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ミトコンドリア病の診療水準やQOL向上を目指した  
調査研究

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ミトコンドリア病の診療水準やQOL向上を目指した調査研究

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**研究要旨** ミトコンドリア病は指定難病の1つで、患者数は約2,000人である。その症状は神経症状を中心とするものの多臓器に及ぶため、代謝性疾患、心筋疾患、腎疾患等としても重要な病気であり、診療科横断的な対応が求められる。さらに、医療の進歩が影響して小児期発症の患者が成人年齢に至る例が増加し、小児科から成人診療科への移行期医療、縦断的診療連携も必要である。本研究班ではミトコンドリア病の正確な診断とそれに基づく適切な治療をめざして、多領域の専門家により診断基準や重症度分類の検討、診断・治療体制の整備を進めている。令和5年度は診療マニュアルの改訂・発行を行い、診療の標準化・均霑化を目指して研究会や学術集会でのシンポジウムの企画し開催した。診断体制の整備においては、遺伝学的検査の保険収載された遺伝学的検査を開始して臨床実装化した。また、患者レジストリの整備・拡大、ミトコンドリア病パンフレットの改訂版作成を実施し、関係機関に配布した。アウトリーチ活動については、AMED研究班と協働して市民公開フォーラムを開催した。生殖補助医療については、出生前診断や基礎研究の推進を支援した。疫学研究においては、National Databaseを活用して有病者数等について記述的な統計解析を行う調査を開始した。

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A. 目的

本研究の目的は、ミトコンドリア病に診療に関する新しい知見に基づく診断基準の検証や改訂、診断・治療体制の整備、医療者や一般社会への最新情報の周知、疾患啓発を行い、広く医療行政に貢献することである。

ミトコンドリア病は指定難病の1つで、患者数は約2,000人である。その症状は神経症状を中心とするものの多臓器に及ぶため、代謝性疾患、心筋疾患、腎疾患、眼疾患等としても重要な病気であり、診療科横断的な対応が求められる。さらに、医療の進歩が影響して小児期発症の患者が成人年齢に至る例が増加し、小児科から成人診療科への移行期医療、縦断的診療連携も必要である。本研究では、多領域の専門家により診断基準や重症度分類の検討、診断・治療体制の整備を進める。特に、ミトコンドリア病の遺伝学的検査と遺伝カウンセリングの重要

性が増しており、わが国全体のゲノム医療の推進に対応して遺伝子検査を位置づけてゆく。

ミトコンドリア病の確定診断には、遺伝子、生化学、病理検査等の専門性の高い検査が必要であり、集約的診断体制が必要である。令和4年にミトコンドリア病の遺伝学的検査が保険収載されたことを受け、包括的な診断体制を構築するために、検査受注のシステムを整備する。また、診療マニュアルの改訂、発行を行い、その周知、啓発活動を通して診療レベルの向上、標準化、均霑化を推進する。着床前診断、核移植などの生殖補助医療の課題を検討し、その内容を広く社会に広報する。また、これまでに構築した患者レジストリの拡充を推進し、超希少疾患については海外活動と連携するとともに、ミトコンドリア病の疫学的研究を行う。

## B. 方法

### 1) 診断体制の整備【三牧、村山、岡崎、西野、後藤、今澤、武田】

ミトコンドリア病の確定診断には種々の検査が必要だが、中でも令和4年に保険収載されたミトコンドリア病の遺伝学的検査については、検査体制の整備が喫緊の課題である。ミトコンドリア病の場合、核DNA上の病因遺伝子が350以上報告されており、ミトコンドリアDNAの量的変化や、点変異や欠失・重複等の質的变化も原因となる特殊性があるため、これらの病因を網羅的に検索するためには困難を伴う。研究班としてこの課題に対応するために、これまで全国から遺伝学的検査を依頼されてきた順天堂大学と国立精神・神経医療研究センター（NCNP）とで協議を行い、まず順天堂大学にて血液検体を用いて病因の確定しているバリエーションを調べる遺伝子検査を開始することとした。NCNPでは、筋生検症例を中心に研究として遺伝学手検査を継続する方針とした。また、遺伝学的検査に必要な遺伝カウンセリングに資する「ミトコンドリア病ハンドブック」の改訂作業を行った。

### 2) 治療体制の整備【三牧、村山、小坂、八ツ賀、井

### 川、今澤、武田】

本研究班では、日本ミトコンドリア学会の協力のもと、AMED難治性疾患実用化研究班（村山圭代表）と協働して「ミトコンドリア病診療マニュアル2017」の改訂作業を進めた。さらに、医療者を対象とした研究会等を開催し、マニュアルの周知等を通して診療の標準化、均霑化を図る活動を展開している。また、多彩な症状を呈するミトコンドリア病に対応すべく、心筋症や腎症の専門家を委員に加え、診断基準や重症度分類の改訂に向けた検討を行っている。

また、治療可能なミトコンドリア病の一つである原発性コエンザイムQ10欠乏症の早期治療介入に繋げるため、早期診断法についての研究を行った。

### 3) 患者レジストリの運用拡大【小牧、村山、大竹、西野】

患者レジストリについては、AMED実用化研究班と連携して行うこととし、小児患者のレジストリシステム（J-MOバンク）においてLeigh症候群等の患者登録を進めている。NCNPにおいてはRemudy方式の患者レジストリを構築し、三大病型（MELAS、CPEO、MERRF）等の登録を、成人症例を中心に進めている。さらなる登録数の増加にむけた方策を検討し、システムの改変について準備した。超希少疾患については、海外活動と連携する。

### 4) アウトリーチ活動【三牧、村山、小坂、井川】

患者会との連携を深化させ、患者・家族のニーズを把握し、その想いを医療者・研究者が共有する環境を醸成させるために、令和6年2月にミトコンドリア病研究公開フォーラムを開催した。また、医療者、患者・患者家族へ最新の情報を提供するために、難病情報センターのホームページの情報更新を行った。

### 5) ミトコンドリア病に対する生殖補助医療の検討【立花、大竹、後藤】

重篤なミトコンドリア病に対する出生前診断が国内外で進んでいる。本研究班では、埼玉医科大学にて、難治性のミトコンドリア病に対する出生前診

断、着床前診断を推進している。マウス受精卵を用いたPGT基礎研究も計画しており、今後より安全で妊娠率の高い方法の確立を目指していく。

また、ミトコンドリアDNA変異で発症する疾患に対し、健康な次子を得るために核移植治療が海外で始まっている。本邦では政府の科学技術・学術会議生命倫理・安全部会特定胚等研究専門委員会にて、ミトコンドリア病の研究を目的としたヒト受精卵（余剰胚）への核置換技術を用いた基礎的研究について、令和3年に承認された。これらの新技術の科学性、効果や安全性、倫理課題についても本研究班で検討している。また、凍結融解卵を用いた極体移植によるミトコンドリア置換の効率を、マウス卵とヒト凍結融解卵を用いて検討した。

## 6) 疫学研究【藤野、ハツ賀】

これまで、わが国ではミトコンドリア病を含む希少疾患の疫学調査の多くがアンケート方式によって行われてきた。しかし、アンケート用紙を使用する調査方法は、人的・時間的・費用的に負担の大きいものである。本研究では、厚生労働省が管理するレセプト情報である、特定健診等情報データベース（NDB, National Database）を用いて、ミトコンドリア病患者の有病者数および有病者割合の推定をはじめとした疫学調査を行うこととした。

## C. 結果と考察

### 1) 診断体制の整備

令和4年度に保険収載された遺伝学的検査を、令和5年秋より順天堂大学にて開始した。血液検体を受け付け、ミトコンドリアDNAの全周配列解析、およびミトコンドリア病の原因遺伝子を搭載した核遺伝子パネル解析を開始し、臨床実装した。ミトコンドリアDNAのバリエーションは血液検体のみでは検出されないことがあるため、NCNPにて筋生検症例を中心に研究としてミトコンドリアDNAの全周解析を行い、点変異や欠失・重複の検出を継続した。両施設を中心として、生化学的、病理学的解析も用いた包括的診断体制を維持しており、今後の難病行政に対応することにし

ている。

また、医師が患者に遺伝学的検査の説明を行う際に使用するハンドブックを改訂した。今回は令和6年公表の診断基準をはじめ新しい情報を記載した。冊子体を全国遺伝子医療部門連絡会議に参加している施設を中心に幅広く医療機関に配布し、難病情報センターのHPからダウンロードできるようにした

([https://www.nanbyou.or.jp/wp-content/uploads/2023/12/mt\\_handbook\\_v2.pdf](https://www.nanbyou.or.jp/wp-content/uploads/2023/12/mt_handbook_v2.pdf))。

### 2) 治療体制の整備

AMED実用化研究班と協働して「ミトコンドリア病診療マニュアル2017」の改訂作業を進め、令和5年6月に「ミトコンドリア病診療マニュアル2023」を発行した。診療レベルの向上を目指し、令和5年11月には、医療者向けのミトコンドリア病研究会を開催した。日本神経学会や日本小児神経学会等の協力を得て、診療マニュアルの周知や、成人診療科と小児科の連携を図るための啓発活動を行うために、令和6年度の日本小児神経学会の学術集会等でのシンポジウムを企画している。小児科から成人診療科への移行期医療、縦断的診療連携についての検討を継続し、今後の診断基準の改訂に対応する。

原発性コエンザイムQ10の早期診断、早期治療介入を可能とするために、非侵襲的診断法の検討を行った。しかしながら、血液検体由来のリンパ芽球では、コエンザイムQ10の低下を検出できなかった。診断のためには筋生検や皮膚生検が必要だと考えられる。

### 3) 患者レジストリの運用拡大

小児患者のレジストリシステムにおいては、令和6年3月までに155例を登録した。NCNPのRemedy方式の患者レジストリでは、令和5年12月までに36名の成人患者の登録が完了した。さらなる登録数の増加にむけ、新規登録や年次情報更新に医師の関与が必須でない条件とし、Webベースで登録が完了できるようなシステムの改変を計画している。

超希少疾患に関する海外活動との連携も行っている。令和5年度は、11月に開催された日本ミトコンド

リア学会年會に、POLG Foundation（ミトコンドリア病の一病型であるPOLG遺伝子異常による疾患の医学研究の推進を目的とする海外の団体）の代表者を招待するとともに、本邦での調査を行った。日本では極めて稀な疾患であることが明らかとなり、同年会で報告した。

#### 4) アウトリーチ活動

AMED実用化研究班と共同で、令和6年2月にミトコンドリア病研究公開フォーラムを開催した。オンライン開催により約160名の参加があった。当研究班の研究分担者、遺伝学的検査や創薬に関わる研究者、さらには患者会の代表が講演し、活発な質疑応答、意見交換が行われた。今後も患者家族、医療従事者、研究者、関連企業を含むオールジャパン体制による開催を継続し、患者・市民参画の充実を目指すことが重要である。また、医療者、患者・患者家族へ最新の情報を提供するために、難病情報センターのホームページの情報更新を行った。

#### 5) ミトコンドリア病に対する生殖補助医療の検討

埼玉医科大学にて、29家系41妊娠(核DNA変異38名、mtDNA変異3名)に対し出生前診断を行い、13名(40%)に発端者と同じ病的バリエーションを認めた。並行し着床前診断も5名(核遺伝子3名、ミトコンドリア遺伝子2名)に実施し、m. T8993G変異の1名で妊娠が確認され継続中である。今後も倫理的配慮を行ったうえで生殖補助医療を推進し、知見を蓄積していく。ミトコンドリアDNA変異で発症する疾患に対する核移植治療についても、引き続き検討している。凍結融解卵を用いた極体移植によるミトコンドリア置換の効率の検討においては、マウスでは凍結融解前に極体移植を行うことによりミトコンドリア置換の効率があがること、ヒトにおいては凍結融解卵でも移植可能であることが示された。

#### 6) 疫学研究

NDBを用いたミトコンドリア病患者の有病者数および有病者割合の推定をはじめとした疫学調査のために、ミトコンドリア病に関する傷病名コードを議論の上で決定した。令和6年1月に厚生労働省にミト

コンドリア病に関するNDBデータの提供について申し出を行い、審査の結果、無条件承諾であることが通知された。今後、完成した集計表を厚生労働省から受領し、ミトコンドリア病患者の有病者数について記述的な統計解析を行う予定である。

#### D. 結論

本研究班の活動はAMED実用化研究班(村山班)と連携しながら進めている。オールジャパン体制で診断システムの整備を行った。診療マニュアルの改訂版を発行するとともに、治療の標準化、均霑化のために、その周知、啓発活動を展開した。市民公開フォーラム等の開催を通して、患者会との交流を行い、患者・市民参画を進めた。生殖補助医療を推進しつつ、核移植についての情報収集と基礎研究の支援を行い、日本での実現可能性についての議論を継続した。患者レジストリの登録数を増やしたが、自然歴の把握や臨床試験に役立てるために、今後さらに拡充していく必要がある。

#### E. 健康危険情報

なし

#### F. 研究発表

##### 1. 論文発表

著書、総説

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#### G. 知的財産権の出願・登録状況（予定を含む）

##### 1. 特許取得

なし

##### 2. 実用新案登録

なし

##### 3. その他

なし

厚生労働科学研究費補助金（難治性疾患政策研究事業）

分担研究報告書

## ミトコンドリア病における診療マニュアルの策定と治療体制の構築

研究分担者 井川 正道 福井大学学術研究院医学系部門・教授

### 研究要旨

ミトコンドリア病における診療マニュアルの策定を行い、治療体制の構築を図るとともに、患者・医療者への疾患啓発を行う。

#### A. 研究目的

ミトコンドリア病は稀少疾患であり、多彩で多臓器に及ぶ症状を呈するため、診療には専門的な知識や経験が必要である。本研究では診療マニュアルの策定を行うことで、治療体制の構築および患者・医療者への疾患啓発につなげることを目的とする。

#### B. 研究方法

以前に刊行した「ミトコンドリア病診療マニュアル2017」の改訂により診療マニュアルの策定を行う。特に専門領域である脳神経内科領域を中心に担当する。

（倫理面への配慮）

診療マニュアルの策定を主な目的とするため、倫理審査は不要である。

#### C. 研究結果

診療マニュアルの策定においては、ミトコンドリア病の一病型であるMERRFの項目を担当し、「ミトコンドリア病診療マニュアル2023」として発刊した。

#### D. 考察

診療マニュアルの策定によって、治療体制の構築および患者・医療者への疾患啓発につながる事が期待される。

#### E. 結論

診療マニュアルを踏まえ、今後は治療体制の構築や患者・医療者への疾患啓発を行う。

#### F. 健康危険情報

（総括研究報告書にまとめて記入）

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H. 知的財産権の出願・登録状況  
(予定を含む。)

1. 特許取得  
なし

2. 実用新案登録  
なし

3. その他  
なし

厚生労働科学研究費補助金（難治性疾患政策研究事業）  
分担研究報告書

ミトコンドリア病のレジストリと生殖補助医療に関する研究

分担研究者：大竹 明 埼玉医科大学病院 特任教授

研究要旨

小児期を中心にミトコンドリア病レジストリ（J-MO bank）を拡張しつつ難病プラットフォームへの提供も継続し、今年度末現在でリー脳症を中心に155名の登録をいただいた。2023年12月には倫理の修正申請（病2021-094）を行い、分担研究施設60、研究協力施設281からなる日本を挙げた研究体制とし、国内では535名に対し新規登録依頼状を発送し、現在その追加登録の集計を行なっている。さらに国際レジストリ（GENOMIT）への登録も始めた。

29家系41妊娠（核DNA変異38名、mtDNA変異3名）に対し遺伝カウンセリング後に出生前診断を行い、13名（40%）に発端者と同じ病的バリエーションを認めた。並行し着床前診断も5名（核遺伝子3名、Mt遺伝子2名）に実施し、m. T8993G変異の1名で妊娠が確認され継続中である。マウス受精卵を用いたPGT基礎研究も計画しており、今後より安全で妊娠率の高い方法の確立を目指す。

A. 研究目的

ミトコンドリア病の患者レジストリを構築し、日本発を中心に各種新規治療薬の開発に協力する。患者系に出生前診断、着床前診断を提供し、並行してマウス受精卵を用いたPGT基礎研究も行い、今後より安全で妊娠率の高い方法の確立を目指す。

B. 研究方法および研究結果

主治医と協力して登録シートに患者さんご自身で記載いただき、ご自身で投函いただく。絨毛または羊水を用いた遺伝子検査による出生前診断と行なう。体外受精を応用した着床前遺伝学的検査を行なう。

（倫理面への配慮）

埼玉医科大学病院IRBにて承認済み承認済（病2021-094）

C. 研究結果

小児期を中心にミトコンドリア病レジストリ（J-MO bank）を拡張しつつ難病プラットフォームへの提供も継続し、今年度末現在でリー脳症を中心に155名の登録をいただいた。2023年12月には倫理の修正申請（病2021-094）を行い、分担研究施設60、研究協力施設281からなる日本を挙げた研究体制とし、国内では535名に対し新規登録依頼状を発送し、現在その追加登録の集計を行っている。さらに国際レジストリ（GENOMIT）への登録も始めた。

29家系41妊娠（核DNA変異38名、mtDNA変異3名）に対し遺伝カウンセリング後に出生前診断を行い、13名（40%）に発端者と同じ病的バリエーションを認めた。並行し着床前診断も5名（核遺伝子3名、Mt遺伝子2名）に実施し、m. T8993G変異の1

名で妊娠が確認され継続中である。

マウス受精卵を用いたPGT基礎研究も計画しており、今後より安全で妊娠率の高い方法の確立を目指す。

#### D. 考察

今後も診断患者への登録票送付を加速し、一層の新規患者登録数の増加を目指す。その中で今まで以上に難病プラットフォームへの登録拡大も目指すと同時に、GENOMITを中心に国際レジストリとの協力も進め、日本発を中心に各種新規治験薬の開発にも協力して行きたい。マウス受精卵を用いたPGT基礎研究も計画しており、今後より安全で妊娠率の高い着床前診断法の確立を目指す。

#### E. 結論

ミトコンドリア病レジストリ (J-MO Bank)への登録は順調に増加しており、今後は定期的安否の確認も継続し、国際レジストリへの協力も加速する。

着床前診断については、マウス受精卵を用いた基礎研究も計画しており、今後より安全で妊娠率の高い方法の確立を目指す。

#### F. 健康危険情報 なし

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厚生労働科学研究費補助金（難治性疾患政策研究事業）  
分担研究報告書

原発性コエンザイム Q10 欠乏症の  
早期診断に向けた非侵襲的診断方法の検討  
研究分担者：小坂 仁 自治医科大学医学部小児科学

研究要旨

【目的】 原発性コエンザイム Q10 欠乏症は、CoQ10 生合成に関わる遺伝子異常によって引き起こされる。腎合併症により、未治療では腎不全に至る CoQ10 補充療法が予後を改善するため、早期診断が重要であり、筋生検や皮膚生検に変わる、侵襲性の低い診断法の確立が求められている。

【方法】 COQ 遺伝子変異を持つ 6 例の患者を対象に、血清 CoQ10 を液体クロマトグラフ質量分析計で定量した。全患者が CoQ10 を内服していたため、芽球化したリンパ球の CoQ10 を測定した。

【結果】 リンパ芽球 CoQ10 値は、対照群で平均 232.08 nmol/g protein であった。一方、患者群では COQ6 遺伝子変異 1 例と COQ8B 遺伝子変異 2 例で低下を認めしたが、3 例は正常範囲内だった。

【まとめ】 CoQ 低値の証明にはリンパ芽球 CoQ10 値は、COQ8B 遺伝子変異のある一部の患者で低下していたのみであり、筋生検や皮膚生検が必要である。

A. 研究目的

原発性 CoQ10 欠乏症は、CoQ10 生合成に関与する酵素が欠損し、CoQ10 低値をきたす疾患で、脳症、心筋症、ステロイド抵抗性ネフローゼ症候群（SRNS）、多臓器障害など多彩な臨床症状を呈する。CoQ10 補充療法により症状の進行を抑制できることから、早期診断が重要である。確定診断は生検筋の CoQ10 低値の証明だが侵襲性が高い。我々は、皮膚線維芽細胞で、還元型・酸化型 CoQ10 を個別に定量する系を構築し、これまで原発性 CoQ10 欠乏症を含め、ミトコンドリア病患者の皮膚繊維芽細胞で還元型・酸化型 CoQ10 患者細胞測定した。今回、より侵襲性の低い血液での診断が可能か検討した。

B. 研究計画・方法（概要）

COQ 遺伝子変異がある患者 6 例 (Table; COQ6 遺伝子変異 1 例、COQ8B 遺伝子変異 5 例) を対象とした。患者は全例 CoQ10 を内服していたため、血清 CoQ10 は薬剤の影響があると考えられた。そのため芽球化を行ったのに、そのリンパ球 CoQ10 を、液体クロマトグラフ質量分析計を用いて定量した。

芽球化リンパ球から CoQ10 を抽出し、重水素で標識した 2H9-CoQ10 を内部標準として、液体クロマトグラフ-タンデム質量分析計（HPLC-MS/MS）で測定した。

Table 1

DNA mutation	variants
COQ6	c.[782C > T]; [- (total deletion)]
COQ8B	c.[737G>A];[737G>A]
COQ8B	c. [737G>A];[532C>T]
COQ8B	c.[532C>T];[737G>A]
COQ8B	c.[737G>A];[737G>A]
COQ8B	c.[737G>A];[737G>A]

C. 研究結果.

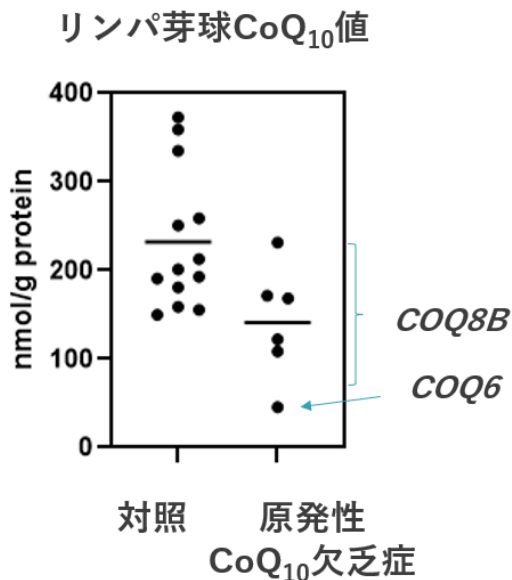


図 1. リンパ芽球の総 CoQ10 値

リンパ芽球 CoQ10 値は、対照では 232.08±77.86 nmol/g protein (n=13, mean±2SD, 149.72-372.55) であった。患者では COQ6 遺伝子変異例 (45.59 nmol/g protein) および COQ8B 遺伝子変異 2 例 (108.31, 122.10 nmol/g protein)、で低下を認めたが、COQ8B 遺伝子変異 3 例は正常範囲内であった。

D. 考察

原発性 CoQ10 欠乏症は、遺伝性 SRNS の約 1~2%を占める。病理所見としては、巣状分節性糸球体硬化症 70~80%, 異型ミトコンドリア 10~30% であり、腎予後としては、成人に達した時点での腎生存率 20~25%, 腎移植 25~30% であり、予後の悪い疾患である。CoQ10 治療により腎予後は改善するが、今回のリンパ芽球を用いた検討では、CoQ10 値は、COQ8B 遺伝子変異 3 例で低下を認めなかった。今後、皮膚線維芽細胞を入手し、CoQ10 値との相関も検討する。

E. 結論

皮膚線維芽細胞の還元型・酸化型 CoQ10 個別測定系を構築し、総 CoQ10 値と還元型 CoQ10/総 CoQ10 比の評価が可能になった。本測定法は、原発性 CoQ10 欠乏症の診断と迅速な治療に貢献でき、さらに CoQ10 還元状態評価のバイオマーカーとしての応用や病態解析にも有用と考えられる。

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Leigh 脳症に対する合成アポルフィンアルカロイドの有効性の検討。第 22 回日本ミトコンドリア学会年会 2023 年 11 月 14 日つくば国際会議場

2) 渡邊 知佳, 渡邊 美有紀, 今澤 俊之, 村山 圭, 大竹 明, 小坂 仁  
ミトコンドリア病患者細胞における CoQ10 酸化還元状態の検討。第 22 回日本ミトコンドリア学会年会。2023 年 11 月 15 日つくば国際会議場

#### H. 知的財産権

なし

分担研究報告書

診療体制の整備、生殖補助医療

研究分担者 後藤 雄一 国立精神・神経医療研究センターMGC 特任研究部長

**研究要旨** 本研究班ではミトコンドリア病の正確な診断とそれに基づく QIL の向上、適切な治療をめざして、特に遺伝学的検査を中核とする診断体制の構築について活動を行った。令和6年4月改訂の診断基準に合わせて、ミトコンドリア病パンフレットの改訂版作成を実施した。生殖補助医療については、総合科学技術・イノベーション会議（CSTI）の方針に基づき、令和3（2021）年6月に、「クローン技術規正法」に基づく「特定胚の取扱いに関する指針」が改正され、余剰胚の利用した核置換技術の基礎研究が容認されたのに加えて、続いて令和6（2024）年2月に、「ヒト受精胚の作成を行う生殖補助医療研究に関する倫理指針」（ART 指針）及び「ヒト受精胚に遺伝情報改変技術を用いる研究に関する倫理指針」（ゲノム編集指針）が改正され、新規胚を用いたミトコンドリア病の核置換技術を用いた研究が容認された。

A. 目的

ミトコンドリアはすべての細胞内において、エネルギーを産生する小器官である。ミトコンドリアに異常があると、大量のエネルギーを必要とする神経・筋、循環器、代謝系、腎泌尿器系、血液系、視覚系、内分泌系、消化器系などに障害が起こる。平成27年1月にミトコンドリア病が指定難病に認定され認定基準を制定したが、多様な臨床症状に加え、最近の遺伝学的検査の重要性を鑑み、それらを踏まえた診断基準の変更を必要としていた。

また英国では、ミトコンドリア病の一部の病型で、核移植を用いた生殖補助医療の適用が本格的に試みられ（Nature 465: 82-85, 2010）、少数ながら症例報告がなされている。そのようなグローバルな研究や医療の流れに遅れをとらないような本邦での調査研究が不可欠である。

本研究では、ミトコンドリア病の検査手段（病理検査、生化学検査、DNA 検査）の標準化と集約的診断体制の確立をめざす。また、生殖補助医療の情報収集と本邦での臨床応用の妥当性を吟味することを目的とする。

B. 方法

1) 遺伝学的検査の実施と診断体制の整備

AMED 難治性疾患実用化研究事業の村山班と協力して、国立精神・神経医療研究センター、順天堂大学、埼玉医科大学などを中心として、mtDNA 検査と核 DNA 上の原因遺伝子の検査について、医療の中でどのように組み込むかを明確にする。また、先端的遺伝学的検査（出生前診断）や適切な遺伝カウンセリングの提供体制を整備する。

2) 生殖補助医療の情報収集と見解のまとめ

ミトコンドリア病、特にミトコンドリア DNA 変異で発症するリー脳症においては、出生前診断や受精卵診断が欧米では行われている。この技術の有用性や倫理的問題について、本研究班で検討する。

C. 結果と考察

1) 遺伝学的検査

最近の遺伝学的検査法の進歩により、診断可能な疾病が大幅に増加し、令和3年度からミトコンドリア病の遺伝学的検査が保険収載された。一方で、ミトコンドリア病の場合は、核 DNA 上の病因遺伝子が 300 以上にのぼり、またミトコンドリア DNA の量的・質的变化も病因となる。今回の保険収載で規定された検査料で

はこれらを網羅的に行う事は困難である。そのため、検査会社が担う部分は遺伝学的検査の一部にならざるを得ず、そこに含まれない遺伝学的検査をどのような体制で行うかが重要な課題となっている。

研究班としてこの課題に対応するために、これまで全国から遺伝学的検査を依頼されてきた国立精神・神経医療研究センターと順天堂大学が協議を行い、まずは順天堂大学が病因の確定しているバリエーションを調べるパネル検査を衛生検査所登録のある病院ラボで行うこととなった。その上で、未確定の症例については、研究としてさらなる解析を行う体制とした。一方、国立精神・神経医療研究センターでは、衛生検査所登録が遅れており、従来通り、筋生検症例を中心に研究として主にミトコンドリア DNA 検査を実施した。近いうちに、衛生検査所登録を行い、全国からの診断依頼に応える準備を始めた。

このような遺伝学的検査に必要な「遺伝カウンセリング」に資するハンドブックを改訂した。初版を2012年に出版し、今回は令和6年4月に改訂される新しい診断基準を記した内容になっており、冊子体とともに、難病情報センターのHPからダウンロードできるようにした。



遺伝学的検査に加えて、生化学検査や病理検査のできる施設を確保し、我が国の診断体制を維持しており、今後の難病行政に対応することとしている。

## 2) 生殖補助医療の情報収集と政府見解のまとめ

平成28年10月に、米国ニューヨークの不妊クリニックが、「核移植治療」でミトコンドリア DNA の

m.8993T>G 変異をもつリー脳症の第1子をもつ母から健常な子が産まれたと発表した。この方法では、父と母(核ゲノム)に加えて別の女性(ミトコンドリアゲノム)が関わっており、「3人の親」がいる子となる。英国内でも、英国外でも倫理的問題があると議論されてきており、米国では禁止された行為であった。

日本においては、本件に関して、政府の科学技術・学術会議声明倫理・安全部会特定胚等研究専門委員会での議論が行われ、研究代表者の後藤が委員として参加した。

会議の結果、令和3(2021)年6月に、「クローン技術規正法」に基づく「特定胚の取扱いに関する指針」が改正され、ミトコンドリア病研究を目的とし、ヒト受精卵(余剰胚)への核置換技術を用いた基礎的研究を行うことについて、一定の要件が確保されることを個別の研究計画において適切に確認することを前提に容認された。

さらに、令和4(2022)年2月のCSTIにおいて、ミトコンドリア病研究を目的とする研究用新規作成胚(配偶子に核置換技術を用い、受精させる場合を含む。)及びその際の卵子提供に係る課題が検討され、新規胚を用いた基礎研究も容認され、令和6(2024)年2月に、「ヒト受精卵の作成を行う生殖補助医療に関する倫理指針」(ART指針)及び「ヒト受精卵に遺伝情報改変技術を用いる研究に関する倫理指針」(ゲノム編集指針)が改正され、新規胚を用いたミトコンドリア病の核置換技術を用いた研究が容認された。

以上から、これらは、受精卵を作成した日から14日以内の取扱期間や胎内への移植等が禁止され、文科大臣認定を要する研究になるものの、ミトコンドリア病における受精や発生初期の動態を研究できる基盤ができたことを意味し、今後の研究に期待する。

## D. 結論

本研究班の活動はAMED難治性疾患実用化研究班(村山班)と連携しながら進めている。全国レベルの診断体制の整備は喫急の課題であり今後も重点的に検討する。また、余剰胚、新規胚での核移植等の基礎研究が我が国で可能となった。今後の基礎研究に期待する

とともに、欧米での核移植症例の情報等を収集して、我が国でも核置換治療の是非をさらに検討してゆく。

E. 健康危険情報  
なし

F. 研究発表

1. 論文発表

著書、総説

後藤雄一：後藤雄一：MELAS の遺伝学的検査とその注意点は？ ミトコンドリア病診療マニュアル 2023（編集：日本ミトコンドリア学会）診断と治療社，東京，pp149-151，2023 June

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その他

国立精神・神経医療研究センター病院ゲノム診療部 遺伝カウンセリング科：ミトコンドリア病ハンドブック—ミトコンドリア病をもつ患者さんとそのご家族

のために—第2版. 10. 30, 2023

【難病情報センターHP からダウンロード可能  
[https://www.nanbyou.or.jp/wp-content/uploads/2023/12/mt\\_handbook\\_v2.pdf](https://www.nanbyou.or.jp/wp-content/uploads/2023/12/mt_handbook_v2.pdf)】

2. 学会発表

国際学会

なし

国内学会

なし

G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

ミトコンドリア病レジストリの構築に関わる検討

研究分担者 小牧宏文

国立精神・神経医療研究センター トランスレーショナル・メディカルセンター長

研究要旨

ミトコンドリア病レジストリの登録事業を前期研究班から行っており、今期も事業を継続している。2023年12月現在登録患者数は36例であり、増加はしているものの登録数は伸び悩んでいる。今後登録促進策を講じることが重要であり、現在神経筋疾患のレジストリの改良と平行してレジストリそのものの改良に関する検討を行っている。

A. 研究目的

希少疾病を対象に臨床開発を行っていくうえでレジストリなどを用いて疫学情報を得ていくことは非常に重要であり、臨床開発がすすみつつあるミトコンドリア病もレジストリの整備、拡充が急務である。

B. 研究方法

神経筋疾患を対象とし、NCNPが運営しているレジストリであるRemudyの基盤を活用し、ミトコンドリア病レジストリを構築運営している (<https://remudy.ncnp.go.jp/>)。今期はそれを引き継ぐこと、並びに課題を整理することを行った。

C. 研究結果

2023年12月時点の登録内容は以下となる。

- ・登録数：2022年度末、30例、2023年12月現在36例（仮登録含む）
- ・性別：女性15例、男性21例
- ・年齢：登録時年齢中央値29歳（0-76歳）、発症年齢中央値19歳（0-58歳）
- ・病名：MELAS14例、CPEO8例、MERRF：4例、Leigh脳症2例、ミトコンドリア病2例、ミオパチー2例、ピルビン酸脱水素酵素欠損症1例、ミトコンドリア糖尿病1例、DNM1L変異症1例、ミトコンドリア劣性運動失調症候群 1例

D. 考察

登録数は増加しているが、微増といわざるを得ない状況である。その要因としては、患者・家族・医

師への案内が不足していること、他疾患でもレジストリ事業が多く、登録作業を行う医師の負担が多くなっておりレジストリを患者・家族に案内するに至らないこと、ミトコンドリア病の臨床開発の情報が得られにくいことなどが挙げられる。

Remudyはデュシェンヌ型筋ジストロフィー、筋強直性ジストロフィーなど5疾患を対象にレジストリ事業を行っているが、登録数の伸び悩みという共通の課題を有している。これまでのような新規登録、年次情報更新のいずれにおいても医師の関与が必須である条件、ならびに紙ベースでの登録方法などの体制に由来することは間違いなく、他のRemudy対象疾患と同様に新規登録、ならびに年次情報更新のいずれにおいても医師の関与が必須ではない条件とし、Web（スマートフォン）ベースで登録が完了できるような大幅な見直しが必要と考えており、現在検討を進めている。

E. 結論

今年度で全体の見直し方針は定まったので次年度以降具体的な検討を進めていく。

F. 健康危険情報

特になし

G. 研究発表

なし

H. 知的財産権の出願・登録状況

なし

凍結融解卵を用いた極体移植によるミトコンドリア（細胞質）置換に関する研究

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**研究要旨**

第一極体ゲノムを用いたミトコンドリア置換の有用性の報告があるが、凍結融解卵を用いた検証はなされていない。また、極体ゲノムをもちいた卵子複製の可能性を検討した。

**A. 研究目的**

凍結融解操作を含む第一極体移植（PB1T）の効率を検討した。

胞を増加させる可能性が示唆された。ヒトにおいては生物学的特徴が異なり、凍結融解卵でも遂行可能であった。

**B. 研究方法**

マウス卵を用いて、PB1T 効率を検証し、極体由来の紡錘体とMII 紡錘体の比較検討、並びにヒト凍結融解卵を用いたPB1T の実現可能性を評価した。  
（倫理面への配慮）  
マウス、ヒトともに施設内倫理委員会の承認を得た。

**F. 健康危険情報**

該当なし

**C. 研究結果**

マウス卵第一極体（PB1）は凍結融解に脆弱であり、PB1T は凍結融解前に行う必要があった。PB1T は元のMII 卵子に加えて、受精可能な卵子を76%、胚盤胞を47%増加させた。しかしながら、PB1T 卵子はMII 卵子に比較して胚盤胞への発生能が優位に低く、19%のPB1由来の紡錘体は形態異常を示し、1/4 のPB1T 胚が染色体異常を示した。ヒトPB1 は凍結耐性を示した。

**G. 研究発表**

1. 論文発表

RMB 原著論文投稿中

日大医学雑誌 総説投稿中

2. 学会発表

2023/7 月第41 回日本受精着床学会

指定演者講演

2023/9 月IFFS2023

JSRM symposium

**D. 考察**

本PB1 由来の紡錘体形成不全は、染色体異常と胚盤胞発生能低下に繋がっている可能性が示唆された。

**H. 知的財産権の出願・登録状況**

（予定を含む。）

1. 特許取得

出願予定なし

2. 実用新案登録

登録予定なし

3. その他

なし

**E. 結論**

PB1T はマウスにおいては凍結融解前に行うことにより、ミトコンドリア置換のみならず、受精卵、胚盤

厚生労働科学研究費補助金 難治性疾患政策研究事業

分担研究報告書

NDB を利用したわが国におけるミトコンドリア病患者の疫学調査

研究分担者：藤野善久 産業医科大学 産業生態科学研究所 環境疫学教授

研究要旨

これまで、わが国ではミトコンドリア病を含む希少疾患の疫学調査の多くがアンケート方式によって行われてきた。しかし、アンケート用紙を使用する調査方法は、人的・時間的・費用的に負担の大きいものである。近年、既存レセプトデータの利用が促進され、希少疾患の疫学調査にも用いられるようになった。本研究の目的は、厚生労働省が管理するレセプト情報である、特定健診等情報データベース(NDB, National Database)を用いて、ミトコンドリア病患者の有病者数および有病者割合の推定をはじめとした疫学調査を行うことである。

2024年1月に厚生労働省にミトコンドリア病に関するNDBデータの提供について申し出を行った。4月に審査の結果、無条件承諾であることが通知された。今後、完成した集計表を厚生労働省から受領し、ミトコンドリア病患者の有病者数について記述的な統計解析を  
行なう。

A. 研究目的

従来では、わが国のミトコンドリア病を含む希少疾患の疫学調査の多くはアンケート方式によって行われてきた。しかし、この調査方法は研究者および実地臨床家にとって人的、時間的、および費用的に負担の大きいものであった。しかし、近年わが国では研究目的をはじめとした既存レセプトデータの利用環境が整備されてきており、希少疾患の疫学研究に利用されるケースも出てきている。本研究では厚生労働省が管理するレセプト情報・特定健診等情報データベース(NDB, National Database)を用いて、ミトコンドリア病患者の有病者数および有病者割合の推定をはじめとした疫学調査

を行う。

B. 研究方法

レセプト情報・特定健診等情報データベース(NDB)は、平成20年4月から施行されている「高齢者の医療の確保に関する法律」に基づき、医療費適正化計画の作成、実施及び評価のための調査や分析などに用いるデータベースとして、レセプト情報及び特定健診・特定保健指導情報を格納・構築しているものである。本研究では、厚生労働省がNDBに格納している約225億の医療レセプトデータを利用する。ミトコンドリア病に関連する傷病名コードを専門医と議論した上で決定し、さらに、表1のように9つ

のサブグループを定義した。その後、厚生労働省に対して、表2のような形式で、ミトコンドリア病の傷病名コードに紐づく外来データの抽出と、ミトコンドリア病に対する性別、年齢階級別、都道府県別等のデータの集計表の作成を依頼した。完成した集計表はCSV形式でDVDに格納され、厚生労働省から受領する。

厚生労働省から受領した集計表を用いて、抽出されたミトコンドリア病患者に対する、性別、年齢階級別、都道府県別等のデータを記述的に確認する。各都道府県別のデータについては、有病者数と標準化有病者割合比(SPR, Standardized Prevalence Ratio)を求める。

本研究では情報の取り扱いが厚生労働省が行い、申請者らは集計結果のみを受領するため、個人情報の加工は必要なく、倫理面への配慮は特に要さない。

#### C. 研究結果

2024年1月に厚生労働省にミトコンドリア病に関するNDBデータの提供に

ついての申出を行った。4月に審査の結果、無条件承諾であることが通知された。今後、完成した集計表を厚生労働省から受領し、ミトコンドリア病患者の有病者数について記述的な統計解析を行う予定である。

#### D. 考察

集計表を受領し、統計解析を実施後に考察する。

#### E. 結論

集計表を受領後に、統計解析を行う予定である。

#### F. 研究発表

1. 論文発表  
なし
2. 学会発表  
なし

#### G. 知的財産権の出願・登録状況

1. 特許取得  
なし
2. 実用新案登録  
なし
3. その他  
なし

表 1.

ミトコンドリア病のサブグループ	請求番号	病名	患者数
いずれかのミトコンドリア病			***
①Leigh脳症			
G318	8840933	リー症候群	***
②ミトコンドリア肝症			
E888	8846972	ミトコンドリア肝症	***
G318	8842457	アルパース病	***
③ミトコンドリア心筋症			
E888	8846224	ミトコンドリア心筋症	***
④MELAS			
E888	8846079	M E L A S 症候群	***
⑤MERRF			
E888	8846080	M E R R F 症候群	***
⑥CPEO/KSS			
H494	8846059	慢性進行性外眼筋麻痺症候群	***
H498	8831018	カーズ・セイアー症候群	***
⑦Leber遺伝性視神経症			
H472	8848684	レーベル遺伝性視神経症	***
⑧ミトコンドリア糖尿病			
E888	8849469	ミトコンドリア糖尿病	***
E888	8849470	ミトコンドリア糖尿病・眼合併症あり	***
E888	8849471	ミトコンドリア糖尿病・ケトアシドーシス合併あり	***
E888	8849472	ミトコンドリア糖尿病・昏睡合併あり	***
E888	8849473	ミトコンドリア糖尿病・神経学的合併症あり	***
E888	8849474	ミトコンドリア糖尿病・腎合併症あり	***
E888	8849475	ミトコンドリア糖尿病・多発糖尿病性合併症あり	***
E888	8849476	ミトコンドリア糖尿病・糖尿病性合併症なし	***
E888	8849477	ミトコンドリア糖尿病・糖尿病性合併症あり	***
E888	8849478	ミトコンドリア糖尿病・末梢循環合併症あり	***
⑨その他・非特異的概念			
E888	8845613	ミトコンドリア病	***
G713	8841409	ミトコンドリアミオパチー	***
G713	8841410	ミトコンドリア脳筋症	***
E888	8849678	フマラーゼ欠損症	***
D640	8846217	ピアソン症候群	***
E888	8846084	M N G I E	***
E888	8849839	ミトコンドリアDNA 枯渇症候群	***
E888	8849840	ミトコンドリア呼吸鎖複合体欠損症	***
E744	8848412	ピルビン酸脱水素酵素複合体欠損症	***
E744	8849677	ピルビン酸カルボキシラーゼ欠損症	***
E888	8849783	スクシニル-CoA リガーゼ欠損症	***

表 2.

年齢階級・性別	合計	サブグループ										
		Leifの強度	ミトコンドリア強度	ミトコンドリアの強度	MELAS	MERRF	CPRO/KSS	Ldbrn遺伝性神経症	ミトコンドリア神経病	その他・非特異的疾患		
0-4歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5-9歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10-14歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15-19歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20-29歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30-39歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
40-49歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
50-59歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
60-69歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
70-79歳	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
80歳以上	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
性別別												
男性												
女性												
地域別												
北海道												
青森県												
岩手県												
宮城県												
秋田県												
山形県												
福島県												
茨城県												
栃木県												
群馬県												
埼玉県												
千葉県												
東京都												
神奈川県												
新潟県												
富山県												
石川県												
福井県												
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厚生労働科学研究費補助金（難治性疾患克服研究事業）  
分担研究報告書

ミトコンドリア病における AMED 実用化研究班との連携、診断・治療体制の整備

分担研究者： 村山 圭（順天堂大学難治性疾患診断・治療学 教授）

研究要旨

ミトコンドリア病は指定難病の1つであり、本邦での患者数は約2000人である。本政策研究の目的は、これまでに策定した診断基準の検証や改訂、医療者や一般社会への周知、疾患啓発、診療体制の整備を行い、広く医療行政に貢献することである。

これを達成するために、AMED 実用化研究班（村山班）と密に連携を取りながら研究を進めていく必要がある。以前から2つの班が連携して、今年度その成果として「ミトコンドリア病診療マニュアル 2023」が改訂版として出版された。引き続き連携をしながら診療マニュアルの啓蒙等を進めている。2024年度に開催される日本小児神経学会、日本人類遺伝学会、日本ミトコンドリア学会等でシンポジウムを企画している。診断体制に関しても研究班と村山班でも共有し、その問題点や課題を解決してきている。また医療研究開発における患者・市民参画（PPI: Patient and Public Involvement）に関して、これまで「ミトコンドリア病研究公開フォーラム」を AMED 研究班で行ってきたが、今年度から本研究班と共同して企画・開催を行った。

研究協力者氏名

・杉山洋平

所属機関名及び所属機関における職名

・順天堂大学小児科 助手

A. 研究目的

ミトコンドリア病は希少疾患でありその研究は、診断体制の確立、遺伝子の同定、病態解明、診療ガイドラインの策定、患者登録やそれによる自然歴の検討など多岐にわたる。このため、実用化研究と政策研究が共同して進めていくことが必須である。さらに患者会と協力して進めていかなければならないことも多い。こうしたことを実行するために、現在 AMED 実用化研究として進められている村山班と連携して本研究班の活動を行うことを目的とする。これにより、ミトコンドリア病診療マニュアルの策定、診断体制の整備、レジストリ活動、患者・市民参画活動を遂行していく。

B. C. 研究方法および研究結果

診療マニュアル 2023 の出版

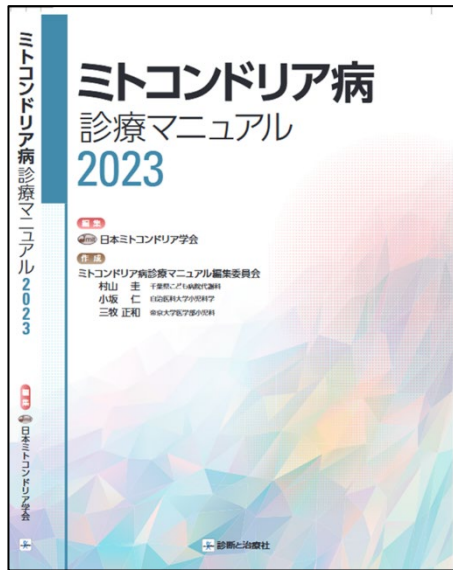
2021 年から政策研究班（旧後藤班）、AMED 村山班と連携し、会議を重ねて「診療マニュアル 2017」の改訂作業を行ってきた。これにより 2023 年 6 月に共同の成果として「ミトコンドリア病診療マニュアル 2023」が出版された。

前マニュアルで取り上げた、

1. Leigh 脳症
2. ミトコンドリア肝症
3. ミトコンドリア心筋症
4. 新生児ミトコンドリア病
5. MELAS
6. CPEO/KSS
7. MERRF

の 7 疾患に加えて、今回新たに以下の病型を加えた。

8. ミトコンドリア難聴
9. ミトコンドリア腎症
10. ミトコンドリアニューロパチー
11. ミトコンドリア糖尿病
12. Leber 病



診療体制に関しては2023年11月から順天堂大学臨床検査部によって遺伝学的検査（保険点数8000点）が開始され、各遺伝子367種類+mtDNA全周検査が行われている。

患者・市民参画（PPI: Patient and Public Involvement）の機会として、AMED村山班と共同して第8回ミトコンドリア病研究公開フォーラムを開催した。

《プログラム》

開催日 2024年2月3日

開催方法 Web配信 (Zoom/YouTube)

講演1. ミトコンドリア総論

国立精神・神経医療研究センター  
後藤 雄一

講演2. ミトコンドリア病の遺伝学的検査について

順天堂大学  
八塚 由紀子

講演3. 変異ミトコンドリアDNAを標的とする薬剤開発

千葉県がんセンター  
高取 敦志

講演4. ミトコンドリア創薬

ルカ・サイエンス株式会社  
菅沼 正司

講演5. MA-5の治療開発について

東北大学  
阿部 高明

講演6. 患者主導型情報プラットフォームJ-RARE 10周年とRDD 15周年

ASrid  
西村 邦裕

講演7. MCMの会へようこそ

ミトコンドリア病患者・家族の会  
高橋 和恵

講演8. みどりの会 ミトコンドリア力を信じて

みどりの会  
伊藤千恵子

講演9. 特定非営利活動法人ミトコンドリア病医療推進機構の活動

帝京平成大学  
高柳 正樹

講演10. ミトコンドリア雑感

順天堂大学  
太田 成男

**第8回ミトコンドリア病研究 公開フォーラム**

日時 2024年2月3日(土) 12:30~16:30 (予定)

Web開催。ご家族そろってご参加ください!

