A, associated with MG53

oligomers when conditions included NEM in the presence or absence of Ca^{2+} (Fig. 3). However, the V67D mutation altered the calcium sensitivity of the association between C2A-GST and MG53 dimers; specifically, V67D-C2A-GST could associate with MG53 when conditions did not include NEM in the absence of Ca^{2+}. In contrast, W52R-C2A-GST did not associate with MG53 when conditions did not include NEM in the presence or absence of Ca^{2+}. These results revealed that the V67D mutation in the dysferlin C2A domain altered the Ca^{2+}-dependence of the association between dysferlin and MG53 dimers.