ミトコンドリア病診療のこれから 2024

予定プログラム

- **ミトコンドリア病の総論**
  - ミトコンドリア総論 --- 後藤 謙一先生(国立精神・神経医療研究センター)
  - ミトコンドリア病の遺伝学的検査について --- 八塚 由紀子先生(順天堂大学)
- **創薬**
  - 変異ミトコンドリアDNAを標的とする薬剤開発 --- 高取 敦志先生(千葉県がんセンター)
  - ミトコンドリア創薬 --- 菅沼 正司先生(ルカサイエンス株式会社)
  - MA-5の治療開発について --- 阿部 高明先生(東北大学)
- **患者会**
  - ASrid・MCMの会・みどりの会 より
- **ミトコンドリア雑感**
  - 太田 成男先生(順天堂大学)

ミトコンドリア病研究会

参加申し込み: y.konno.bn@juntendo.ac.jp にメールにてお申し込みください。  
 参加費無料 ◆ 事前登録: 2024年1月20日まで  
 要参加申込み ◆ 詳細は、JAMP-MITホームページ <http://jamp-mit.or.jp/index.html>  
 J-MO Bankホームページ <http://jmo-bank.com/> を参照ください。

主催: 特定非営利活動法人 ミトコンドリア病医療推進機構 JAMP-MIT (理事長: 高柳 正樹)  
 共催: 日本医療研究開発機構(AMED) 難治性疾患実用化研究事業 ミトコンドリア病研究 (研究者代表: 村山 圭)  
 厚生労働省 難治性疾患実用化研究事業 ミトコンドリア病研究(研究者代表: 三致 正和)  
 日本先天代謝学会 患者登録委員会 JASMA事務局 (代表: 小須賀 龍彦)

## 【参加者】

患者会 91家族 111名  
医療関係者 32名  
演者、座長、スタッフ 21名  
参加者総数 164名

## 【参加患者会一覧】

- ・ミトコンドリアみどりの会
- ・MCMの会
- ・NPO法人 全国ポンペ病患者と家族の会
- ・ひだまりたんぼぼ
- ・認定NPO法人ALDの未来を考える会
- ・先天性代謝異常症患者会
- ・シトリン財団
- ・小児神経伝達物質病家族会

以上8患者会・親の会

## (倫理面への配慮)

例年通り患者の個人名、疾患名などは講演においても、ホームページ上においても明らかにしていない。フォーラムをインターネット配信したが、その画像などに患者が映らないように留意するなど、個人情報の管理に十分配慮した。

## D. 考察

政策研究班とAMED研究班の共同で本フォーラムを開催した。最初に前政策研究班代表の後藤雄一先生からミトコンドリア総論を話していただき、現在のミトコンドリア病診療の課題について解説していただいた。続いて、保険収載されたことによって、新しく始まったミトコンドリア病の遺伝学的検査について、実際に行っている順天堂大学の八塚由紀子先生にわかりやすく解説していただいた。

創薬研究に関してはこれまでのアンケートからも患者家族のニーズが高い分野である。今回は変異ミトコンドリアDNAを標的とする薬剤開発(ポリアミド化合物)に関して、開発者である千葉県がんセンター高取敦志先生に、またミトコンドリア移植についてルカサイエンス株式会社の菅沼健司先生に、さらにAMED研究において開発されているMA-5について、東北大学の阿部高明先生からそれぞれ開発の現状につい

て話をしていただいた。

続いて、レジストリに関連した話題として、患者主導型情報プラットフォーム(J-RARE)について、これまで情報収集を行ってきたNPO法人ASrid(Advocacy Service for Rare and Intractable Diseases' multi-stakeholders in Japan)の西村邦裕氏から「J-RARE 10周年とRDD 15周年」としてお話いただいた。2024年2月29日に開催される世界希少・難治性疾患の日(RDD)のイベントについての情報共有もしていただいた。同イベントにはミトコンドリア病患者会の方々も参加された。

患者家族会からは日本で活動している2つの団体(MCMの会、ミトコンドリアみどりの会)の代表の方(高橋和恵さん、伊藤千恵子さん)から現在の活動状況についてご紹介いただいた。そして本フォーラムの主催者であるNPO法人ミトコンドリア病医療推進機構の高柳正樹代表から活動報告と入会案内について話をいただいた。

フォーラムの最後に、ミトコンドリア学会前々理事長の太田成男先生に「ミトコンドリア雑感」としてまとめていただいた。

今回はWeb形式という形でフォーラムを円滑に開催することができた。約160名の方々にフォーラムに参加して頂くことができた。かつては現地参加で行っていた本フォーラムであるが、コロナ禍を契機としてWeb開催が定着してきている。引き続き患者家族、医療従事者、研究者、関連企業を含むオールジャパン体制での確立や財政面での安定性の保証などを十分に検討、協議して、持続可能な本フォーラムを企画していく必要がある。

## E. 結論

2023年度ミトコンドリア病研究公開フォーラムを実施した。今年度もハイブリッド形式での開催となったが引き続き多くの方々にご参加頂けた。今後もミトコンドリア病の研究は患者会との綿密な協力のもと実施する意義は十分にある。

## F. 研究発表

1. 論文発表  
なし
2. 学会発表

なし

G. 知的財産権の出願・登録状況  
(予定を含む。)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（難治性疾患政策研究事業）  
分担研究報告書

ミトコンドリア病の新規バイオマーカーGDF15の有用性の検討

研究分担者 八ツ賀 秀一 福岡大学小児科 准教授

研究要旨

ミトコンドリア病の新規バイオマーカーとして、GDF15の診断カットオフ値および治療効果判定指標、交絡因子を明らかにする。患者血液をELISA法にてGDF15を測定する。患者は、遺伝学的に診断されたミトコンドリア病のLeigh脳症とMELASを各10名。遺伝学的に明確なミトコンドリア病の診断補助としてGDF15のカットオフ値が明らかになると推測している。また現在、様々な治療薬開発が進んでいるが、その治療効果判定として、GDF15が有用になることが予想される。

A. 研究目的

ミトコンドリア病の診療水準ならびにQOL向上のため、新規バイオマーカーGDF15 (growth differentiation factor 15) の診断カットオフ値および治療効果判定指標、交絡因子を明らかにする。

B. 研究方法

ミトコンドリア病患者、特に患者数の多いLeigh脳症10名、MELAS10名の患者から血液を1-2mL採取し、ELISA法にてGDF15を測定する。同時に、従来のバイオマーカーである乳酸、ピルビン酸、乳酸/ピルビン酸比、アラニン、CKを測定する。診療録から、年齢、発症年齢、罹病期間、治療内容を調査する。

採取した血液は、速やかに3000rpmで5分間遠心分離を行い、GDF15を測定するまで-80度で冷凍保管する。

GDF15の測定は、R&D systems社のものを使用する (Yatsuga S, et al. Ann Neurol. 2015)。

1検体につき2ウェル使用する。1プレート(キット)あたり38検体測定できる。測定感度以上の値が出た場合は、10倍に希釈して計測する。そのため、1-2プレート(キット)余分に使用する可能性はある。

ミトコンドリア病の定義は、遺伝学的、酵素学的、病理学的にミトコンドリア病の診断基準を満たすものとする(難病情報センター;ミトコンドリア病(指定難病21))。

<倫理面への配慮>

C. 研究結果

現在、実施可能性につき検討しており、令和6年度中に倫理委員会に研究計画書を提出する予定である。

D. 考察

研究実施可能性の検討中であり、かつ倫理委員会申請前であるが、新規バイオマーカーGDF15の診断カットオフ値および治療効果判定指標、交絡因子の意義について起こり得る結果についての考察を記載する。

GDF15は、ミトコンドリア病のバイオマーカーとして、感度/特異度がともに98%であることが報告されている (Yatsuga S, et al. Ann Neurol. 2015)。しかし、臨床的診断によるミトコンドリア病を中心とした検体であり、遺伝学的な検証が十分にされていない。今回、明確な遺伝子異常を伴ったミトコンドリア病患者の検体を検査することで、従来のバイオマーカーとの相関性、交絡因子を見つけることができると考える。

E. 結論

遺伝学的に明確なミトコンドリア病の診断補助としてGDF15のカットオフ値が明らかになると推測している。また現在、様々な治療薬開発が進んでいるが、その治療効果判定として、GDF15が有用になることが予想される。

F. 健康危険情報

総括研究報告書にまとめて記入。

G. 研究発表

1. 論文発表

該当なし。

2. 学会発表

該当なし。

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。

3. その他

該当なし。

ミトコンドリア心筋症の診療体制の整備、重症度分類に関する研究

研究分担者 武田 充人 北海道大学大学院医学研究院小児科学教室・講師

**研究要旨**

ミトコンドリア心筋症の診療体制整備に関して免疫染色法を用いたミトコンドリア心筋症病理診断の開発を行い、非常に有用性が高いことが判明している。

**A. 研究目的**

ミトコンドリア心筋症(MCM)は原因遺伝子や表現型が多彩で、小児から成人まであらゆる年齢層において発症し、しばしば見過ごされがちである(E. Braunwald, EHJ 2023)。本症は病理学、遺伝学、生化学的検査によって診断されるが、これまで病理は電子顕微鏡(EM)からしか疑うことができなかった。我々は、呼吸鎖複合体抗体を用いた免疫組織化学染色法(IHC)によるMCMの病理診断を開発した(A. Takeda et. al., J Clin Path 2021)。本報告では、この方法で病理診断されたMCMの遺伝学的特徴と臨床的特徴について述べる。

**B. 研究方法**

IHCによりミトコンドリア心筋症と診断された23例のうち、遺伝子検査、組織生化学、EMのいずれかが実施可能であった13例を後方視的に検討した。呼吸鎖複合体酵素活性とIHCとの相関関係について、双方の検査が行われた13例(ミトコンドリア心筋症7例、残りは他の心筋症)について調べた。

(倫理面への配慮)

本研究は大学IRBで倫理審査済である

**C. 研究結果**

IHC: MCMと診断された12例のうち、3例は複合体Iのみ欠損、3例は複合体IVのみ欠損、残りの7例

は複合体Iと複合体IVの両方が欠損であった。また、呼吸鎖複合体酵素活性を7例に実施したところ、5例に欠損が認められ、酵素活性とIHCの結果には有意な相関がみられた。11名の患者が遺伝子検査を受け、全例がMCMの病原性遺伝子バリエントを有していた。5例はm.3243A>G、他はm.4300A>G, m.14453A>G, m.12264C>T, MT-ND6, GARS, GTPBP3, TOP3Aであった。すべての患者にEMにおけるミトコンドリア異常所見がみられた。

**D. 結論**

IHCはMCMの病理診断に大きな役割を果たすものと期待される。

**E. 健康危険情報**

該当なし

**F. 研究発表**

1. 論文発表

なし

2. 学会発表

10th Association for European Cardiovascular Pathology (AECVP) Biennial Meeting 21-23 Sep. 2023 Padua, Italy

Role of immunostaining methods in the diagnosis of mitochondrial cardiomyopathy

Atsuhito Takeda, Hirokuni Yamazawa, Ayako Chida-

Nagai, Daisuke Sasaki, Asuka Takahata

出願予定なし

G. 知的財産権の出願・登録状況

2. 実用新案登録

(予定を含む。)

登録予定なし

1. 特許取得

3. その他

なし

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# ミトコンドリア病 診療マニュアル 2023

編集

 日本ミトコンドリア学会

作成

ミトコンドリア病診療マニュアル編集委員会

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## ■編集 日本ミトコンドリア学会

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## ■作成 ミトコンドリア病診療マニュアル編集委員会

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日本小児栄養消化器肝臓学会	日本新生児成育医学会
日本小児循環器学会	日本先天代謝異常学会
日本小児神経学会	日本糖尿病学会
日本神経学会	

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# ミトコンドリア病ハンドブック

ミトコンドリア病をもつ患者さんとそのご家族のために

第2版

国立精神・神経医療研究センター病院  
ゲノム診療部 遺伝カウンセリング科

## はじめに

### ミトコンドリアとミトコンドリア病

#### ミトコンドリア

ミトコンドリアの機能障害・検査法・診断名

ミトコンドリア病の診断基準 (1) (2)

### ミトコンドリア病の検査

#### ミトコンドリア病の検査

ミトコンドリア病のDNA (遺伝子) 検査

核DNA・染色体・遺伝子・タンパク質

ミトコンドリアDNA・遺伝子・タンパク質

代表的なミトコンドリアDNAの変化

ホモプラスミーとヘテロプラスミー

ミトコンドリア病の病理検査

ミトコンドリア病の生化学検査

### ミトコンドリア病の症状

ミトコンドリア病の主な症状

卒中様症状を伴うミトコンドリア病

ミオクローヌスを伴うミトコンドリア病

慢性進行性外眼筋麻痺症候群

Leigh脳症

### ミトコンドリア病の治療

ミトコンドリア病の治療法 (対症療法) (原因療法)

### ミトコンドリア病の患者・家族のための社会資源

代表的な医療・福祉制度

情報サイト

患者登録制度

### ミトコンドリア病の遺伝

ミトコンドリアDNAの遺伝=母系 (母性) 遺伝

ミトコンドリアDNAの新しくおきた変化

核DNAの遺伝=メンデル遺伝 顕性 (優性) と潜性 (劣性)

常染色体潜性遺伝 (劣性遺伝) 常染色体顕性遺伝 (優性遺伝)

X連鎖潜性遺伝 (劣性遺伝) X連鎖顕性遺伝 (優性遺伝)

出生前診断・着床前診断



## はじめに



このハンドブックは、ミトコンドリア病をもつ患者さんとそのご家族に、病気への理解を深めるときの参考としていただくために作成しました。各項目について、イラストと文章での説明があります。

イラストは、医療者から当事者の方へ説明するときの資料として、文章は、当事者の方がご自身で読んで理解するときの補足説明として、利用していただけるようになっています。

分かりにくいところがあれば、医療者へお尋ねください。

また、ミトコンドリア病の症状は多様ですので、すべての患者さんには当てはまらない内容もたくさん書かれています。

病気とうまく付き合っていくためには、

それぞれの状況に合わせて対応することが大切ですので、

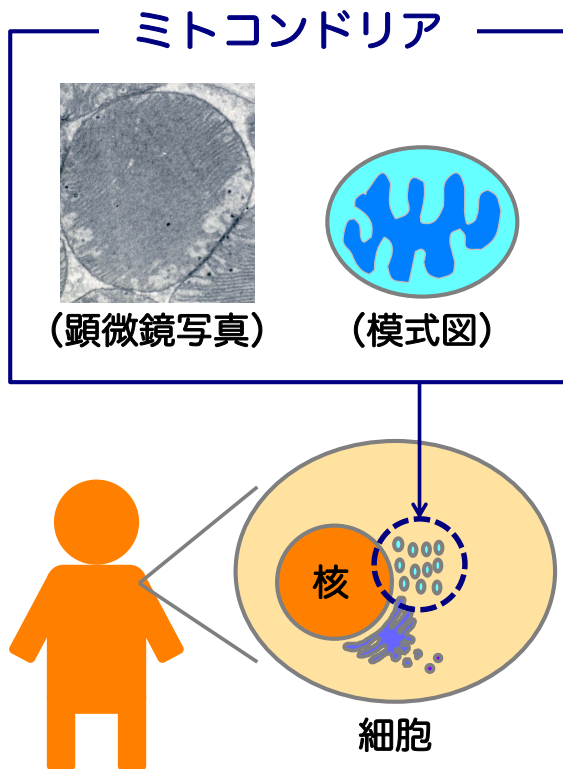
ご自身の病気については、担当の医療機関等でご相談ください。



# ミトコンドリアとミトコンドリア病



# ミトコンドリア



《細胞内での働き》

エネルギーの合成

活性酸素の発生

アポトーシス

カルシウムの貯蔵

感染の防御

ミトコンドリアの機能障害  
→ ミトコンドリア病

私たちの体は、たくさんの細胞でできています。

その細胞の一つ一つの中にミトコンドリアは存在しています。

一つの細胞に数百個のミトコンドリアが入っていて、

細胞に必要なエネルギーを作り出しています。

そのため、ミトコンドリアに機能障害が生じると細胞の働きが悪くなり、

さまざまな症状が現れます。これがミトコンドリア病です。

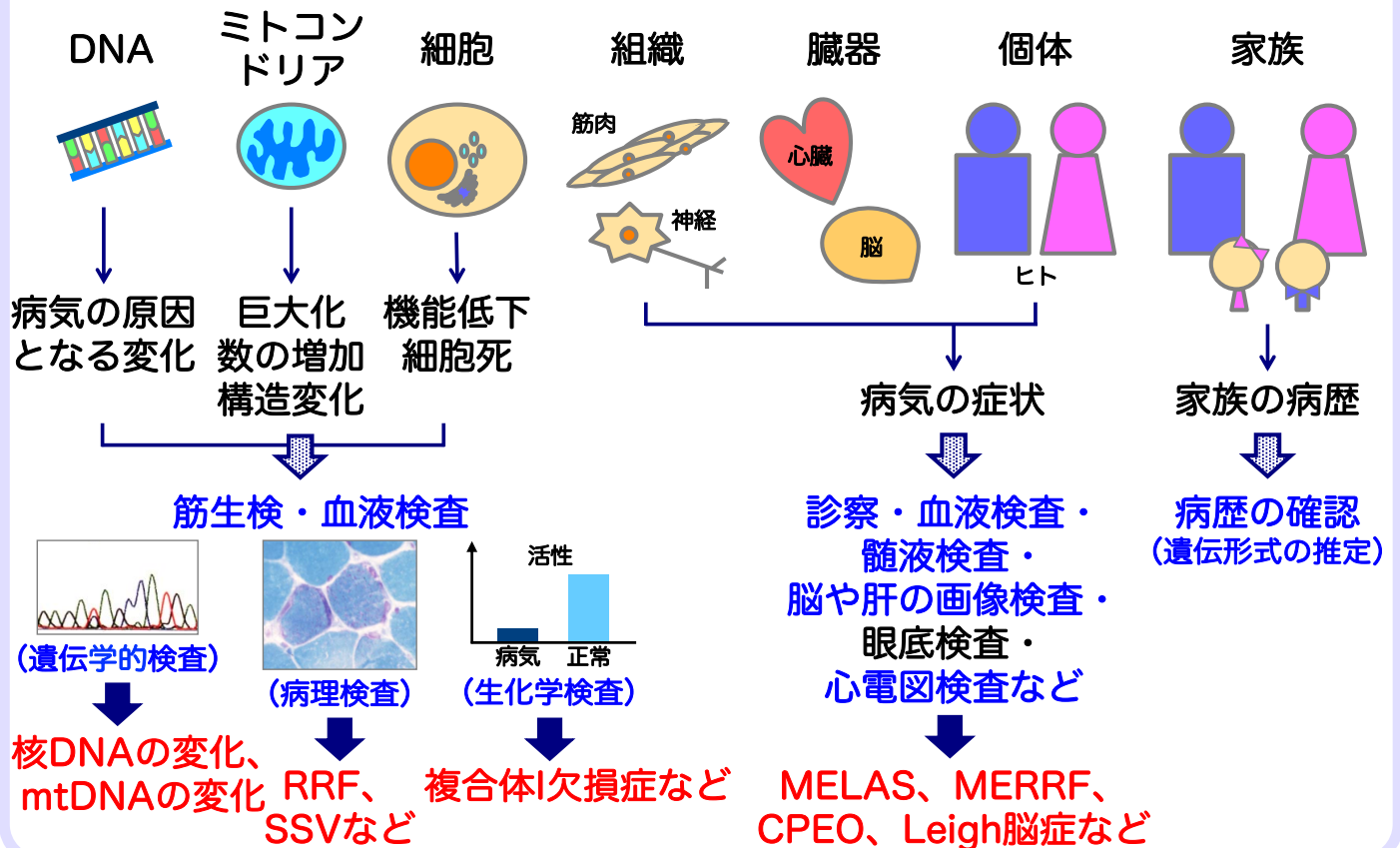
体のどこのミトコンドリアに機能障害が生じるかによって症状は異なります。

また、ミトコンドリアは、活性酸素の発生、アポトーシス（細胞死）、

カルシウムの貯蔵、感染の防御などにも関わっていて、

ミトコンドリア病以外のさまざまな病気にも関与しています。

# ミトコンドリアの機能障害・検査法・診断名



ミトコンドリアの機能障害を調べる検査には多くの種類があります。それは、ミトコンドリアの機能障害がさまざまな形で現れるためです。たとえば、設計図であるDNAには、病気の原因となる変化（病的バリエーション）が生じます。DNAに病気の原因となる変化があるミトコンドリアは、大きさや数、構造が変化し、そのミトコンドリアをもつ細胞は、機能が低下したり、死んだりします。そして私たちの体のさまざまな部分（臓器）に症状となって現れます。DNAは先祖から代々受け継がれるものなので、家族の中に同じような症状をもつ方がいる場合もあります。

## 指定難病医療費助成制度

# ミトコンドリア病の診断基準（1）

### （1）主症状

- ① 進行性の筋力低下、横紋筋融解症又は外眼筋麻痺を認める。
- ② 知的退行、記銘力障害、痙攣、精神症状、一過性麻痺、半盲、皮質盲、ミオクローヌス、ジストニア、小脳失調などの中枢神経症状のうち、1つ以上を認める。または手足のしびれなどの末梢神経障害を認める。
- ③ 心伝導障害、心筋症などの心症状、肺高血圧症などの呼吸器症状、糸球体硬化症、腎尿細管機能異常などの腎症状、強度の貧血などの血液症状、又は肝障害、黄疸、凝固能低下などの肝症状、体重増加不良、繰り返す嘔吐・下痢・便秘等の消化器症状を認める。
- ④ 低身長、甲状腺機能低下症などの内分泌症状や糖尿病を認める。
- ⑤ 強度視力低下、網膜色素変性などの眼症状、感音性難聴などの耳症状を認める。
- ⑥ 新生児期または乳児期に、発育異常、発達遅延を認める。

ミトコンドリア病は、平成27年（2015年）1月に、国の難病対策の一つである指定難病医療費助成制度の対象に認められました。

この事業は、一定の条件を満たす病気を対象に、その患者さんの医療費を助成し、原因の究明や治療法の開発などに向けた調査研究を推進しようとする制度です。

指定難病としての「ミトコンドリア病」と診断されるためには定められた診断基準を満たす必要があります。

ここに掲げた診断基準は、令和6年4月から施行される予定のものです。

具体的には、主症状として筋肉、中枢神経、心臓、肺、腎臓、血液、肝臓、内分泌、膵臓、眼、耳のいずれかに症状があることが要件となります。

新生児期や乳児期には、あきらかな臓器の症状が現れていないこともあります。

いずれの場合も、次に示す「検査・画像所見」が明確に存在する時にミトコンドリア病の診断が付けられます。

## 指定難病医療費助成制度

# ミトコンドリア病の診断基準 (2)

### (2) 検査・画像所見

- ① ミトコンドリアDNAの質的、量的異常又はミトコンドリア関連分子をコードする核遺伝子変異を認める。 (遺伝学的検査所見)
- ② 骨格筋生検や培養細胞又は症状のある臓器の細胞や組織でミトコンドリアの病理異常を認める。 (病理検査所見)
- ③ ミトコンドリア関連酵素の活性低下、又はコエンザイムQ10などの中間代謝物の欠乏を認める。または、ミトコンドリアDNAの発現異常を認める。 (生化学検査所見)
- ④ 安静臥床時の血清又は髄液の乳酸値が繰り返して高い、又はMRスペクトロスコピーで病変部に明らかな乳酸ピークを認める。
- ⑤ 脳CT/MRIにて大脳基底核、脳幹に両側対称性の病変等を認める。 (脳画像所見)
- ⑥ 眼底検査にて、急性期においては蛍光漏出を伴わない視神経乳頭の発赤・腫脹、視神経乳頭近傍毛細血管蛇行、網膜神経線維腫大、視神経乳頭近傍の出血のうち1つ以上の所見を認めるか、慢性期（視力低下の発症から通常6か月以降）における視神経萎縮所見を両眼に認める。 (眼底検査所見)
- ⑦ 腹部エコー、CT/MRI、あるいは肝組織所見にて、脂肪肝、あるいは肝硬変の所見を認める。 (肝画像所見)

主症状と検査・画像所見の中で、満たす項目の種類と数により  
**Definite (確実)**、**Probable (ほぼ確実)**、**Possible (可能性あり)** に分類される

本制度で医療費助成の対象になるのは、**Definite** か **Probable** と診断され、さらに「**重症度分類**」に照らして病状の程度が一定程度以上の場合です。

初めて指定難病の申請をする際には、難病指定医による「**臨床調査個人票**」と呼ばれる診断書の作成が必要となりますので、主治医の先生にご相談ください。

また、18歳未満の患者さんの場合には、小児慢性特定疾病医療費助成を受けられる場合があります。

申請する際には、小児慢性特定疾病指定医による「**医療意見書**」と呼ばれる診断書の作成が必要となりますので、主治医の先生にご相談ください。

なお、引き続き治療が必要と認められる場合には、有効期間終了前に継続手続を行うことにより、20歳未満まで延長することができます。

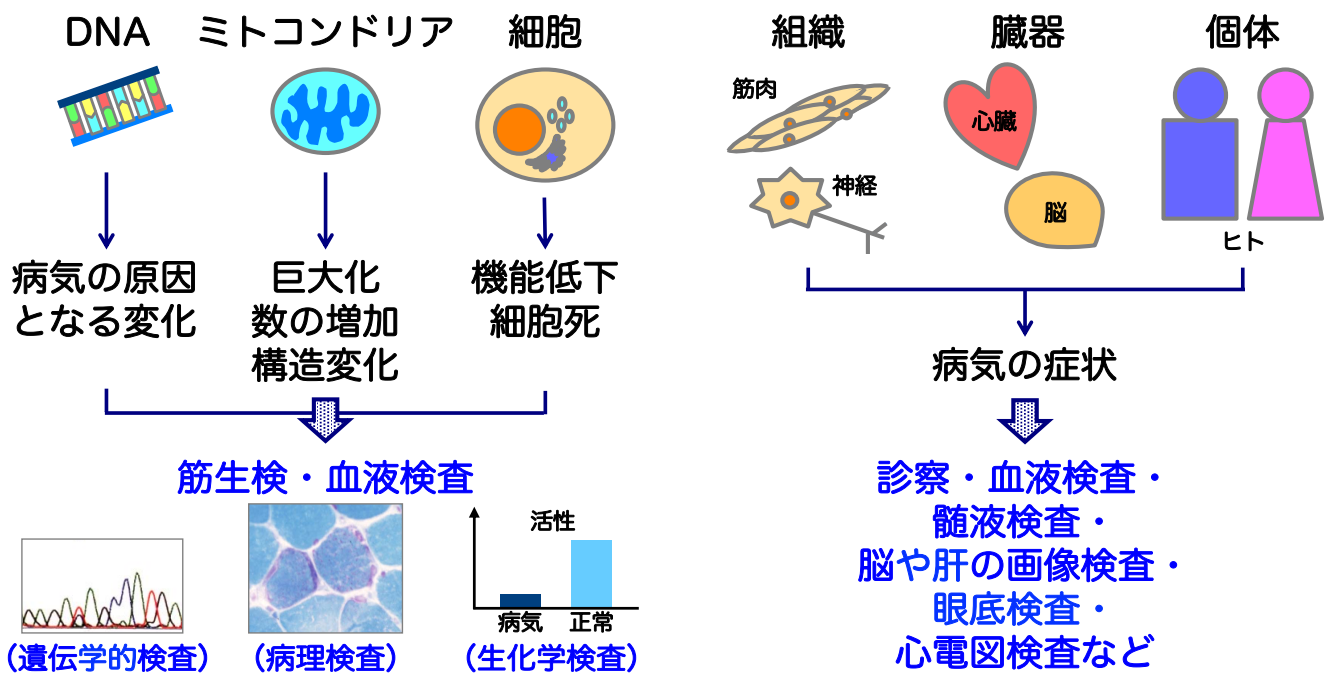


## ミトコンドリア病の検査

# ミトコンドリア病の検査

ミトコンドリア機能障害があるかどうか

どのような症状が出ているか



ミトコンドリア病の検査は、その目的によって大きく二つに分けられます。一つは、どのような症状が出ているかを調べるための検査（右）です。脳や肝の画像検査や心電図検査などを行い、さまざまな臓器に異常があるかどうかを調べます。

もう一つは、ミトコンドリアの機能障害を調べるための検査（左）です。筋生検や血液検査で採取した検体を用いて、DNA（遺伝学的）検査、病理検査、生化学検査を実施します。これによって、ミトコンドリアのDNA、形、働きを詳しく調べることができます。

# ミトコンドリア病のDNA（遺伝学的）検査

## 核DNAの変化

## ミトコンドリアDNAの変化

酵素の一部となるタンパク質に  
関係する遺伝子の変化

ミトコンドリアへのタンパク質の  
輸送に関係する遺伝子の変化

ミトコンドリアの生合成に  
関係する遺伝子の変化

ミトコンドリアDNAの働きに  
関係する遺伝子の変化

ミトコンドリアDNA  
の一塩基の変化

ミトコンドリアDNA  
の単一欠失/重複

ミトコンドリアDNA  
の欠乏

ミトコンドリアDNA  
の多重欠失/重複

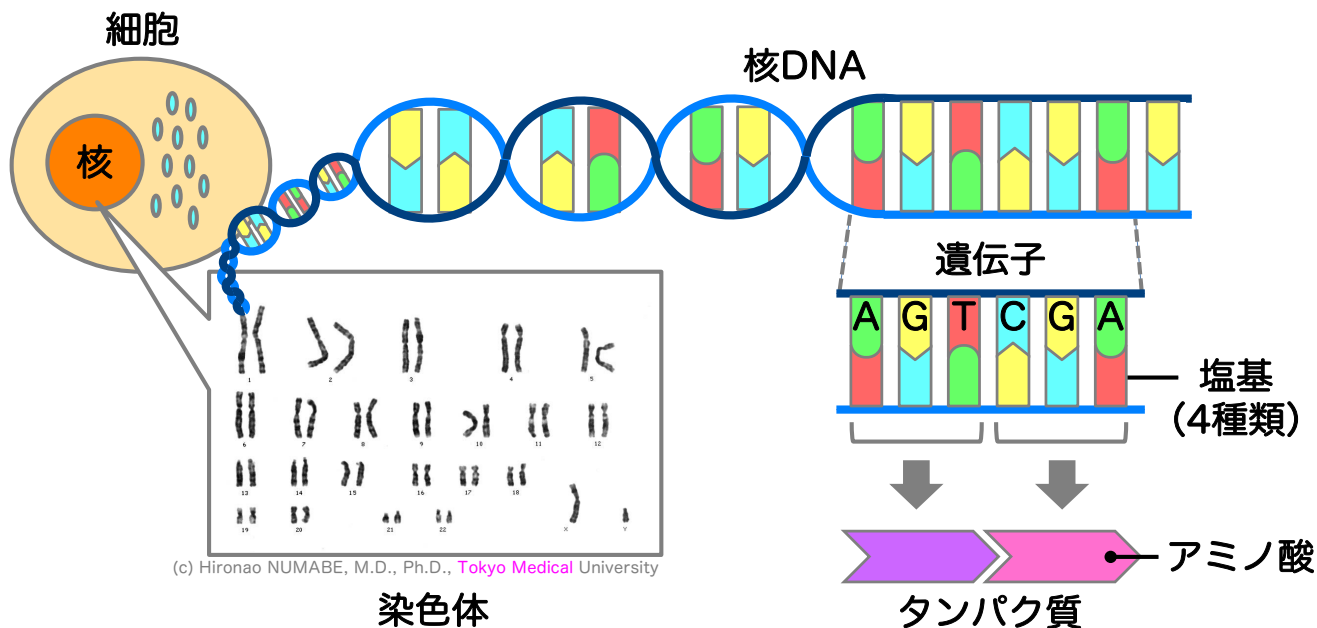
ミトコンドリアの機能低下=ミトコンドリア病

DNA（遺伝学的）検査は、筋生検や採血によって採取した細胞からDNAを取り出して、特定の遺伝子に病気の原因となる変化があるかどうかを調べます。

病気の原因となる変化が見つかれば、それが症状の原因であるということが分かります。

ミトコンドリア病の原因となる遺伝学的な変化は、核DNAの場合とミトコンドリアDNAの場合があります。どちらのDNAにどのような変化が生じているかによって、その由来や次世代への遺伝の仕方が異なります。

# 核DNA・染色体・遺伝子・タンパク質

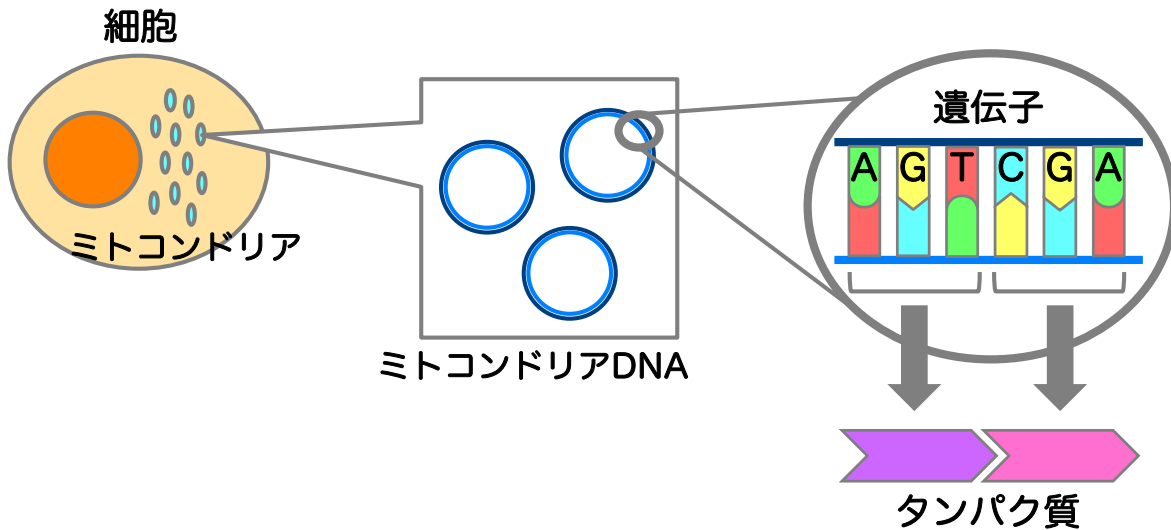


- ミトコンドリアに必要なタンパク質のうち、1500種類が核DNAの情報をもとに作られる
- 核DNAはミトコンドリアDNAの働きにも関与している

核DNAは、核という部分に入っているDNAで、一般的には単に「DNA」と呼ばれることが多いようです。核DNAは2本のひもが螺旋状に絡まったような構造をしています。時期によって複数の棒のような状態で存在していて、染色体と呼ばれます。DNAには、私たちの体を作ったり維持したりするのに必要な情報がたくさんつまっていて、その一つ一つを遺伝子と呼んでいます。塩基という4種類の物質 (A・G・C・T) を暗号としてアミノ酸が作られ、それが連なってタンパク質となります。つまり遺伝子は私たちの体の設計図のようなものだと言えます。

ミトコンドリアに必要なタンパク質のうち、約1500種類が核DNAの情報をもとに作られています。核DNAはミトコンドリアDNAの働きにも関与しています。

## ミトコンドリアDNA・遺伝子・タンパク質

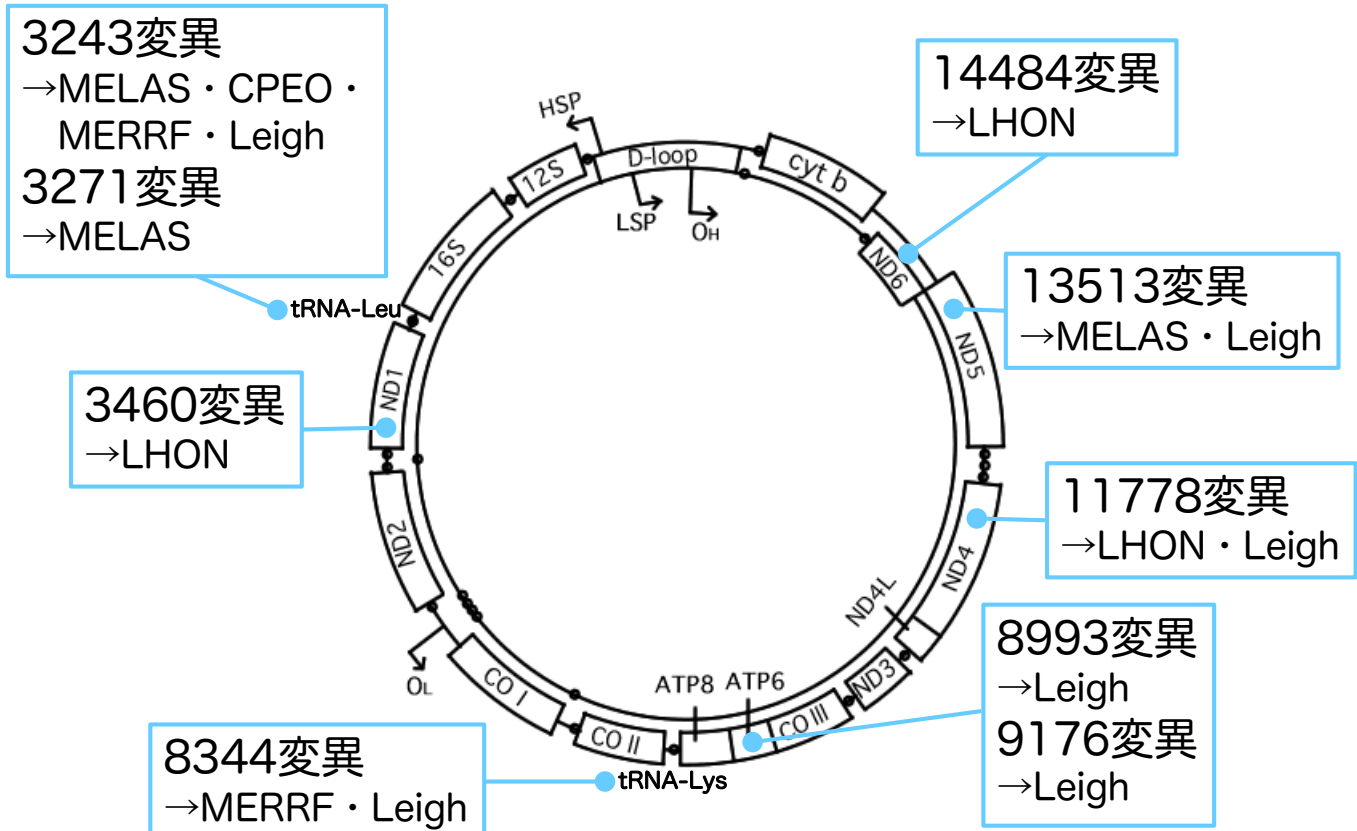


- 一つの細胞に数百～数千個のミトコンドリアDNAが入っている
- ミトコンドリアに必要なタンパク質のうち、13種類がミトコンドリアDNAの情報をもとに作られる
- ミトコンドリアDNAは核DNAの約10倍変化しやすい

ミトコンドリアDNAは、ミトコンドリアの中に入っているDNAです。一つの細胞にはミトコンドリアが数百個存在していて、それぞれにミトコンドリアDNAが複数個ずつ入っているため、一つの細胞には数百～数千個のミトコンドリアDNAが入っています。その構造は、2本のひもが輪になったような形をしています。

ミトコンドリアに必要なタンパク質のうち、13種類がミトコンドリアDNAの情報をもとに作られます。ミトコンドリアDNAは変化が生じやすいことが知られていて、その頻度は核DNAの約10倍とされています。

## 代表的なミトコンドリアDNAの変化



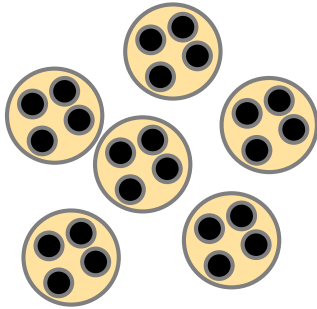
ミトコンドリア病の患者さんのミトコンドリアDNAを調べると、さまざまな変化が見つかります。

そのうちいくつかは病気との関連が明らかになっています。

また、同じ変化によって異なる病気を発症したり、同じ病気が異なる変化によって発症したりすることが分かっています。

※病気の原因となる変化のことを「変異」と呼ぶため、上図では「変異」と記載しています。

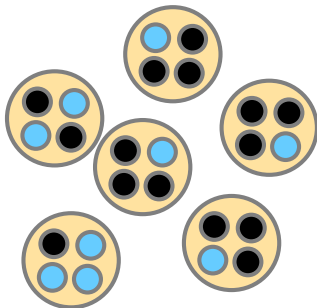
# ホモプラスミーとヘテロプラスミー



## ホモプラスミー

細胞中のミトコンドリアDNAがすべて同じ（**病気の原因となる変化** または 正常）

- 変化あり
- 変化なし



## ヘテロプラスミー

細胞中に正常なミトコンドリアDNAと病気の原因となる変化をもつミトコンドリアDNAが混在している  
細胞や組織によって割合が異なる〈細胞／組織特異性〉  
病気の原因となる変化をもつミトコンドリアDNAの割合が一定以上になると機能が障害される  
〈閾値（しきいち／いきち）効果〉  
病気の原因となる変化をもつミトコンドリアDNAの割合は細胞分裂のときに変化する

ミトコンドリアDNAの変化によって生じるミトコンドリア病の多くは、正常なミトコンドリアDNAと病気の原因となる変化をもつミトコンドリアDNAが混在している「ヘテロプラスミー」の状態で見症します。

この場合、

細胞や組織によって病気の原因となる変化をもつミトコンドリアDNAの割合が異なること（細胞／組織特異性）、

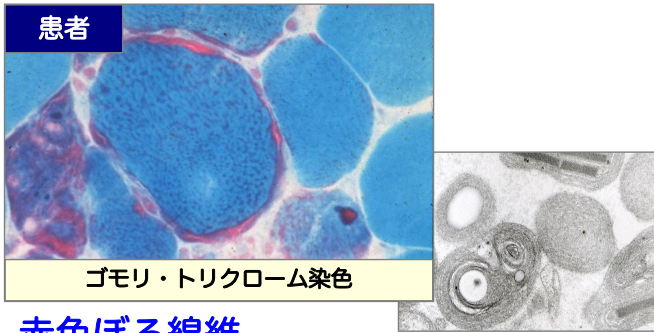
病気の原因となる変化をもつミトコンドリアDNAの割合が一定以上になると機能が障害されること（閾値効果）が特徴として挙げられます。

また、病気の原因となる変化をもつミトコンドリアDNAの割合は、細胞分裂のときに変化します。

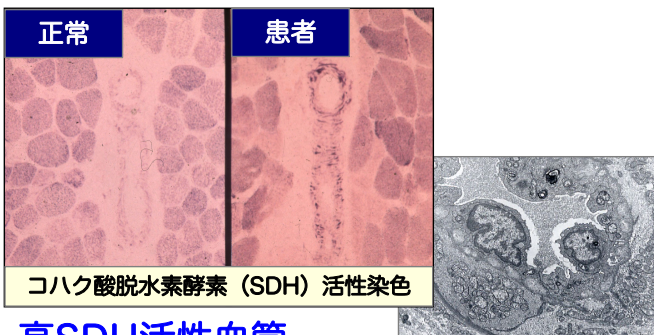
つまり、一人の患者さんでも、体のどの部分にどのくらいの割合で病気の原因となる変化をもつミトコンドリアDNAをもっているかによって症状は異なり、さらにその割合は変化すると考えられています。

また、親から子へと伝わる時にも病気の原因となる変化をもつミトコンドリアDNAの割合は変化します。

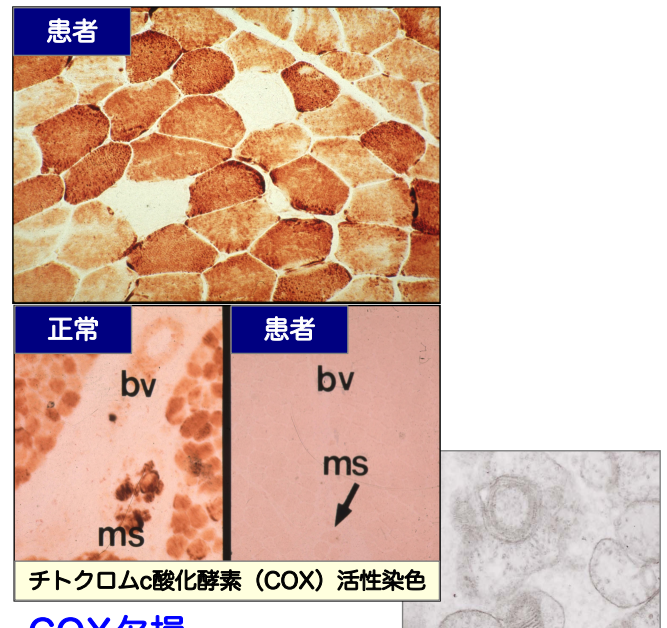
# ミトコンドリア病の病理検査 (左：光学／右：電子顕微鏡下の所見)



赤色ぼろ線維  
Ragged Red Fiber; RRF



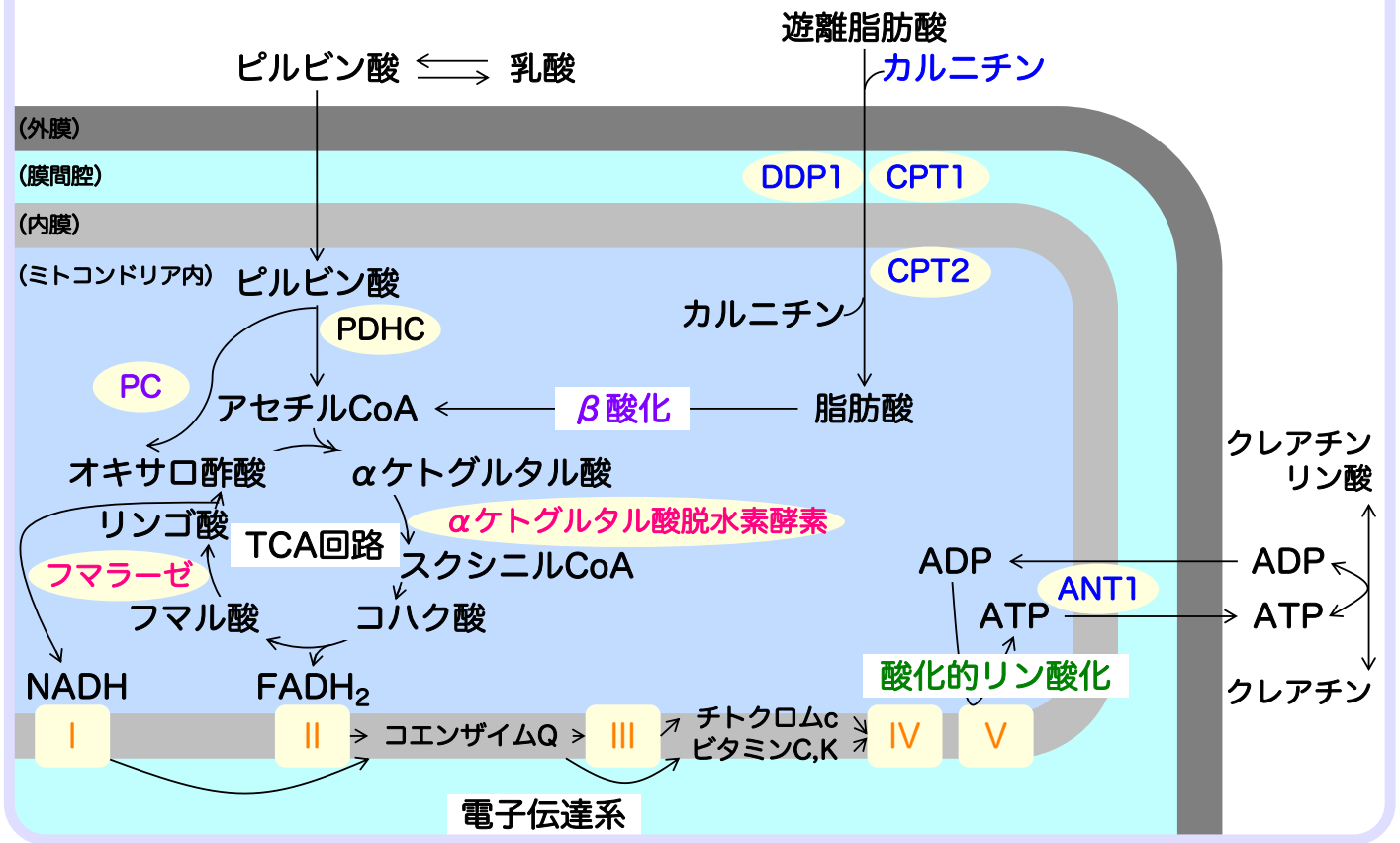
高SDH活性血管  
Strongly SDH-reactive blood vessel; SSV




COX欠損  
COX deficiency

病理検査は、筋生検などをおこなって細胞を採取し、薬品（染色液や反応液）で処理した後、顕微鏡で細胞の特徴を観察します。ミトコンドリア病の患者さんでは写真のような特徴的な所見が見られます。これらの所見があればミトコンドリアや細胞に変化があるということが分かります。

# ミトコンドリア病の生化学検査

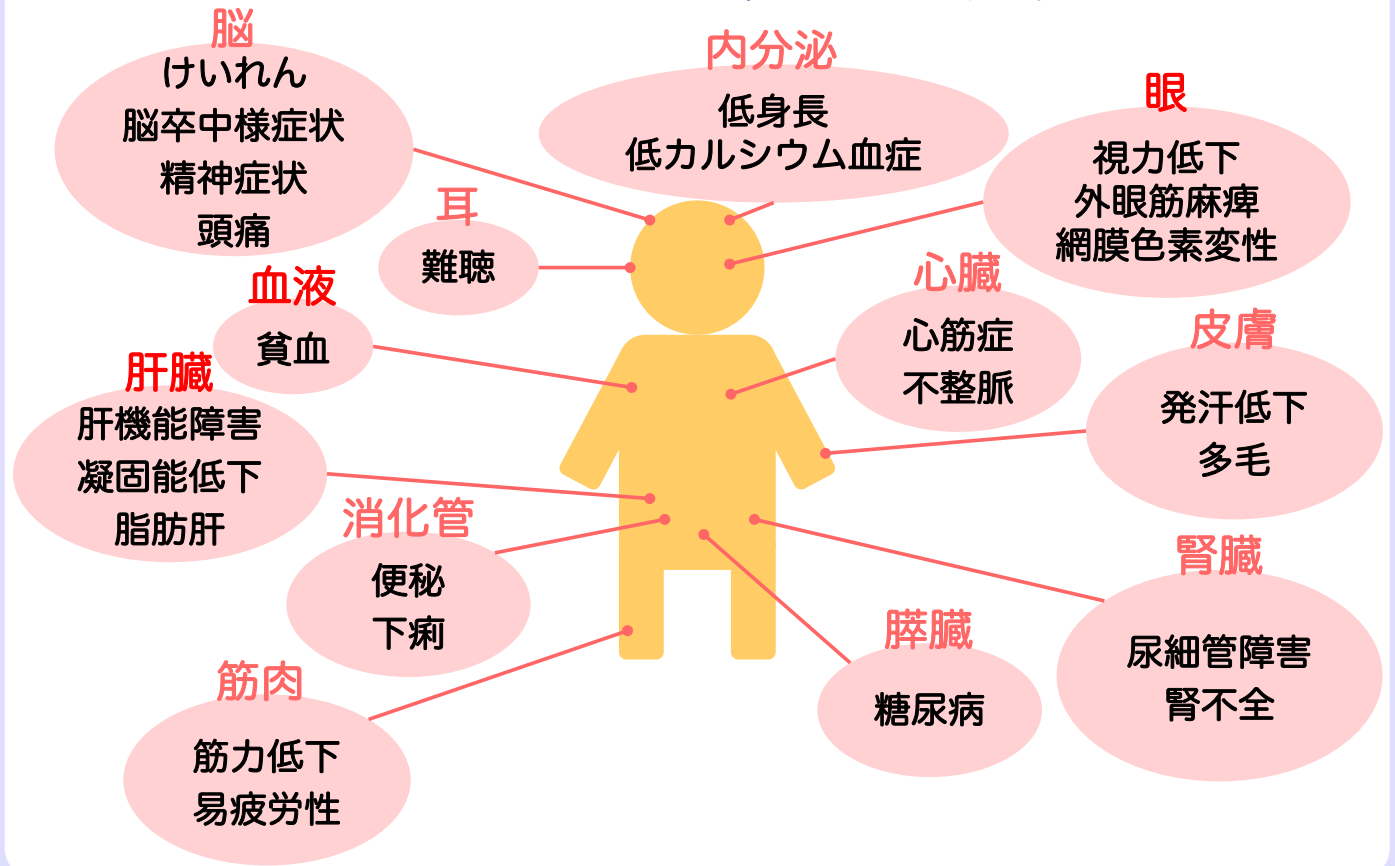


生化学検査では、筋肉や培養細胞を用いて、ミトコンドリアの働きを調べます。ミトコンドリアでは、私たちが摂取した栄養素が、酵素と呼ばれるたんぱく質の働きによって順序よく分解されていきます。その過程でエネルギーが合成されます。このエネルギー合成に関わっている酵素や、合成中に消費・産生される物質を測定することによって、どの段階に機能障害があるのかを調べることができます。



## ミトコンドリア病の症状

## ミトコンドリア病の主な症状



ミトコンドリアは体中の細胞に存在しているため、ミトコンドリア病の症状は体のさまざまなところに現れます。そのため、一人の患者さんがいくつもの症状をもっている場合には、ミトコンドリア病が疑われることとなります。特にエネルギーを多く必要とする脳や筋肉などは症状が出やすいことが知られていて、ミトコンドリア病は「ミトコンドリア脳症」「ミトコンドリア脳筋症」と呼ばれることもあります。

症状によって、卒中様症状を伴うミトコンドリア病 (MELAS)、ミオクローヌスを伴うミトコンドリア病 (MERRF)、慢性進行性外眼筋麻痺症候群 (CPEO)、Leigh脳症など、さまざまな病気に分類されます。

# 卒中様症状を伴うミトコンドリア病

Mitochondrial encephalomyopathy with lactic acidosis  
and stroke-like episodes; **MELAS** (メラス)

〈主な症状〉



発症時期 = 小児～成人

〈遺伝学的検査〉

mtDNAの一塩基の変化

=母系遺伝、まれに新しくおきた変化

核DNAの変化

=メンデル遺伝

〈病理〉

特徴的変化あり

〈生化学〉

髄液・血中乳酸値が中～高度に上昇

電子伝達系酵素複合体活性 (I、IV、複数) が低下

〈画像〉

異常所見あり

卒中様症状を伴うミトコンドリア病 (MELAS) には、

急激な意識障害や運動麻痺など脳卒中に似た症状が現れるという特徴があります。

心臓や膵臓、耳、内分泌器官などの臓器に症状がおこる場合もあります。

症状の種類や程度は患者さんごとに異なり、

一人の患者さんでも時間とともに変化することがあります。

多くの患者さんで、ミトコンドリアDNAの一塩基の変化が見つかるため、

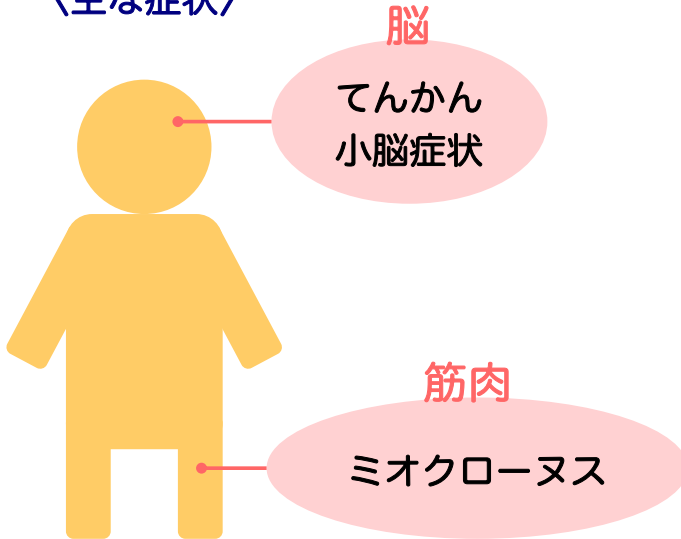
通常は母系遺伝すると考えられますが、中には当てはまらない人もいます。

(遺伝については後のページに詳しい解説があります。)

# ミオクローヌスを伴うミトコンドリア病

Myoclonic epilepsy with ragged-red fibers; **MERRF** (マーフ)

## 〈主な症状〉



発症時期 = 小児～成人

## 〈遺伝学的検査〉

mtDNAの一塩基の変化

=母系遺伝、まれに新しくおきた変化

核DNAの変化

=メンデル遺伝

## 〈病理〉

特徴的変化あり

## 〈生化学〉

血中乳酸値が中～高度に上昇

ミオクローヌスを伴うミトコンドリア病（MERRF）の症状は、主に脳と筋肉に現れます。体がふらついたり（小脳症状）、自分の意思とは関係なく筋肉が動いたりします（ミオクローヌス）。まれに脳卒中のような症状がおこることもあります。多くの患者さんで、ミトコンドリアDNAの一塩基の変化が見つかるため、通常は母系遺伝すると考えられますが、中には当てはまらない人もいます。

# 慢性進行性外眼筋麻痺症候群

Chronic progressive external ophthalmoplegia;

**CPEO** (シーピーイーオー)

〈遺伝学的検査〉

mtDNA単一欠失

=新しくおきた変化、まれに母系遺伝  
mtDNAの一塩基の変化

=母系遺伝、まれに新しくおきた変化  
mtDNA重複

=新しくおきた変化または母系遺伝  
mtDNA多重欠失 (核DNAの変化)

=新しくおきた変化またはメンデル遺伝

〈病理〉

特徴的变化あり

〈生化学〉

血中乳酸値が軽度上昇

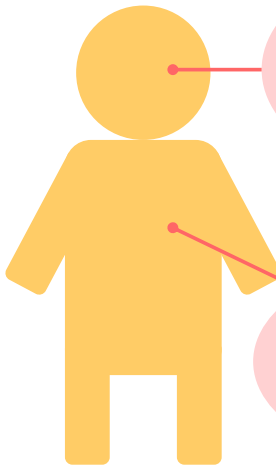
〈主な症状〉

目

外眼筋麻痺  
眼瞼下垂  
網膜色素変性\*

心臓

心伝導障害\*



発症時期 = 小児～成人

\* = Kearns-Sayre (カーンズ・セイヤー) 症候群

慢性進行性外眼筋麻痺症候群 (CPEO) の主な症状は、

目の周りの筋肉が麻痺して眼球を動かせなくなることです。

これに網膜の変性と心臓の伝導障害を合併する場合、

Kearns-Sayre症候群と呼ばれます。

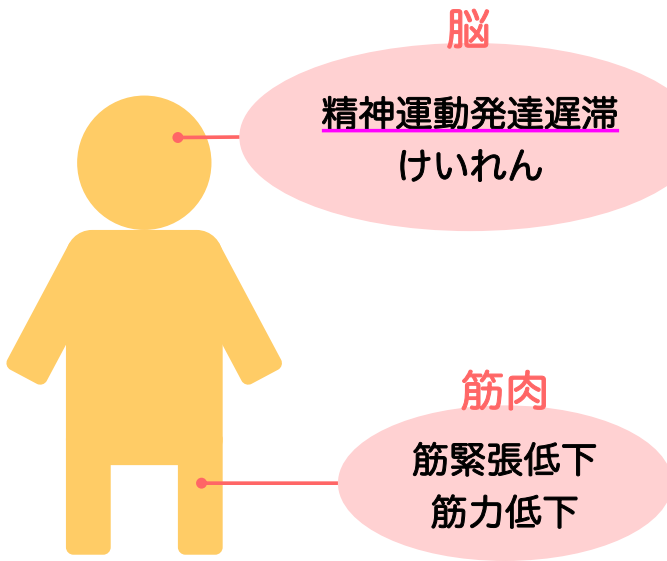
骨格筋、膵臓、腎臓、消化管などの臓器に症状が現れることもあります。

遺伝学的検査では、多くの場合、ミトコンドリアDNAの変化が認められます。

変化の種類によって、由来や次世代への遺伝の仕方が異なります。

# Leigh (リー) 脳症

## 〈主な症状〉



発症時期 = 乳幼児～小児

## 〈遺伝学的検査〉

mtDNAの一塩基の変化  
= 母系遺伝、まれに新しくおきた変化  
核DNAの変化  
= メンデル遺伝

## 〈病理〉

特徴的な変化なし

## 〈生化学〉

髄液・血中乳酸値が高度に上昇  
電子伝達系酵素複合体活性 (I、II、IV、V、複数) が低下  
ATP合成が低下

## 〈画像〉

特徴的な所見あり

Leigh脳症では、脳と筋肉に主な症状が現れます。

通常は乳幼児期に発症し、精神運動発達の遅れや退行、けいれん、筋緊張や筋力の低下といった症状が見られます。

ただし発症時期や進行の度合いは患者さんによってさまざまです。

原因は、ミトコンドリアDNAに変化がおきている場合と核DNAに変化がおきている場合があります。

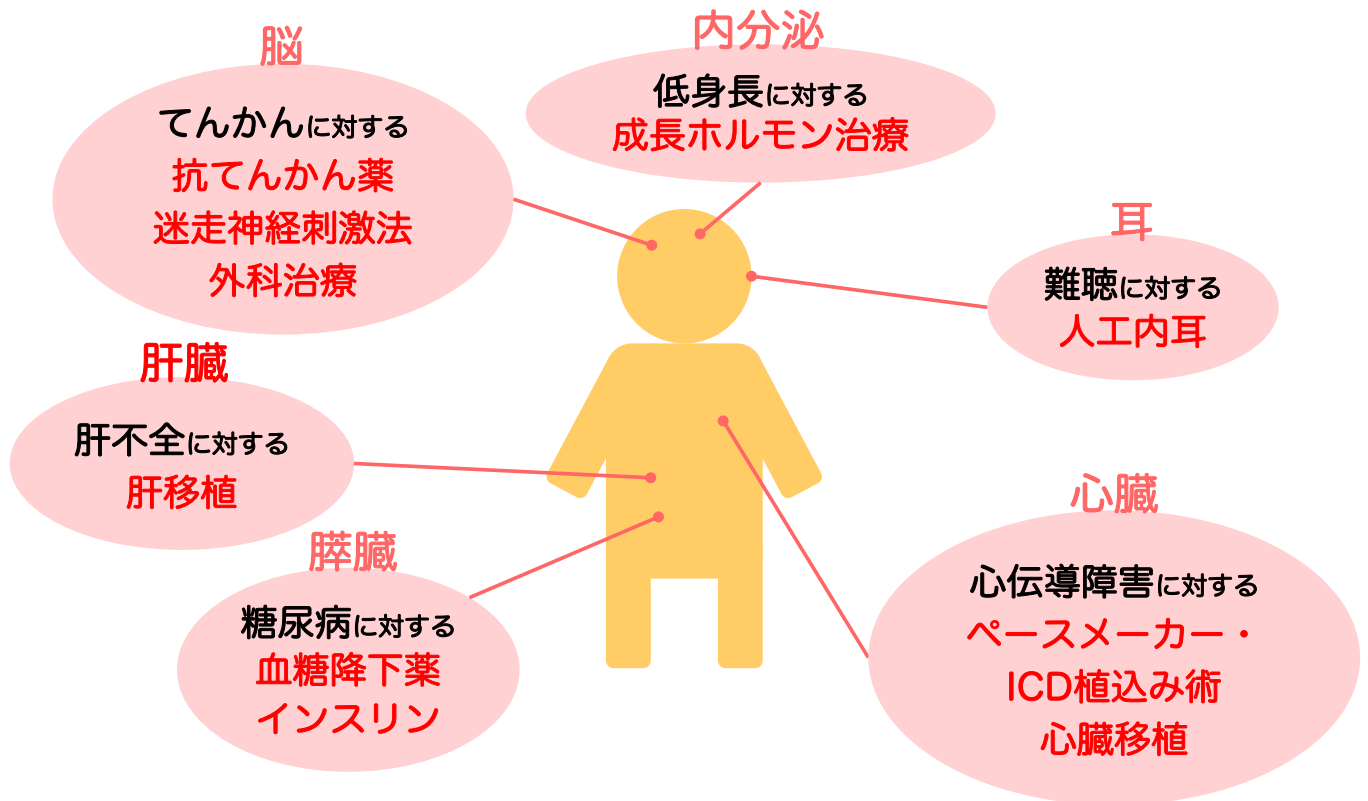
原因によって、由来や次世代への遺伝の仕方が異なります。

※退行：お座りや歩行、ことばなど、できていたことができなくなる



## ミトコンドリア病の治療

## ミトコンドリア病の治療法（対症療法）



ミトコンドリア病の治療法は、大きく二つに分かれます。  
一つは、現れている症状を和らげる対症療法です。

糖尿病、てんかん、低身長、肝不全など、  
有効な治療法が確立されている症状に対しては、  
その治療法が用いられます。

難聴や心伝導障害については、医療機器を利用することで、  
低下した臓器の機能を補うことができます。

対症療法は、各臓器の専門医に診てもらうことが望ましいですので、  
診療科の多い病院を中心として医療を受けることをお勧めします。

## ミトコンドリア病の栄養療法



### 食事の注意点

- ・日常生活の活動度に応じたカロリー量を計算する
- ・ビタミンを多く含むバランスのよい食事をとる

### 〈呼吸鎖複合体Ⅰ欠損症〉

高脂質・低炭水化物栄養療法が有効な場合がある

### 〈PDHC欠損症〉

ケトン食が有効な場合がある

### 〈小児難治性てんかん〉

登録外特殊ミルクの適応疾患

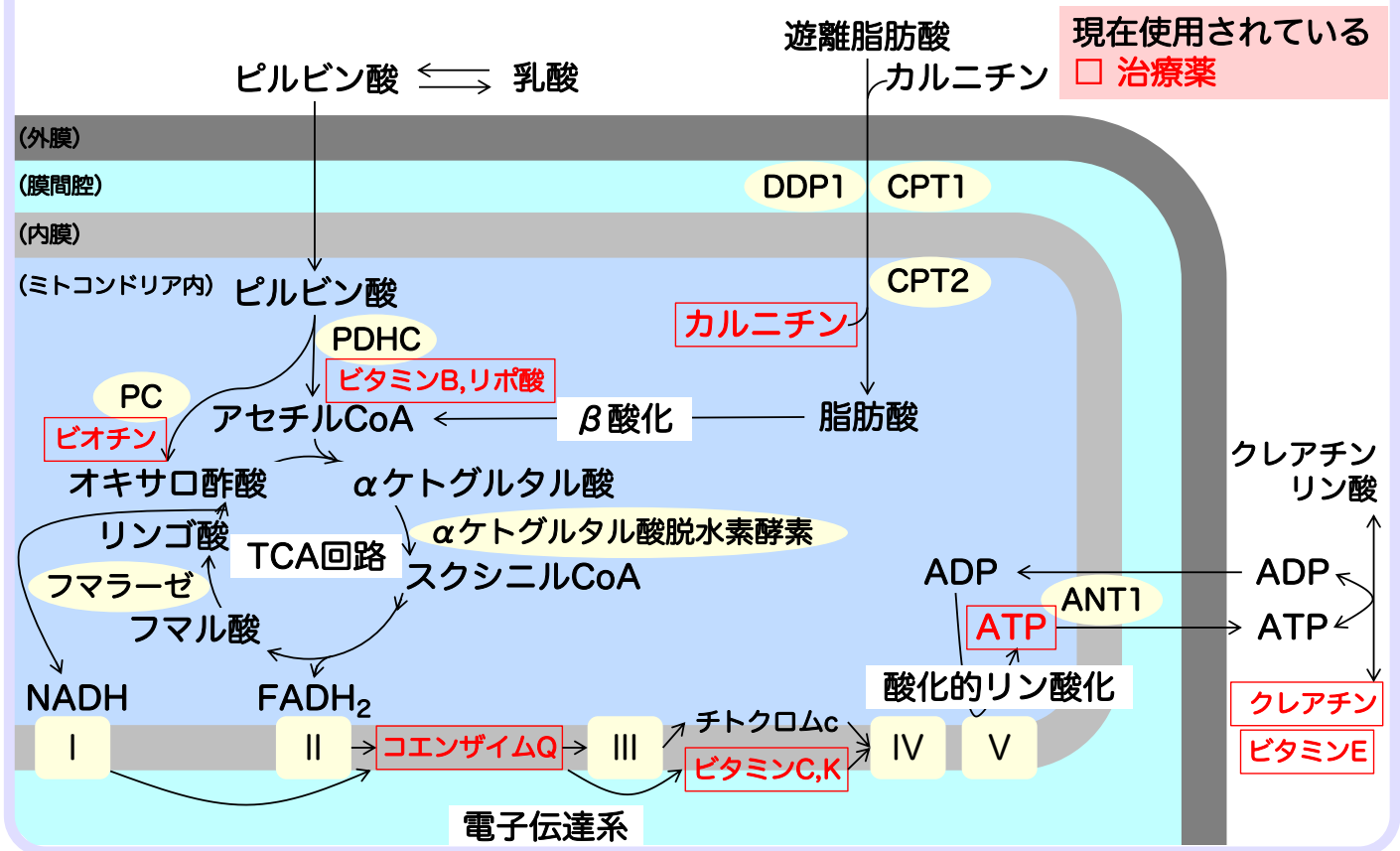
現在、ミトコンドリア病に対する栄養療法として確立されたものではありません。

さまざまな重症度があるため、日常生活の活動度に応じたカロリー量を計算し、ビタミンを多く含んだバランスのよい食事をとることが推奨されます。

脱水や電解質異常、低血糖などには注意しましょう。

高脂質・低炭水化物栄養療法やケトン食、特殊ミルクなどの栄養療法は医師の指導のもとに行われますので、主治医の先生に指示を仰ぎましょう。

# ミトコンドリア病の治療法（原因療法）



もう一つの治療法は、

病気の原因であるミトコンドリア機能の低下を改善させる原因療法です。

ミトコンドリアでの代謝に関わる物質やビタミンなども使用されていますが、現時点では、有効性が証明されているものは限られます。

MELASという病気の患者さんに対しては、タウリンの内服により脳卒中様発作を抑制する効果が認められ、保険薬として承認されています。

また、生活上の注意点としてミトコンドリア機能を低下させるような行動は避けることが望ましいと考えられます。

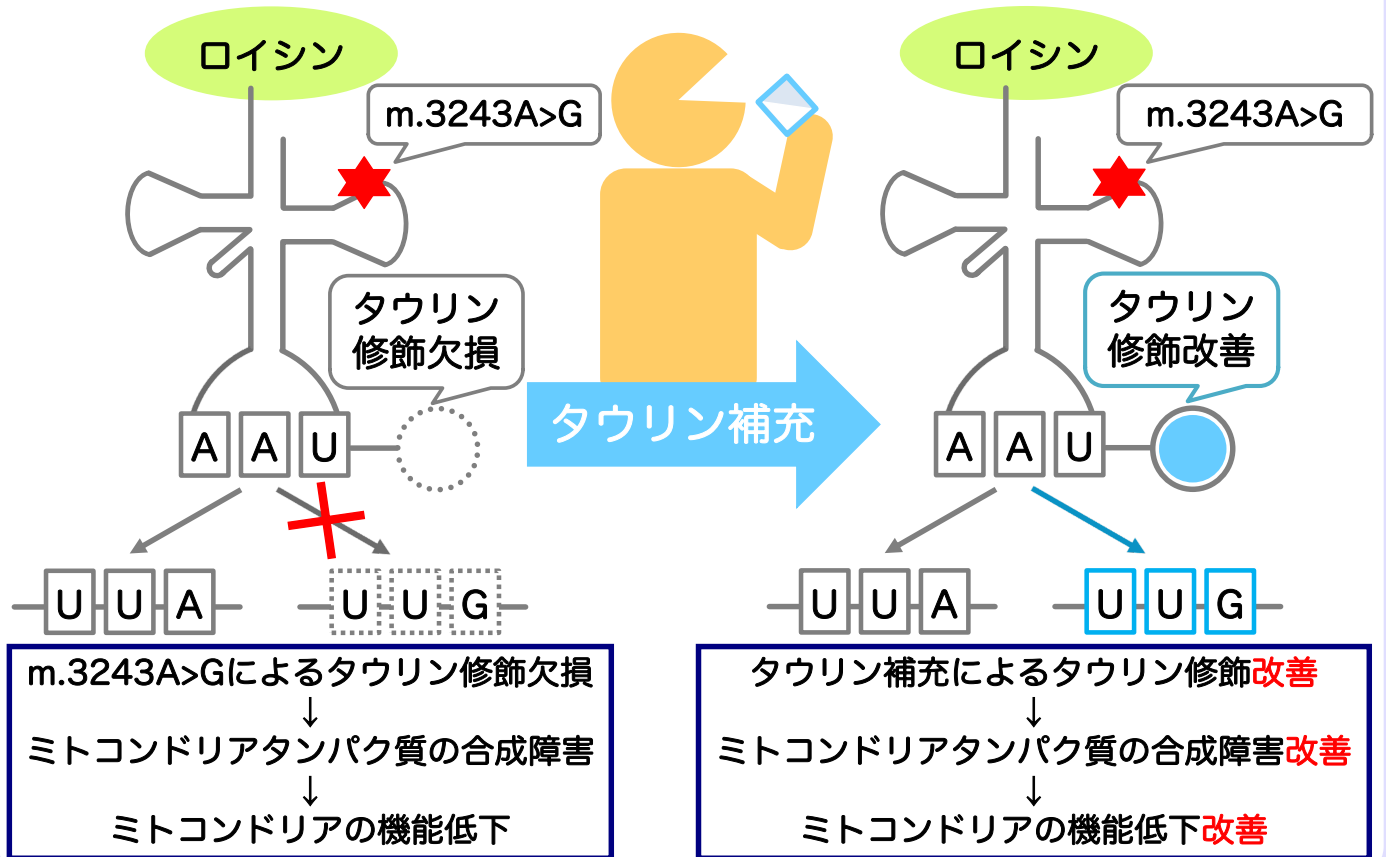
飲酒、過食や飢餓は、ミトコンドリアに負担をかけます。

睡眠は十分とることが大切です。

感染をきっかけに症状が悪化することもありますので、注意しましょう。


栄養バランスのよい、ビタミンの多い食事と適度の運動、生活リズムを整えることが、なにより重要です。

## タウリン補充治療の仕組み




ミトコンドリアDNAのm.3243A>Gという変化をはじめ、いくつかの一塩基の変化をもつMELASの患者さんは、ミトコンドリア転移RNAのタウリン修飾が阻害されることが分かりました。タウリン修飾が阻害されると、ミトコンドリア内のタンパク質が合成されなくなり、病気が発症する一因と考えられています。

タウリン補充療法では、タウリンを大量に服用することで、ミトコンドリア転移RNAのタウリン修飾欠損が改善し、ミトコンドリア内のタンパク質合成と機能が改善すると考えられています。



ミトコンドリア病の  
患者・家族のための社会資源



## 代表的な医療費助成制度

### 指定難病医療費助成制度

対象：

指定難病の診断基準を満たし「重症度分類」に照らして病状の程度が一定以上の人  
(軽症者でも高額な医療※を継続することが必要な人は対象) ※条件あり

内容：

医療費の助成

### 小児慢性特定疾病医療費助成制度

対象：

小児慢性特定疾病の認定基準を満たす症状をもつ18歳未満の子ども  
(18歳到達後も引き続き治療が必要と認められる場合、20歳未満の人も対象)

内容：

医療費の助成

### 高額療養費制度

対象：

同一月(1日から月末まで)にかかった医療費の自己負担額が一定額を超えた人

内容：

医療費の払い戻し

ミトコンドリア病患者さんが利用を検討できる  
代表的な医療費助成制度をご紹介します。

指定難病医療費助成制度、小児慢性特定疾病医療費助成制度は、  
それぞれ指定の医療機関を受診し、  
申請に必要な書類を指定医に記載してもらう必要があります。  
病気の状態によって、助成制度の対象外となる人もいます。  
詳しくは、主治医の先生や通院している病院の医療福祉相談室、  
お住まいの市町村の担当窓口や保健所にご相談ください。

また、高額療養費制度の支給申請方法はご自身が加入されている  
公的医療保険(健康保険組合・協会けんぽ・共済組合など)に  
お問い合わせください。

## 代表的な福祉制度

### 障害福祉サービス

対象：

身体または知的障害のある人（児童含む）、精神障害のある人、難病患者等で一定の障害のある人

内容：

介護給付（居宅介護や重度訪問介護など）

訓練等給付（自立訓練や就労移行支援など）

### 介護保険

対象：

介護が必要と認定された一定年齢以上の人

内容：

ケアプランにもとづいた介護サービスの利用

### 身体障害者手帳

対象：

認定基準を満たす障害をもつ人

内容：

補装具の交付・税金の減免・交通費の割引など

### 障害年金

対象：

障害をもつ年金加入者

内容：

年金の給付

その他：特別障害者手当・障害児福祉手当など（所得制限あり）

ミトコンドリア病患者さんが利用を検討できる

代表的な福祉制度をご紹介します。

利用できる制度は、病気の状態やお住まいの地域などによって異なります。

自治体が独自に実施している制度もありますので、

詳しくは、お住まいの市区町村の担当窓口、保健所、

通院している病院の医療福祉相談室などでご相談ください。

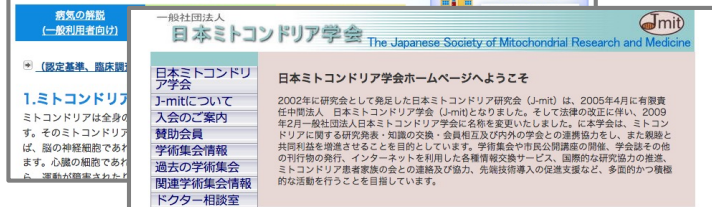
制度をよく理解して有効に利用することで、

療養生活をより快適に過ごすことができるでしょう。

# 情報サイト



難病情報センター  
病気の解説や研究班の活動など  
<http://www.nanbyou.or.jp/>



日本ミトコンドリア学会  
ドクター相談室や学術活動など  
<http://www.j-mit.org/>



ミトコンドリア病  
患者・家族の会  
病気に関する情報共有など  
<http://mcm.sakura.ne.jp/wpnew/>

インターネット上の情報源には、  
国の「難病情報センター」や専門家による「ミトコンドリア学会」、  
当事者による「ミトコンドリア病患者・家族の会」のページがあります。  
ミトコンドリア病についてのさまざまな情報が公開されています。



## ミトコンドリア病の患者登録制度

# 患者登録制度

## Remudy (レムディ)

対象：  
ミトコンドリア病患者さん

ホームページ：  
<http://www.remudy.jp/mitd/>



## J-MO Bank

対象：  
新生児期・小児期発症の  
ミトコンドリア病患者さん

ホームページ：<http://mo-bank.com/>



ミトコンドリア病患者さんを対象とした患者登録制度が始まっています。

患者登録制度は2つあり、どちらも治療研究および新薬開発の促進と、円滑な臨床試験・治験実施などを目的としています。

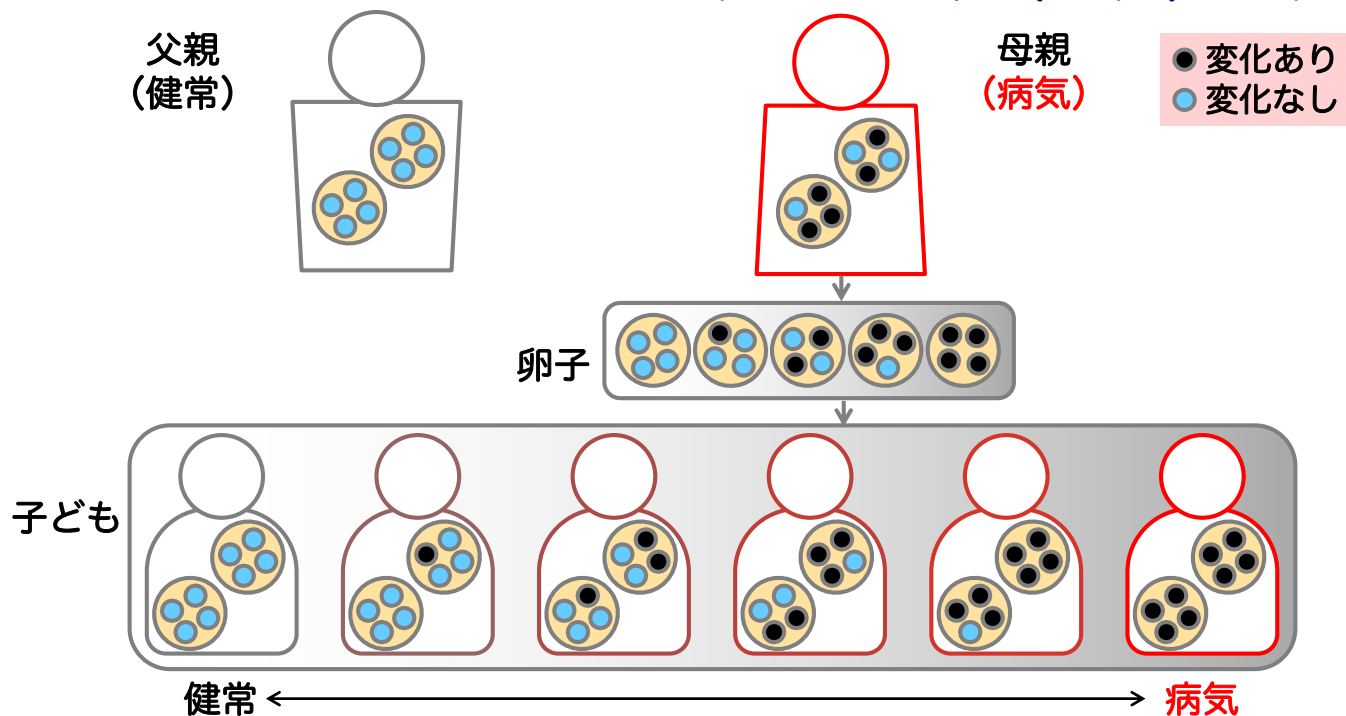
登録を希望される患者さん、あるいはご家族が登録の手続きをする仕組みで臨床情報の記入は主治医の先生に協力してもらう必要があります。

患者登録を検討される場合には、それぞれの患者登録制度のホームページで登録方法をご確認ください。



## ミトコンドリア病の遺伝

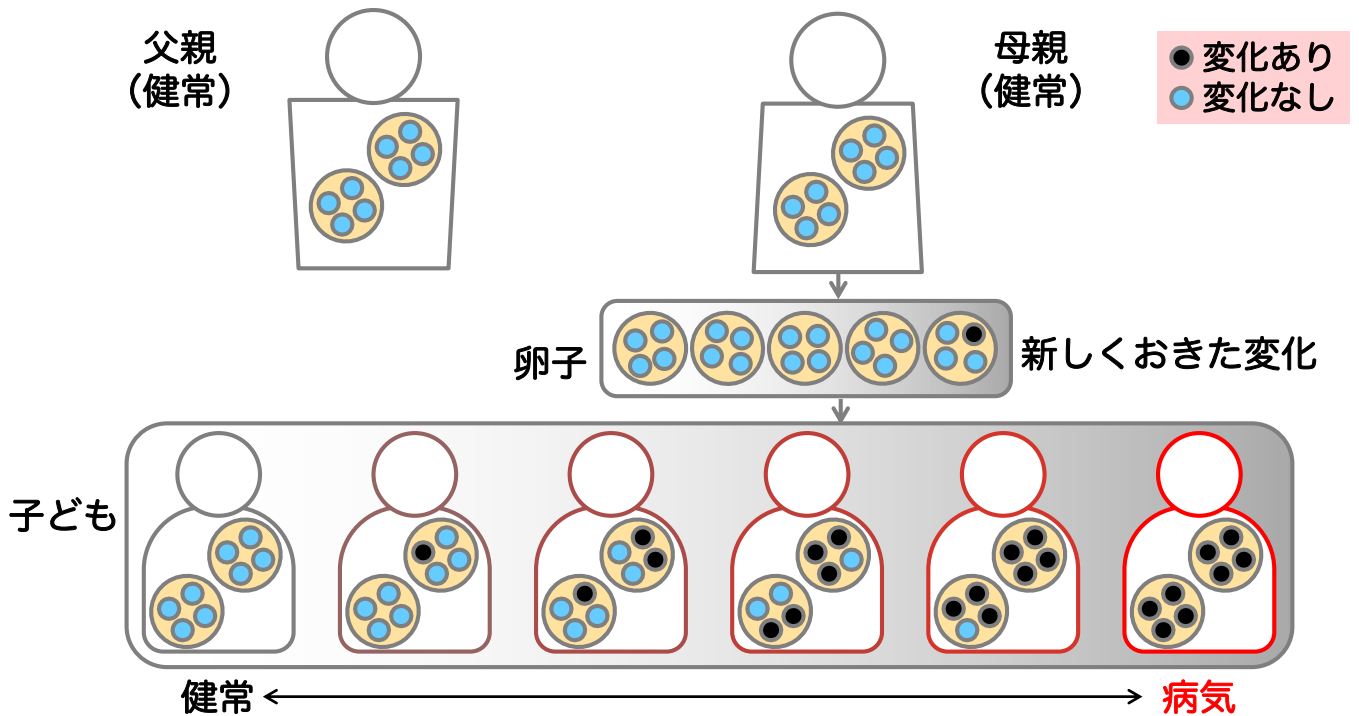
# ミトコンドリアDNAの遺伝 = 母系（母性）遺伝



- ・ 子どもへは母親のミトコンドリアが受け継がれる（父系遺伝は1例のみ）
- ・ 子どもの症状の有無や程度は、ミトコンドリアDNAの変化が、どの細胞にどのくらいの割合で存在するかによって異なるため、予測が難しい

ミトコンドリア病の多くは、  
ミトコンドリアDNAの変化が原因で発症します。  
ミトコンドリアDNAは、母親から子どもに受け継がれます。  
これを母系（母性）遺伝と呼びます。  
父親のミトコンドリアは子どもへは通常受け継がれません。  
（これまでに1例のみ報告されています。）  
子どもの症状の有無や程度は、ミトコンドリアDNAの変化が、  
どの細胞にどのくらいの割合で存在するかによって異なるため、  
予測が難しいと考えられています。

## ミトコンドリアDNAの新しくおきた変化



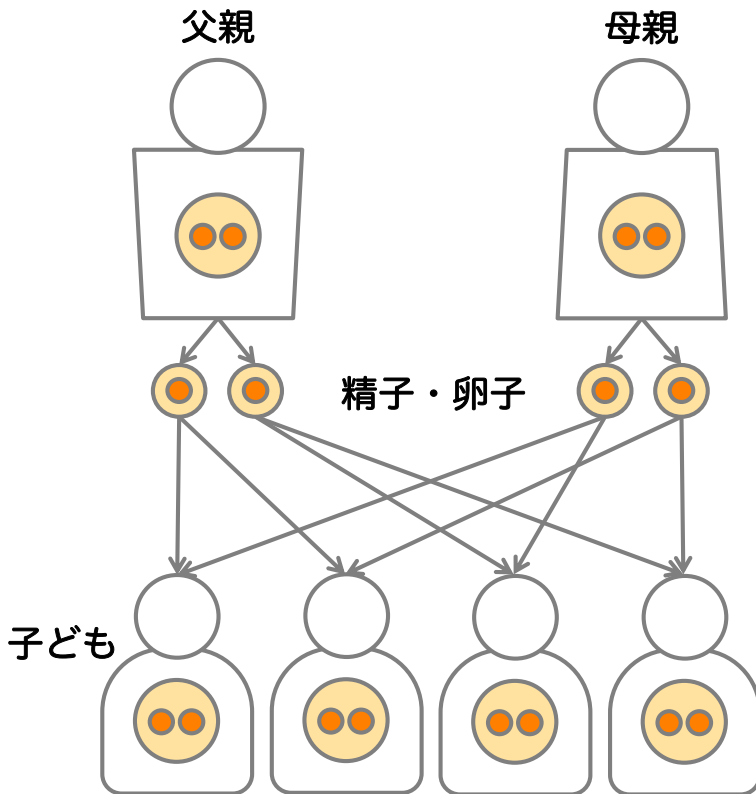
- ・ 少なくとも出生児200人に1人はミトコンドリアDNA変異をもっている
  - ・ 変化が新しくおきる確率は10万人に107人 (1,000人弱に1人) である
- Hannah, R. et al. AJHG 2008

ミトコンドリアDNAの変化は、母親由来ではなく新しくおきた変化である可能性もあります。

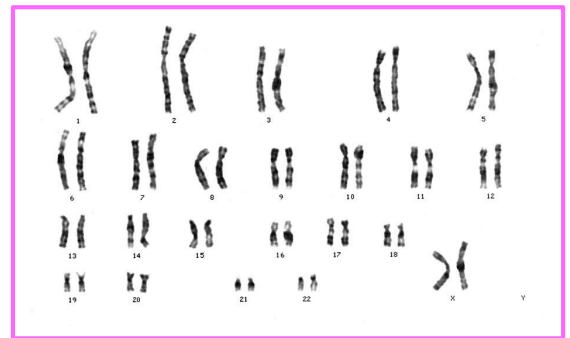
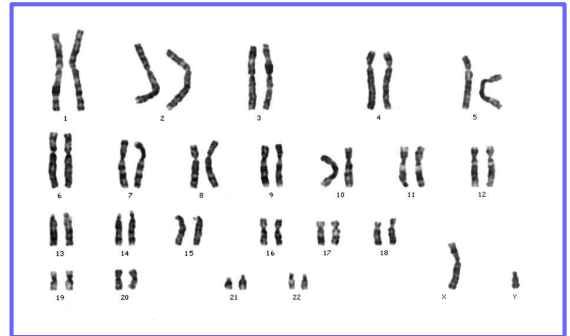
ある調査によると、少なくとも新生児200人に1人はミトコンドリアDNA変異をもっていることが分かっています。

変化が新しくおきる確率は、10万人に107人と計算されています。

# 核DNAの遺伝 = メンデル遺伝



染色体写真 (上: 男性、下: 女性)



(c) Hironao NUMABE, M.D., Ph.D., Tokyo Medical University

核DNAは、両親から子どもに受け継がれます。

私たちは両親から一つずつDNAを受け継ぎますので、

同じ種類の遺伝子を二つずつもっています。

そしてそれを子どもに伝えるときには、どちらか一つだけを渡します。

染色体も同じ種類のものが二つずつあります。

その中には、22番までの番号が振られた男女共通の染色体（常染色体）と性別を決める染色体（性染色体）があります。

男性の場合はX染色体とY染色体を一つずつ、

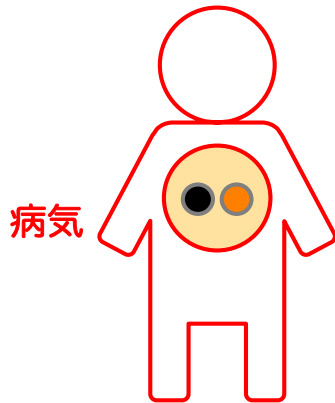
女性の場合はX染色体を二つもっています。

性染色体に入っている遺伝子は男女間で数が異なるため、その遺伝子に病気の原因となる変化が生じた場合には、

性別によって発症の仕方が異なります。

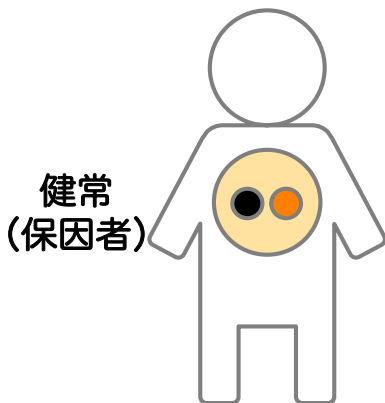
## 顕性（優性）と潜性（劣性）

● 変化あり  
● 変化なし



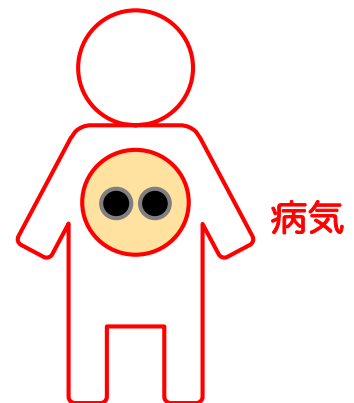
### 顕性（優性）

1対の遺伝子のうち  
片方に変化がおこると  
症状が現れる



### 潜性（劣性）

1対の遺伝子の  
両方に変化がおこると  
症状が現れる



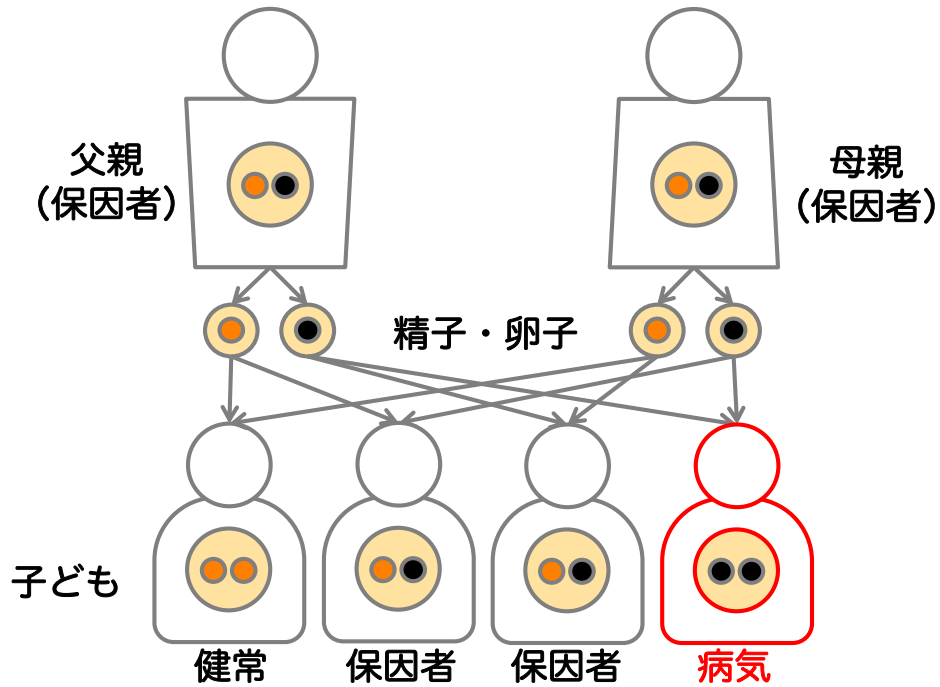
二つある遺伝子のうち、  
片方に病気をおこす変化をもつと症状が現れる場合を顕性（優性）、  
両方に病気をおこす変化をもつと初めて症状が現れる場合を潜性（劣性）  
と言います。

潜性（劣性）の場合に、片方の遺伝子に病気をおこす変化をもつ人を  
保因者と呼びます。

保因者は通常発症しませんが、  
病気によっては何らかの症状が現れることもあります。  
病気の原因となる遺伝子が入っているのが常染色体か性染色体か、  
その遺伝子の変化が顕性（優性）か潜性（劣性）かによって、  
次世代への遺伝の仕方が異なります。

## 常染色体潜性遺伝（劣性遺伝）

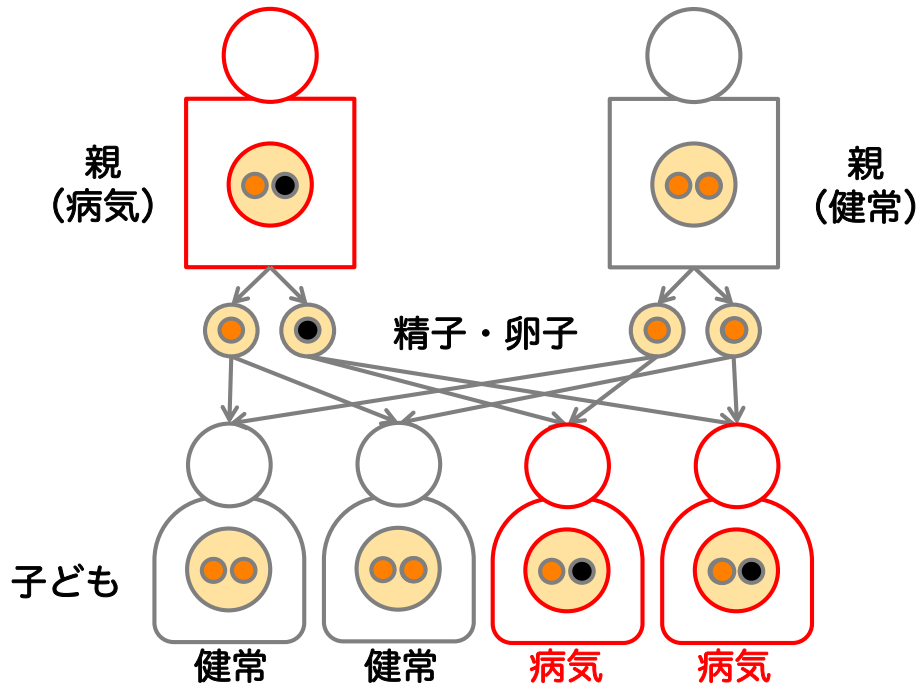
● 変化あり  
● 変化なし



- ・ 両親とも保因者であると考えられる
- ・ 次の子が病気をもつ確率は $1/4$  (25%)、保因者の確率は $1/2$  (50%)

核DNAの変化が原因で起こるミトコンドリア病のほとんどは、常染色体潜性遺伝（劣性遺伝）と呼ばれる形式で伝わります。両親が一つずつもっている遺伝子の変化が、両方とも子どもに伝わることで発症すると考えられます。つまり両親は保因者です。したがって、次の子が病気をもつ確率は4分の1 (25%)、保因者となる確率は2分の1 (50%) です。

## 常染色体顕性遺伝（優性遺伝）

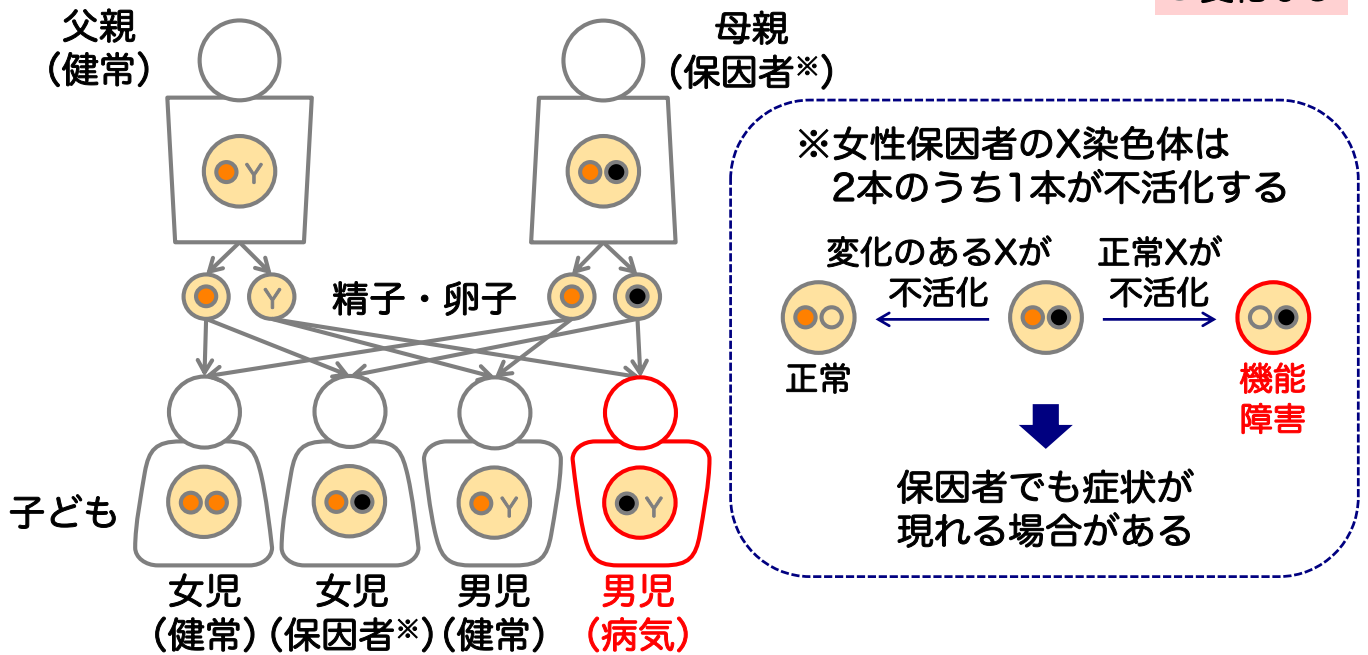


- ・ 両親いずれかが遺伝子の変化をもつ場合、子に伝わる確率は1/2 (50%)
- ・ 新しくおきた変化の場合、次の子が病気をもつ確率は一般と同じ

ミトコンドリア病のうち、常染色体顕性遺伝（優性遺伝）と呼ばれる形式で伝わる病気はまれで、CPEOの一部などに限られます。この形式では、両親のいずれかが遺伝子の変化をもつ場合、子どもには2分の1（50%）の確率でそれが伝わります。両親が遺伝子の変化をもっておらず、新しくおきた変化によって子どもが発症した場合には、次の子が病気をもつ確率は一般の人と同じと考えられます。

## X連鎖潜性遺伝（劣性遺伝）

● 変化あり  
○ 変化なし



- ・ 母親が保因者の場合、男の子の1/2が病気、女の子の1/2が保因者となる
- ・ 新しくおきた変化の場合、次の子が病気をもつ確率は一般と同じ
- ・ 女性保因者でも不活化の影響によって症状が現れる場合がある

ミトコンドリア病のうち、

X連鎖潜性遺伝（劣性遺伝）と呼ばれる形式で伝わる病気は、

DDP 遺伝子の変化によって生じる難聴とジストニアなどに限られます。

男性のX染色体は1本なので、その中の遺伝子に変化がおこると発症します。

女性は2本あるため保因者となり、通常、症状は現れません。

ただし、2本のうち1本は不活化されていて働かないため、

どちらが不活化されるかによって、女性でも症状が現れる場合があります。

保因者である母親からは、X染色体2本のどちらかが子どもに伝わるため、

男の子の2分の1（50%）が病気、

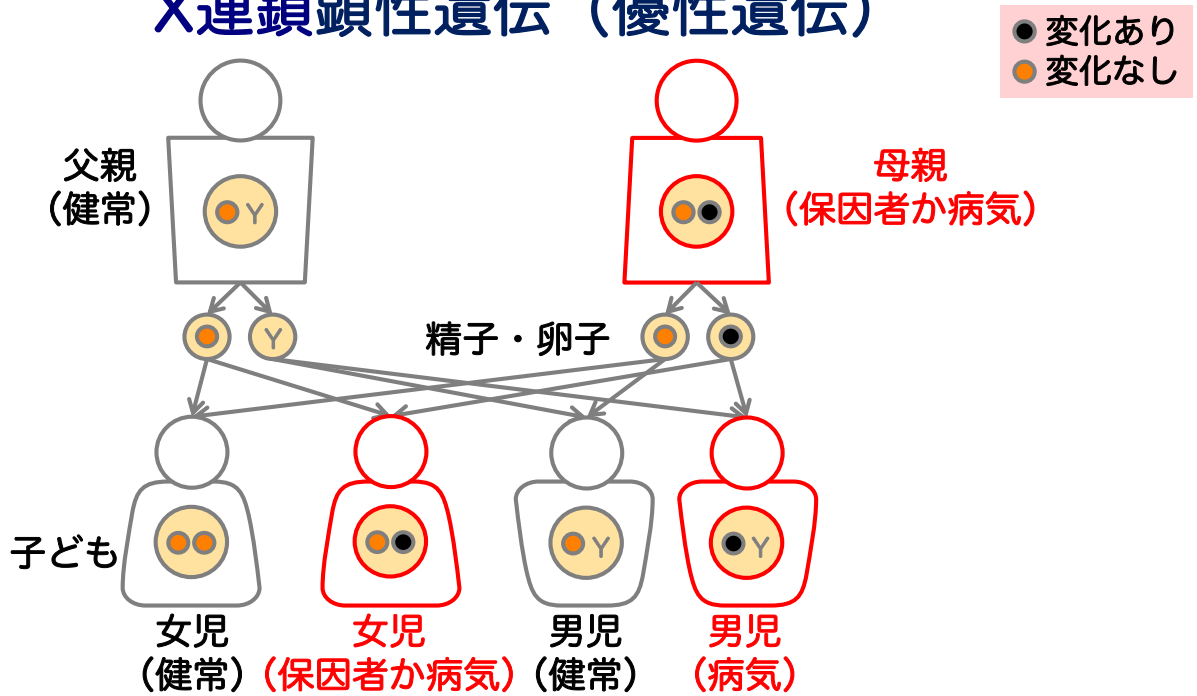
女の子の2分の1（50%）が保因者となります。

病気の子がもっている変化が、

母親由来ではなく新しくおきた変化の場合、

次の子が病気をもつ確率は一般と同じと考えられます。

## X連鎖顕性遺伝（優性遺伝）



- ・ 遺伝子の変化をもつ女性は保因者の場合と病気の場合がある
- ・ 母親が遺伝子の変化をもつ場合、男の子の1/2が病気、女の子の1/2が保因者か病気となる
- ・ 新しくおきた変化の場合、次の子が病気をもつ確率は一般と同じ

ミトコンドリア病のうち、

X連鎖顕性遺伝（優性遺伝）と呼ばれる形式で伝わる病気は、

*PDHA1* 遺伝子の変化によって生じるPDHC欠損症が知られています。

男性のX染色体は1本なので、その中の遺伝子に変化がおこると発症します。

女性は2本あるため症状の現れない保因者となる場合と、

病気になる場合があります。

遺伝子の変化をもつ母親からは、

X染色体2本のどちらかが子どもに伝わるため、

男の子の2分の1（50%）が病気、

女の子の2分の1（50%）が保因者か病気となります。

病気の子がもっている変化が、母親由来ではなく新しくおきた変化の場合、次の子が病気をもつ確率は一般と同じと考えられます。

## 出生前診断・着床前診断

### 出生前診断

- 妊娠中に絨毛または羊水を採取して特定の遺伝子や染色体の異常の有無を調べる
- 流産の可能性あり

### 着床前診断

- 体外受精でできた受精卵を用いて特定の遺伝子や染色体の異常の有無を調べる
- 臨床研究のため申請が必要

- 胎児が重篤な病気をもつ可能性がある場合に限って実施する
- ヘテロプラスミーで発症する病気の場合は正確な診断ができないことがある
- 夫婦が希望していること、検査の意義について十分理解し、同意していることが条件となる
- 結果の解釈について事前に十分検討しておくことが重要
- 胎児の病気を理由に妊娠を諦めてよいという法律はない

子どもが遺伝子の変化をもっているかどうかを生まれる前に調べる方法には、出生前診断と着床前診断があります。出生前診断は、妊娠中に絨毛または羊水を採取して、特定の遺伝子や染色体に変化があるかどうかを調べる技術です。着床前診断は、体外受精でできた受精卵を用いて、特定の遺伝子や染色体に変化があるかどうかを調べる技術です。どちらも技術的な制約や倫理的な問題などがあるため、それらを十分理解した上で実施するかどうかを考えることが重要です。

これらの診断について詳しく知りたい方は、通っておられる病院の遺伝カウンセリングをご利用ください。

このハンドブックは  
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「ミトコンドリア病の調査研究」班（研究代表者：後藤雄一）初版  
「ミトコンドリア病の診断水準やQOL向上を目指した調査研究」班  
（研究代表者：三牧正和）第2版 の  
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# 第8回ミトコンドリア病研究 公開フォーラム

日時

2024年2月3日(土) 12:30~16:30(予定)



Web開催。  
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ご参加ください！

ミトコンドリア病診療のこれから 2024

## 予定プログラム

### \* ミトコンドリア病の強度

- ・ミトコンドリア総論
- ・ミトコンドリア病の遺伝学的検査について

- …後藤 諒一先生(国立精神・神経医療研究センター)
- …八塚 由紀子先生(順天堂大学)

### \* 創薬

- ・変異ミトコンドリアDNAを標的とする薬剤開発
- ・ミトコンドリア創薬
- ・MA-5の治療開発について

- …高取 敦志先生(千葉県がんセンター)
- …菅沼 正司先生(ルカ・サイエンス株式会社)
- …阿部 高明先生(東北大学)

### \* 患者会

- …ASrid・MCMの会・みどりの会 より

### \* ミトコンドリア雑感

- …太田 成男先生(順天堂大学)

♪ミトコンドリア・ミニコンサート♪

**参加費無料**  
要参加申込み

- ◆ 参加申し込み : [y.konno.bn@juntendo.ac.jp](mailto:y.konno.bn@juntendo.ac.jp) にメールにてお申し込みください。
- ◆ 事前登録 : 2024年1月29日まで
- ◆ 詳細は、JAMP-MITホームページ <http://iamp-mit.org/index.html>  
J-MO Bankホームページ <http://mo-bank.com/> を参照ください。

主催: 特定非営利活動法人 ミトコンドリア病医療推進機構 JAMP-MIT(理事長: 高柳 正樹)  
共催: 日本医療研究開発機構(AMED) 難治性疾患実用化研究事業 ミトコンドリア病研究(研究者代表: 村山 圭)  
厚生労働省 難治性疾患政策研究事業 ミトコンドリア病研究(研究者代表: 三牧 正和)  
日本先天代謝学会 患者登録委員会 JaSMn事務局(代表: 小須賀 基通)



## Total and reduced/oxidized forms of coenzyme Q<sub>10</sub> in fibroblasts of patients with mitochondrial disease

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Forward electron transport  
Reverse electron transport

### ABSTRACT

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) is involved in ATP production through electron transfer in the mitochondrial respiratory chain complex. CoQ<sub>10</sub> receives electrons from respiratory chain complex I and II to become the reduced form, and then transfers electrons at complex III to become the oxidized form. The redox state of CoQ<sub>10</sub> has been reported to be a marker of the mitochondrial metabolic state, but to our knowledge, no reports have focused on the individual quantification of reduced and oxidized CoQ<sub>10</sub> or the ratio of reduced to total CoQ<sub>10</sub> (reduced/total CoQ<sub>10</sub>) in patients with mitochondrial diseases.

We measured reduced and oxidized CoQ<sub>10</sub> in skin fibroblasts from 24 mitochondrial disease patients, including 5 primary CoQ<sub>10</sub> deficiency patients and 10 respiratory chain complex deficiency patients, and determined the reduced/total CoQ<sub>10</sub> ratio.

In primary CoQ<sub>10</sub> deficiency patients, total CoQ<sub>10</sub> levels were significantly decreased, however, the reduced/total CoQ<sub>10</sub> ratio was not changed. On the other hand, in mitochondrial disease patients other than primary CoQ<sub>10</sub> deficiency patients, total CoQ<sub>10</sub> levels did not decrease. However, the reduced/total CoQ<sub>10</sub> ratio in patients with respiratory chain complex IV and V deficiency was higher in comparison to those with respiratory chain complex I deficiency.

Measurement of CoQ<sub>10</sub> in fibroblasts proved useful for the diagnosis of primary CoQ<sub>10</sub> deficiency. In addition, the reduced/total CoQ<sub>10</sub> ratio may reflect the metabolic status of mitochondrial disease.

### 1. Introduction

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>), also known as ubiquinone, is a lipophilic molecule composed of a redox-active benzoquinone head group and species-specific isoprenoid side chain (10 subunits in humans) [1,2]. CoQ<sub>10</sub> takes three forms depending on the redox state of the benzoquinone ring; oxidized (CoQ<sub>10</sub>, Ubiquinone), fully-reduced (CoQ<sub>10</sub>H<sub>2</sub>, Ubiquinol), and semi-reduced (CoQ<sub>10</sub><sup>•</sup>, Semiubiquinone) forms [2]. It

presents ubiquitously in all cellular membranes and cells [3]. The amount of CoQ<sub>10</sub> and the proportion of reduced CoQ<sub>10</sub> differ between organs and cells; CoQ<sub>10</sub> is distributed in high amounts in the heart, kidneys, liver, and muscles, and the proportion of reduced CoQ<sub>10</sub> is lower in the brain and lungs [3]. In cells, it is mostly localized in the mitochondria [3].

CoQ<sub>10</sub> has multiple functions. One of the main roles of CoQ<sub>10</sub> is as a component of the mitochondrial respiratory chain. As a mobile electron

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carrier, CoQ<sub>10</sub> accepts electrons from complex I and II and transfers them to complex III [2]. In the mitochondrial inner membrane, CoQ<sub>10</sub> is proposed to exist as two independent pools: a CoQ<sub>NADH</sub> pool in the super-complex (CI, CIII, CIV) involved in the oxidation of NADH; and a CoQ<sub>FADH</sub> pool involved in the oxidation of CII and other enzymes that use CoQ as a cofactor [4]; CoQ<sub>NADH</sub> receives electrons from NADH, and CoQ<sub>FADH</sub> receives electrons from FADH<sub>2</sub> and other enzymes, such as glycerol phosphate dehydrogenase (GPDH), choline dehydrogenase (CHDH), sulphide:quinone oxidoreductase (SQOR), dihydroorote dehydrogenase (DHODH), and electron transfer flavoprotein dehydrogenase (ETFHDH), and is reduced [5]. In respiratory chain complex III, electrons are transferred from reduced CoQ<sub>10</sub> to cytochrome *c* in a process called the Q cycle. The Q cycle results in the oxidation of two molecules of reduced CoQ<sub>10</sub>, the reduction of two molecules of cytochrome *c*, and the formation of one additional molecule of reduced CoQ<sub>10</sub> [2]. This normal forward electron transfer results in the creation of an electrical gradient and a pH gradient (proton gradient) between the mitochondrial matrix and intermembrane space. The energy generated by this proton motive force enables complex V(ATP synthase) to synthesize ATP. On the other hand, reverse electron transfer (RET) of CoQ<sub>10</sub> is also known to occur; in RET, electrons from reduced CoQ<sub>10</sub> are returned to complex I, reducing NAD<sup>+</sup> to NADH, and generating ROS [6] (Supplemental Fig. 1).

Another important role of CoQ<sub>10</sub> is as an antioxidant. CoQ<sub>10</sub> is the sole lipid-soluble antioxidant that is endogenously synthesized. Reduced CoQ<sub>10</sub> inhibits both the initiation and the propagation of lipid peroxidation [7]. NADH-quinone oxidoreductase 1 and cytochrome *b5* reductase, have been known to be the major oxidoreductases in the plasma membrane [8]. In addition, ferroptosis suppressor protein 1 (FSP1) was also found to be an important oxidoreductase. FSP1 reduces extra-mitochondrial CoQ<sub>10</sub> and acts as a lipophilic radical-trapping antioxidant to suppress lipid peroxides, resulting in the inhibition of cell death, called ferroptosis [9,10]. Moreover, reduced CoQ<sub>10</sub> also regenerates the other antioxidants— $\alpha$ -tocopherol and ascorbate—into an active reduced form [7]. CoQ<sub>10</sub> is also involved in the  $\beta$ -oxidation of fatty acids [11], de novo pyrimidine biosynthesis [12], sulfide oxidation [13], an essential cofactor for uncoupling proteins (UCPs) [14], and modulation of the mitochondrial permeability transition pore [15]. Thus, the importance of the distinct state of CoQ<sub>10</sub> is gaining growing attention [5].

Primary CoenzymeQ<sub>10</sub> (CoQ<sub>10</sub>) deficiency is an autosomal recessive mitochondrial disease caused by a decrease in CoQ<sub>10</sub> due to mutations in genes involved in CoQ<sub>10</sub> biosynthesis (*COQ* genes) [16]. To date, defects in at least 10 *COQ* genes (*COQ2*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *COQ8A*, *COQ8B*, *COQ9*, *PDSS1*, *PDSS2*) have been found to cause this disease [17]. Primary CoQ<sub>10</sub> deficiency was first reported in 1989 as familial mitochondrial encephalomyopathy [18]. Currently, over 280 patients from 180 families have been reported [17]. Secondary CoQ<sub>10</sub> deficiencies occur in a wider variety of pathologies, including mitochondrial disease [19,20]. Measuring the CoQ levels is known to be useful for diagnosing CoQ<sub>10</sub> deficiency [21]. As described above, the ratio of reduction varies among organs and cells. As a result, in addition to the total amount of CoQ<sub>10</sub>, evaluating the reduced/oxidized CoQ<sub>10</sub> ratio may also be useful for further elucidating various pathophysiological states in cells.

Methods to measure CoQ<sub>10</sub> in fibroblast and the reduced/oxidized state of CoQ<sub>10</sub> have been reported [22,23]. However, to our knowledge, there are no reports on the measurement of the reduced/oxidized CoQ<sub>10</sub> ratio in primary CoQ<sub>10</sub> deficiency or mitochondrial diseases. Therefore, we decided to measure the total CoQ<sub>10</sub> levels as well as the levels of reduced and oxidized CoQ<sub>10</sub> in skin fibroblasts from patients affected by various mitochondrial diseases.

## 2. Material, methods, and patients

### 2.1. Subjects

We studied fibroblasts from 24 patients with mitochondrial disease, including primary CoQ<sub>10</sub> deficiency (Table 1). The inclusion criterion for patients with primary CoQ<sub>10</sub> deficiency was childhood onset with biallelic pathogenic variants in the *COQ* gene encoding proteins for the biosynthesis of CoQ<sub>10</sub> [16]. Fibroblasts were obtained from patients at Kanagawa Children's Medical Center, Chiba Children's Medical Center, and Jichi Medical University under the approval of the Ethics Committee of Jichi Medical University. Written informed consent was obtained from the parents of each patient.

We obtained fibroblasts from five patients with primary CoQ<sub>10</sub> deficiency. One patient carries biallelic *COQ2* variants with the c.[349G > C];[912 + 1G > del] (Case 1). Four patients had biallelic *COQ4* mutations: one with compound heterozygous biallelic variants c.[718C > T];[421C > T] (Case 2) [24], one with c.[431C > A];[718C > T] (Case 3) [25], and two with c.[190C > T];[479G > A] (Cases 4, 5).

Ten patients had disorders of mitochondrial respiratory chain subunits. Seven patients had mutations related to complex I: one with a c.[55C > T] mutation in *NDUFA1* (Case 6) [26], one with an m.10158 T > C mutation in *MT-ND3* (Case 7) [26], Three with an m.13513G > A mutation in *MT-ND5* (Case 8) [27], (Case 9), (Case 10) [28], one with a c.[811 T > G];[1766-2A > G] mutation in *ACAD9* (Case 11), and one with a c.[1150G > A];[1817 T > A] mutation in *ACAD9* (Case 12) [28]. *NDUFA1*, *MT-ND3*, and *MT-ND5* are subunits of complex I, and *ACAD9* is the assembly factor of complex I. Two patients had mutations related to complex IV: one with a c.[743C > A] mutation in *SURF1* (Case 13) [29], and one with a c.[367\_368delAG]; [572delC] mutation in *SURF1* (Case 14) [25]. *SURF1* is the assembly factor of complex IV. One patient had an m.8993 T > G mutation in *MT-ATP6* (Case 15) [25]. *MT-ATP6* is a subunit of complex V.

Three patients had mitochondrial DNA (mtDNA) depletion syndrome: one with a c.[143-307\_170del335];[143-307\_170del335] mutation in *DGUOK* (Case 16) [30], one with a c.[451dupC];[308\_310del] mutation (Case 17) [30] and one with a c.[148C > T];[149G > A] mutation in *MPV17* (Case 18) [30]. Both *DGUOK* and *MPV17* are involved in the maintenance of mtDNA. One patient had Kearns-Sayre syndrome with a single mtDNA deletion (5513 bp del; m.8290–13,802) (Case 19). Two patients had MELAS: one with an m.3243 A > G mutation of tRNA-Leu (Case 20) [26] and one with an m.5541C > T, mutation of tRNA-Trp (Case 21) [26]. One patient had short-chain enoyl-CoA hydratase (*ECHS1*) deficiency with heterozygous mutations in maternal c.[832G > A] in *ECHS1* (Case 22) [31]. *ECHS1* plays a role in valine and fatty acid catabolism in mitochondria. Two patients had c.[287A > G]; [287A > G] mutation in *BOLA3* (Cases 23) [28], (Case 24). *BOLA3* is related to iron-sulfur cluster production and is involved in the assembly of the mitochondrial respiratory chain complex.

Five fibroblasts from healthy individuals were purchased: two fibroblasts from the PromoCell Company (#C-12300, GmbH, Heidelberg, Germany), two fibroblasts from Japanese Collection of Research Bioresources Cell Bank (#TIG-120, #HT-2020, Japan), and fibroblasts from Lonza Japan (#CC-2509, Tokyo, Japan). Another five fibroblasts from patients without mitochondrial disease were used as controls. Cells from passages 4–29 were used for assays.

### 2.2. Cell culture and growth conditions

The fibroblasts were maintained in 1.0 g/L low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cells were incubated at 37 °C under 5% CO<sub>2</sub>.

**Table 1**  
Fibroblast cell lines from patients with mitochondrial disease, including primary CoQ<sub>10</sub> deficiency.

Case [ref]	diagnosis	DNA mutation	variants, heteroplasmy rate <sup>a</sup>	function
1	primary CoQ <sub>10</sub> deficiency	<i>COQ2</i>	c.[349G > C];[912 + 1G > del]	CoQ <sub>10</sub> biosynthesis
2 [24]	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[718C > T];[421C > T]	CoQ <sub>10</sub> biosynthesis
3 [25]	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[431C > A];[718C > T]	CoQ <sub>10</sub> biosynthesis
4	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[190C > T];[479G > A]	CoQ <sub>10</sub> biosynthesis
5	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[190C > T];[479G > A]	CoQ <sub>10</sub> biosynthesis
6 [26]	Leigh syndrome	<i>NDUFA1</i>	c.[55C > T], 100% (X-linked)	Respiratory chain subunits, complex I
7 [26]	Leigh syndrome	<i>MT-ND3</i>	m.10158 T>C, heteroplasmic (F; 90%)	Respiratory chain subunits, complex I
8 [27]	neonatal cardiomyopathy	<i>MT-ND5</i>	m.13513G > A, heteroplasmic (F; 78.87%)	Respiratory chain subunits, complex I
9	infantile mitochondrial disease	<i>MT-ND5</i>	m.13513G > A, heteroplasmic (B; 77%)	Respiratory chain subunits, complex I
10 [28]	Leigh syndrome	<i>MT-ND5</i>	m.13513G > A, heteroplasmic (F; 26%)	Respiratory chain subunits, complex I
11	mitochondrial cardiomyopathy	<i>ACAD9</i>	c.[811 T > G];[1766-2A > G]	Respiratory chain assembly factor, complex I
12 [28]	non-lethal infantile mitochondrial disease	<i>ACAD9</i>	c.[1150G > A];[1817 T > A]	Respiratory chain assembly factor, complex I
13 [29]	Leigh syndrome	<i>SURF1</i>	c.[743C>A], homoplasmy	Respiratory chain assembly factor, complex IV
14 [25]	Leigh syndrome	<i>SURF1</i>	c.[367_368delAG];[572delC]	Respiratory chain assembly factor, complex IV
15 [25]	Leigh syndrome	<i>MT-ATP6</i>	m.8993 T > G, homoplasmy	Respiratory chain subunits, complex V
16 [30]	mtDNA depletion syndrome	<i>DGUOK</i>	c.[143-307_170del335];[143-307_170del335]	Deoxynucleotide triphosphate synthesis
17 [30]	mtDNA depletion syndrome	<i>MPV17</i>	c.[451dupC];[308_310del]	mitochondrial protein synthesis
18 [30]	mtDNA depletion syndrome	<i>MPV17</i>	c.[148C > T];[149G > A]	mitochondrial protein synthesis
19	Kearns-Sayre syndrome		Single mtDNA deletion (5513 bp del; m.8290–13,802)	
20 [26]	MELAS	(tRNA-Leu)	m.3243 A > G, heteroplasmic (F; 21%)	Mitochondrial tRNA
21 [26]	MELAS	(tRNA-Trp)	m.5541C > T, heteroplasmic (F; 49%)	Mitochondrial tRNA
22 [31]	ECHS1 deficiency	<i>ECHS1</i>	c.[832G > A]	Metabolism of toxic compounds
23 [28]	cardiomyopathy	<i>BOLA3</i>	c.[287A > G];[287A > G]	Iron-sulfur protein assembly
24	cardiomyopathy	<i>BOLA3</i>	c.[287A > G];[287A > G]	Iron-sulfur protein assembly

<sup>a</sup> F; fibroblasts, B; blood

### 2.3. CoQ<sub>10</sub> measurement in fibroblasts

#### 2.3.1. CoQ extraction

The methods for the extraction CoQ<sub>10</sub> were based on a previously reported method with slight modifications [32]. To extract CoQ<sub>10</sub> from fibroblast in 60 mm dishes, cells were washed twice with PBS, and pellets were re-suspended in 500  $\mu$ L of lysis buffer (0.25 mM Sucrose, 2 mM EDTA, 10 mM Tris, and 100 UI/mL heparin, pH 7.4.), and sonicated twice for 5 s. These homogenates were also used to citrate synthase and protein quantification. To measure CoQ<sub>10</sub>, nine hundred microliters of ethanol containing internal standard CoQ<sub>10</sub>-d9 (IsoSciences, Ambler, PA) and 20  $\mu$ M *tert*-butyl hydroquinone (TBHQ) (FUJIFILM Wako, Osaka, Japan) was added to 100  $\mu$ L of homogenates. TBHQ was added to prevent oxidation of reduced CoQ<sub>10</sub>. The cell suspensions were vortexed and centrifuged at 15,700  $\times$ g for 10 min (4 °C).

#### 2.3.2. Reduction of ubiquinone

Reduced CoQ<sub>10</sub> was required for use in the calibration curve measurement. However, since reduced CoQ<sub>10</sub> is easily oxidized, reduced CoQ<sub>10</sub> was prepared just before the analysis by reducing oxidized CoQ<sub>10</sub> following a previously reported method with slight modification [33]. Briefly, 50  $\mu$ L of CoQ<sub>10</sub> was diluted in 1.95 mL hexane in a glass tube. Twenty milligrams of NaBH<sub>4</sub> was added and followed by the addition of 100  $\mu$ L methanol, vortexed for 3 min, then placed in the dark for 5 min at room temperature. After reduction, 1 mL of water containing 100  $\mu$ M EDTA was added to stop the reaction, vortexed for 1 min, and centrifuged 1500  $\times$ g for 5 min at 4 °C. The upper layer containing reduced CoQ<sub>10</sub> was transferred to a glass tube.

#### 2.3.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The method for measuring reduced and oxidized CoQ<sub>10</sub> was based on the previously reported method with slight modifications [23]. An LC-MS/MS analysis was performed on an LC-electrospray ionization-MS (LC-ESI-MS) with triple quadrupole (Nexera X2 and LCMS-8060, Shimadzu, Kyoto, Japan). A Kinetex C18 column (100 mm  $\times$  2.1 mm, 2.6  $\mu$ m, Phenomenex) and a guard column filled with the same packing material were used. The column temperature was kept at 40 °C. The mobile phase was isocratic with 2 mM ammonium formate in methanol.

The flow rate was 0.8 mL/min, the injection volume was 2  $\mu$ L, and the run time was 6 min. The interface temperature was 300 °C, the desolvation line temperature was 250 °C, and the heat block temperature was 400 °C. The nebulizing gas flow was 3 L/min, the heating gas flow was 10 L/min, and the drying gas flow was 10 L/min. The samples were kept at 4 °C before injection by the autosampler. The MS/MS conditions for each target were optimized using the automated multiple reaction monitoring (MRM) optimization procedures in LabSolutions (Shimadzu). The MRM used for quantification was *m/z* 880.5 > 197.1 for oxidized CoQ<sub>10</sub>, 882.4 > 197.0 for reduced CoQ<sub>10</sub>, and 890.4 > 206.2 for CoQ<sub>10</sub>-d9 (internal standard). Standards and samples were quantified using the LabSolutions software program to determine the peak area for oxidized CoQ<sub>10</sub>, reduced CoQ<sub>10</sub>, CoQ<sub>10</sub>-d9, and the standard curves were used to determine the total amount of CoQ present in the samples.

Intra-assay coefficients of validation (CVs) and relative errors (REs), as measurements of precision and accuracy, respectively, were determined in five parallel analyses of the same cell. To evaluate inter-assay precision and accuracy, one cell line was independently evaluated on three different days. Precision was calculated as (standard deviation/mean concentration)  $\times$  100 (%), and accuracy was calculated as (quantitative value/theoretical value)  $\times$  100 (%). The intra-assay precision (CV) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 0.58% and 1.39%, respectively. The intra-assay accuracy (RE) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 4.20% and 2.50%, respectively. The inter-assay precision (CV) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 1.27% and 1.84%, respectively. The intra-assay accuracy (RE) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 10.54% and 1.18%, respectively (Supplemental Table 1, QC1).

#### 2.3.4. Citrate synthase and protein quantification

Fibroblast CoQ<sub>10</sub> levels were expressed as citrate synthase (CS) activity (measured CoQ<sub>10</sub> values/CS units, nmol/CS units). CS activity was measured spectrophotometrically referring to the method described by Srere (1969), with 0.1 mM DTNB, 0.3 mM Acetyl-CoA, 0.5 mM Oxaloacetate, and 12–20  $\mu$ g protein in 200  $\mu$ L total incubation volume. CS units are determined as follows: CS Units ( $\mu$ mol/min/mL) = ( $\Delta A_{412}$ /min  $\times$  V (mL)  $\times$  dil)/13.6  $\times$  L (cm)  $\times$  V<sub>enz</sub> (mL), V (mL); the reaction volume, dil; the dilution factor of the original sample, 13.6 (mM<sup>-1</sup> cm<sup>-1</sup>); the extinction coefficient of TNB at 412 nm, L (cm); pathlength for

absorbance measurement (0.552 cm),  $V_{enz}$ (mL); the volume of the enzyme sample. Protein concentrations were quantified using Qubit™ Protein Assay Kits and a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

#### 2.4. Statistical analyses

Statistical analyses were performed using the GraphPad Prism software program (version 9.01, GraphPad Software Inc., La Jolla, CA). Comparisons between samples were performed using a one-way ANOVA. The results are expressed as the mean (standard deviation). *P* values of <0.05 were considered to indicate statistical significance.

### 3. Results

#### 3.1. Total CoQ<sub>10</sub> levels were observed to decrease in all patients with primary CoQ<sub>10</sub> deficiency

We showed the reduced, oxidized, and total (sum of reduced and oxidized) CoQ<sub>10</sub> values corrected for the CS unit (nmol/CS unit) (Table 2). In addition, we also measured the CoQ<sub>10</sub> values corrected for protein levels (nmol/g protein) (Supplemental Table 2). Six patients showed decreased the total CoQ<sub>10</sub> values (<70% of the control): five with primary CoQ<sub>10</sub> deficiency (Cases 1–5), and one with Kearns-Sayre syndrome (Case 19) (Table 2). The total CoQ<sub>10</sub> levels were significantly decreased in primary CoQ<sub>10</sub> deficiency than in controls (primary CoQ<sub>10</sub> deficiency ( $n = 5$ )  $1.00 \pm 0.19$  nmol / CS unit (mean  $\pm$  SD), controls ( $n = 10$ )  $2.30 \pm 0.24$  nmol / CS unit,  $p < 0.0001$ ) (Fig. 1). However, total CoQ<sub>10</sub> levels were the same in mitochondrial disease other than primary CoQ<sub>10</sub> deficiency as in controls (mitochondrial disease ( $n = 19$ )  $2.23 \pm 0.26$  nmol / CS unit,  $p = 0.93$ ).

**Table 2**

Reduced and oxidized CoQ<sub>10</sub> values and total CoQ deficiency in fibroblasts.

Case	reduced CoQ <sub>10</sub>		oxidized CoQ <sub>10</sub>		total CoQ <sub>10</sub> (nmol/CS unit)		% CoQ deficiency (%)
	mean	SD	mean	SD	mean	SD	
1	0.37	0.15	0.37	0.02	0.74	0.13	32
2	0.12	0.00	0.16	0.00	0.28	0.00	12
3	0.57	0.45	0.59	0.18	1.16	0.63	50
4	0.64	0.03	0.74	0.20	1.38	0.17	60
5	0.78	0.01	0.64	0.02	1.42	0.01	62
6	0.48	0.01	1.65	0.00	2.13	0.01	
7	1.03	0.04	1.00	0.32	2.03	0.29	
8	1.22	0.04	0.82	0.18	2.04	0.22	
9	1.40	0.54	0.96	0.03	2.36	0.51	
10	0.86	0.16	1.34	0.30	2.20	0.46	
11	1.02	0.08	2.17	0.53	3.19	0.44	
12	1.18	0.56	1.31	0.17	2.49	0.38	
13	1.33	0.03	0.57	0.02	1.90	0.00	
14	1.77	0.31	0.58	0.10	2.35	0.21	
15	1.54	0.09	0.71	0.00	2.25	0.09	
16	0.86	0.02	1.46	0.01	2.32	0.01	
17	1.37	0.40	1.28	0.14	2.65	0.25	
18	1.01	0.20	1.52	0.08	2.53	0.29	
19	0.57	0.01	0.72	0.02	1.29	0.02	56
20	1.41	0.40	0.51	0.11	1.92	0.51	
21	1.24	0.28	0.65	0.28	1.89	0.56	
22	1.19	0.11	0.87	0.23	2.06	0.34	
23	0.81	0.19	1.84	0.46	2.65	0.27	
24	0.92	0.01	1.23	0.00	2.15	0.01	
Reference (n = 10)	1.19	0.15	1.11	0.10	2.30	0.24	

% CoQ deficiency: <70% of control CoQ<sub>10</sub> value.

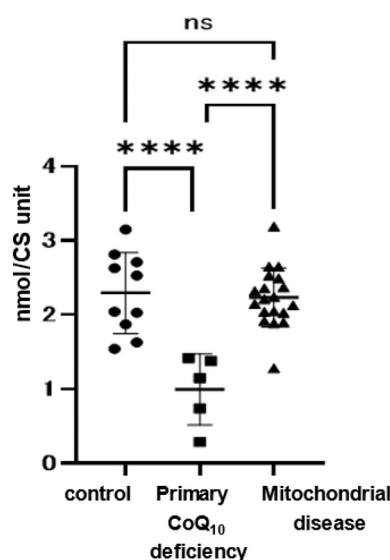
#### 3.2. The reduced/total CoQ<sub>10</sub> ratio was unchanged in primary CoQ<sub>10</sub> deficiency, but was higher in complex IV or V deficiency

We showed the ratio of reduced CoQ<sub>10</sub> to total CoQ<sub>10</sub> (reduced/total CoQ<sub>10</sub>) of fibroblasts (Table 3). The ratio of reduced and oxidized CoQ<sub>10</sub> to total CoQ<sub>10</sub> in control fibroblasts was 52% and 48%, respectively (Table 3). In primary CoQ<sub>10</sub> deficiency, the reduced/total CoQ<sub>10</sub> ratio did not change compared to the control (reduced/total CoQ<sub>10</sub> ratio of primary CoQ<sub>10</sub> deficiency ( $n = 5$ )  $49 \pm 7\%$  (mean  $\pm$  SD), controls ( $n = 10$ )  $52 \pm 1\%$ ,  $p = 0.92$ ) (Fig. 2). Regarding the cases with respiratory chain complex deficiency, there was no difference between control and complex I deficiency (reduced/total CoQ<sub>10</sub> ratio of complex I deficiency ( $n = 7$ )  $44 \pm 7\%$ ,  $p = 0.37$ ). However, the reduced/total CoQ<sub>10</sub> ratio in complex IV or V deficiency was increased in comparison to the control (complex IV or V deficiency ( $n = 3$ )  $71 \pm 3\%$ ,  $p = 0.022$ ). In addition, the reduced/total CoQ<sub>10</sub> ratio in complex IV or V deficiency was higher in comparison to complex I deficiency and primary CoQ<sub>10</sub> deficiency (complex IV or V deficiency vs. complex I deficiency:  $p = 0.0021$ , complex IV or V deficiency vs. primary CoQ<sub>10</sub> deficiency:  $p = 0.015$ ).

In individual cases, the reduced/total CoQ<sub>10</sub> ratio decreased (<80% of the reduced/total CoQ<sub>10</sub> ratio in the control) in six cases; three cases with complex I deficiency (Cases 6, 10, 11), two cases with mtDNA depletion syndrome (Cases 16, 18), one case with BOLA3 mutation (Case 23) (Table 3). On the other hand, the reduced/total CoQ<sub>10</sub> ratio increased (>120% of the reduced/total CoQ<sub>10</sub> ratio in the control) in five cases; two cases with complex IV deficiency (Cases 13, 14), two cases with MELAS (Cases 20, 21), one case with complex V deficiency (Case 15) (Table 3).

### 4. Discussion

We showed here that the total CoQ<sub>10</sub> values of fibroblasts were significantly lower in patients with primary CoQ<sub>10</sub> deficiency. In this syndrome, early recognition and therapy can stop progression and improve the prognosis; however, established severe symptoms cannot be reversed [16,34]. Biochemical measurements of low CoQ<sub>10</sub> levels in muscle biopsy have been utilized for the diagnosis of this syndrome



**Fig. 1.** Total CoQ<sub>10</sub> values.

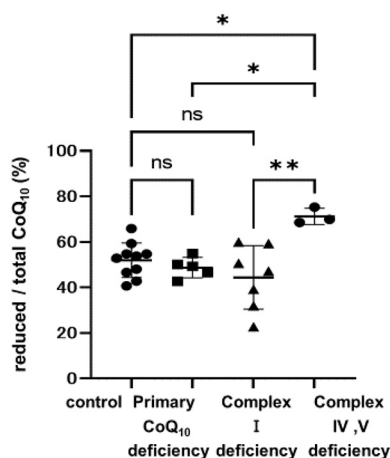
Total (sum of reduced and oxidized) CoQ<sub>10</sub> values of mitochondrial patient fibroblasts. CoQ<sub>10</sub> deficiency (CoQ<sub>10</sub> levels <70% of control CoQ<sub>10</sub>) was found in six cases. All cases of primary CoQ<sub>10</sub> deficiency showed decreased CoQ<sub>10</sub> levels. In mitochondrial disease, CoQ<sub>10</sub> values were not decreased, with the exception of Case 19 (Kearns-Sayre syndrome). Data are expressed as \*\*\*\*  $P < 0.0001$ , and n.s. indicates no significance.

**Table 3**  
Ratio of reduced / total CoQ<sub>10</sub>.

Case	reduced/total CoQ <sub>10</sub> (%)		Reduced/total CoQ <sub>10</sub> in cases versus reduced/total CoQ <sub>10</sub> in controls (%)
	mean	SD	
1	50	11	96
2	43	1	83
3	49	14	95
4	47	8	90
5	55	1	106
6	23	0	44
7	51	9	97
8	60	4	115
9	59	10	114
10	39	1	75
11	32	7	61
12	47	15	91
13	70	1	135
14	75	7	145
15	68	1	131
16	37	1	71
17	52	10	100
18	40	3	77
19	44	0	85
20	74	1	142
21	66	5	127
22	58	4	111
23	30	10	59
24	43	0	83
Reference (n = 10)	52	1	

The reduced/total CoQ<sub>10</sub> ratio decreased (< 80% of control value); cases 6, 10, 11, 16, 18, 23.

The reduced/total CoQ<sub>10</sub> ratio increased (120% < of control value); cases 13, 14, 15, 20, 21.



**Fig. 2.** Comparison of the reduced/total CoQ<sub>10</sub> ratio.

Comparison of the reduced/total CoQ<sub>10</sub> ratio in cases with primary CoQ<sub>10</sub> deficiency, complex I deficiency, and complex IV or V deficiency. In primary CoQ<sub>10</sub> deficiency, the reduced/total CoQ<sub>10</sub> ratio was the same as that of the controls. In complex I deficiency, 3/7 cases showed a decreased (< 80% of control value) reduced/total CoQ<sub>10</sub> ratio. In complex IV or V deficiency, 3/3 cases showed an increased (120% < of control value) reduced/total CoQ<sub>10</sub> ratio. Data are expressed as \**P* < 0.05, \*\**P* < 0.01, and n.s. indicates no significance.

[16]. Moreover, the identification of biallelic pathogenic variants in the *COQ* genes, which encode proteins involved in coenzyme Q biosynthesis, enables a definitive diagnosis [16]. However, the invasiveness of muscle biopsy hampers this procedure and can delay the diagnosis. CoQ<sub>10</sub> levels in fibroblasts were examined and implicated in their usefulness [21]. Our data with LC-MS/MS for the measurement of CoQ<sub>10</sub> from fibroblasts also supported the usefulness for detecting CoQ<sub>10</sub>

deficiency [32,35]. The fibroblasts from Case 2 showed the lowest CoQ<sub>10</sub> concentration and showed a very severe phenotype. A correlation between CoQ<sub>10</sub> levels and phenotype has been suggested [24,36,37]. Therefore, the CoQ<sub>10</sub> value from skin fibroblast may reflect the clinical severity.

In addition to primary CoQ<sub>10</sub> deficiency, various mitochondrial diseases have also been reported to decrease CoQ<sub>10</sub> in fibroblasts and muscle, particularly in mtDNA depletion syndrome [20,38]. However, in our analysis, three patients with mtDNA depletion, including *DGUOK* and *MPV17* mutations, showed no decrease in CoQ<sub>10</sub> (Cases 16–18). Only one patient with large deletions of mtDNA showed markedly decreased CoQ<sub>10</sub> (Case 19). Therefore, decreased CoQ<sub>10</sub> was only a constant feature in primary CoQ<sub>10</sub> deficiency syndrome in our analysis. Our results support the widely accepted idea that early CoQ<sub>10</sub> therapy should be therefore indicated if decreased levels of CoQ<sub>10</sub> are found in the fibroblasts of patients with suspected mitochondrial disease.

To our knowledge, this is the first report to describe the reduced/total CoQ<sub>10</sub> ratios in patients with mitochondrial diseases, including primary CoQ<sub>10</sub> deficiency. In primary CoQ<sub>10</sub> deficiency, the reduced/total CoQ<sub>10</sub> ratio did not change. On the other hand, the reduced/total CoQ<sub>10</sub> ratio was decreased in 3/7 of cases of complex I deficiency and was increased in 3/3 of cases of complex IV or V deficiency. Primary CoQ<sub>10</sub> deficiency is caused by impaired CoQ<sub>10</sub> biosynthesis, which results in a decrease in the absolute value of CoQ<sub>10</sub>; however, as expected, our data suggest that it does not affect the redox reaction in mitochondria. Since CoQ<sub>10</sub> changes from the oxidized form to the reduced form by accepting electrons from complex I, complex II and other dehydrogenases, the reduced/total CoQ<sub>10</sub> ratio is expected to decrease in complex I deficiency. However, only 3 out of 7 complex I patients in our cohort showed disturbed Q reduced/total ratios. In contrast, CoQ<sub>10</sub> changes from the reduced form to the oxidized form in complex III, and the reduced/total CoQ<sub>10</sub> ratio is expected to increase in complex III and later complex deficiencies (Supplemental Fig. 1, left panel). In fact, the reduced CoQ<sub>10</sub> ratio was significantly decreased with complex I inhibitor, whereas the reduced CoQ<sub>10</sub> ratio was increased with complex IV inhibitor [23]. Some (but not all) of our results support these observations. Among complex I deficiency patients, patients with isolated complex I deficiency tended to have a decreased reduced/total CoQ<sub>10</sub> ratio (3/4 cases; Cases 6, 10, 11) (Supplemental Table 3). On the other hand, the reduced/total CoQ<sub>10</sub> ratio was unchanged in a patient who also had a decreased complex III and IV enzyme activity (Case 8), in a patient without a decreased CI enzyme activity in fibroblasts (Case 12), and in a patient with a decreased CI enzyme activity in muscle (Case 7).

In recent years, it has been reported that the reduced/oxidized CoQ<sub>10</sub> ratio may be a marker of mitochondrial metabolic status [39]. In situations where CoQ<sub>FADH</sub> can be excessively reduced, RET is induced, and the production of reactive oxygen species (ROS) from complex I is stimulated, which causes complex I destruction [4,6]. On the other hand, oxidizing the CoQ pool by alternative oxidase (AOX) of *Ciona intestinalis* xenotopically expressed in mouse mitochondria induces forward electron transport (FET) from RET [40]. Our system of measuring the reduced/total CoQ<sub>10</sub> ratio of skin fibroblasts provide the amount and redox states of CoQ<sub>10</sub>.

As a limitation of our study, CoQ<sub>10</sub> is mostly localized in the mitochondria in subcellular fractions but it has also been shown to localize in Golgi, lysosomes, and other organelles [3]. In this study, we measured the whole cell CoQ<sub>10</sub> level without separating the mitochondrial and non-mitochondrial fractions. Moreover, we examined an only limited number of patients affected by only some mitochondrial diseases, which can be caused by >400 gene mutations [41].

In conclusion, we measured the reduced/total CoQ<sub>10</sub> ratio in fibroblasts from a cohort of patients with mitochondrial disease for the first time. The reduced/total CoQ<sub>10</sub> ratio tended to show no change in many of the cells that we measured. However, the reduced/total CoQ<sub>10</sub> ratio was increased in complex IV or V deficiency, while the reduced/total CoQ<sub>10</sub> ratio tended to decrease in some cases of complex I deficiency.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mgmr.2022.100951>.

### Author contributions

CW, HO contributed to the conceptualization and performance of the statistical analysis of the data and wrote the manuscript; MW, AM, EFJ, TT, HU, OT, ET, and KA performed sampling and data acquisition; YK, YO performed genetic testing; KM, AO recruited patients, provided clinical information, collected samples; TY conducted supervision of the project. All authors read and approved the final manuscript.

### Ethics

All procedures followed were by the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of the World Medical Organization. This study was approved by the Ethics Committee of Jichi Medical University and written informed consent was obtained from all participants.

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### CRedit authorship contribution statement

**Chika Watanabe:** Conceptualization, Writing – original draft. **Hitoshi Osaka:** Conceptualization, Writing – review & editing, Funding acquisition. **Miyuki Watanabe:** Investigation. **Akihiko Miyauchi:** Investigation. **Eriko F. Jimbo:** Investigation. **Takeshi Tokuyama:** Resources. **Hideki Uosaki:** Resources. **Yoshihito Kishita:** Resources. **Yasushi Okazaki:** Resources. **Takanori Onuki:** Resources. **Tomohiro Ebihara:** Resources. **Kenichi Aizawa:** Resources. **Kei Murayama:** Resources. **Akira Ohtake:** Resources. **Takanori Yamagata:** Supervision.

### Declaration of Competing Interest

None.

### Data availability

No data was used for the research described in the article.

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## Case Report

Focal segmental glomerulosclerosis with a mutation in the *mitochondrially encoded NADH dehydrogenase 5* gene: A case report

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## ABSTRACT

NADH dehydrogenase 5 (ND5) is one of 44 subunits composed of Complex I in mitochondrial respiratory chain. Therefore, a *mitochondrially encoded ND5 (MT-ND5)* gene mutation causes mitochondrial oxidative phosphorylation (OXPHOS) disorder, resulting in the development of mitochondrial diseases. Focal segmental glomerulosclerosis (FSGS) which had podocytes filled with abnormal mitochondria is induced by mitochondrial diseases. An *MT-ND5* mutation also causes FSGS. We herein report a Japanese woman who was found to have proteinuria and renal dysfunction in an annual health check-up at 29 years old. Because her proteinuria and renal dysfunction were persistent, she had a kidney biopsy at 33 years of age. The renal histology showed FSGS with podocytes filled with abnormal mitochondria. The podocytes also had foot process effacement and cytoplasmic vacuolization. In addition, the renal pathological findings showed granular swollen epithelial cells (GSECs) in tubular cells, age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs), and red-coloured podocytes (ReCPos) by acidic dye. A genetic analysis using peripheral mononuclear blood cells and urine sediment cells detected the m.13513 G > A variant in the *MT-ND5* gene. Therefore, this patient was diagnosed with FSGS due to an *MT-ND5* gene mutation. Although this is not the first case report to show that an *MT-ND5* gene mutation causes FSGS, this is the first to demonstrate podocyte injuries accompanied with accumulation of abnormal mitochondria in the cytoplasm.

## 1. Introduction

Mitochondrial diseases/disorders are rare and occur every 1 in 5000 births [1]. Mitochondria play a key role in the biosynthesis of adenosine triphosphate (ATP), the main energy source of cells, through oxidative phosphorylation (OXPHOS) using the mitochondrial respiratory chain

(MRC) complex. Given that the genes related to the MRC complex are encoded in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), mitochondrial diseases can occur in cases of mtDNA or nDNA mutations [2,3]. As ATP is mandatory for all cells that require energy, the clinical phenotypes of mitochondrial diseases are versatile and are expressed as encephalopathy [4], myopathy [5], cardiomyopathy [6], hepatopathy

**Abbreviations:** ND5, NADH dehydrogenase 5; *MT-ND5*, mitochondrially encoded ND5; OXPHOS, oxidative phosphorylation; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; FSGS, focal segmental glomerulosclerosis; GSECs, granular swollen epithelial cells; AiDIVs, age-inappropriately disarranged and irregularly sized vascular smooth muscle cells; ReCPos, red-coloured podocytes; ATP, adenosine triphosphate; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; Cr, creatinine; sCr, serum creatinine; eGFR, estimated glomerular filtration rate; COX IV, cytochrome c oxidase subunit 4.

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[7], deafness [8], and diabetes mellitus [9]. Mitochondrial nephropathy, which occurs because of a mitochondrial disorder in nephrons, has also been reported [10–12].

Focal segmental glomerulosclerosis (FSGS) is a histological term, rather than a specific disease category and diagnosed by the presence of sclerosis in parts of at least one glomerulus in the kidney biopsy specimen [13]. It can be classified into primary, secondary, genetic, and unknown forms of FSGS [14]. Among causative genes of mitochondrial nephropathy, m.3243A > G pathogenic variant in *MT-TL1* gene on mtDNA, which encodes mitochondrial transfer RNA leucine 1, is majority [12]. The m.3243A > G mutation leads to genetic cause of FSGS with accumulation of abnormal mitochondria in podocytes [11,15]. Because podocyte injury plays a key role in the pathogenesis of FSGS [16–18], impaired mitochondrial function in podocytes should induce FSGS [19–22].

The pathogenic variants in *mitochondrially encoded NADH dehydrogenase 5 (MT-ND5)* gene cause mitochondrial diseases such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [23–25]. ND5 is one of the 44 subunits of mitochondria respiratory complex I. The pathogenic variants in *MT-ND5* gene also cause FSGS [26–29]. However, in all cases with FSGS lesions presented in the past reports, abnormal mitochondria or their accumulation were not identified in podocytes. In another recent report of three cases with nephropathy due to *MT-ND5* gene mutation, one of the cases showed FSGS lesion. The authors of this report concluded that FSGS lesion of their case was secondary [28]. The pathogenesis of the secondary FSGS is considered to involve intraglomerular hypertension caused by hypertension or obesity, by adaptation to nephron loss due to progression of glomerulosclerosis or by a low nephron number due to low birth weight [30,31]. Therefore, it has not been evident whether genetic FSGS occurs with a pathogenic variant in *MT-ND5* gene. Here, we report the case with an *MT-ND5* mutation with apparent FSGS lesions. In this case, foot process effacement and cytoplasmic vacuolization in podocytes with accumulation of abnormal mitochondria were observed.

## 2. Case presentation

This case occurred in a Japanese woman born at 39 weeks of

gestation with a birth weight of 3210 g. She had no remarkable medical history. Annual health check-ups showed no proteinuria on urinalysis until age 29. At 29 years of age, the annual health check-up revealed proteinuria (1+) for the first time using a urine dipstick and a high serum creatinine (sCr) level of 1.01 mg/dL. Her estimated glomerular filtration rate (eGFR) using the Japanese Eq. [32] was calculated to be 54.0 mL/min/1.73 m<sup>2</sup>. At the same check-up, she was also found to have a slight hearing disturbance. Additionally, she had a headache once a week from 30 years of age and was diagnosed by a neurologist with migraine, uncontrolled with triptan. Her health check-up at 32 years of age again showed proteinuria (1+) and increased sCr (1.06 mg/dL). Hematuria has never been noted before. At 33 years of age, she was referred to the Yamanashi Prefectural Central Hospital by her primary doctor to examine the reason for her proteinuria and decreased eGFR. At the first visit, proteinuria (1.09 g/gCr), elevated sCr (1.28 mg/dL), and elevated uric acid (7.5 mg/dL) were detected. Febuxostat and sodium bicarbonate were prescribed for the treatment of hyperuricemia. Because of the continued presentation of proteinuria and decreased renal function, she was admitted for a kidney biopsy, at 6 months following the initial visit.

Upon admission, her blood pressure was 114/70 mmHg. Physical examination showed a height of 152 cm, body weight of 43.8 kg, and body mass index of 19.0. No crackles or murmurs were detected on chest auscultation. No abnormal neurological findings were observed. Neither skin lesions nor pitting oedema were detected. The laboratory data on admission are summarised in Table 1, which shows decreased eGFR and proteinuria. Hematuria was not observed. In the data we measured, there were no data to suggest the presence of tubular dysfunction such as Fanconi's syndrome or distal tubular acidosis. The long × short axis of the kidneys measured 101 × 38 mm on the left and 104 × 50 mm on the right, indicating no renal atrophy. The electrocardiogram test result was normal, and echocardiography revealed normal cardiac function. The standard pure-tone hearing test showed a right-ear value of 38.8 dB and a left-ear value of 40.0 dB. She was therefore diagnosed with sensorineural hearing loss by an otolaryngologist. No visual field defects were noted. Furthermore, because of hearing loss and headache symptoms, a brain magnetic resonance imaging (MRI) was also performed to examine for brain lesions, but no abnormal findings were found.

Her parents had died because of cancer (mother, breast cancer,

**Table 1**  
Laboratory data on admission.

Blood cell count			Blood chemistry			Immunology			Urinalysis		
WBC	5400	/μL	TP	6.8	g/dL	CH50	54	U/mL	Gravity	1.01	
RBC	373	×10 <sup>4</sup> /μL	Alb	3.9	g/dL	C3	76.1	mg/dL	pH	7.0	
Hb	11.5	g/dL	AST	21	IU/L	C4	19.3	mg/dL	RBC	1–4	/HPF
Ht	35.1	%	ALT	11	IU/L	IgG	1340.3	mg/dL	WBC	<1	/HPF
MCV	94.1	fl	LDH	186	IU/L	IgA	225.7	mg/dL	<u>Protein</u>	<u>2.04</u>	<u>g/gCr</u>
MCHC	32.8	%	ALP	149	IU/L	IgM	180.1	mg/dL	Glucose	(–)	
Plt	25.7	×10 <sup>4</sup> /μL	Tbil	0.56	mg/dL	ANA	(–)		NAG	8.6	U/L
			<u>BUN</u>	<u>21.8</u>	mg/dL	M protein	(–)				
Coagulation			<u>Cr</u>	<u>1.26</u>	mg/dL	HBs Ag	(–)				
APTT	34.0	sec	<u>eGFR</u>	<u>40.8</u>	mL/min/1.73 m <sup>2</sup>	HCV Ab	(–)				
PT	99.0	%	K	5.0	mEq/L						
INR	0.97	INR	P	3.1	mg/dL						
Fib	373.0	mg/dL	UA	5.4	mg/dL						
			Tcho	255	mg/dL						
			CRP	0.01	mg/dL						
			FBS	85	mg/dL						
			HbA1c	5.3	%						

Aberrant values are underlined.

WBC, white blood cells; RBC, red blood cells; Hb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; Plt, platelet; APTT, activated partial thromboplastin time; PT, prothrombin time; INR, international normalized ratio; Fib, fibrinogen; TP, total protein; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; Tbil, total bilirubin; BUN, blood urea nitrogen; Cr, creatinine; eGFR, estimated glomerular filtration rate (by the Japanese equation); K, potassium; P, phosphate; UA, uric acid; Tcho, total cholesterol; CRP, C-reactive protein; FBS, fasting blood sugar; HbA1c, haemoglobin A1c (NGSP); CH50, 50% haemolytic complement activity; C3, complement 3; IgG, immunoglobulin G; ANA, antinuclear antibody; M protein, monoclonal protein; HBs Ag, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; NAG, N-acetyl glucosaminidase; HPF, high power field.

father, colon cancer) without kidney disease or any hearing difficulty. Neither of her two older brothers had any abnormalities in kidney function, urine, or hearing. She had a 7-year-old daughter and a 5-year-old son. Her daughter's birth weight was 2840 g at 41 weeks and 2 days of gestation, and her son's birth weight was 2632 g at 39 weeks of gestation. Her daughter was born using suction delivery, whereas her son was born through normal delivery. None of her children had any urinary abnormalities or hearing difficulties, as examined during medical check-ups. However, the children sometimes complained of headaches that had not yet been diagnosed by any doctor.

A percutaneous kidney biopsy was performed under ultrasound guidance. Light microscopy photographs of renal biopsy specimens are shown in Fig. 1. Nine glomeruli were observed in the specimens. One glomerulus was globally sclerosed. Furthermore, segmental sclerosis was observed at the perihilar area in two glomeruli, compatible with the perihilar variant in the Columbia classification of FSGS (Fig. 1a) [13,30]. Interestingly, red-coloured podocytes (ReCPos), whose cytoplasm was dyed red, were observed using AZAN trichrome staining (Fig. 1a and b). Neither mesangial proliferation nor hypercellularity were detected. Interstitial fibrosis and tubular atrophy were observed in almost 30% of the interstitium. There were no abnormal findings such as thickening or lamellation of the tubular basement membrane. Characteristically, granular swollen epithelial cells (GSECs), reported to be specific to mitochondrial diseases [33,34], were markedly observed in the distal tubular cells using AZAN trichrome staining (Fig. 1c). Such GSECs were emphasised by staining with anti-cytochrome c oxidase subunit 4 (COX IV) antibody [mouse anti-COX IV antibody (20E8C12)] (ab14744; Abcam PLC., Cambridge, UK; Fig. 1d). COX IV staining is recognised as a loading control for mitochondria [34,35]. Furthermore, the sizes of the vascular smooth muscle cells in arterioles were irregular, and their arrangement was disorganized in a manner similar to that seen in older patients, despite the younger age (33 years) of the patient, without any medical history that may have caused arteriosclerosis (Fig. 1e). Such age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) were also noticeable in the interlobular arteries (Fig. 1f). Immunofluorescence analysis revealed IgM, C3 and C1q deposits in the glomeruli (Fig. 1g-i). IgG and IgA were negative in observed glomeruli. Electron microscopy analyses (Fig. 2a–e) also showed podocytes with increased mitochondria that had lost their normal cristae structure. Foot processes of such podocytes with accumulation of abnormal mitochondria were effaced (Fig. 2b and d). A vacuolization was also observed in the cytoplasm in the podocyte with mitochondrial accumulation (Fig. 2b). We also detected cells with increased abnormal mitochondria in the parietal epithelial cells of the Bowman's capsule (Fig. 2f). Although the glomerular basement membrane was partially thin at the part along with the podocytes with abnormal mitochondria, the membranes at other parts showed normal thickness (> 250 nm) without lamination or reticulation. Electron dense deposits were not observed in mesangium or glomerular basement membranes. These renal pathology findings strongly suggest genetic FSGS due to mitochondrial disease.

The serum lactate and pyruvate levels were slightly elevated at 21.0 mg/dL (normal: 3.0–17.0 mg/dL) and 1.34 mg/dL (normal: 0.30–0.94 mg/dL), respectively. The pathological finding of accumulation of abnormal mitochondria in glomerular epithelial cells and tubules, the presence of sensorineural hearing difficulty, and elevated blood lactate led us to perform a comprehensive genetic analysis of mitochondrial diseases.

After obtaining informed consent from the patient according to protocol and permission from our ethics committees, genomic DNA extracted from the patient's peripheral mononuclear blood cells and urine sediment cells was analysed by targeted resequencing coupled with next-generation sequencing, followed by Sanger sequencing. The DNA was subjected to fragmentation and library preparation using the Lotus DNA Library Prep Kit (#10001074; Integrated Device Technology, Inc., San Jose, CA, USA) according to the manufacturer's instructions.

Target enrichment was performed with xGen Human mtDNA Research Panel (#1075705; Integrated Device Technology) targeting the whole mtDNA and with custom xGen® Predesigned Gene Capture Pools (Integrated Device Technology)/xGen Lockdown Probe pool (Integrated Device Technology) targeting the exons of 367 nuclear-encoded genes that cause mitochondrial diseases. The library was then sequenced on the Illumina MiSeq platform using the MiSeq Reagent Kit v2 (MS-102-2002; Illumina, Inc., San Diego, CA, USA). A pathogenic mtDNA variant of m.13513G > A was detected in both the patient's blood cells and urine sediment cells (Fig. 3). The Minor Variant Finder (MVF) software program (Thermo Fisher Scientific, Waltham, MA, USA) revealed that the heteroplasmy rates of the blood cells and urine sediment cells were 10.3% (forward: 11.6%/reverse: 9.0%) and 62.2% (forward: 63.7%/reverse: 60.7%), respectively. Therefore, we diagnosed her with renal dysfunction and proteinuria due to FSGS caused by an *MT-ND5* mutation.

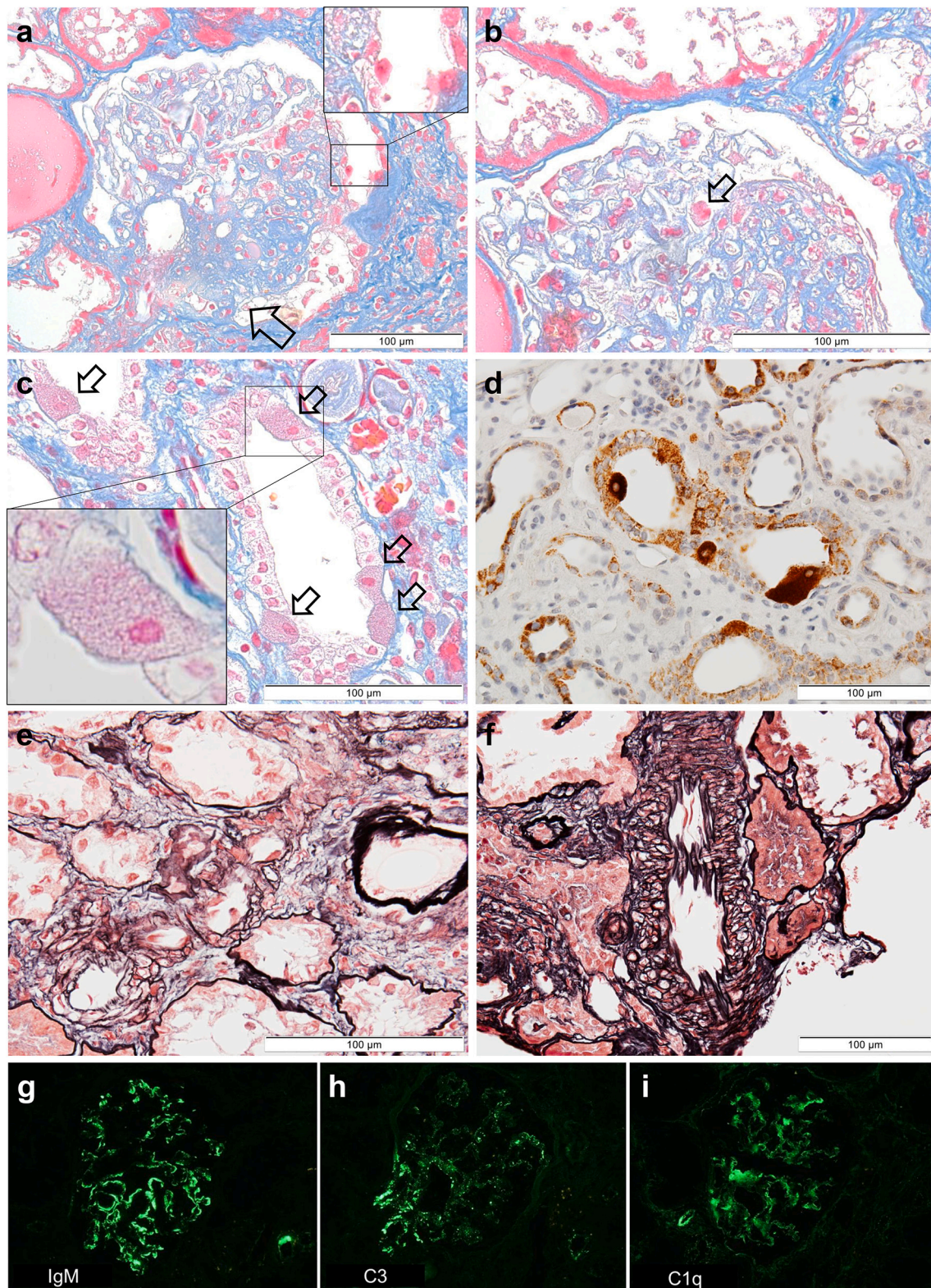
### 3. Discussion and conclusions

*MT-ND5* gene encodes NADH dehydrogenase 5 (ND5), which is a subunit of mitochondria respiratory complex I. Therefore, *MT-ND5* mutations cause mitochondrial diseases such as MELAS [23,36,37], Leigh syndrome [36–39], and Leber hereditary optic neuropathy [37]. Our case with the m.13513G > A variant in the *MT-ND5* gene showed FSGS lesions with injured podocytes that had accumulation of abnormal mitochondria in the cytoplasm. The m.13513G > A has already been confirmed as a pathogenic variant of the mitochondrial disease using MITOMAP, a human mitochondrial genome database (<https://www.mitomap.org/MITOMAP>).

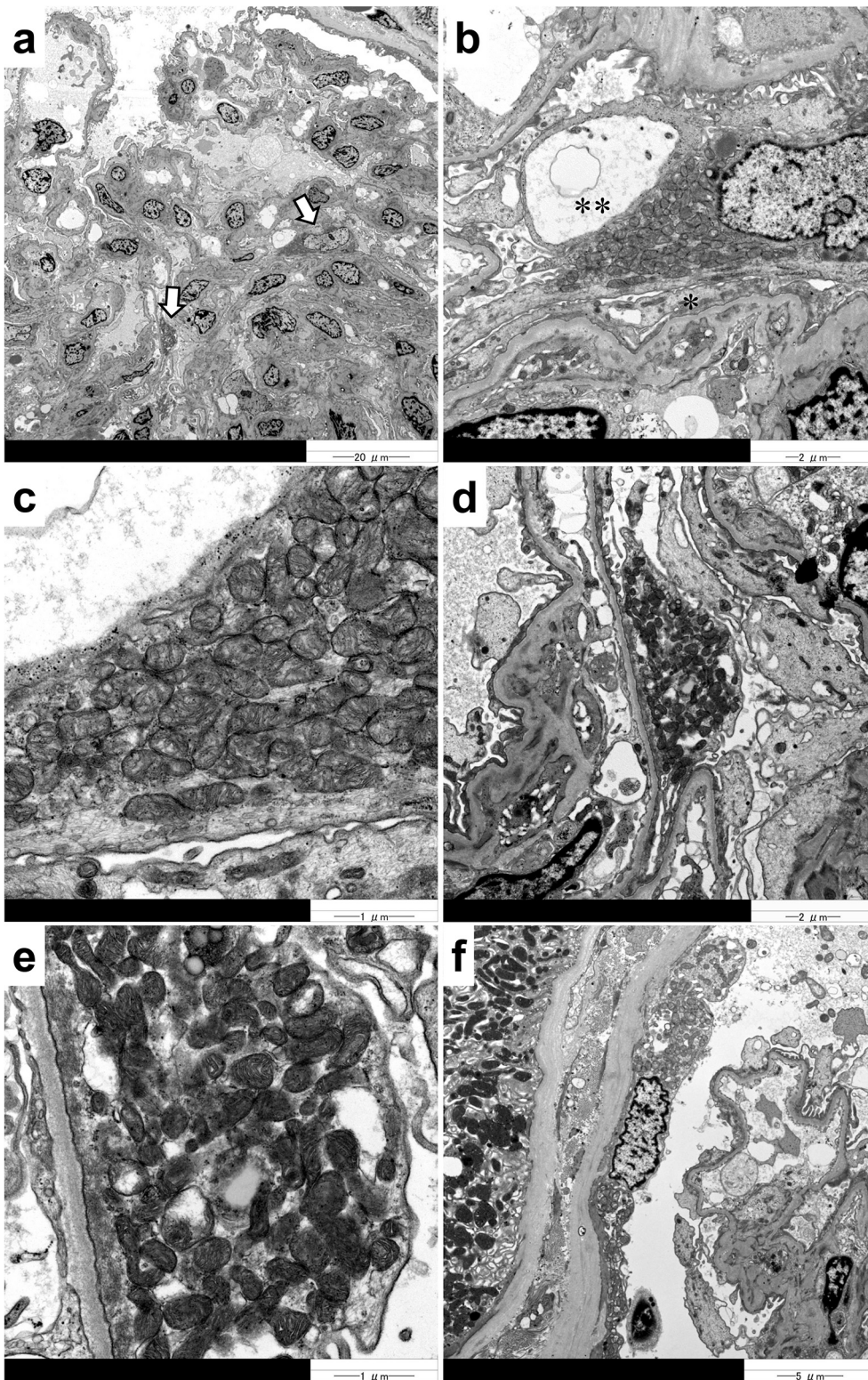
Mitochondrial diseases cause genetic FSGS [11,12,15]. Its etiology is postulated to be caused by genetically disrupted mitochondrial function in podocytes [19,22]. As an indication of this, cases with FSGS due to m.3243A > G mutation had podocytes filled with mitochondria with abnormal cristae structure [10,11]. In the past, several cases of mitochondrial diseases due to *MT-ND5* mutations have been also reported to show FSGS lesions [26–29]. However, in these cases, pathological findings of podocyte injuries with accumulation of abnormal mitochondria were not confirmed, leaving the possibility of secondary FSGS caused by intraglomerular hypertension [14,28,30,31]. In contrast, our present case showed apparent FSGS lesions, and we detected increased abnormal mitochondria in the glomerular podocytes that also had pathological findings indicating their injuries such as foot process effacement and the cytoplasmic vacuolization. Our patient did not have hypertension, obesity, or a history of low birth weight. Furthermore, the diameters of all glomeruli observed under light microscopy in this patient were < 250 µm (mean ± SD; 149 ± 72 µm). Therefore, we deemed the etiology of FSGS, in this case, to be a disorder of the mitochondrial OXPHOS system due to an *MT-ND5* mutation and not due to 'secondary' by intraglomerular hypertension [14,30,31]. The pathological picture of tubulointerstitial nephropathy with GSECs was also observed in this case. This tubulointerstitial damage may be due to the *MT-ND5* mutation, as has been reported in the past [28].

In general, mitochondrial diseases induced by mtDNA mutations vary markedly based on differences in the rates of heteroplasmy by cell type or organ [40,41]. Therefore, while the reason for the differences between the present and the previous cases with the pathogenic variant in *MT-ND5* gene could not be completely ascertained, the heteroplasmy rate of mtDNA with the pathogenic variant in podocytes might have been higher in our case than that in the previous cases.

In this case, IgM, C3, and C1q were positive in glomeruli (Fig. 1g-i). Since neither hematuria, mesangial cell proliferation, nor electron dense deposits were observed, we considered that it was unlikely that IgM nephropathy or C1q nephropathy was complicated [42,43]. On the other hand, glomerular IgM and C3 deposits frequently accompany FSGS [44]. In addition, IgM and C3 were positive in glomeruli of two out of three cases report by Bakis et al., too [28]. Therefore, IgM and C3



**Fig. 1.** Light microscopy and immunofluorescence findings (a): The segmental sclerosis lesion at the perihilar area is indicated by an arrow. A red-coloured podocyte (ReCPo) is surrounded by a square, magnified at the top right (AZAN stain). (b): The ReCPo is indicated by an arrow (AZAN stain). (c): GSECs in the distal tubules are indicated by arrows (AZAN stain). One GSEC is magnified at the lower left. (d): Staining of COX IV emphasizes tubular cells, including many mitochondria, which correspond to GSECs. (e): In the afferent arteriole that connects to the glomerulus of (a), the sizes of the vascular smooth muscle cells are irregular, and their arrangement is disorganized, similar to those seen in older patients (PAM-HE stain). (f): Age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) are observed in an interlobular artery (PAM-HE stain). (g): IgM deposits are detected in glomeruli. (h): IgG deposits are detected in glomeruli. (i): C1q deposits are detected in glomeruli.

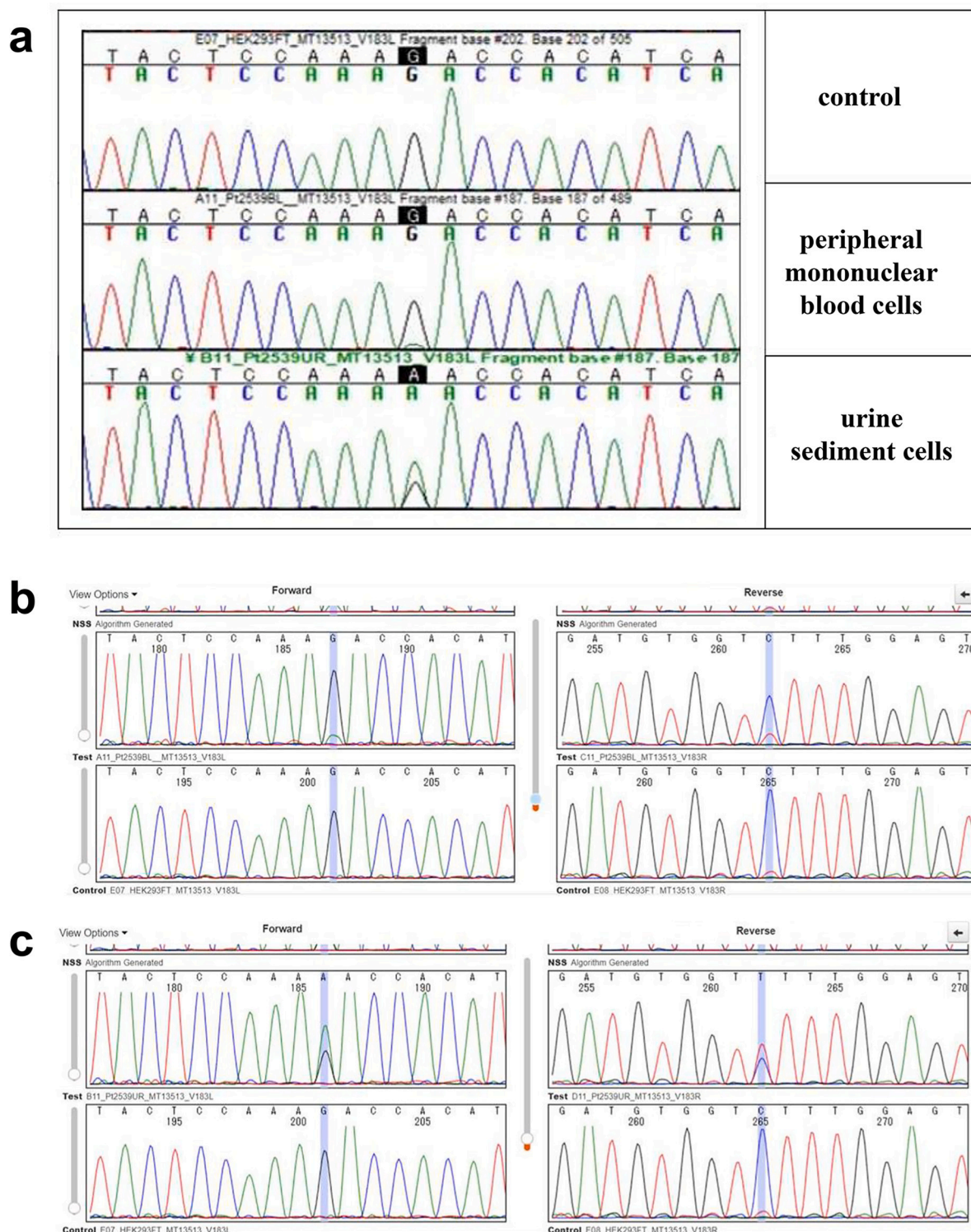


**Fig. 2.** Electron microscopy findings (a): Arrows indicate podocytes filled with increased mitochondria. (b): In a podocyte, abnormally high numbers of mitochondria are seen in the cytoplasm. Foot process effacement (\*) and cytoplasmic vacuolization (\*\*) are observed in this podocyte. (c): Magnification of (b) reveals that the increased mitochondria have lost their organized cristae structure. (d): A podocyte is filled with mitochondria. The foot process of this podocyte is effaced. (e): Magnification of (d) reveals that mitochondria with disorganized cristae increase. (f): The parietal epithelial cells in Bowman's capsule are also filled with abnormal mitochondria.

deposition may be related to a part of the pathogenesis of FSGS caused by *MT-ND5* mutation. Although the reason for C1q deposition in glomeruli is also not clear. Because C1q is reported to bind to mitochondria, it may be associated with the pathogenesis of FSGS by driving oxidative stress [45–47].

This case should be suggestive because it had several characteristic light-microscopical findings of mitochondrial nephropathy. In our case, GSECs with red-coloured cytoplasm were observed with AZAN

trichrome staining. As mitochondria are dyed red by acidic dyes such as AZAN, red-coloured GSECs indicate that they abnormally include many mitochondria [33]. In addition, as COX IV is a subunit of complex IV, COX IV staining can be used to stain mitochondria [35] and may help confirm the presence of abnormally increased numbers of mitochondria [34,48]. Furthermore, age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) were observed in the arterioles and intralobular arteries in this case. Such findings have also been



**Fig. 3.** Genetic analysis (a): The electropherogram from Sanger sequencing indicates that the patient has the m.13513 G > A variant in her peripheral mononuclear blood and urine sediment cells. (b): The rate of heteroplasmy calculated by the MVF software program is 10.3% (forward: 11.6%/reverse: 9.0%) in peripheral mononuclear blood cells (upper panel: patient, lower panel: control). (c): The rate of heteroplasmy is 62.2% (forward: 63.7%/reverse: 60.7%) in urine sediment cells (upper panel: patient, lower panel: control).

reported in other cases of mitochondrial nephropathy [12,34]. Interestingly, red-coloured podocytes (ReCPo) were detected using AZAN trichrome staining, as we recently reported in a case of mitochondrial nephropathy [49]. Like GSECs, ReCPo abnormally include many mitochondria in their cytoplasm [49]. In summary, in cases of FSGS or glomerulosclerosis with unknown aetiologies, the existence of GSECs,

AiDIVs, and ReCPo should be carefully evaluated. COX IV staining may aid in the detection of abnormally increased mitochondrial counts.

We describe the weaknesses of our report. In this case, because mitochondrial disease was suspected based on the pathological findings of abnormal mitochondria, the presence of hearing loss, and high blood lactate levels, we comprehensively examined the genes associated with

mitochondrial diseases. Therefore, genetic FSGS due to other gene mutation [50] cannot be completely ruled out. In addition, because genetic testing was performed only on the patient but not on other family members, it could not be investigated whether they carried the heteroplasmic mitochondrial variant. The parents were already deceased and the two healthy brothers did not consent to genetic analysis. The two her children were still under 18 years old and the patient did not wish genetic test. Furthermore, measurement of MRC enzyme activity using kidney biopsy specimens is an effective for confirming mitochondrial dysfunction [51,52]. However, it could not be performed due to the paucity of kidney specimens for this analysis.

In conclusion, this is the first case report which indicates that the pathogenic variant an *MT-ND5* gene causes accumulation of abnormal mitochondria in podocytes, which leads to forming FSGS.

### Ethics approval and consent to participate

The original study to perform a genetic analysis for mitochondrial diseases was approved by the Ethics Committee of Chiba Children's Hospital (2014-11-05). In addition, the analysis of this case was also approved by the Ethics Committee of Yamanashi Prefectural Central Hospital (2020-16). The patient provided informed consent and permission to participate in the study.

### Consent for publication

Written informed consent was obtained from the patient for the publication of this case report and accompanying images. A copy of the written consent is available for review by the editor of this journal. All authors consented to the publication of the manuscript in *BMC Nephrology*.

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### Authors' contributions

TN drafted the manuscript. TI was involved in the coordination of the study and draft preparation. IN and MW provided clinical information. KH performed the pathological analysis. YY and YK performed molecular genetic studies. YO performed sequence alignment and data curation. KM was involved in the coordination of the study and data curation. YJ was involved in the coordination of the study and clinical information. All authors read and approved the final manuscript.

### Declaration of Competing Interest

The authors declare that they have no competing interests.

### Data availability

Most of the clinical data used and analysed in this case report are presented in this manuscript. More detailed information is available from the corresponding author upon request.

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Original research

# Strategic validation of variants of uncertain significance in *ECHS1* genetic testing

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## ABSTRACT

**Background** Enoyl-CoA hydratase short-chain 1 (*ECHS1*) is an enzyme involved in the metabolism of branched chain amino acids and fatty acids. Mutations in the *ECHS1* gene lead to mitochondrial short-chain enoyl-CoA hydratase 1 deficiency, resulting in the accumulation of intermediates of valine. This is one of the most common causative genes in mitochondrial diseases. While genetic analysis studies have diagnosed numerous cases with *ECHS1* variants, the increasing number of variants of uncertain significance (VUS) in genetic diagnosis is a major problem.

**Methods** Here, we constructed an assay system to verify VUS function for *ECHS1* gene. A high-throughput assay using *ECHS1* knockout cells was performed to index these phenotypes by expressing cDNAs containing VUS. In parallel with the VUS validation system, a genetic analysis of samples from patients with mitochondrial disease was performed. The effect on gene expression in cases was verified by RNA-seq and proteome analysis.

**Results** The functional validation of VUS identified novel variants causing loss of *ECHS1* function. The VUS validation system also revealed the effect of the VUS in the compound heterozygous state and provided a new methodology for variant interpretation. Moreover, we performed multiomics analysis and identified a synonymous substitution p.P163= that results in splicing abnormality. The multiomics analysis complemented the diagnosis of some cases that could not be diagnosed by the VUS validation system.

**Conclusions** In summary, this study uncovered new *ECHS1* cases based on VUS validation and omics analysis; these analyses are applicable to the functional evaluation of other genes associated with mitochondrial disease.

## INTRODUCTION

*ECHS1* encodes short-chain enoyl-CoA hydratase 1 (SCEH), which is responsible for the degradation of branched chain amino acids and fatty acids.<sup>1</sup> Abnormalities in valine metabolism particularly impact the pathogenesis of SCEH deficiency (MIM #616277); accumulation of valine metabolites such as S-(2-carboxypropyl) cysteine (SCPC) and S-(2-carboxypropyl) cysteamine derived from methacrylyl-CoA, and S-(2-carboxyethyl) cysteine (SCEC), S-(2-carboxyethyl) cysteamine

## WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Mitochondrial short-chain enoyl-CoA hydratase 1 deficiency is caused by variants in *ECHS1* gene.
- ⇒ *ECHS1* is the most frequent causative gene of mitochondrial diseases in Japan.

## WHAT THIS STUDY ADDS

- ⇒ High-throughput and efficient assay system was constructed to validate the variants of uncertain significance (VUS) in *ECHS1* and 15 variants were evaluated.
- ⇒ Combination of multiomics analysis and VUS assay led to the diagnosis of five new cases.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Our methodology accelerates VUS evaluation, resulting in increased diagnostic rates and speed of diagnosis.

and 2-methyl-2,3-dihydroxybutyric acid, derived from acrylyl-CoA, is often observed.<sup>2</sup> Pathogenic variants in *ECHS1* mainly cause Leigh encephalopathy presenting elevated plasma lactate and brain MRI abnormalities.<sup>3–7</sup> In addition, mitochondrial respiratory chain complex abnormalities have also been reported to cause mitochondrial dysfunction in cases with *ECHS1* pathogenic variants.<sup>5–7</sup> Many Japanese cases with *ECHS1* pathogenic variants have also been reported.<sup>4 5 7–10</sup> Numerous *ECHS1* variants have been reported all over the world and pathogenic variants are located throughout the *ECHS1* gene. A variant frequently reported in Asians is c.176A>G (NM\_004092.4): Asn59Ser (NP\_004083.3). The expansion in the clinic of genetic testing has resulted in the rapid accumulation of variants of uncertain significance (VUS). That is no exception in mitochondrial diseases, and the VUS number in *ECHS1* is increasing. On the other hand, recent studies have also shown that a valine-restricted diet is effective for cases with *ECHS1* pathogenic variants.<sup>11 12</sup> Quick diagnosis is important for early treatment of SCEH deficiency.

Recently, various approaches have been tried to solve VUS. Functional analysis using cultured cells and model organisms is a powerful validation



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method, providing strong evidence of pathogenicity according to the American College of Medical Genetics and Genomics guidelines.<sup>13</sup> Especially, high-throughput assays combining CRISPR/Cas9 and genome-sequencing technologies are being used for VUS verification in cancer-causing genes.<sup>14–15</sup> Although VUS have been validated for *IVD*, *ACADVL* and *ACAD9*, all causative genes of inborn errors of metabolism,<sup>16–18</sup> there is little research on VUS verification in rare diseases.

In this study, we focused on VUS in *ECHS1*, common in many cases of mitochondrial diseases.<sup>8,9,19</sup> In Japan, Tohoku Medical Megabank Organization (ToMMo) has a genome project for healthy subjects.<sup>20,21</sup> In recessive diseases, the causative variant is often present at a certain frequency within a given race. Therefore, in addition to the VUS found in our previous studies of patients with mitochondrial diseases, we conducted VUS validation of rare variants registered in the Japanese Multi-Omics Reference Panel (jMorp) operated by ToMMo.

Here, we constructed an assay system with ATP measurement using cells deficient in the *ECHS1* gene for systematic VUS verification and validation of heterozygotic variants. Furthermore, we found a novel *ECHS1* variant by multiomics analysis. Coincidentally, the uncharacterised variants were verified by the assay system as being caused by *ECHS1*.

## MATERIALS AND METHODS

### Cell culture and knockout cell generation

Cells were cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (with 4.5 g/L glucose; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Single guide RNAs (sgRNAs) were designed using CRISPRdirect software.<sup>22</sup> The target sequence was as follows: 5'-GGGCCTTGGGCGGTTTCAGT-3'. gRNA oligonucleotides were inserted into a pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene 62988) plasmid as previously described.<sup>23</sup> HEK293FT cells were transfected with PX459 including *ECHS1* targeted sgRNA. Cells were selected using 2 µg/mL puromycin and single cells were isolated. Genomic DNA was extracted from isolated cells, and sgRNA target sites were amplified using KOD FX Neo (Toyobo). Primer sequences are as follows: 5'-CCCATGACCGTCTTCACTCG-3' and 5'-ACATCCCTTCCCACTCTC-3'. PCR products were purified and directly sequenced.

### ATP assay

HEK293FT wild-type (WT) or *ECHS1* knockout (KO) cells were seeded in a collagen-coated 96-well plate (354650, Corning, Arizona, USA) at 1 × 10<sup>4</sup> cells/well with growth medium containing 25 mM glucose. For VUS validation, *ECHS1* KO cells were transfected with 20 ng of expression vectors encoding WT or *ECHS1* variants. One day after plating or transfection, the medium was replaced with 25 mM glucose or 10 mM galactose medium supplemented with dialysed 10% fetal bovine serum (04-011-1A, Biological industries, KibbutzBeit-Haemek, Israel) and L-valine (13046-62, Nacalai Tesque, Kyoto, Japan). Four days after culture in galactose or glucose medium, the ATP content was measured using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega) with a VICTOR Nivo multimode microplate reader (PerkinElmer, Massachusetts, USA).

### RNA-seq

RNA was purified from fibroblasts by the Maxwell RSC simplyRNA Cells Kit and a Maxwell RSC Instrument (Promega).

After quality check by Agilent 2100 and Qubit 2.0, the mRNA was enriched using oligo(dT) beads and rRNA was removed using the Ribo-Zero kit. The mRNA was fragmented randomly by adding fragmentation buffer; then, cDNA was synthesised by using the mRNA template and random hexamer primers, followed by addition of a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I to initiate second-strand synthesis. Second, after a terminal repair, a ligation and sequencing adaptor ligation, the double-stranded cDNA library was completed through size selection and PCR enrichment. Sequencing was performed using 150 bp paired-end reads on a NovaSeq6000 (Illumina). Fastq files were aligned to the GRCh38/hg38 genome by STAR. Gene read counts were quantified by STAR quantMode GeneCounts function. The aligned BAM files were loaded into the Integrated Genomics Viewer and visualised using a Sashimi plot for mRNA splicing analysis.

### Proteome

Samples from fibroblasts were prepared as described previously.<sup>24</sup> The samples were measured in both data-dependent and data-independent modes performed on the Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) as previously described.<sup>24</sup> Finally, 5979 proteins were detected in 16 samples including 2 healthy controls and 14 patients with mitochondrial disease. Then, outlier protein expression analysis was performed using OUTRIDER.<sup>25</sup>

### Western blotting analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were performed as previously described.<sup>19</sup> Five days after transfection, cells were directly harvested by 1x SDS sample buffer (62.5 mM Tris-HCl pH6.8, 2% SDS, 5% sucrose) containing protease inhibitor cocktail (Nacalai Tesque). After sonication, 2-mercaptoethanol was added to the samples to a final concentration of 5%. Prepared samples were denatured for 5 min at 95°C and separated by SDS-PAGE on the 5%–20% gradient polyacrylamide gel (2331730, ATTO, Tokyo, Japan). Proteins were transferred to polyvinylidene fluoride (PVDF) membrane and subjected to western blotting (WB). Each antibody was obtained as follows: *ECHS1* (11 305-1-AP, Proteintech, Illinois, USA), GAPDH (G9545, Sigma-Aldrich), beta-actin (A5441, Sigma-Aldrich).

### Statistics

Data are expressed as mean ± SEM. The statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test using Prism V.9 (GraphPad Software, California, USA).

## RESULTS

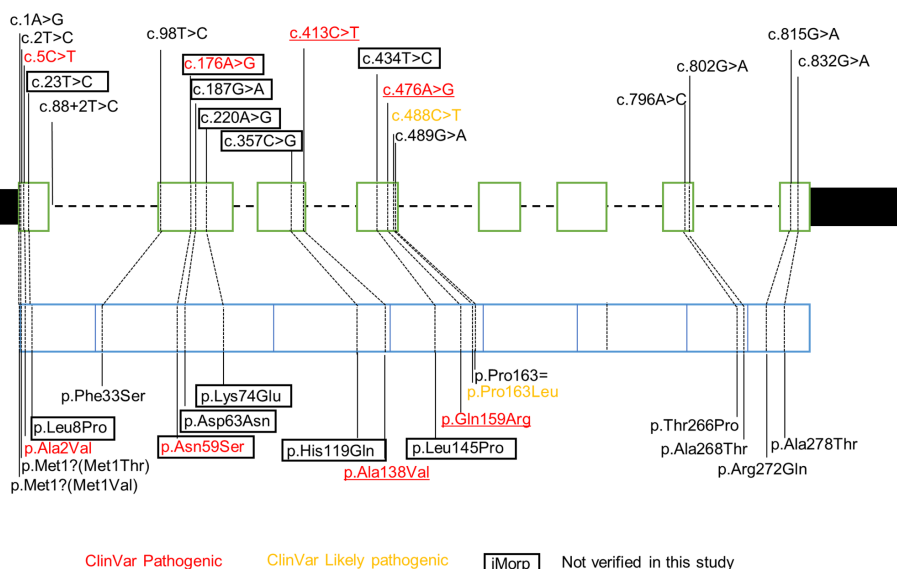
### Cases with *ECHS1* variants

In previous genomic studies on patients with mitochondrial disease including suspected cases (1221 cases), direct Sanger sequencing (4 cases), gene panel sequencing (857 cases) and whole exome sequencing (603 cases (243 cases of panel sequencing have been performed)) led to the discovery of 8 variants in 15 *ECHS1* cases (figure 1A). In Japanese people, the most frequently identified variant was c.176A>G(p.Asn59Ser), followed by c.5C>T(p.Ala2Val)<sup>6,7,9</sup> (figure 1A,B). These variants are common in other Asian cases; the allele frequencies of c.176A>G(p.Asn59Ser) and c.5C>T(p.Ala2Val) are 0.0005769 and 0.0001927 in the gnomAD v3.1.2 East Asian, respectively. Several of these cases also had VUS in ClinVar (as of 20220715,

**A**

Patient ID	DNA (NM_004092.4)	Protein(NP_004083.3)	Patient ID	DNA (NM_004092.4)	Protein(NP_004083.3)
0207	c.5C>T	p.Ala2Val	1038ES	c.5C>T	p.Ala2Val
0207	c.176A>G	p.Asn59Ser	1038ES	c.176A>G	p.Asn59Ser
0255	c.176A>G	p.Asn59Ser	1038YS	c.5C>T	p.Ala2Val
0255	c.413C>T	p.Ala138Val	1038YS	c.176A>G	p.Asn59Ser
0346ES	c.176A>G	p.Asn59Ser	1135ES	c.5C>T	p.Ala2Val
0346ES	c.476A>G	p.Gln159Arg	1135ES	c.176A>G	p.Asn59Ser
0346YS	c.176A>G	p.Asn59Ser	1135YS	c.5C>T	p.Ala2Val
0346YS	c.476A>G	p.Gln159Arg	1135YS	c.176A>G	p.Asn59Ser
0376	c.98T>C	p.Phe33Ser	1553	c.5C>T	p.Ala2Val
0376	c.176A>G	p.Asn59Ser	1553	c.176A>G	p.Asn59Ser
0536	c.1A>G	p.Met1?	2521	c.23T>C	p.Leu8Pro
0536	c.5C>T	p.Ala2Val	2521	c.176A>G	p.Asn59Ser
0775	c.5C>T	p.Ala2Val	2637	c.5C>T	p.Ala2Val
0775	c.88+2T>C		2637	c.176A>G	p.Asn59Ser
			2816	c.23T>C	p.Leu8Pro
			2816	c.176A>G	p.Asn59Ser

**B**



**Figure 1** Summary of *ECHS1* variants. (A) *ECHS1* variants identified from genomic analysis. (B) Gene structure (top) and corresponding amino acids (bottom) of *ECHS1*. Variants registered as pathogenic (red) and likely pathogenic (yellow) in ClinVar and jMorp (square) are shown. The two underlined variants were identified from our genomic analysis study; no experimental variants of uncertain significance verification was performed. *ECHS1*, enoyl-CoA hydratase short-chain 1; jMorp, Japanese Multi-Omics Reference Panel.

and the same after here), such as c.1A>G(p.Met1Val) and c.23T>C(p.Leu8Pro).<sup>8,26</sup> In addition, we selected rare variants (minor allele frequency <0.005 in gnomAD) without taking into account zygosity. We also found heterozygous variants in *ECHS1* with conflicting interpretations of pathogenicity (c.796A>C:p.Thr266Pro and c.832G>A:p.Ala278Thr), VUS (c.815G>A:p.Arg272Gln) or unreported (c.802G>A:p.Ala268Thr). All these variants are rare and may be disease-causing, but no experimental verification of the variants has been performed so far. Variants with low allele frequency have been identified, and a number of them have been designated as VUS. Accurate and quick interpretation of these variants is essential for improved diagnosis.

### VUS validation in *ECHS1*

To perform VUS validation of *ECHS1*, we first generated cells deficient in the *ECHS1* gene by targeting *ECHS1* using the CRISPR/Cas9 system in HEK293FT cells. The *ECHS1* KO cell line had an in-frame deletion of 18 bases in exon 2 of the *ECHS1* gene (figure 2A). This in-frame deletion resulted in loss of *ECHS1* mRNA and protein, as confirmed by qRT-PCR and WB (figure 2B). We also confirmed abnormalities in

the mitochondrial function in *ECHS1* KO cells. In galactose medium, ATP production is largely dependent on mitochondrial respiration.<sup>27</sup> Accordingly, we measured ATP levels of WT and *ECHS1* KO cells after incubation in glucose and galactose media. The ratio of ATP levels under galactose and glucose conditions was stable, and no significant difference was observed between *ECHS1* KO and WT cells under these conditions (figure 2C). The cellular toxicity of *ECHS1* deficiency is due to the accumulation of intermediates in valine metabolism. That explains why a valine-restricted diet is recommended to patients harbouring *ECHS1* pathogenic variants.<sup>12</sup> Consistently, valine addition to the galactose medium led to further reduced ATP levels in *ECHS1* KO cells, in a dose-dependent manner (figure 2D,E). These results suggest that the abnormal mitochondrial function in *ECHS1* KO cells results from the abnormal valine metabolism, as shown in human patients, making it a suitable model to validate VUS of *ECHS1*. Accordingly, we performed a functional verification of VUS using *ECHS1* KO cells expressing VUS under additional valine supplementation.

First, 15 variants identified from previous genomic studies and jMorp were selected for functional validation. Six rare



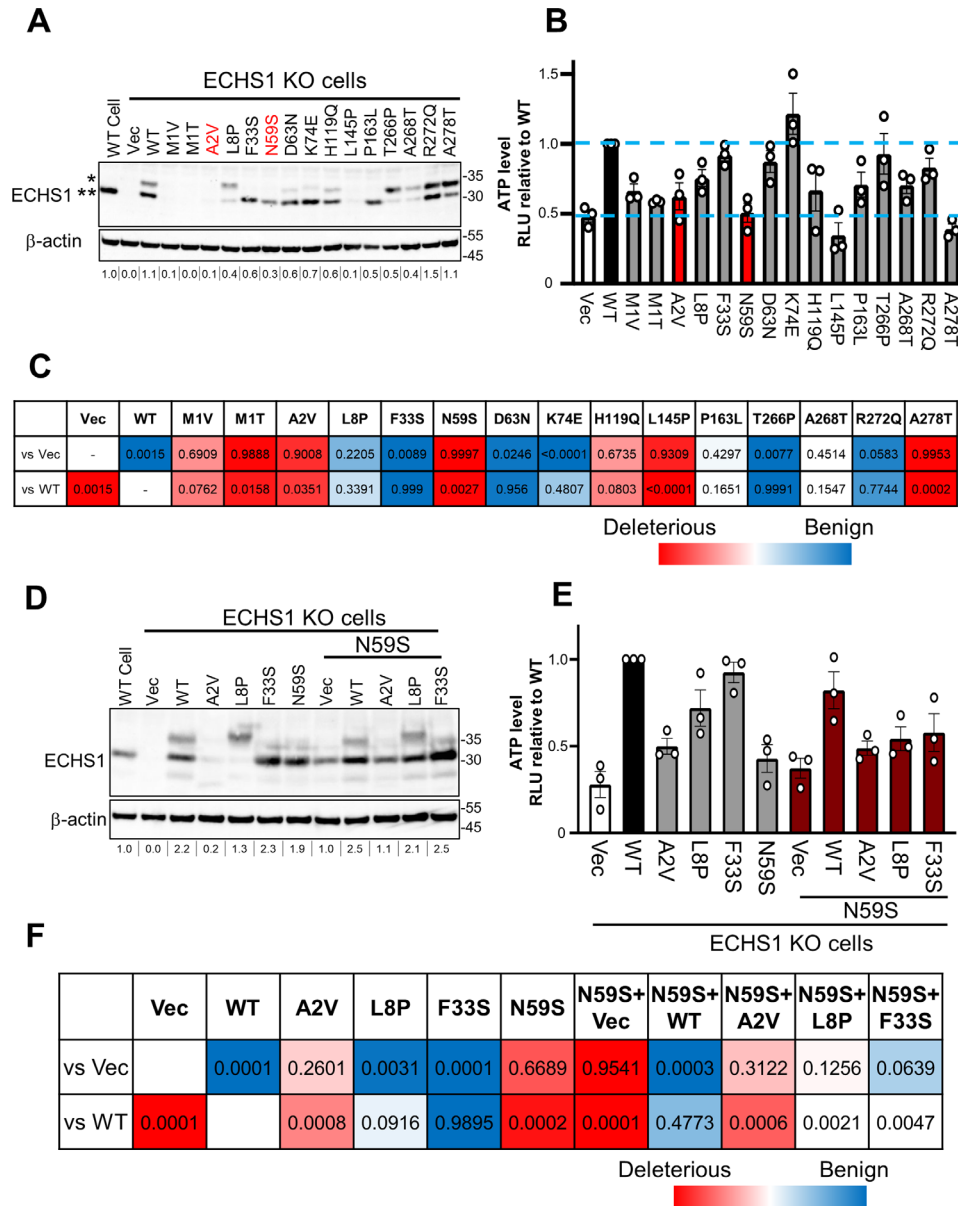
**Figure 2** Characterisation of *ECHS1* KO cells. (A) Genomic analysis of *ECHS1* KO cells by Sanger sequencing. (B) *ECHS1* expression levels in WT and *ECHS1* KO HEK293FT cells were quantified by qRT-PCR (left). Whole cell extracts from WT and *ECHS1* KO HEK293FT cells analysed by immunoblotting using the indicated antibodies. Single and double asterisks indicate uncleaved and cleaved forms, respectively (right). (C) ATP assay of WT and *ECHS1* KO HEK293FT cells cultured in glucose or galactose medium. The graph shows the ATP level in galactose medium divided by galactose. (D, E) ATP assay of WT and *ECHS1* KO HEK293FT cells treated with the indicated L-valine concentrations under glucose (D) and galactose (E) medium. Bar graphs represent the average ATP level in each condition from three biological independent experiments. Error bars:  $\pm$ SEM. Statistical analysis was performed using analysis of variance followed by Dunnett's test. KO, knockout; RLU, relative luciferase unit; WT, wild-type.

variants out of the 15 variants were registered in jMorp (4.7KJPN released on 20190902; [figure 1B](#), online supplemental table 1). The c.23T>C(p.Leu8Pro) and c.176A>G(p.Asn59Ser) variants were found in our patients as well as registered in jMorp (4.7KJPN). Other four variants were not identified in our genome analysis of patients with mitochondrial disease. *ECHS1* is a mitochondria-localised protein with a mitochondrial targeting sequence (MTS) at the N-terminal 1–22 amino acid residues. Four variants were located at the translation initiation site and the MTS. c.176A>G(p.Asn59Ser) and c.5C>T(p.Ala2Val), two of the most frequent variants, were selected as positive controls for pathogenic variants. We compared *ECHS1* KO cells transfected with an empty vector and *ECHS1* WT cDNA to *ECHS1* KO cells transfected with an *ECHS1* gene including VUS.

Exogenously expressed *ECHS1* shows uncleaved and cleaved forms ([figure 3A](#), single and double asterisks, respectively). Some variants were superiorly expressed in an uncleaved form. Further,

we found a significant decrease in the expression level for vectors expressing variants located in the MTS and enoyl-CoA hydratase conserved site (131–151 residues) including at leucine 145 ([figure 3A](#)).

Next, we examined VUS functions in the mitochondria. *ECHS1* WT expression restored ATP levels in *ECHS1* KO cells ([figure 3B](#)). The ATP levels of KO cells transfected with each variant were compared with that of KO cells transfected with empty vector or *ECHS1* WT expression vector; changes in ATP levels were evaluated by one-way ANOVA followed by Dunnett's test ([figure 3C](#)). In addition to p.Ala2Val(c.5C>T) and p.Asn59Ser(c.176A>G), reported as pathogenic variants, p.Met1Val(c.1A>G) and p.Met1Thr(c.2T>C) altered start codons, and p.Leu145Pro(c.434T>C) failed to restore ATP levels probably due to significantly reduced protein expression. p.Leu8Pro(c.23T>C) showed a significant reduction in the lower band considered to correspond to mature *ECHS1*, but partially restored ATP levels in KO cells. p.His119Gln(c.357C>G),

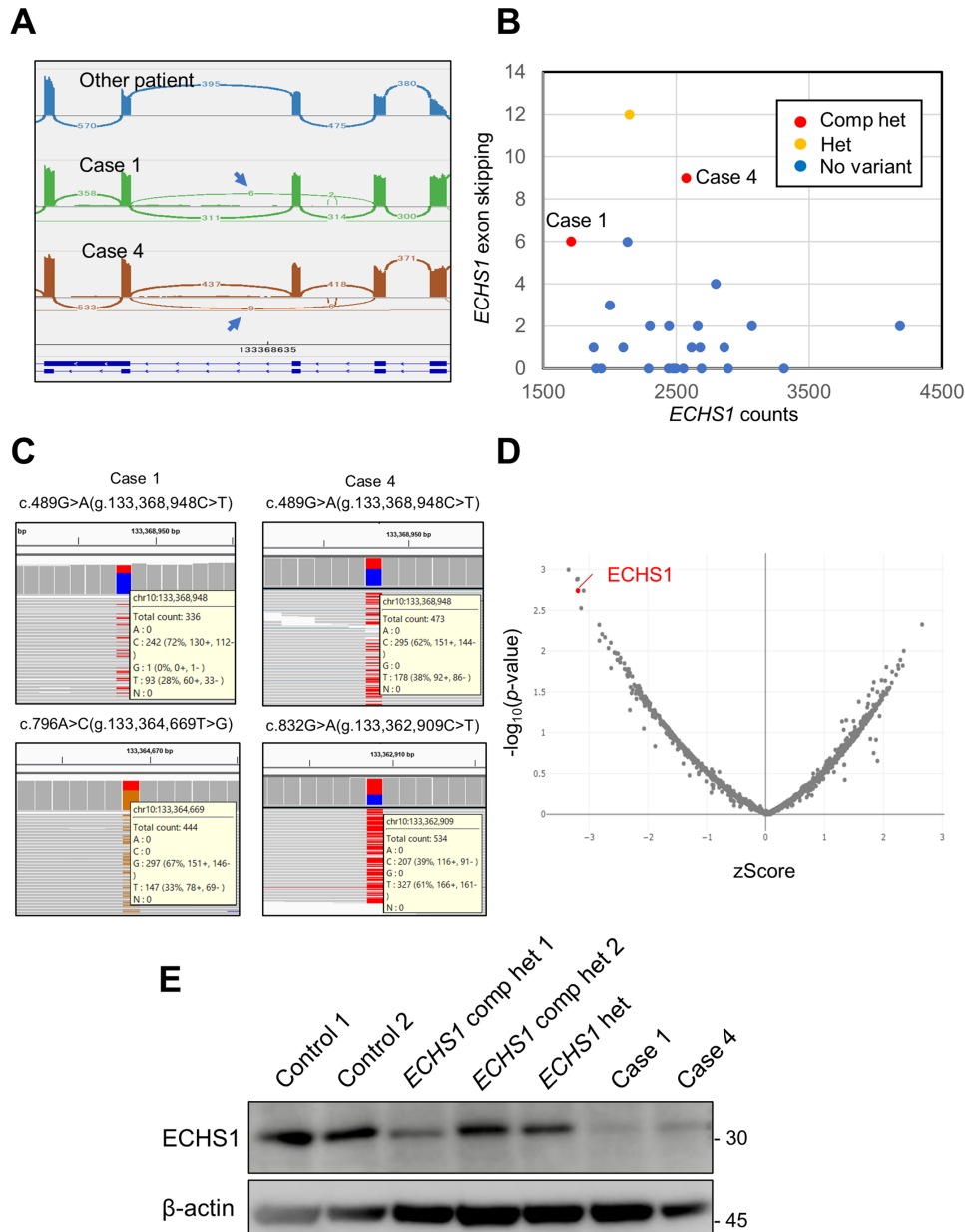


**Figure 3** Validation of *ECHS1* VUS. WT and *ECHS1* KO HEK293FT cells transfected with the indicated expression vectors subjected to immunoblotting analysis (A) and ATP assay (B). (A) Whole cell extracts were analysed by immunoblotting using the indicated antibodies. Single and double asterisks indicate uncleaved and cleaved forms, respectively. Quantification analysis of *ECHS1*/β-actin relative to WT cell is shown below blotting for β-actin. (B) ATP assay 4 days after treatment with L-valine (0.8 mM). Bar graphs represent the average ATP level in each condition from three biological independent experiments. Error bars: ±SEM. Red bars: pathogenic variants. Grey bars: VUS. (C) Statistical analysis of (B) using ANOVA followed by Dunnett’s test. The colour scale shows the p value compared with vector (vs Vec, statistically different red to blue) and WT *ECHS1* (vs WT, statistically different blue to red). (D) Whole cell extracts from WT and *ECHS1* KO HEK293FT cells transfected with the indicated expression vectors analysed by immunoblotting using the indicated antibodies. Quantification analysis of *ECHS1*/β-actin relative to WT cell is shown below blotting for β-actin. (E) ATP assay of *ECHS1* KO HEK293FT cells expressing the indicated expression vectors treated with additional L-valine (0.8 mM) for 4 days. Bar graphs represent the average ATP level in each condition from three biological independent experiments. Error bars: SEM. (F) Statistical analysis of (B) using ANOVA followed by Dunnett’s test. The colour scale shows the p value compared with vector (vs Vec, statistically different red to blue) and WT *ECHS1* (vs WT, statistically different blue to red). ANOVA, analysis of variance; KO, knockout; RLU, relative luciferase unit; VUS, variants of uncertain significance; WT, wild-type.

p.Pro163Leu(c.488C>T), p.Ala268Thr(c.802G>A) and p.Ala278Thr(c.832G>A) variants had higher expression levels but failed to fully improve ATP levels equivalent to WT, suggesting that these variants were deleterious.

Although p.Leu8Pro(c.23T>C) and p.Phe33Ser(c.98T>C) were reported as pathogenic variants,<sup>19,26</sup> ATP assays demonstrate a mild functional decline. Since p.Leu8Pro(c.23T>C) and p.Phe33Ser(c.98T>C) were identified as a compound heterozygote with p.Asn59Ser(c.176A>G), a non-functional

variant (figure 3B), we hypothesised that p.Leu8Pro(c.23T>C) and p.Phe33Ser(c.98T>C) might be mild deleterious variants capable of restoring KO cells but not KO cells expressing highly deleterious variants. To test this hypothesis, we reproduced compound heterozygous genotypes by transfecting *ECHS1* KO cells with two different variants (figure 4A). The ATP assay demonstrated that WT restored KO cells on co-transfection with p.Asn59Ser(c.176A>G), whereas, as expected, all three variants did not show recovery (figure 3E,F). These results demonstrate



**Figure 4** Abnormal splicing and allele-biased gene expression resulting aberrant protein expression. (A) RNA-seq data from two patients with *c.489G>A* (p.P163=) showing reads suggestive of splicing abnormalities in exons with *c.489G>A*. (B) Read counts of the *ECHS1* gene are plotted on the horizontal axis and the number of detected exon skipping is plotted on the vertical axis. Gene counts were calculated by STAR, and the number of exon skipping was extracted from the Sashimi plot data. (C) The ratio of *c.489G>A* and *c.796A>C* and *c.832G>A* variants on the IGV viewer. In two cases, the allele expression with *c.489G>A* was decreased. (D) OUTRIDER analysis illustrating protein expression in a volcano plot. *ECHS1* was detected as a protein with a large decrease in expression. (E) Western blotting for *ECHS1* in patients with *ECHS1* pathogenic variants and controls. Compared with previously reported *ECHS1* cases, case 1 and case 4 showed significantly reduced *ECHS1* expression.  $\beta$ -actin was detected as a loading control. *ECHS1*, enoyl-CoA hydratase short-chain 1.

that our system is potentially valuable to validate the pathogenic nature of the compound heterozygous state.

#### Validation of *ECHS1* variants from omics analysis

Multimiomics analysis is an effective and useful method in the search for causative genes. In this study, multiomics analysis was conducted together with VUS validation to improve the efficiency and speed of genetic diagnosis. Here, we identified *ECHS1* decreases in two cases (case 1 and case 4) with multiomics analysis and found a common *c.489G>A* (p.Pro163=) variant. We further re-searched for cases with *c.489G>A* (p.Pro163=) from

our previous genome analysis data and finally identified three cases (cases 2, 3 and 5). In case 1, *c.796A>C* (p.Thr266Pro) variant was identified; in case 2, *c.802G>A* (p.Ala268Thr) variant was identified; in cases 3 and 4, *c.832G>A* (p.Ala278Thr) variant was identified; in case 5, *c.833C>T* (p.Ala278Val) variant was identified (table 1, online supplemental figure 2). In addition, these cases also had *c.489G>A* (p.Pro163=) variant, identified in a Samoan family and a very frequent variant (0.01156) in gnomAD v3.1.2 East Asian populations, and suggested to exhibit splicing abnormalities.<sup>28</sup> In the present study, RNA-seq analysis was performed on case 1 and case 4. Then, we identified

**Table 1** Patient summary of cases with *ECHS1* variants

Case ID	Sex	Age range at onset (years)	Symptoms	Complex deficiency	OCR	Var 1	Var 2
1	Female	<1	Poor suckling, metabolic acidosis	CIV deficiency (Fb)	Down (Fb)	c.796A>C (p.Thr266Pro)	c.489G>A (p.Pro163=)
2	Male	>3–5	Gait disorder, nystagmus, mental retardation, MRI abnormality	Normal (Fb)	N.T.	c.802G>A (p.Ala268Thr)	c.489G>A (p.Pro163=)
3	Male	1–3	Listlessness, mental retardation, regression after exanthema subitum, MRI abnormalities, deafness, fatigue, hypotonia	Normal (Fb)	Down (Fb)	c.832G>A (p.Ala278Thr)	c.489G>A (p.Pro163=)
4	Male	<1	Developmental delay, convulsion, MRI abnormalities, regression	Normal (Fb)	Down (Fb)	c.832G>A (p.Ala278Thr)	c.489G>A (p.Pro163=)
5	Male	<1	Leigh syndrome, increase of 2-methyl-2,3-dihydroxybutyric acid	Normal (Fb)	Down (Fb)	c.833C>T (p.Ala278Val)	c.489G>A (p.Pro163=)

Complex enzyme activity was defined by <40% decrease. For OCR, a value <71.6% was used as a diagnostic criterion. CIV, mitochondrial respiratory chain complex IV; Fb, fibroblast; OCR, oxygen consumption rates.

sequence reads showing exon skipping as a consequence of the c.489G>A(p.Pro163=) variant (figure 4A). To examine whether exon skipping actually increased in cases with c.489G>A(p.Pro163=), we plotted RNA-seq counts of the *ECHS1* gene and the number of detected reads showing exon skipping (figure 4B). We analysed 26 RNA-seq data, including case 1 and case 4, as well as one case with heterozygous c.489G>A(p.Pro163=); exon skipping increased in these samples. We also found that exon skipping is detectable in case 1 and case 4 fibroblasts and that blocking nonsense-mediated mRNA decay using puromycin and cycloheximide significantly increased its levels by RNA-seq and qRT-PCR analysis (online supplemental figure 3). In addition, after counting the allele numbers at the c.796A>C(p.Thr266Pro) and c.832G>A(p.Ala278Thr) variant positions showed a bias in allele expression (figure 4C). This indicates that the significantly reduced expression of alleles with c.489G>A(p.Pro163=) in case 1 and case 4 fibroblasts. Proteome analysis in case 1 fibroblasts confirmed a significant decrease in *ECHS1* expression (figure 4D). Furthermore, previous studies experimentally demonstrated that case 4 leads to decreased *ECHS1* expression and enzymatic activity, as well as accumulation of intermediate products of valine metabolism.<sup>29</sup> WB analysis further confirmed a marked decrease in protein expression in case 1 and case 4 fibroblasts (figure 4E). We here concluded that synonymous and non-synonymous substitution combinations are disease-causing in these cases.

## DISCUSSION

*ECHS1* is one of the most frequent genes found in patients with mitochondrial diseases. In addition to our genetic study,<sup>7–9</sup> *ECHS1* has been reported as a frequent cause of mitochondrial disease in genetic studies of Leigh encephalopathy in Asian countries such as China<sup>30</sup> and Korea.<sup>31</sup> The length of the coding region is 873 bp. About 250 variants have been reported in ClinVar, of which about 40 are pathogenic/likely pathogenic and nearly 80 have been registered as VUS or with conflicting interpretations of pathogenicity. However, the molecular mechanism by which *ECHS1* variants cause disease is poorly understood. In this study, we established a system to functionally validate *ECHS1* variants, finding some potential pathogenic variants.

Resolving VUS is a major challenge in various diseases, because VUS continue to accumulate under circumstances where genome analysis is becoming more common. Genome sequence projects in healthy individuals have revealed allele frequencies in various races. The accumulation of genome analysis information

is expected to lead to the discovery of novel pathogenic variants, since rare variants also found in healthy individuals can be pathogenic. Against this background, it may be possible to make a proactive evaluation of variants not yet associated with the disease. Based on the above, we could identify novel pathogenic variants in this study. Furthermore, the combination of RNA-seq, proteomics and conventional genomic analysis enabled a reliable diagnosis.

*ECHS1* is a nuclear-encoded protein transported into the mitochondria by the MTS.<sup>32</sup> MTS locates at the N-terminus and is cleaved in the mitochondria by mitochondrial proteases.<sup>33</sup> There are three points involved in pathogenicity: (1) expression level, (2) location and (3) function. As reported that *ECHS1* is degraded through ubiquitin-proteasome pathway in cancer cells,<sup>34</sup> inhibition of proteasome degradation by MG132 leads to subtle increase in the protein level of all *ECHS1* variants tested in this study (online supplemental figure 1C). As shown in figure 3A, p.Met1Val (c.1A>G), p.Met1Thr(c.2T>C) and p.Ala2Val(c.5C>T) were weakly expressed. Consistently, these variants failed to restore ATP decrease in *ECHS1* KO cells. The p.Leu8Pro(c.23T>C) variant was detected superiorly in the uncleaved form (figure 3A), suggesting mistargeting to the mitochondria. p.Leu145Pro(c.434T>C) variant showed a weak signal at the estimated molecular weight (figure 3A) and was detected at the high molecular weight (higher than 70 kDa; online supplemental figure 1C). *ECHS1* is known to form a dimer of 2 trimers,<sup>35–36</sup> but p.Leu145Pro(c.434T>C) failed to restore KO cells, suggesting that these high molecular weight forms are abnormal oligomerisation or aggregation. As with p.Asn59Ser(c.176A>G), p.Leu145Pro(c.434T>C) is likely to be a functionally disrupted variant. Interestingly, c.434T>C(p.Leu145Pro) is a variant reported only in jMorp and not previously identified in patients with the disease. The variant registered in jMorp has not been reported as a cause of SCEH deficiency at this time, but it is expected to be found in Japanese cases with this variant in the future. We propose that those who identify variants in *ECHS1* by the genetic test will refer to our variant evaluation study as evidence and that this will lead to a solution to the cause of the disease in the future.

p.Leu8Pro(c.23T>C) and p.Phe33Ser(c.98T>C), considered 'Likely benign' variants from VUS validation experiments (figure 3B,C), were validated by coexpressing with the p.Asn59Ser(c.176A>G) variant (figure 3E,F). By validating

the assumption of compound heterozygosity in combination with a pathogenic variant, we could obtain data accurately reflecting the functional evaluation of hypomorphic variants. The assay was effective for variants in the borders, for which very subtle validation results were obtained. Despite its higher experimental complexity, the assay can be performed while maintaining the conventional throughput, and the validation targets can be expanded. In our reported *ECHS1* mutant cases, the most common combinations are c.176A>G(p.Asn59Ser) and c.5C>T(p.Ala2Val). Since c.176A>G(p.Asn59Ser) has a higher allele frequency than c.5C>T(p.Ala2Val), homozygous cases for c.176A>G(p.Asn59Ser) should be more frequent, but no patients homozygous for c.176A>G(p.Asn59Ser) have been found. This suggests that c.176A>G(p.Asn59Ser) may be so harmful that in homozygosity it may result in severe developmental abnormalities in the prenatal period. The high number of c.176A>G(p.Asn59Ser) and c.5C>T(p.Ala2Val) combinations may be due to c.5C>T(p.Ala2Val) having a smaller functional loss than p.Asn59Ser(c.176A>G), as shown experimentally in this study. c.176A>G(p.Asn59Ser) is only viable in a compound heterozygous state with less deleterious variants. The combination with milder variants such as p.Leu8Pro(c.23T>C) and p.Phe33Ser(c.98T>C), verified in this study, might allow normal development until birth. In other words, this combined assay could first accurately indicate functional abnormalities for variants with intermediate abnormality between normal and completely defective, being a very effective and essential method for variant evaluation.

Mostly, in silico predictions and experimental validation of most variants are comparable. On the other hand, in silico predictions for some variants differ from our validation experiments; therefore, validation experiments are important for such variants. For example, p.Ala2Val(c.5C>T) reported as pathogenic, was not highly damaging on in silico predictions (online supplemental table 1). However, the validation results suggest that although the experimental data indicate an effect on protein gene expression itself, the functional effect may not be significant. A similar trend was observed for p.Leu8Pro(c.23T>C), expected to have a significant effect on protein localisation but relatively little effect on protein function. p.His119Gln(c.357C>G) was also rated mostly tolerant or benign in silico, but experimental validation suggested that the variant negatively affects gene function. The major achievement of this study is providing quantitative experimental validation data for each variant on the same functional platform. We are expanding the validation of other genes; in particular, VUS validation of genes involved in respiratory chain complex I confirmed this to be a reproducible and efficient analysis.

In this validation, we focused on variants specific to Japanese and Asian populations. Naturally, this should be further expanded to include variants from other ethnicities. Our assay system is based on a very simple method and can easily be expanded to other VUS, by including gnomAD and ClinVar variants. In the present study, VUS was verified for jMorp (4.7KJPN) variants, but since then, the jMorp data have been updated and the number of registrations has increased. While planning our validation experiment, the variant data of 4.7KJPN were registered, from which we extracted variants with very low allele frequencies (online supplemental table 2). However, there are now 38KJPN, with 20 more registrations from our validated variants. The data are being updated at an accelerated pace, with 14KJPN in 2021 and 38KJPN in 2022. Given the constant

updates to the database, it is important to verify these variants in the future.

*ECHS1* protein loss is considered to have a threshold of 30%–40% for disease.<sup>28</sup> This is shown by the analysis with c.489G>A(p.Pro163=) and c.832G>A(p.Ala278Thr) from the study of Simon *et al.* Those homozygous for c.489G>A (p.Pro163=) did not develop the disease, despite showing a protein expression decrease around 40%. On the other hand, the combination of p.Pro163=(c.489G>A) and p.Ala278Thr(c.832G>A) showed <30% protein expression and developed the disease. p.Ala278Thr(c.489G>A) is considered highly damaging in the previous study, as it was found to be deleterious in the VUS validation experiment. Although p.Ala278Val(c.833C>T) has not been validated, we think it likely to be as deleterious as p.Ala278Thr(c.832G>A) since many scores in silico also indicated damaging effects. For p.Thr266Pro(c.796A>C), no significant decrease in ATP levels was observed in VUS validation. However, abnormalities at the protein level were evident from the proteome and immunoblotting. VUS expression experiments with the p.Thr266Pro(c.796A>C) variant also showed reduced *ECHS1* expression (figure 3A). Considering these results, p.Thr266Pro(c.796A>C) might have less functional loss and more impact on protein expression. In addition, the forced expression system showed a protein decrease not expected to significantly reduce ATP levels. In protein expression validation (online supplemental figure 1), the amount of mature *ECHS1* synthesised from p.Thr266Pro(c.796A>C) was lower than that of immature *ECHS1*, suggesting that this variant has a significant effect on protein expression and maturation. For p.Ala268Thr(c.802G>A), a mild decrease in ATP levels was observed. Since the value of p.Ala268Thr(c.802G>A) was similar to that of p.Pro163Leu(c.488C>T), reported as likely pathogenic in ClinVar, p.Ala268Thr(c.802G>A) was considered to have an effect on protein function. Case 2 with the c.802G>A(p.Ala268Thr) variant had later onset and milder symptoms than other *ECHS1* cases, suggesting that the variant itself has a milder effect. c.489G>A(p.Pro163=), despite its high allele frequency (0.01156 in gnomAD v3.1.2 East Asian, 0.00789 in the jMorp (38KJPN)), is a possible causative variant; its combination with other pathogenic variants leads to disease development. We found five cases with c.489G>A(p.Pro163=) and another rare variant in this study. There have been very few reports of such a high frequency variant in studies of mitochondrial diseases; this variant might be responsible for the increased frequency of cases with *ECHS1* pathogenic variants. However, variants without amino acid substitutions have been overlooked as benign in previous genetic diagnoses. Given the high potential number of patients with c.489G>A(p.Pro163=), it is expected that the number of cases with c.489G>A(p.Pro163=) will further increase because of our findings.

Our VUS verification system has some limitations. Even pathogenic variants may be missed in this experimental system. In fact, p.Pro163Leu(c.488C>T), registered as likely pathogenic in ClinVar, showed a mild score. Thus, variants with intermediate ratings could be pathogenic. In such cases, it would be necessary to validate them using an assay system that assumes compound heterozygosity. Moreover, since this is a forced expression system, variants that cause protein stability or splicing abnormalities may be missed. To extract these variants, other assay systems and functional experiments using patient specimens are required.

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## BRIEF COMMUNICATION



# Novel *ITPA* variants identified by whole genome sequencing and RNA sequencing

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Approximately 80% of rare diseases have a genetic cause, and an accurate genetic diagnosis is necessary for disease management, prognosis prediction, and genetic counseling. Whole-exome sequencing (WES) is a cost-effective approach for exploring the genetic cause, but several cases often remain undiagnosed. We combined whole genome sequencing (WGS) and RNA sequencing (RNA-seq) to identify the pathogenic variants in an unsolved case using WES. RNA-seq revealed aberrant exon 4 and exon 6 splicing of *ITPA*. WGS showed a previously unreported splicing donor variant, c.263+1G>A, and a novel heterozygous deletion, including exon 6. Detailed examination of the breakpoint indicated the deletion caused by recombination between Alu elements in different introns. The proband was found to have developmental and epileptic encephalopathies caused by variants in the *ITPA* gene. The combination of WGS and RNA-seq may be effective in diagnosing conditions in proband who could not be diagnosed using WES.

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Identifying the differentiating disease based on symptoms and general examination is often difficult in rare intractable diseases. Comprehensive genetic testing is usually essential to make a definitive diagnosis of an incurable disease. In recent years, whole-exome sequencing (WES) and whole-genome sequencing (WGS) have aided in determining the molecular cause of rare and undiagnosed diseases. WES has enabled the diagnosis of conditions in ~35% of the patients [1]. However, the large majority of these patients have undiagnosed conditions. Variants in deep intronic/intergenic regions are usually undetectable because they are not covered by WES. On the contrary, WGS provides comprehensive information on the human genome, including deep intronic/intergenic regions. The technique has been reported to be appropriate for increasing the diagnostic rates of rare disorders [2, 3]. RNA-seq could detect aberrant expression [4], aberrant splicing, and mono-allelic expression, which makes it a powerful method for variant interpretation. We performed WGS and RNA-seq on a case that could not be diagnosed using WES, which revealed a novel splicing variant and a deletion of ~3.4 kb via recombination between two Alu elements in *ITPA*. Variants in *ITPA* are known to cause developmental epileptic encephalopathy 35 (MIM:616647). In addition to neurological symptoms, the family had recurrent episodes of dilated cardiomyopathy caused by *ITPA* deficiency.

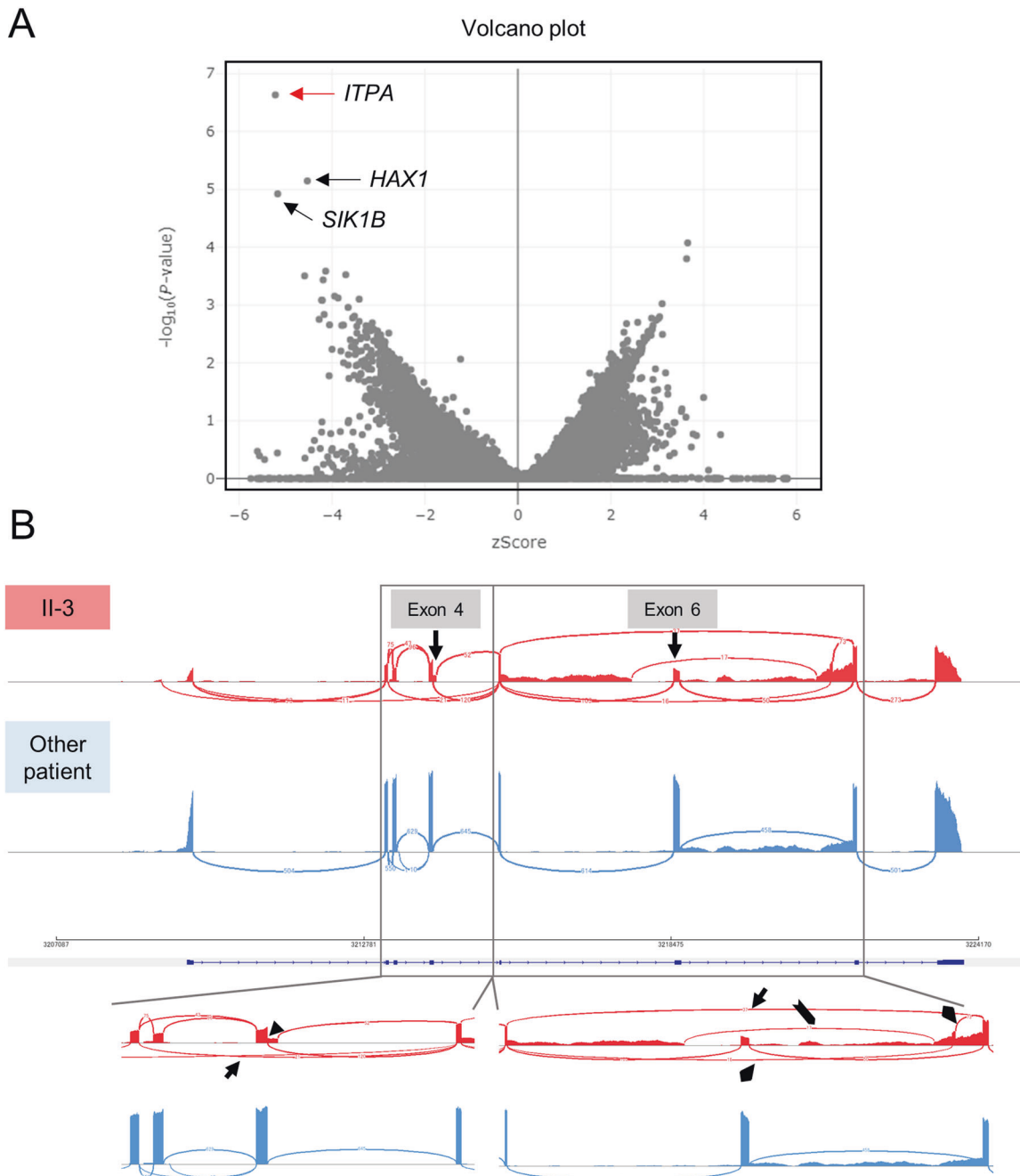
The proband (II-3) was the third child of nonconsanguineous parents. She was born at the 39th gestational week via spontaneous vaginal delivery with a birth weight of 2565 g (−1.6 SD), a body length of 48.8 cm (−0.3 SD), and an occipito-frontal circumference of 32.5 cm (−0.6 SD). The Apgar score was 8/9. She had hypotonia, mild feeding difficulties, and refractory epilepsy at birth. At 11 months of age, the proband had not yet acquired a fixed neck. She is now 6 years old and has refractory epilepsy associated with severe developmental delay and requires percutaneous endoscopic gastrostomy for nutritional support. Her symptoms are similar to those of dilated cardiomyopathy, with the left ventricular ejection fraction being ~45% that has been stabilized by β-blocker treatment. Her two elder brothers had a similar presentation. The eldest brother was born at 40 weeks of gestational age with a birth weight of 3392 g (0.7 SD). He exhibited myotonia, feeding difficulties, and poor weight gain. Febrile seizures began at 10 months of age, followed by refractory epilepsy at 14 months of age. The second brother was born at 39 weeks of gestational age with a birth weight of 2730 g (−1.1 SD). He was diagnosed with congenital hypotonia at birth and, then at 4 months of age, began having involuntary movements, seizures, and epileptic gaze deviations. Finally, her eldest brother and the second brother died of dilated cardiomyopathy at 5 and 3 years of age, respectively.

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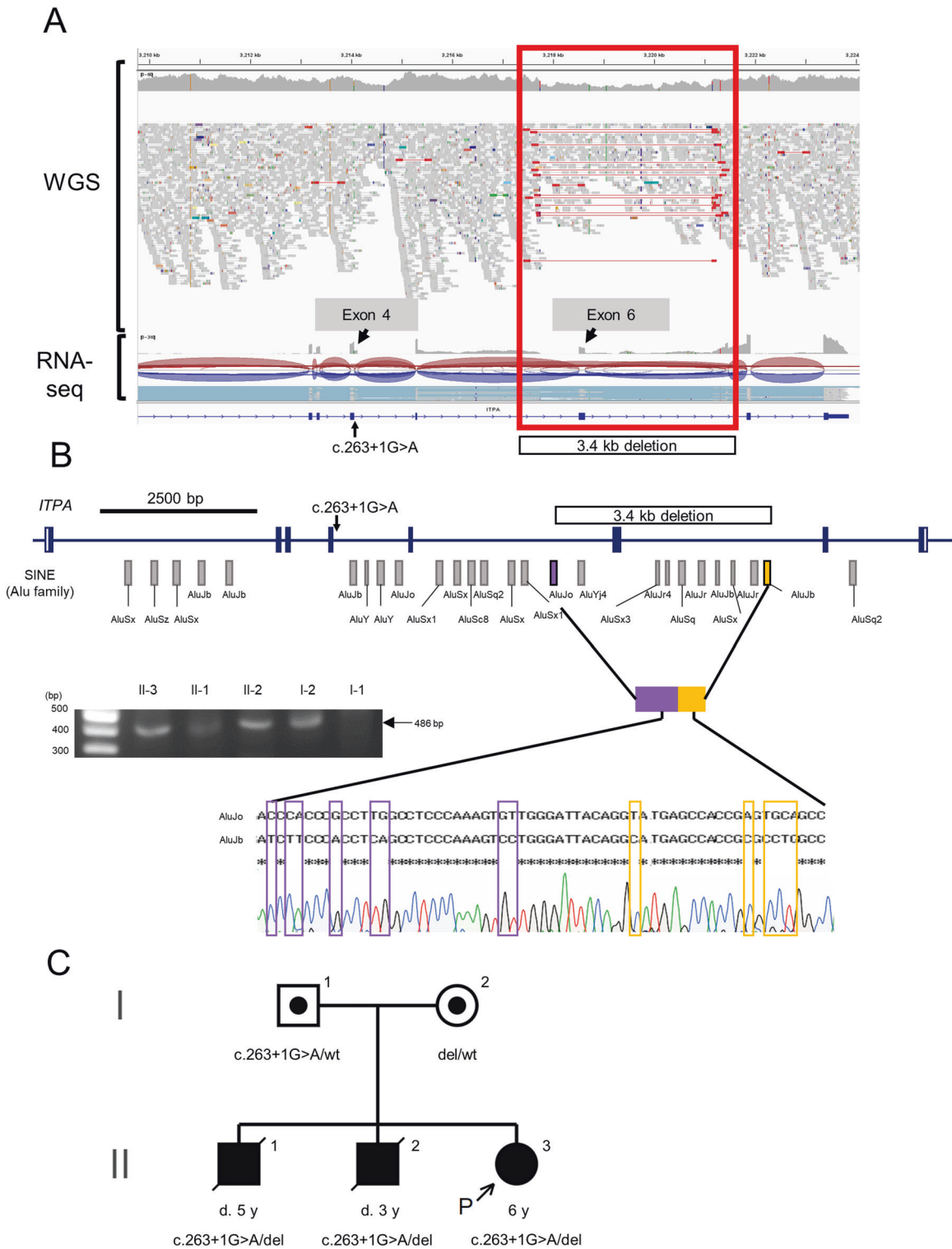
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**Fig. 1** Abnormal expression of *ITPA* revealed using RNA-seq. **A** Volcano plot at gene level from OUTRIDER analysis. RNA-seq is performed on 35 patients with suspected mitochondrial disease (undiagnosed cases) including the proband. The RNA-seq data of proband was compared with those of 34 cases. We used the patient population as a control and extracted outlier genes by using OUTRIDER software. *ITPA*, *HAX1* and *SIK1B* were genes that were significantly downregulated. **B** IGV Sashimi plot of RNA-seq data of II-3 and control at the whole *ITPA* gene (upper panel). At the bottom are zoomed-in views of exon 4 and exon 6. The arrows indicate exon skipping, and the arrowhead indicates exon extension. Diamond-shaped arrows indicate abnormal splicing events. The chevron arrow shows the location of the deletion

The disease-causing variants could not be determined by the trio-WES analysis. Initially, we performed RNA-seq using the proband's fibroblast cell (F1431). RNA-seq analysis using OUTRIDER software [4] revealed decreased expression of *ITPA* (Fig. 1A). We first found exon elongation on the 3' side of exon 4 (Fig. 1B), and exon skipping of exon 3 and 4 were detected as well (Fig. 1B and Supplementary Fig. 1). We observed a decrease in the read depth of exon 6 when compared with the other exons (Fig. 1B). In addition to exon 6 depth reduction, exon skipping and several abnormal splicing events were also detected (Fig. 1B). The Sashimi plot revealed skipping of exon 6 and other connections indicating abnormal splicing in the middle of introns

(Fig. 1B and Supplementary Fig. 1). By performing reverse transcription-PCR in patient cells with suppressed nonsense-mediated mRNA decay, we found that these aberrant transcripts undergo degradation (Supplementary Fig. 2). A decrease in the expression of *HAX1* and *SIK1B* was also observed (Fig. 1A). The pathogenic heterozygous variant c.256C>T (p.Arg86Ter) in *HAX1* (NM\_006118.4) was identified from WGS. *HAX1* has been reported to cause Neutropenia, severe congenital 3, autosomal recessive (MIM: 610738). The blood tests showed mild decrease in neutrophils (1585 cells/ $\mu$ l; neutropenia is defined as less than 1500 cells/ $\mu$ l) that might be caused by the heterozygous variant in *HAX1*. *SIK1B* encodes a putative serine/threonine-protein kinase



**Fig. 2** Variants in *ITPA* revealed using WGS. **A** The deletion around exon 6 is indicated by IGV. Red indicates paired reads in distant regions. **B** The region surrounding exon 6 was amplified using PCR and sequenced using Sanger sequencing. The deletion was probably caused by recombination between AluJo (purple) in intron 5 and AluJb (orange) in intron 6. The Alu sequences were present in a mixed state, with purple derived from AluJo and orange from AluJb. **C** Pedigree of the family. Affected members are indicated with filled symbols. Individuals labeled with dots are carriers. The arrow indicates the index proband

protein, but its function is unknown. No variant in *SIK1B* has been detected by WGS. An association between *ITPA* deficiency and decreased expression of these genes has not been reported to date.

We performed WGS to search for deep intronic and intergenic variants. Analysis of structural variation (SV) and copy number

variation (CNV) from the WGS data using Manta and Lumpy identified a heterozygous deletion of 3.4 kbp containing exon 6 (Fig. 2A, B). Furthermore, we found a heterozygous variant c.263+1G>A (NM\_033453.4), in *ITPA*, which is likely to be involved in the extension of exon 4. The integrative genomics viewer (IGV)

screen snapshot showed a prominent decrease in depth around intron 5, exon 6, and intron 6. Read pairs showing deletions at the paired-end were also detected.

Trio Sanger sequencing confirmed that c.263+1G>A is of paternal origin (Fig. 2C). The PCR of a deletion region containing exon 6 revealed short fragments in DNA from the proband and the mother, which confirmed NC\_000020.11: g.(3217736\_3217747)\_(3221159\_3221170) del. The two elder brothers who died of dilated cardiomyopathy had the same genotype as the proband. To determine the detailed sequence of the breakpoint, PCR fragments were analyzed using Sanger sequencing, which revealed that the deletion was caused by homologous recombination between AluJo and AluJb (Fig. 2B).

We performed a multi-omics analysis for the proband whose condition could not be diagnosed using WES and finally identified compound heterozygous variants in *ITPA*. *ITPA* encodes inosine triphosphate pyrophosphohydrolase (ITPase) that hydrolyzes inosine triphosphate (ITP) and deoxyinosine triphosphate. Variants in *ITPA* cause developmental epileptic encephalopathy 35 (DEE35) [5] or Inosine triphosphatase deficiency [6]. DEE35 is a rare neurodegenerative disease characterized by developmental delay, microcephaly, feeding difficulties, early-onset intractable seizures, subsequent psychomotor retardation, and early childhood fatality [7]. In this family, siblings with *ITPA* mutations have died of dilated cardiomyopathy. Cardiac involvement has been noted in 28.6% of patients with *ITPA* deficiency, and dilated cardiomyopathy is the most common cause of cardiac involvement [7]. Furthermore, dilated cardiomyopathy has been reported in *ITPA* gene-disrupted mouse [8, 9]. Thus, in this proband and her brothers, dilated cardiomyopathy is thought to be caused by variants in *ITPA*. The other clinical features of the proband were consistent with those of DEE35.

Our analysis identified a novel Alu recombination-mediated deletion (ARMD). Deletions due to ARMD have been reported to account for 0.3% of human genetic disorders [10]. To the best of our knowledge, this is the first report of ARMD with DEE35. Such ARMDs are thought to have been missed by previous genetic testing because Alu is present in introns, which are difficult to cover with WES and panel sequencing. We must actively promote genetic testing methodologies to identify Alu-mediated recombination, such as ARMD. A recent study reported that capture-seq and long-read sequencing specifically examine recombination between Alu or L1 [11]. We believe that improving the accuracy of methods for detecting CNV and SV from WGS data and applying techniques to specifically detect transposable elements such as Alu, for the genetic testing of diseases are necessary to improve diagnostic accuracy. RNA-seq enables more effective identification of genes showing aberrant expression and abnormal splicing, which is handy when combined with WGS. In our previous studies, Alu-mediated deletions have been identified using RNA-seq and WGS [12, 13]. These methods should be considered for WES-negative cases to detect pathogenic variants missed by WES.

## DATA AVAILABILITY

Raw data are available from the corresponding author upon reasonable request. Some genomic information that could be used to identify individuals cannot be shared due to ethical constraints.

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## AUTHOR CONTRIBUTIONS

NO and YK wrote the manuscript. NO and YK performed the experiments. NO, YK, KR, TF and YY analyzed the data. MN, HS, AT, EK, YS, KI and KM acquired clinical information. YK, AO, KM, and YO supervised the study. All authors discussed the results and commented on the manuscript.

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## COMPETING INTERESTS

The authors declare no competing interests.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the regional Ethics Committees of Juntendo University, Saitama Medical University, Chiba Children's Hospital, and Kindai University. Written informed consent was obtained from the parents. All methods were performed by relevant guidelines and regulations.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s10038-023-01156-y>.

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表2 ミトコンドリア病の原因遺伝子に合わせた特異的治療法

疾患名	原因遺伝子	治療	治療効果
Brown-Vialetto-Van Laere症候群/ Fazio-Londe症候群 (難聴など)	SLC52A2, SLC52A3, SLC52A1	リボフラビン (経口: 10~50 mg/kg/ day)	良好
ピオチン反応性基底核症 (Leigh)	SLC19A3	チアミン (経口: 10~20 mg/kg/day), ピオチン (経口: 10~15 mg/kg/day)	良好
ピオチン欠損症 (脳筋症)	BTBD	ピオチン (経口: 5~10 mg/kg/day)	良好
ホロカルボキシラーゼ合成酵素欠損 症 (新生児 Mito)	HLCSD	ピオチン (経口: 10~20 mg/kg/day)	不安定だが良好
チアミンピロホスホキナーゼ欠損症 (脳症)	TPK1	チアミン (経口: 20 mg/kg/day)	不安定 (これまで10 名以上に投与)
ACAD9欠損症 (脳筋症)	ACAD9	リボフラビン (経口: 10~20 mg/kg/ day)	不安定
マルチプルアシル CoA 脱水素酵素欠 損症 (乳児 Mito)	ETFA, ETFB, ETFDH, SLC25A32, FLAD1	リボフラビン (経口: 10 mg/kg/day)	良好
チアミン反応性PDHC欠損症 (Leigh)	PDHA1	リボフラビン (経口: 30~40 mg/kg/ day)	不安定
コエンザイムQ10欠損症 (腎症, 脳 筋症)	PDSS1, PDSS2, COQ2, COQ4, COQ6, COQ7, ADCK3, ADCK4, COQ9	コエンザイムQ10 (経口: 10~30 mg/ kg/day)	とても不安定 (異常が なにかに依る)
シトクロムc酸化酵素欠損症 (Leigh 他)	SCO2, COA6	ヒスチジン銅 (投与量不明; 500 mgの皮 下注射が提案されている)	不明, SCO2異常症一 名のみ投与
モリブデン補酵素欠損症 (新生児 Mito)	MOCS1, MOCS2, GPHN	環状ピラノプテリンモノホスファート (静脈注射: 80~320 mg/kg/day)	タイプAのモリブデン 補酵素欠損症に対して 良好
3-ヒドロキシイソ酪酸-CoA加水分解 酵素欠損症 (Leigh)	HIBCH	バリン制限食	不明, 数名にのみ投与
エノイルCoAヒドラーゼ欠損症 (Leigh)	ECHS1	バリン制限食	不明, 数名にのみ投与
チオレドキシン2欠損症 (脳症)	TXN2	抗酸化療法 (例: イデベノン20 mg/kg/ day)	明らかに良好 (患者は 一名のみ)
エチルマロン酸脳症 (新生児 Mito)	ETHE1	メトロニダゾール, N-アセチル-システ イン (グルタチオン前駆体), 肝臓移植	不安定

赤字はわれわれの遺伝子解析でも見つかっている遺伝子異常。文献4より引用 (ただし赤字への変更は著者らによる)。

胞を使用することも多い。呼吸鎖酵素活性の評価では、各呼吸鎖複合体の酵素欠損症や、複数の酵素の活性が同時に低下する疾患を見出すことができる。ウェスタンブロット法は呼吸鎖酵素の構成タンパク質の量的低下の評価において、Blue Native電気泳動(BN-PAGE解析)は呼吸鎖複合体の量的・質的評価においてそれぞれ有用である。皮膚線維芽細胞を用いた酸素消費量解析では、生きた細胞を用いてリアルタイムに測定を行え、酵素活性よりも鋭敏にミトコンドリア障害を判別できる。

近年、遺伝子診断によって病態が解明され、治療可能なミトコンドリア病があり(表2)<sup>4)</sup>、補酵素代謝異常を伴うミトコンドリア病は、この種の治療可能な疾患の大部分を占めている。したがってできるだけ早期に遺伝子診断を行うことがますます重要になってきている。

核DNA・mtDNAも含めた遺伝子の種類を分類すると、以下ようになる<sup>2)</sup> 5)。

- ① 各呼吸鎖酵素複合体のサブユニットの異常やセンブリー因子の異常

- ② mtDNAおよびそれに関係するRNAやタンパク質合成の異常
- ③ 呼吸鎖の上流反応にあたる、基質産生の異常(TCA回路や脂肪酸酸化など)
- ④ 呼吸鎖に関連する補因子(cofactor)の異常
- ⑤ ミトコンドリアのホメオスタシスの異常(ミトコンドリアの分裂・融合など)
- ⑥ 呼吸鎖に対する阻害物質の産生

乳児・小児ミトコンドリア病の遺伝子検査に関しては、mtDNAおよび核DNAを含む包括的遺伝子解析<sup>※2</sup>が必要な場合が多い。本邦でも2016年に神田らが日本人ミトコンドリア病140超例の包括的遺伝子解析の報告をしている<sup>6)</sup>。さらにLeigh脳症やミトコンドリア肝症など比較的候補遺伝子がある程度絞られる場合は遺伝子パネルを用いることがある。一部のミトコンドリア病に関して、その病態を踏まえた特異的な治療法が行われており、核遺伝子も含めた包括的遺伝子検査の重要性が、年々重要になってきている。

## 2 世界での遺伝子診断やミトコンドリア病バイオバンクレジストリの動向

オーストラリアではultra-rapid exome sequencingとよばれる、単一遺伝子の異常が疑われる重症な小児に対して迅速に遺伝学的な診断に迫る研究が公的な医療システムで行われた<sup>7)</sup>。このシステムでは、サンプル受領から結果報告までの平均時間は3.3日(95% CI, 3.2~3.5日)であった。また診断のついた症例の76%、診断のつかなかった症例の11%で臨床での管理方針に影響を与えたと考えられた。

また、ドイツでは2009年からGerman Network for Mitochondrial Diseases (mitoNET)が、ドイツ連邦教育研究省の資金援助で運用されている。この団体は、

### ※2 包括的遺伝子解析

従来の遺伝子解析であるmtDNA全周解析、ミトコンドリア病遺伝子パネル検査に加え、RNAシーケリング、全エクソーム解析/全ゲノム解析、プロテオーム解析などの多階層オミクス技術を用い、病因遺伝子を探っていく手法。詳細についてはわれわれの実施した研究課題 (<https://kaken.nii.ac.jp/ja/grant/KAKENHI-PROJECT-19H03624/>)も参照してほしい。

特に患者登録とバイオバンクを運営し、ミトコンドリア病の自然史に関する研究を行っており、1,700人以上の患者さんのデータが登録されている<sup>8)</sup>。

さらにEUが資金提供するプロジェクトGENOMITでは、国際的なデータ収集のためのグローバルミトコンドリア病レジストリが開設されている。現在GENOMITにはドイツ、オーストリア、フランス、イタリア、英国、米国そして日本がグローバルパートナーとして参画している<sup>9)</sup>。

ミトコンドリア病は、日本や欧米諸国では出生5,000人に1人の割合で発生するといわれている<sup>10) 11)</sup>。しかし、ある特定の遺伝子に関連した症例は、日本全国で1人か数人であり、超希少疾患といえる。したがって、ミトコンドリア病研究において、遺伝子探索、病態解明、創薬、レジストリなどさまざまな面で国際協力がきわめて重要である。この種のレジストリ登録により、多数の患者を対象としたミトコンドリア病の表現型および遺伝子型スペクトルに関する新たな洞察を得ることができ、臨床試験への参加に関して適切な患者に迅速に連絡することが可能になる。さらに、定期的なフォローアップによって疾患の自然史をモニターすることは、緊急に必要とされる無作為化治療試験の計画にとって非常に重要である。また、前述のような国際連携を進めるとともに、国内でのミトコンドリア病の各種病態の認知を上げ、診断体制を整え、診断の質を向上させ、幅を広げていくことが同時に重要である。われわれはミトコンドリア病診療マニュアルの改訂を行うとともに、国内で新生児や小児のミトコンドリア病の症例登録システムを構築している(J-MO Bank)<sup>12)</sup>。臨床・生化学・遺伝学的データを共有し、原因遺伝子の特定や創薬につなげるため、これらのシステムを発展させ、欧米の研究者もアクセス可能なグローバルなミトコンドリア病症例登録の構築を計画している。さらに、国立精神・神経医療研究センターにおいて、成人のミトコンドリア病レジストリを構築する予定である。

## 3 ミトコンドリア病の遺伝子診断

本項ではわれわれの研究グループ(千葉県こども病院・埼玉医科大学・順天堂大学)において実際に行っているミトコンドリア病の遺伝子診断を紹介する。

ミトコンドリア病研究の長年にわたる成果として国内最大規模のミトコンドリア病患者コホートを有し、全国の病院施設から紹介されるミトコンドリア病疑い症例は年々増加傾向にある。各症例の臨床情報・検体受付状況・DNA/RNA サンプルの情報・生化学診断や遺伝子診断の結果等が一覧できる研究用匿名化データベースが完成しており、3施設間でスムーズな情報連携を図っている。

年間200件ほどのミトコンドリア病疑い症例の診断依頼を受け入れ、これまでの解析件数はWGS = 228症例、WES = 707症例、ミトコンドリア病遺伝子パネルシーケンス = 954症例にものぼる。また、新規原因遺伝子の報告は通算13遺伝子を数え、近年はマルチオミクス解析 (WGS + RNA-seq + Proteome) においても着実に成果を出している。

ミトコンドリアゲノムは母親から遺伝することから、「ミトコンドリア病 = 母系遺伝」と誤解されることがあるが、ミトコンドリア病の発症にはミトコンドリア内に存在するミトコンドリアゲノム (mtDNA) と核にコードされたミトコンドリア病原因遺伝子との両方がかかわっている。つまり、ミトコンドリア病の遺伝子診断を行うにはmtDNAと核遺伝子の両方を調べる必要がある。また、発症時期による差異も明らかになってきており、新生児期/小児期発症のミトコンドリア病では核遺伝子に変異を認める常染色体潜性遺伝の症例が多い<sup>13)</sup>。われわれの研究グループはミトコンドリア病のなかでも特に新生児期/小児期発症のミトコンドリア病に焦点をあてて研究を進めてきたことから多数の核遺伝子異常を明らかにしており、そのことが新規原因遺伝子の報告にもつながっている。

#### 4 ミトコンドリア病遺伝子パネルシーケンス

ミトコンドリア病疑い症例の遺伝子解析をはじめにあたり、まず最初にミトコンドリア病遺伝子パネルシーケンスを行っている。順天堂大学難病の診断と治療研究センターでは、核ゲノムにコードされた既知のミトコンドリア病原因遺伝子 (合計367遺伝子) とmtDNA全周の両方を1回の実験で調べることができる解析システムを開発し、運用してきた。本解析システ

ムはターゲットキャプチャー方式を採用しているため (アンプリコン方式では検出が難しい) mtDNAの数kb単位の単一欠失を正確なブレイクポイントとともに検出することが可能である。本解析システムを稼働した2020年1月から現在までの解析結果を集計し、図に示した。

##### 1) 解析対象

ミトコンドリア病の疑いがある0~64歳の合計449症例 (499サンプル) について解析を行った。他の遺伝学的検査と同様、主に血液由来DNA (72.9%) が使用されるが、mtDNA変異を疑う症例においては罹患臓器のDNAの他に尿沈渣DNA (11.2%) の使用も非常に有用である (図右下)。尿沈渣DNAの有用性については別資料に詳しく記載してあるので参考にさせていただきたい<sup>14)</sup>。

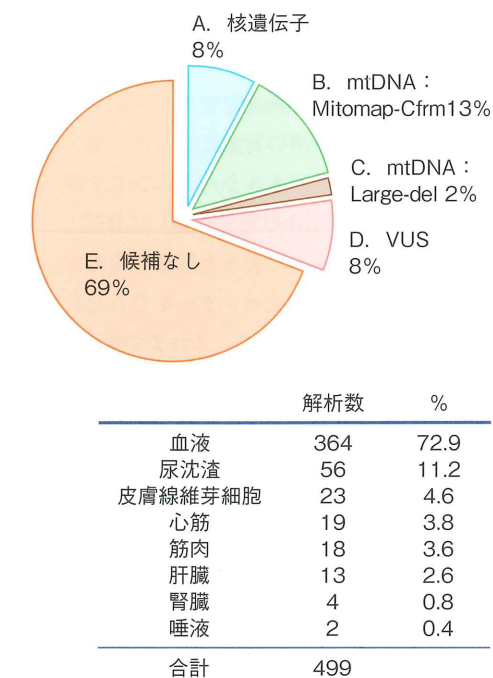
##### 2) 解析結果

449症例に対してミトコンドリア病遺伝子パネルシーケンスを行い、98症例の遺伝子診断が確定した。その内訳は核遺伝子に病的バリエーションを認めた症例が合計38 (8%)、mtDNAにMitomap-Cfrmバリエーション (MITOMAP<sup>15)</sup> に検証済と登録されている疾患関連バリエーションを認めた症例が合計60 (13%)、mtDNAに単一欠失を認めた症例が合計7 (2%) であった (図左上・右上)。また、年齢別に集計すると核遺伝子に病的バリエーションを認めた症例は0~1カ月齢が合計16症例と最も多く、次いで1カ月齢~1歳が合計13症例であった (図左下)。この集計結果から核遺伝子に異常をもつ常染色体潜性遺伝形式のミトコンドリア病は新生児期/小児期発症のより重篤なタイプのミトコンドリア病であることが読みとれる。

#### 5 マルチオミクス解析

前述のミトコンドリア病遺伝子パネルシーケンスやWESで解決しなかった症例に対しては、WGS・RNA-seq・Proteome等を行って横断的にデータを精査するマルチオミクス<sup>\*3</sup>解析へと入っていく。ただし、これらの追加解析は全例に対して行われるわけではない。その年度の獲得研究費に応じた実施計画が立てられるため自ずと解析数には上限が発生する。①臨床症状および生化学診断からミトコンドリア病である可能

A. 核遺伝子	症例数	B. Mitomap-Cfrm	症例数
<i>PDHA1</i>	8	3,243 A > G	28
<i>COQ4, ECHS1</i>	3	13,513 G > A	6
<i>FBXL4, NDUFA4, QRSL1</i>	2	8,993 T > G	4
		14,487 T > C	3
<i>ACAD9, AGK, AIFM1, DNM1L, EARS2, HSD17B10, IARS2, LARS, NAXE, NDUFAF6, OPA1, POLG, SCO2, SLC25A13, SUCLG1, SURF1, TARS2, TOP3A</i>	1	8,344 A > G	3
		1,555 A > G	2
		1,644 G > A	2
		8,851 T > C	2
		11,778 G > A	2
		14,453 G > A	2
合計	38	14,459 G > A	2
		4,300 A > G	1
		9,176 T > C	1
		9,185 T > C	1
		14,674 T > C	1
		合計	60



Age	0-1m	>1m	>1y	>6y	>20y	N/A
Nuclear gene (38)	16	13	7	2	0	0
mtDNA : Mitomap-Cfrm (60)	12	11	10	6	15	6
mtDNA : Large-del (7)	2	1	0	4	0	0
VUS (173)	16	5	7	3	4	0

図 ミトコンドリア病遺伝子パネルシーケンス 2020.01 ~ 2022.11 集計, 449 症例 (499 サンプル)。

性が高く、②解析に堪え得る高品質サンプルが準備可能である (例えば皮膚線維芽細胞樹立済)。等を判断材料として進めていくことになる。

われわれはマルチオミクス解析によって遺伝子異常が明らかになり、遺伝子診断確定に至った症例を多数経験しているが<sup>16)~19)</sup>、ここでは2021年にHuman Mutation誌に発表した*NDUFV2*遺伝子異常をもつ症例<sup>18)</sup>を紹介する。当該症例は生化学的にミトコンドリア呼吸鎖I活性低下の診断が付され、臨床的にもミト

コンドリア病に特徴的な中枢神経症状と先天性高乳酸血症を呈していた。WESでミトコンドリア呼吸鎖I複合体のサブユニットである*NDUFV2*にヘテロ接合型のアミノ酸置換バリエーションが1つ検出されたため、常染色体潜性遺伝を想定し、対立アレルの遺伝子異常を明らかにする目的でマルチオミクス解析が施行された。その結果、ミトコンドリア病の原因としては世界初の報告となる、Alu因子 (レトロトランスポソンの1種) を介した非常に珍しい遺伝子欠失が同定され、本症例の遺伝子診断が確定するとともにミトコンドリア病の遺伝子診断におけるマルチオミクス解析の重要性が示された。この症例を契機にわれわれのミトコンドリア病コホートのなかから、その後4例のAlu因子を介した挿入や欠失による異常が見つかった。

##### ※3 マルチオミクス解析

Multi (多数の) とOmics (オミクス) を合わせた造語であり、人体の機能を司るさまざまな生体物質 (DNA, RNA, タンパク質, 代謝物など) を包括的に分析する手法を指している。

## おわりに—今後の展望

令和4年度診療報酬改定でミトコンドリア病の遺伝学的検査が保険診療の対象となった。順天堂大学では4で述べた遺伝子パネルシーケンスを順天堂医院の臨床検査部 (ISO15189 認定取得) に移管し、保険診療としてスタートすべく着々と準備を進めている。他の病院施設や各臨床検査センターもそれぞれ対応を検討していると聞き及んでいる。これまで研究の範疇で行ってきた遺伝子パネルシーケンスが保険診療に切り替わるとともにミトコンドリア病の遺伝子解析を行う基礎・医学研究者にはより高度で複雑な解析への貢献が期待される。マルチオミクス解析までもが社会実装される将来をめざして日々の研究に精進したいと思う。

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特集 代謝 I. 代謝と臓器・疾患

# ミトコンドリア病の病態解明と治療法の開発

八塚 由紀子 岡崎 康司

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**Key words:** ミトコンドリア病, 呼吸鎖複合体, マルチオミクス解析, 5-アミノレブリン酸(5-ALA), ピロールイミダゾールポリアミド(PIポリアミド)

ミトコンドリア病とはミトコンドリアの機能低下によって発症する疾患の総称である。発症時期が新生児期～成人期と広く、臨床症状や遺伝形式も多岐にわたる。そして、ミトコンドリアDNAと核DNAのいずれに存在する遺伝子変異も発症の原因となり得る<sup>1)</sup>。このように、臨床的にも遺伝的にも診断が非常に難しい疾患ではあるが、筆者らは、生化学診断とゲノム解析を組み合わせることによる体系的な病態解明と有効な治療法の開発に取り組んでいる。

ミトコンドリアは細胞が必要とするエネルギー(アデノシン三リン酸; ATP)の95%を産生しており、ミトコンドリアの機能低下はエネルギー不足に直結する。ゆえにミトコンドリア病では、エネルギー需要の高い組織(脳, 神経, 心筋, 骨格筋など)に異常を来すことが多い。ミトコンドリア病を疑った際には、ミトコンドリアの機能異常を証明するための生化学的な特殊検査および遺伝学的検査を行う。次世代シーケンサーの普及以降、遺伝学的検査を先行する“genetic-first”な診断フローが選択される場面が増えたが、病態解明のためには生化学的検査と遺伝学的検査の両方が必要不可欠である。

## ■ミトコンドリア病の診断と病態

先天代謝異常症(ミトコンドリア病を含む)を疑った際には一般生化学検査, アミノ酸分析, 尿中有機酸分析, アシルカルニチン分析などのスクリーニング検査から開始し, 次いでミトコンドリア病を明らかにするための生化学的検査として, ミトコンドリアの呼吸鎖酵素活性の評価, Blue Native PAGE/ウェスタンブロット法による呼吸鎖複合体構成タンパク質の量的・質的低下の評価, 患者由来皮膚線維芽細胞を用いた酸素消費量

測定の評価などが行われる。一方でミトコンドリア病の遺伝学的検査としては、まず遺伝子パネルシーケンス[筆者らは核DNAのミトコンドリア病原因遺伝子367個とミトコンドリアDNA(mtDNA)全周16.6kbを一括で解析している]を行い、遺伝子診断が不十分である場合には全ゲノムシーケンス, RNAシーケンス, プロテオームなどによるマルチオミクス解析に入っていく。

ミトコンドリア病の病態を遺伝子異常から大別すると、①呼吸鎖複合体の異常, ②mtDNAおよびそれに関連するRNAやタンパク質合成の異常, ③TCAサイクルや脂肪酸β酸化などの基質産生異常, ④ミトコンドリアの分裂・融合異常, ⑤呼吸鎖に対する阻害物質の産生, となり、各遺伝子異常に対応した治療法の開発が期待されるが、現時点では有効な根治療法がなく対症療法にとどまる。

## ■ミトコンドリア病の治療薬

ミトコンドリア病の治療薬として承認されている薬剤は欧州ではイデベノン(レーベル遺伝性視神経症), わが国ではタウリン(MELAS症候群)のみである。現在開発中の治療薬としては、5-アミノレブリン酸(5-ALA)とクエン酸第一鉄ナトリウム(SFC)の組合せ使用, mtDNAを標的とするピロールイミダゾールポリアミド(PIポリアミド), 既存薬とは異なる作用機序が期待される新規化合物mitochonic acid-5(MA-5), パーキンソン病の治療薬であるアポモルフィンのミトコンドリア病への適応拡大, などがある。治験に関する公開情報がわかりやすくまとめられたページがある([https://genex.co.jp/clinical\\_trial\\_mitochondrial\\_disease/](https://genex.co.jp/clinical_trial_mitochondrial_disease/))。

以下、5-ALA+SFCとPIポリアミドの開発状況について紹介する。

5-ALA: 体内に存在するアミノ酸の一種で、ミトコンドリア内ではヘムとして呼吸鎖複合体II, III, IVおよびシトクロムCを構成するエネルギー代謝に必須の物質である。経口投与後は上部消化管ではほぼすべて吸収され全身に分布し、血液脳関門をも通過するという優れた体内動態をとる。培養細胞に対する5-ALA+SFC投与実験において、ヒト正常皮膚線維芽細胞では呼吸鎖複合体I, II, III, IVの代表的サブユニットの遺伝子発現量に用量依存的な増加, 更にLeigh脳症患者由来の複数症例の皮膚線維芽細胞では、最大酸素消費量およびATP産生量の有意な上昇が確認された<sup>2)</sup>。脳神経症状を中心とするミトコンドリア病患者に対する医師主導型治験においては、残念ながら主要評価項目に有意差を認めなかったものの、少なくとも一部の症例に格段のQOL改善を認めていた。引き続き開発を進めるべく、ブラッシュアップした臨床治験を計画中である。

PIポリアミド: mtDNAの変異は、ミトコンドリア病だけでなく糖尿病などの成人生活習慣病やがん, 更には痴呆や老化にも関与する。変異したmtDNA配列を直接標的とし、ミトコンドリア品質管理機構(MQC)に認識させて除去する、という理想的な治療法を目指して開発された化合物がPIポリアミドである。mtDNAが存在するミトコンドリアへと送達させることを目的として、脂溶性カチオンであるトリフェニルホスホニウムカチオン(TPP)とPIポリアミドの複合体(PIP-TPP)を合成・精製する<sup>3)</sup>。わが国で最も症例数が多いmtDNA変異m.3243A>Gに対して設計したPIP-TPPは、変異特異的にミトコンドリアの活性酸素種の産生, マイトファジー, およびアポトーシスを誘導した<sup>4)</sup>。現在、AMED難治性疾患実用化研究事業(リン班)として臨床応用を目指して進めているところである。

## ■おわりに

筆者らは長年にわたり疾患ゲノム解析に携わってきたが、本格的にミトコンドリア病研究というものに焦点を置いたのは文部科学省セルイノベ

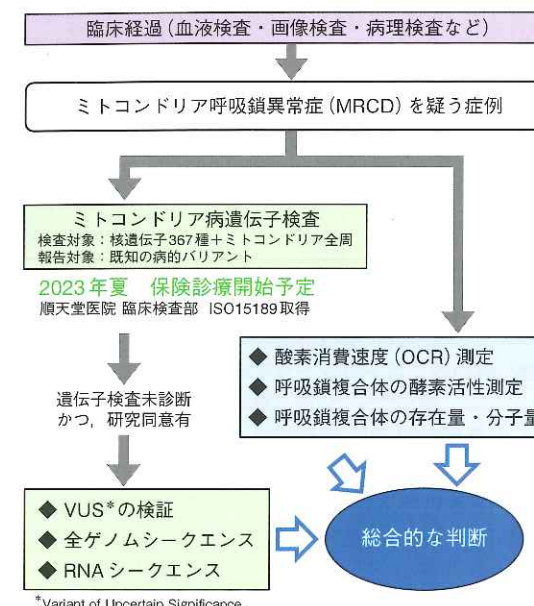


図 ミトコンドリア病の診断フローチャート

ションプログラム「神経細胞機能に着目した、ミトコンドリア呼吸鎖異常を起こす遺伝子変異の系統的な探索」に採択された平成22年以降である。当時100個程度とされていたミトコンドリア病の原因遺伝子数は今では400個を超え、そのなかには筆者らの研究グループが世界に先駆けて発表した新規原因遺伝子が13個含まれる。ミトコンドリアの正常な機能を維持するのに必要な遺伝子は1,500個とも言われ<sup>5)</sup>、今後も新規原因遺伝子の報告が予想されるが、重要なことは各遺伝子異常の関与を明らかにすることでいかに病態解析を推進し、有効な治療薬の開発に結びつけるかという点である。

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## Total and reduced/oxidized forms of coenzyme Q<sub>10</sub> in fibroblasts of patients with mitochondrial disease

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Forward electron transport  
Reverse electron transport

### ABSTRACT

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) is involved in ATP production through electron transfer in the mitochondrial respiratory chain complex. CoQ<sub>10</sub> receives electrons from respiratory chain complex I and II to become the reduced form, and then transfers electrons at complex III to become the oxidized form. The redox state of CoQ<sub>10</sub> has been reported to be a marker of the mitochondrial metabolic state, but to our knowledge, no reports have focused on the individual quantification of reduced and oxidized CoQ<sub>10</sub> or the ratio of reduced to total CoQ<sub>10</sub> (reduced/total CoQ<sub>10</sub>) in patients with mitochondrial diseases.

We measured reduced and oxidized CoQ<sub>10</sub> in skin fibroblasts from 24 mitochondrial disease patients, including 5 primary CoQ<sub>10</sub> deficiency patients and 10 respiratory chain complex deficiency patients, and determined the reduced/total CoQ<sub>10</sub> ratio.

In primary CoQ<sub>10</sub> deficiency patients, total CoQ<sub>10</sub> levels were significantly decreased, however, the reduced/total CoQ<sub>10</sub> ratio was not changed. On the other hand, in mitochondrial disease patients other than primary CoQ<sub>10</sub> deficiency patients, total CoQ<sub>10</sub> levels did not decrease. However, the reduced/total CoQ<sub>10</sub> ratio in patients with respiratory chain complex IV and V deficiency was higher in comparison to those with respiratory chain complex I deficiency.

Measurement of CoQ<sub>10</sub> in fibroblasts proved useful for the diagnosis of primary CoQ<sub>10</sub> deficiency. In addition, the reduced/total CoQ<sub>10</sub> ratio may reflect the metabolic status of mitochondrial disease.

### 1. Introduction

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>), also known as ubiquinone, is a lipophilic molecule composed of a redox-active benzoquinone head group and species-specific isoprenoid side chain (10 subunits in humans) [1,2]. CoQ<sub>10</sub> takes three forms depending on the redox state of the benzoquinone ring; oxidized (CoQ<sub>10</sub>, Ubiquinone), fully-reduced (CoQ<sub>10</sub>H<sub>2</sub>, Ubiquinol), and semi-reduced (CoQ<sub>10</sub><sup>•</sup>, Semiubiquinone) forms [2]. It

presents ubiquitously in all cellular membranes and cells [3]. The amount of CoQ<sub>10</sub> and the proportion of reduced CoQ<sub>10</sub> differ between organs and cells; CoQ<sub>10</sub> is distributed in high amounts in the heart, kidneys, liver, and muscles, and the proportion of reduced CoQ<sub>10</sub> is lower in the brain and lungs [3]. In cells, it is mostly localized in the mitochondria [3].

CoQ<sub>10</sub> has multiple functions. One of the main roles of CoQ<sub>10</sub> is as a component of the mitochondrial respiratory chain. As a mobile electron

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carrier, CoQ<sub>10</sub> accepts electrons from complex I and II and transfers them to complex III [2]. In the mitochondrial inner membrane, CoQ<sub>10</sub> is proposed to exist as two independent pools: a CoQ<sub>NADH</sub> pool in the super-complex (CI, CIII, CIV) involved in the oxidation of NADH; and a CoQ<sub>FADH</sub> pool involved in the oxidation of CII and other enzymes that use CoQ as a cofactor [4]; CoQ<sub>NADH</sub> receives electrons from NADH, and CoQ<sub>FADH</sub> receives electrons from FADH<sub>2</sub> and other enzymes, such as glycerol phosphate dehydrogenase (GPDH), choline dehydrogenase (CHDH), sulphide:quinone oxidoreductase (SQOR), dihydroorote dehydrogenase (DHODH), and electron transfer flavoprotein dehydrogenase (ETFHDH), and is reduced [5]. In respiratory chain complex III, electrons are transferred from reduced CoQ<sub>10</sub> to cytochrome *c* in a process called the Q cycle. The Q cycle results in the oxidation of two molecules of reduced CoQ<sub>10</sub>, the reduction of two molecules of cytochrome *c*, and the formation of one additional molecule of reduced CoQ<sub>10</sub> [2]. This normal forward electron transfer results in the creation of an electrical gradient and a pH gradient (proton gradient) between the mitochondrial matrix and intermembrane space. The energy generated by this proton motive force enables complex V(ATP synthase) to synthesize ATP. On the other hand, reverse electron transfer (RET) of CoQ<sub>10</sub> is also known to occur; in RET, electrons from reduced CoQ<sub>10</sub> are returned to complex I, reducing NAD<sup>+</sup> to NADH, and generating ROS [6] (Supplemental Fig. 1).

Another important role of CoQ<sub>10</sub> is as an antioxidant. CoQ<sub>10</sub> is the sole lipid-soluble antioxidant that is endogenously synthesized. Reduced CoQ<sub>10</sub> inhibits both the initiation and the propagation of lipid peroxidation [7]. NADH-quinone oxidoreductase 1 and cytochrome *b*<sub>5</sub> reductase, have been known to be the major oxidoreductases in the plasma membrane [8]. In addition, ferroptosis suppressor protein 1 (FSP1) was also found to be an important oxidoreductase. FSP1 reduces extra-mitochondrial CoQ<sub>10</sub> and acts as a lipophilic radical-trapping antioxidant to suppress lipid peroxides, resulting in the inhibition of cell death, called ferroptosis [9,10]. Moreover, reduced CoQ<sub>10</sub> also regenerates the other antioxidants— $\alpha$ -tocopherol and ascorbate—into an active reduced form [7]. CoQ<sub>10</sub> is also involved in the  $\beta$ -oxidation of fatty acids [11], de novo pyrimidine biosynthesis [12], sulfide oxidation [13], an essential cofactor for uncoupling proteins (UCPs) [14], and modulation of the mitochondrial permeability transition pore [15]. Thus, the importance of the distinct state of CoQ<sub>10</sub> is gaining growing attention [5].

Primary CoenzymeQ<sub>10</sub> (CoQ<sub>10</sub>) deficiency is an autosomal recessive mitochondrial disease caused by a decrease in CoQ<sub>10</sub> due to mutations in genes involved in CoQ<sub>10</sub> biosynthesis (*COQ* genes) [16]. To date, defects in at least 10 *COQ* genes (*COQ2*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *COQ8A*, *COQ8B*, *COQ9*, *PDSS1*, *PDSS2*) have been found to cause this disease [17]. Primary CoQ<sub>10</sub> deficiency was first reported in 1989 as familial mitochondrial encephalomyopathy [18]. Currently, over 280 patients from 180 families have been reported [17]. Secondary CoQ<sub>10</sub> deficiencies occur in a wider variety of pathologies, including mitochondrial disease [19,20]. Measuring the CoQ levels is known to be useful for diagnosing CoQ<sub>10</sub> deficiency [21]. As described above, the ratio of reduction varies among organs and cells. As a result, in addition to the total amount of CoQ<sub>10</sub>, evaluating the reduced/oxidized CoQ<sub>10</sub> ratio may also be useful for further elucidating various pathophysiological states in cells.

Methods to measure CoQ<sub>10</sub> in fibroblast and the reduced/oxidized state of CoQ<sub>10</sub> have been reported [22,23]. However, to our knowledge, there are no reports on the measurement of the reduced/oxidized CoQ<sub>10</sub> ratio in primary CoQ<sub>10</sub> deficiency or mitochondrial diseases. Therefore, we decided to measure the total CoQ<sub>10</sub> levels as well as the levels of reduced and oxidized CoQ<sub>10</sub> in skin fibroblasts from patients affected by various mitochondrial diseases.

## 2. Material, methods, and patients

### 2.1. Subjects

We studied fibroblasts from 24 patients with mitochondrial disease, including primary CoQ<sub>10</sub> deficiency (Table 1). The inclusion criterion for patients with primary CoQ<sub>10</sub> deficiency was childhood onset with biallelic pathogenic variants in the *COQ* gene encoding proteins for the biosynthesis of CoQ<sub>10</sub> [16]. Fibroblasts were obtained from patients at Kanagawa Children's Medical Center, Chiba Children's Medical Center, and Jichi Medical University under the approval of the Ethics Committee of Jichi Medical University. Written informed consent was obtained from the parents of each patient.

We obtained fibroblasts from five patients with primary CoQ<sub>10</sub> deficiency. One patient carries biallelic *COQ2* variants with the c.[349G > C];[912 + 1G > del] (Case 1). Four patients had biallelic *COQ4* mutations: one with compound heterozygous biallelic variants c.[718C > T];[421C > T] (Case 2) [24], one with c.[431C > A];[718C > T] (Case 3) [25], and two with c.[190C > T];[479G > A] (Cases 4, 5).

Ten patients had disorders of mitochondrial respiratory chain subunits. Seven patients had mutations related to complex I: one with a c.[55C > T] mutation in *NDUFA1* (Case 6) [26], one with an m.10158 T > C mutation in *MT-ND3* (Case 7) [26], Three with an m.13513G > A mutation in *MT-ND5* (Case 8) [27], (Case 9), (Case 10) [28], one with a c.[811 T > G];[1766-2A > G] mutation in *ACAD9* (Case 11), and one with a c.[1150G > A];[1817 T > A] mutation in *ACAD9* (Case 12) [28]. *NDUFA1*, *MT-ND3*, and *MT-ND5* are subunits of complex I, and *ACAD9* is the assembly factor of complex I. Two patients had mutations related to complex IV: one with a c.[743C > A] mutation in *SURF1* (Case 13) [29], and one with a c.[367\_368delAG]; [572delC] mutation in *SURF1* (Case 14) [25]. *SURF1* is the assembly factor of complex IV. One patient had an m.8993 T > G mutation in *MT-ATP6* (Case 15) [25]. *MT-ATP6* is a subunit of complex V.

Three patients had mitochondrial DNA (mtDNA) depletion syndrome: one with a c.[143-307\_170del335];[143-307\_170del335] mutation in *DGUOK* (Case 16) [30], one with a c.[451dupC];[308\_310del] mutation (Case 17) [30] and one with a c.[148C > T];[149G > A] mutation in *MPV17* (Case 18) [30]. Both *DGUOK* and *MPV17* are involved in the maintenance of mtDNA. One patient had Kearns-Sayre syndrome with a single mtDNA deletion (5513 bp del; m.8290–13,802) (Case 19). Two patients had MELAS: one with an m.3243 A > G mutation of tRNA-Leu (Case 20) [26] and one with an m.5541C > T, mutation of tRNA-Trp (Case 21) [26]. One patient had short-chain enoyl-CoA hydratase (*ECHS1*) deficiency with heterozygous mutations in maternal c.[832G > A] in *ECHS1* (Case 22) [31]. *ECHS1* plays a role in valine and fatty acid catabolism in mitochondria. Two patients had c.[287A > G]; [287A > G] mutation in *BOLA3* (Cases 23) [28], (Case 24). *BOLA3* is related to iron-sulfur cluster production and is involved in the assembly of the mitochondrial respiratory chain complex.

Five fibroblasts from healthy individuals were purchased: two fibroblasts from the PromoCell Company (#C-12300, GmbH, Heidelberg, Germany), two fibroblasts from Japanese Collection of Research Bioresources Cell Bank (#TIG-120, #HT-2020, Japan), and fibroblasts from Lonza Japan (#CC-2509, Tokyo, Japan). Another five fibroblasts from patients without mitochondrial disease were used as controls. Cells from passages 4–29 were used for assays.

### 2.2. Cell culture and growth conditions

The fibroblasts were maintained in 1.0 g/L low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cells were incubated at 37 °C under 5% CO<sub>2</sub>.

**Table 1**  
Fibroblast cell lines from patients with mitochondrial disease, including primary CoQ<sub>10</sub> deficiency.

Case [ref]	diagnosis	DNA mutation	variants, heteroplasmy rate <sup>a</sup>	function
1	primary CoQ <sub>10</sub> deficiency	<i>COQ2</i>	c.[349G > C];[912 + 1G > del]	CoQ <sub>10</sub> biosynthesis
2 [24]	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[718C > T];[421C > T]	CoQ <sub>10</sub> biosynthesis
3 [25]	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[431C > A];[718C > T]	CoQ <sub>10</sub> biosynthesis
4	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[190C > T];[479G > A]	CoQ <sub>10</sub> biosynthesis
5	primary CoQ <sub>10</sub> deficiency	<i>COQ4</i>	c.[190C > T];[479G > A]	CoQ <sub>10</sub> biosynthesis
6 [26]	Leigh syndrome	<i>NDUFA1</i>	c.[55C > T], 100% (X-linked)	Respiratory chain subunits, complex I
7 [26]	Leigh syndrome	<i>MT-ND3</i>	m.10158 T>C, heteroplasmic (F; 90%)	Respiratory chain subunits, complex I
8 [27]	neonatal cardiomyopathy	<i>MT-ND5</i>	m.13513G > A, heteroplasmic (F; 78.87%)	Respiratory chain subunits, complex I
9	infantile mitochondrial disease	<i>MT-ND5</i>	m.13513G > A, heteroplasmic (B; 77%)	Respiratory chain subunits, complex I
10 [28]	Leigh syndrome	<i>MT-ND5</i>	m.13513G > A, heteroplasmic (F; 26%)	Respiratory chain subunits, complex I
11	mitochondrial cardiomyopathy	<i>ACAD9</i>	c.[811 T > G];[1766-2A > G]	Respiratory chain assembly factor, complex I
12 [28]	non-lethal infantile mitochondrial disease	<i>ACAD9</i>	c.[1150G > A];[1817 T > A]	Respiratory chain assembly factor, complex I
13 [29]	Leigh syndrome	<i>SURF1</i>	c.[743C>A], homoplasmy	Respiratory chain assembly factor, complex IV
14 [25]	Leigh syndrome	<i>SURF1</i>	c.[367_368delAG];[572delC]	Respiratory chain assembly factor, complex IV
15 [25]	Leigh syndrome	<i>MT-ATP6</i>	m.8993 T > G, homoplasmy	Respiratory chain subunits, complex V
16 [30]	mtDNA depletion syndrome	<i>DGUOK</i>	c.[143-307_170del335];[143-307_170del335]	Deoxynucleotide triphosphate synthesis
17 [30]	mtDNA depletion syndrome	<i>MPV17</i>	c.[451dupC];[308_310del]	mitochondrial protein synthesis
18 [30]	mtDNA depletion syndrome	<i>MPV17</i>	c.[148C > T];[149G > A]	mitochondrial protein synthesis
19	Kearns-Sayre syndrome		Single mtDNA deletion (5513 bp del; m.8290–13,802)	
20 [26]	MELAS	(tRNA-Leu)	m.3243 A > G, heteroplasmic (F; 21%)	Mitochondrial tRNA
21 [26]	MELAS	(tRNA-Trp)	m.5541C > T, heteroplasmic (F; 49%)	Mitochondrial tRNA
22 [31]	ECHS1 deficiency	<i>ECHS1</i>	c.[832G > A]	Metabolism of toxic compounds
23 [28]	cardiomyopathy	<i>BOLA3</i>	c.[287A > G];[287A > G]	Iron-sulfur protein assembly
24	cardiomyopathy	<i>BOLA3</i>	c.[287A > G];[287A > G]	Iron-sulfur protein assembly

<sup>a</sup> F; fibroblasts, B; blood

### 2.3. CoQ<sub>10</sub> measurement in fibroblasts

#### 2.3.1. CoQ extraction

The methods for the extraction CoQ<sub>10</sub> were based on a previously reported method with slight modifications [32]. To extract CoQ<sub>10</sub> from fibroblast in 60 mm dishes, cells were washed twice with PBS, and pellets were re-suspended in 500 µL of lysis buffer (0.25 mM Sucrose, 2 mM EDTA, 10 mM Tris, and 100 UI/mL heparin, pH 7.4.), and sonicated twice for 5 s. These homogenates were also used to citrate synthase and protein quantification. To measure CoQ<sub>10</sub>, nine hundred microliters of ethanol containing internal standard CoQ<sub>10</sub>-d9 (IsoSciences, Ambler, PA) and 20 µM *tert*-butyl hydroquinone (TBHQ) (FUJIFILM Wako, Osaka, Japan) was added to 100 µL of homogenates. TBHQ was added to prevent oxidation of reduced CoQ<sub>10</sub>. The cell suspensions were vortexed and centrifuged at 15,700 ×g for 10 min (4 °C).

#### 2.3.2. Reduction of ubiquinone

Reduced CoQ<sub>10</sub> was required for use in the calibration curve measurement. However, since reduced CoQ<sub>10</sub> is easily oxidized, reduced CoQ<sub>10</sub> was prepared just before the analysis by reducing oxidized CoQ<sub>10</sub> following a previously reported method with slight modification [33]. Briefly, 50 µL of CoQ<sub>10</sub> was diluted in 1.95 mL hexane in a glass tube. Twenty milligrams of NaBH<sub>4</sub> was added and followed by the addition of 100 µL methanol, vortexed for 3 min, then placed in the dark for 5 min at room temperature. After reduction, 1 mL of water containing 100 µM EDTA was added to stop the reaction, vortexed for 1 min, and centrifuged 1500 ×g for 5 min at 4 °C. The upper layer containing reduced CoQ<sub>10</sub> was transferred to a glass tube.

#### 2.3.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The method for measuring reduced and oxidized CoQ<sub>10</sub> was based on the previously reported method with slight modifications [23]. An LC-MS/MS analysis was performed on an LC-electrospray ionization-MS (LC-ESI-MS) with triple quadrupole (Nexera X2 and LCMS-8060, Shimadzu, Kyoto, Japan). A Kinetex C18 column (100 mm × 2.1 mm, 2.6 µm, Phenomenex) and a guard column filled with the same packing material were used. The column temperature was kept at 40 °C. The mobile phase was isocratic with 2 mM ammonium formate in methanol.

The flow rate was 0.8 mL/min, the injection volume was 2 µL, and the run time was 6 min. The interface temperature was 300 °C, the desolvation line temperature was 250 °C, and the heat block temperature was 400 °C. The nebulizing gas flow was 3 L/min, the heating gas flow was 10 L/min, and the drying gas flow was 10 L/min. The samples were kept at 4 °C before injection by the autosampler. The MS/MS conditions for each target were optimized using the automated multiple reaction monitoring (MRM) optimization procedures in LabSolutions (Shimadzu). The MRM used for quantification was *m/z* 880.5 > 197.1 for oxidized CoQ<sub>10</sub>, 882.4 > 197.0 for reduced CoQ<sub>10</sub>, and 890.4 > 206.2 for CoQ<sub>10</sub>-d9 (internal standard). Standards and samples were quantified using the LabSolutions software program to determine the peak area for oxidized CoQ<sub>10</sub>, reduced CoQ<sub>10</sub>, CoQ<sub>10</sub>-d9, and the standard curves were used to determine the total amount of CoQ present in the samples.

Intra-assay coefficients of validation (CVs) and relative errors (REs), as measurements of precision and accuracy, respectively, were determined in five parallel analyses of the same cell. To evaluate inter-assay precision and accuracy, one cell line was independently evaluated on three different days. Precision was calculated as (standard deviation/mean concentration) × 100 (%), and accuracy was calculated as (quantitative value/theoretical value) × 100 (%). The intra-assay precision (CV) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 0.58% and 1.39%, respectively. The intra-assay accuracy (RE) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 4.20% and 2.50%, respectively. The inter-assay precision (CV) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 1.27% and 1.84%, respectively. The intra-assay accuracy (RE) of reduced CoQ<sub>10</sub> and oxidized CoQ<sub>10</sub> was 10.54% and 1.18%, respectively (Supplemental Table 1, QC1).

#### 2.3.4. Citrate synthase and protein quantification

Fibroblast CoQ<sub>10</sub> levels were expressed as citrate synthase (CS) activity (measured CoQ<sub>10</sub> values/CS units, nmol/CS units). CS activity was measured spectrophotometrically referring to the method described by Srere (1969), with 0.1 mM DTNB, 0.3 mM Acetyl-CoA, 0.5 mM Oxaloacetate, and 12–20 µg protein in 200 µL total incubation volume. CS units are determined as follows: CS Units (µmol/min/mL) = (ΔA<sub>412</sub>/min × V (mL) × dil)/13.6 × L (cm) × V<sub>enz</sub> (mL), V (mL); the reaction volume, dil; the dilution factor of the original sample, 13.6 (mM<sup>-1</sup> cm<sup>-1</sup>); the extinction coefficient of TNB at 412 nm, L (cm); pathlength for

absorbance measurement (0.552 cm),  $V_{enz}$ (mL); the volume of the enzyme sample. Protein concentrations were quantified using Qubit™ Protein Assay Kits and a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

#### 2.4. Statistical analyses

Statistical analyses were performed using the GraphPad Prism software program (version 9.01, GraphPad Software Inc., La Jolla, CA). Comparisons between samples were performed using a one-way ANOVA. The results are expressed as the mean (standard deviation). *P* values of <0.05 were considered to indicate statistical significance.

### 3. Results

#### 3.1. Total CoQ<sub>10</sub> levels were observed to decrease in all patients with primary CoQ<sub>10</sub> deficiency

We showed the reduced, oxidized, and total (sum of reduced and oxidized) CoQ<sub>10</sub> values corrected for the CS unit (nmol/CS unit) (Table 2). In addition, we also measured the CoQ<sub>10</sub> values corrected for protein levels (nmol/g protein) (Supplemental Table 2). Six patients showed decreased the total CoQ<sub>10</sub> values (<70% of the control): five with primary CoQ<sub>10</sub> deficiency (Cases 1–5), and one with Kearns-Sayre syndrome (Case 19) (Table 2). The total CoQ<sub>10</sub> levels were significantly decreased in primary CoQ<sub>10</sub> deficiency than in controls (primary CoQ<sub>10</sub> deficiency ( $n = 5$ )  $1.00 \pm 0.19$  nmol / CS unit (mean  $\pm$  SD), controls ( $n = 10$ )  $2.30 \pm 0.24$  nmol / CS unit,  $p < 0.0001$ ) (Fig. 1). However, total CoQ<sub>10</sub> levels were the same in mitochondrial disease other than primary CoQ<sub>10</sub> deficiency as in controls (mitochondrial disease ( $n = 19$ )  $2.23 \pm 0.26$  nmol / CS unit,  $p = 0.93$ ).

**Table 2**

Reduced and oxidized CoQ<sub>10</sub> values and total CoQ deficiency in fibroblasts.

Case	reduced CoQ <sub>10</sub>		oxidized CoQ <sub>10</sub>		total CoQ <sub>10</sub> (nmol/CS unit)		% CoQ deficiency (%)
	mean	SD	mean	SD	mean	SD	
1	0.37	0.15	0.37	0.02	0.74	0.13	32
2	0.12	0.00	0.16	0.00	0.28	0.00	12
3	0.57	0.45	0.59	0.18	1.16	0.63	50
4	0.64	0.03	0.74	0.20	1.38	0.17	60
5	0.78	0.01	0.64	0.02	1.42	0.01	62
6	0.48	0.01	1.65	0.00	2.13	0.01	
7	1.03	0.04	1.00	0.32	2.03	0.29	
8	1.22	0.04	0.82	0.18	2.04	0.22	
9	1.40	0.54	0.96	0.03	2.36	0.51	
10	0.86	0.16	1.34	0.30	2.20	0.46	
11	1.02	0.08	2.17	0.53	3.19	0.44	
12	1.18	0.56	1.31	0.17	2.49	0.38	
13	1.33	0.03	0.57	0.02	1.90	0.00	
14	1.77	0.31	0.58	0.10	2.35	0.21	
15	1.54	0.09	0.71	0.00	2.25	0.09	
16	0.86	0.02	1.46	0.01	2.32	0.01	
17	1.37	0.40	1.28	0.14	2.65	0.25	
18	1.01	0.20	1.52	0.08	2.53	0.29	
19	0.57	0.01	0.72	0.02	1.29	0.02	56
20	1.41	0.40	0.51	0.11	1.92	0.51	
21	1.24	0.28	0.65	0.28	1.89	0.56	
22	1.19	0.11	0.87	0.23	2.06	0.34	
23	0.81	0.19	1.84	0.46	2.65	0.27	
24	0.92	0.01	1.23	0.00	2.15	0.01	
Reference (n = 10)	1.19	0.15	1.11	0.10	2.30	0.24	

% CoQ deficiency: <70% of control CoQ<sub>10</sub> value.

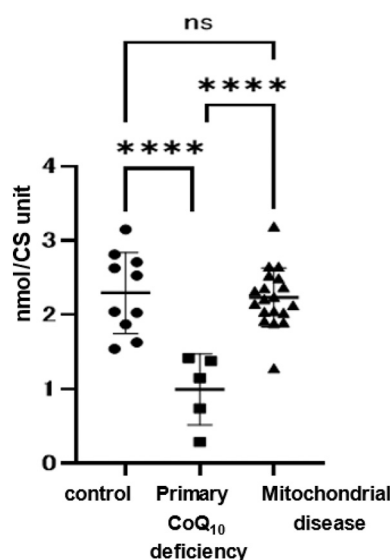
#### 3.2. The reduced/total CoQ<sub>10</sub> ratio was unchanged in primary CoQ<sub>10</sub> deficiency, but was higher in complex IV or V deficiency

We showed the ratio of reduced CoQ<sub>10</sub> to total CoQ<sub>10</sub> (reduced/total CoQ<sub>10</sub>) of fibroblasts (Table 3). The ratio of reduced and oxidized CoQ<sub>10</sub> to total CoQ<sub>10</sub> in control fibroblasts was 52% and 48%, respectively (Table 3). In primary CoQ<sub>10</sub> deficiency, the reduced/total CoQ<sub>10</sub> ratio did not change compared to the control (reduced/total CoQ<sub>10</sub> ratio of primary CoQ<sub>10</sub> deficiency ( $n = 5$ )  $49 \pm 7\%$  (mean  $\pm$  SD), controls ( $n = 10$ )  $52 \pm 1\%$ ,  $p = 0.92$ ) (Fig. 2). Regarding the cases with respiratory chain complex deficiency, there was no difference between control and complex I deficiency (reduced/total CoQ<sub>10</sub> ratio of complex I deficiency ( $n = 7$ )  $44 \pm 7\%$ ,  $p = 0.37$ ). However, the reduced/total CoQ<sub>10</sub> ratio in complex IV or V deficiency was increased in comparison to the control (complex IV or V deficiency ( $n = 3$ )  $71 \pm 3\%$ ,  $p = 0.022$ ). In addition, the reduced/total CoQ<sub>10</sub> ratio in complex IV or V deficiency was higher in comparison to complex I deficiency and primary CoQ<sub>10</sub> deficiency (complex IV or V deficiency vs. complex I deficiency:  $p = 0.0021$ , complex IV or V deficiency vs. primary CoQ<sub>10</sub> deficiency:  $p = 0.015$ ).

In individual cases, the reduced/total CoQ<sub>10</sub> ratio decreased (<80% of the reduced/total CoQ<sub>10</sub> ratio in the control) in six cases; three cases with complex I deficiency (Cases 6, 10, 11), two cases with mtDNA depletion syndrome (Cases 16, 18), one case with BOLA3 mutation (Case 23) (Table 3). On the other hand, the reduced/total CoQ<sub>10</sub> ratio increased (>120% of the reduced/total CoQ<sub>10</sub> ratio in the control) in five cases; two cases with complex IV deficiency (Cases 13, 14), two cases with MELAS (Cases 20, 21), one case with complex V deficiency (Case 15) (Table 3).

### 4. Discussion

We showed here that the total CoQ<sub>10</sub> values of fibroblasts were significantly lower in patients with primary CoQ<sub>10</sub> deficiency. In this syndrome, early recognition and therapy can stop progression and improve the prognosis; however, established severe symptoms cannot be reversed [16,34]. Biochemical measurements of low CoQ<sub>10</sub> levels in muscle biopsy have been utilized for the diagnosis of this syndrome



**Fig. 1.** Total CoQ<sub>10</sub> values.

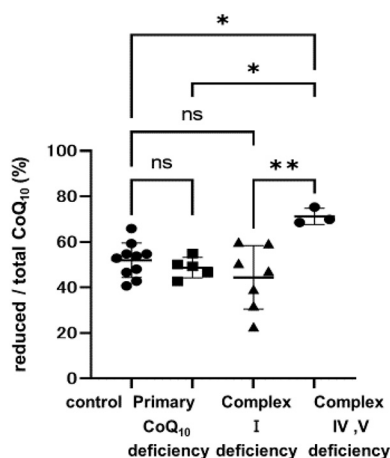
Total (sum of reduced and oxidized) CoQ<sub>10</sub> values of mitochondrial patient fibroblasts. CoQ<sub>10</sub> deficiency (CoQ<sub>10</sub> levels <70% of control CoQ<sub>10</sub>) was found in six cases. All cases of primary CoQ<sub>10</sub> deficiency showed decreased CoQ<sub>10</sub> levels. In mitochondrial disease, CoQ<sub>10</sub> values were not decreased, with the exception of Case 19 (Kearns-Sayre syndrome). Data are expressed as \*\*\*\*  $P < 0.0001$ , and n.s. indicates no significance.

**Table 3**  
Ratio of reduced / total CoQ<sub>10</sub>.

Case	reduced/total CoQ <sub>10</sub> (%)		Reduced/total CoQ <sub>10</sub> in cases versus reduced/total CoQ <sub>10</sub> in controls (%)
	mean	SD	
1	50	11	96
2	43	1	83
3	49	14	95
4	47	8	90
5	55	1	106
6	23	0	44
7	51	9	97
8	60	4	115
9	59	10	114
10	39	1	75
11	32	7	61
12	47	15	91
13	70	1	135
14	75	7	145
15	68	1	131
16	37	1	71
17	52	10	100
18	40	3	77
19	44	0	85
20	74	1	142
21	66	5	127
22	58	4	111
23	30	10	59
24	43	0	83
Reference (n = 10)	52	1	

The reduced/total CoQ<sub>10</sub> ratio decreased (< 80% of control value); cases 6, 10, 11, 16, 18, 23.

The reduced/total CoQ<sub>10</sub> ratio increased (120% < of control value); cases 13, 14, 15, 20, 21.



**Fig. 2.** Comparison of the reduced/total CoQ<sub>10</sub> ratio.

Comparison of the reduced/total CoQ<sub>10</sub> ratio in cases with primary CoQ<sub>10</sub> deficiency, complex I deficiency, and complex IV or V deficiency. In primary CoQ<sub>10</sub> deficiency, the reduced/total CoQ<sub>10</sub> ratio was the same as that of the controls. In complex I deficiency, 3/7 cases showed a decreased (< 80% of control value) reduced/total CoQ<sub>10</sub> ratio. In complex IV or V deficiency, 3/3 cases showed an increased (120% < of control value) reduced/total CoQ<sub>10</sub> ratio. Data are expressed as \**P* < 0.05, \*\**P* < 0.01, and n.s. indicates no significance.

[16]. Moreover, the identification of biallelic pathogenic variants in the *COQ* genes, which encode proteins involved in coenzyme Q biosynthesis, enables a definitive diagnosis [16]. However, the invasiveness of muscle biopsy hampers this procedure and can delay the diagnosis. CoQ<sub>10</sub> levels in fibroblasts were examined and implicated in their usefulness [21]. Our data with LC-MS/MS for the measurement of CoQ<sub>10</sub> from fibroblasts also supported the usefulness for detecting CoQ<sub>10</sub>

deficiency [32,35]. The fibroblasts from Case 2 showed the lowest CoQ<sub>10</sub> concentration and showed a very severe phenotype. A correlation between CoQ<sub>10</sub> levels and phenotype has been suggested [24,36,37]. Therefore, the CoQ<sub>10</sub> value from skin fibroblast may reflect the clinical severity.

In addition to primary CoQ<sub>10</sub> deficiency, various mitochondrial diseases have also been reported to decrease CoQ<sub>10</sub> in fibroblasts and muscle, particularly in mtDNA depletion syndrome [20,38]. However, in our analysis, three patients with mtDNA depletion, including *DGUOK* and *MPV17* mutations, showed no decrease in CoQ<sub>10</sub> (Cases 16–18). Only one patient with large deletions of mtDNA showed markedly decreased CoQ<sub>10</sub> (Case 19). Therefore, decreased CoQ<sub>10</sub> was only a constant feature in primary CoQ<sub>10</sub> deficiency syndrome in our analysis. Our results support the widely accepted idea that early CoQ<sub>10</sub> therapy should be therefore indicated if decreased levels of CoQ<sub>10</sub> are found in the fibroblasts of patients with suspected mitochondrial disease.

To our knowledge, this is the first report to describe the reduced/total CoQ<sub>10</sub> ratios in patients with mitochondrial diseases, including primary CoQ<sub>10</sub> deficiency. In primary CoQ<sub>10</sub> deficiency, the reduced/total CoQ<sub>10</sub> ratio did not change. On the other hand, the reduced/total CoQ<sub>10</sub> ratio was decreased in 3/7 of cases of complex I deficiency and was increased in 3/3 of cases of complex IV or V deficiency. Primary CoQ<sub>10</sub> deficiency is caused by impaired CoQ<sub>10</sub> biosynthesis, which results in a decrease in the absolute value of CoQ<sub>10</sub>; however, as expected, our data suggest that it does not affect the redox reaction in mitochondria. Since CoQ<sub>10</sub> changes from the oxidized form to the reduced form by accepting electrons from complex I, complex II and other dehydrogenases, the reduced/total CoQ<sub>10</sub> ratio is expected to decrease in complex I deficiency. However, only 3 out of 7 complex I patients in our cohort showed disturbed Q reduced/total ratios. In contrast, CoQ<sub>10</sub> changes from the reduced form to the oxidized form in complex III, and the reduced/total CoQ<sub>10</sub> ratio is expected to increase in complex III and later complex deficiencies (Supplemental Fig. 1, left panel). In fact, the reduced CoQ<sub>10</sub> ratio was significantly decreased with complex I inhibitor, whereas the reduced CoQ<sub>10</sub> ratio was increased with complex IV inhibitor [23]. Some (but not all) of our results support these observations. Among complex I deficiency patients, patients with isolated complex I deficiency tended to have a decreased reduced/total CoQ<sub>10</sub> ratio (3/4 cases; Cases 6, 10, 11) (Supplemental Table 3). On the other hand, the reduced/total CoQ<sub>10</sub> ratio was unchanged in a patient who also had a decreased complex III and IV enzyme activity (Case 8), in a patient without a decreased CI enzyme activity in fibroblasts (Case 12), and in a patient with a decreased CI enzyme activity in muscle (Case 7).

In recent years, it has been reported that the reduced/oxidized CoQ<sub>10</sub> ratio may be a marker of mitochondrial metabolic status [39]. In situations where CoQ<sub>FADH</sub> can be excessively reduced, RET is induced, and the production of reactive oxygen species (ROS) from complex I is stimulated, which causes complex I destruction [4,6]. On the other hand, oxidizing the CoQ pool by alternative oxidase (AOX) of *Ciona intestinalis* xenotopically expressed in mouse mitochondria induces forward electron transport (FET) from RET [40]. Our system of measuring the reduced/total CoQ<sub>10</sub> ratio of skin fibroblasts provide the amount and redox states of CoQ<sub>10</sub>.

As a limitation of our study, CoQ<sub>10</sub> is mostly localized in the mitochondria in subcellular fractions but it has also been shown to localize in Golgi, lysosomes, and other organelles [3]. In this study, we measured the whole cell CoQ<sub>10</sub> level without separating the mitochondrial and non-mitochondrial fractions. Moreover, we examined an only limited number of patients affected by only some mitochondrial diseases, which can be caused by >400 gene mutations [41].

In conclusion, we measured the reduced/total CoQ<sub>10</sub> ratio in fibroblasts from a cohort of patients with mitochondrial disease for the first time. The reduced/total CoQ<sub>10</sub> ratio tended to show no change in many of the cells that we measured. However, the reduced/total CoQ<sub>10</sub> ratio was increased in complex IV or V deficiency, while the reduced/total CoQ<sub>10</sub> ratio tended to decrease in some cases of complex I deficiency.

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### Author contributions

CW, HO contributed to the conceptualization and performance of the statistical analysis of the data and wrote the manuscript; MW, AM, EFJ, TT, HU, OT, ET, and KA performed sampling and data acquisition; YK, YO performed genetic testing; KM, AO recruited patients, provided clinical information, collected samples; TY conducted supervision of the project. All authors read and approved the final manuscript.

### Ethics

All procedures followed were by the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of the World Medical Organization. This study was approved by the Ethics Committee of Jichi Medical University and written informed consent was obtained from all participants.

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### CRedit authorship contribution statement

**Chika Watanabe:** Conceptualization, Writing – original draft. **Hitoshi Osaka:** Conceptualization, Writing – review & editing, Funding acquisition. **Miyuki Watanabe:** Investigation. **Akihiko Miyauchi:** Investigation. **Eriko F. Jimbo:** Investigation. **Takeshi Tokuyama:** Resources. **Hideki Uosaki:** Resources. **Yoshihito Kishita:** Resources. **Yasushi Okazaki:** Resources. **Takanori Onuki:** Resources. **Tomohiro Ebihara:** Resources. **Kenichi Aizawa:** Resources. **Kei Murayama:** Resources. **Akira Ohtake:** Resources. **Takanori Yamagata:** Supervision.

### Declaration of Competing Interest

None.

### Data availability

No data was used for the research described in the article.

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OPEN

# Apomorphine is a potent inhibitor of ferroptosis independent of dopaminergic receptors

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Originally, apomorphine was a broad-spectrum dopamine agonist with an affinity for all subtypes of the Dopamine D1 receptor to the D5 receptor. We previously identified apomorphine as a potential therapeutic agent for mitochondrial diseases by screening a chemical library of fibroblasts from patients with mitochondrial diseases. In this study, we showed that apomorphine prevented ferroptosis in fibroblasts from various types of mitochondrial diseases as well as in normal controls. Well-known biomarkers of ferroptosis include protein markers such as prostaglandin endoperoxide synthase 2 (PTGS2), a key gene for ferroptosis-related inflammation PTGS2, lipid peroxidation, and reactive oxygen species. Our findings that apomorphine induced significant downregulation of PTGS2 and suppressed lipid peroxide to the same extent as other inhibitors of ferroptosis also indicate that apomorphine suppresses ferroptosis. To our knowledge, this is the first study to report that the anti-ferroptosis effect of apomorphine is not related to dopamine receptor agonist action and that apomorphine is a potent inhibitor of ferroptotic cell death independent of dopaminergic receptors.

Mitochondrial disease is a genetic disorder consisting of various clinical phenotypes, including Leigh syndrome (LS), myopathy encephalopathy lactic acidosis, stroke-like episodes (MELAS), Kearns-Sayre syndrome (KSS), and mitochondrial cardiomyopathy<sup>1,2</sup>. At present, no curative treatment is available and effective treatment options are eagerly awaited. In our previous study, we screened a chemical library based on drugs already approved for central nervous system (CNS) diseases using fibroblasts from two patients with LS and two patients with MELAS using an L-buthionine-S, R-sulfoximine (BSO)-induced cell viability assay. BSO has been used to induce cell death by inhibiting glutathione (GSH) biosynthesis, which leads to the overproduction of reactive oxygen species (ROS)<sup>3</sup>. In addition, BSO has been recently reported to trigger ferroptosis in various cancer cells<sup>4,5</sup>.

While searching for potential therapeutic drugs for mitochondrial diseases, we identified apomorphine as a potential therapeutic drug for LS and MELAS<sup>6</sup>. Apomorphine is a known treatment for Parkinson's disease (PD) and erectile dysfunction<sup>7-9</sup>. It is a broad-spectrum dopamine agonist for all subtypes ranging from the dopamine D1 receptor to the D5 receptor. Apomorphine exerts protective effects by inhibiting oxytosis and stress-dependent non-apoptotic cell death via activation of the dopamine D4 receptor (D4R) in a micromolar order<sup>10,11</sup>.

Because oxytosis shares some concepts with ferroptosis proposed in recent years, one hypothesis is that the ferroptotic action of apomorphine is related to dopamine receptors. Ferroptosis is a common form of regulated cell death that is distinguished from apoptosis and necroptosis and is a form of lipid- and iron-dependent cell death associated with GSH depletion<sup>12-14</sup>. The mechanism of ferroptosis includes oxidative damage to cellular structures and dysfunction in membrane stability triggered by polyunsaturated fatty acid peroxidation<sup>13,15</sup>. It can be induced by GSH depletion using chemicals such as BSO, cystine-glutamate transporter inhibition, or GPX4 inhibition<sup>12,16</sup>. Among them, RSL3, which can inactivate GPX4, is the most commonly used inducer of ferroptosis, leading to excessive lipid peroxidation, which causes cell death<sup>16</sup>. There are also several biomarkers of ferroptosis,

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including protein markers, such as PTGS2 (which encodes COX-2), lipid peroxidation, and ROS<sup>17–20</sup>. In recent years, ferroptosis has been widely investigated for its involvement in various diseases, including mitochondrial diseases, neurodegenerative diseases, liver toxicity, liver fibrosis, hemochromatosis, and cardiomyopathy<sup>13,21–24</sup>. Also ferroptosis is regulated by various cellular metabolic pathways, including redox homeostasis, mitochondrial activity, and iron metabolism<sup>13,15</sup>.

In the present study, we found that apomorphine inhibited ferroptosis in fibroblasts from patients with mitochondrial disease as well as in normal controls, and the cell-protective effect was not related to the agonistic action of the dopamine receptor.

## Results

### Induction of ferroptosis by GSH depletion by BSO and prevention of BSO-induced ferroptosis by apomorphine in LS patient fibroblasts

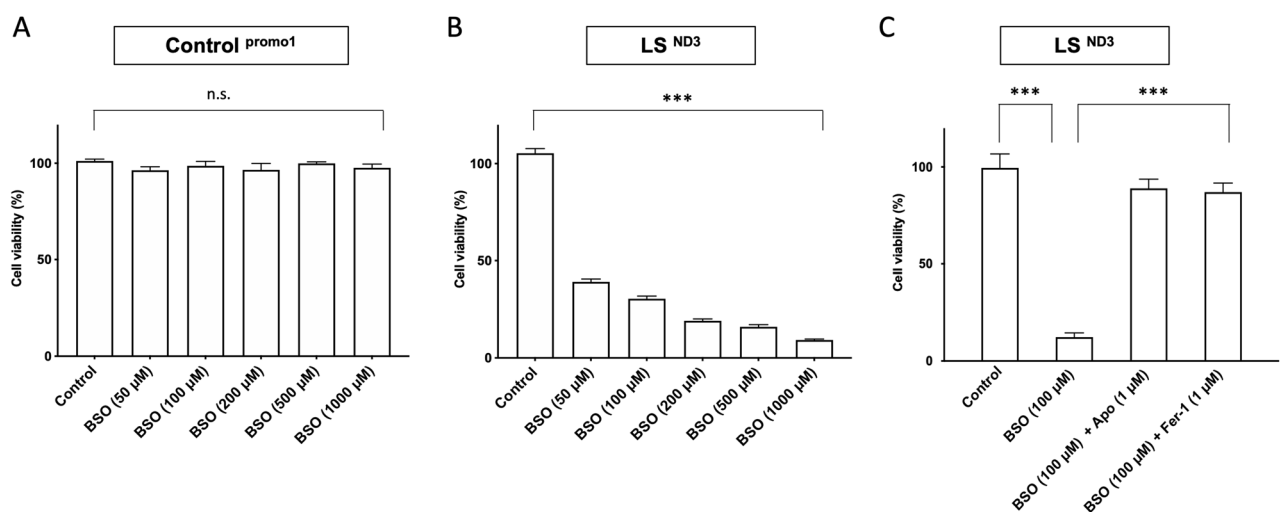
BSO reduced GSH production in a concentration-dependent manner<sup>25</sup>. Under these conditions, fibroblasts from LS patients showed enhanced cell death and were more vulnerable to BSO-induced cell stress than control fibroblasts, as previously described (Fig. 1A,B)<sup>6</sup>. We subsequently performed BSO-induced cell survival assays to investigate whether or not they were suppressed by adding ferrostatin-1 (Fer-1), one of the most common ferroptosis inhibitors<sup>13,16</sup>, to LS patient-derived fibroblasts. Fer-1 showed dramatic cell-protective effects under BSO-induced stress, and apomorphine showed similar effects (Fig. 1C).

### Protective effect of apomorphine against ferroptosis induced by BSO and RSL3

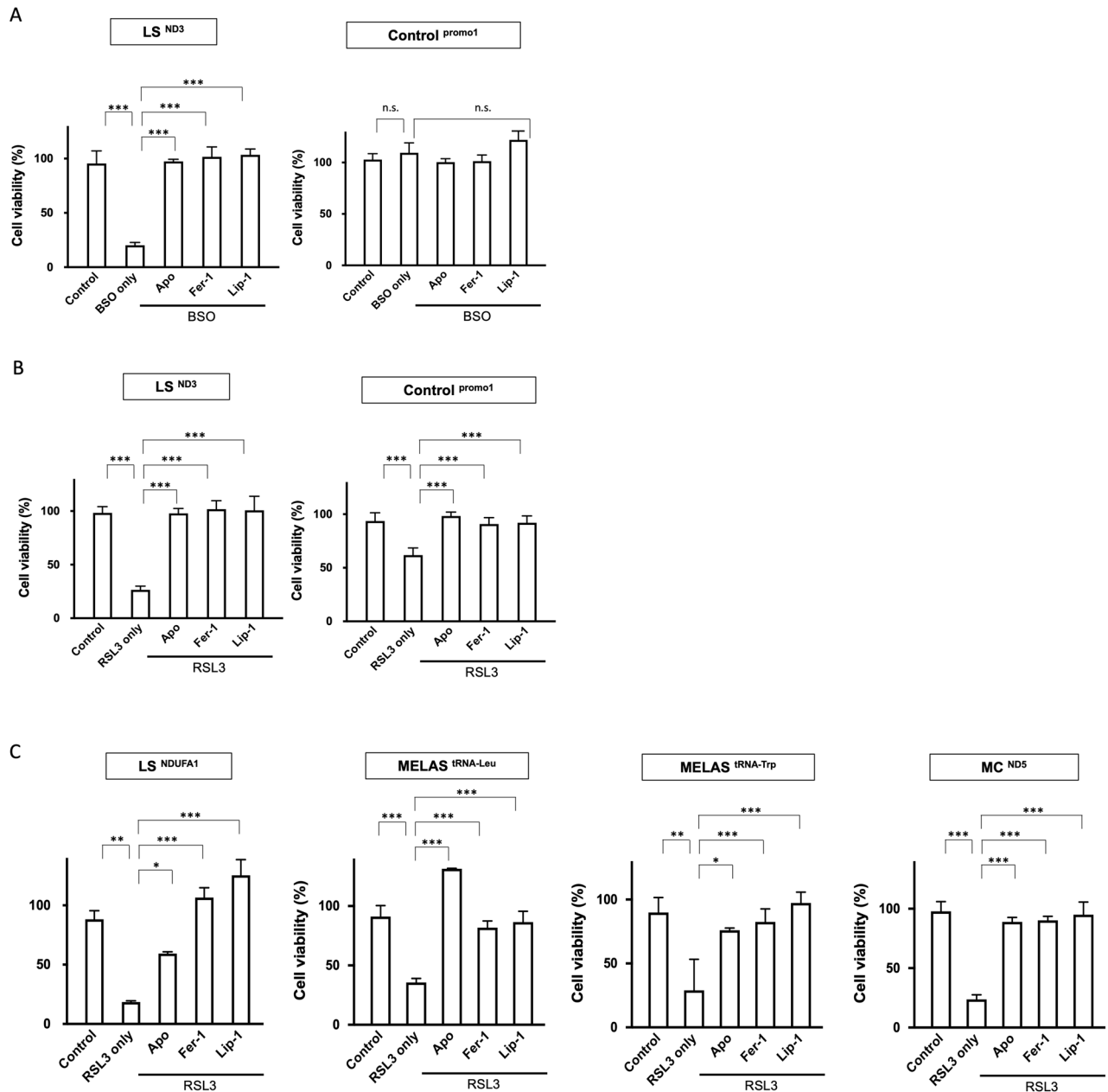
To evaluate the effects of apomorphine against ferroptosis, we examined the survival of LS fibroblasts in the presence of RSL3 as a common assay for ferroptosis screening, in addition to a BSO assay<sup>16,26</sup>. Prior to that, we examined the cell survival rate of control and patient fibroblasts under RSL3-induced stress, an inducer of ferroptosis. Fibroblasts obtained from LS, MELAS, mitochondrial cardiomyopathy, and KSS patients showed enhanced cell death in response to RSL3-induced stress, but this effect was less pronounced than the response to BSO-induced stress (Supplementary Fig. 1A–F).

Subsequently, we compared the cell protective effect of apomorphine with that of Fer-1 and Liproxstatin-1 (Lip-1), well-known specific ferroptosis inhibitors<sup>16,27,28</sup>. Apomorphine in LS fibroblasts under ferroptosis induced by BSO showed cell-protective effects, but we could not investigate the effects of apomorphine in response to BSO-induced stress in control fibroblasts because BSO did not induce cell death in control fibroblasts (Fig. 2A). In the case of RSL3-induced stress, the difference in the survival between control and LS fibroblasts was less than that in response to RSL3-induced stress; apomorphine showed cell-protective effects in both control and LS fibroblasts. The effects of apomorphine were similar to those of Fer-1 and Lip-1 (Fig. 2B). We also examined other fibroblasts from different subtypes of mitochondrial diseases in the presence of RSL3 and obtained similar results (Fig. 2C).

We next examined the effects of various inhibitors of cell death pathways other than ferroptosis to rule out the possibility that apomorphine protects cells via other cell death pathways using GSK-872 (necroptosis inhibitor) or Z-VAD-FMK (apoptosis inhibitor). Although we could not examine the effects of these inhibitors in response to BSO-induced stress in control fibroblasts, BSO did not induce cell death in control fibroblasts, and neither GSK-872 nor Z-VAD-FMK showed any cell-protective effects against cell death induced by BSO and RSL3 (Fig. 3A,B).



**Figure 1.** The cell viability assay for fibroblast cells from LS patients (LS<sup>ND3</sup>) under BSO-induced stress. While the fibroblasts of control subjects were able to maintain almost full viability (A), those from patients with LS showed enhanced BSO dose-dependent cell death (B). Under the conditions of 100 μM BSO treatment, we evaluated the effects of Fer-1 (1 μM), which is one of the most common ferroptosis inhibitors and Apo (1 μM). Fer-1 showed dramatic cell-protective effects, similar to those of Apo (C). Apo apomorphine, Fer-1 Ferrostatin-1. Data (n = 6) are expressed as the mean ± SD \*\*\*P < 0.001. n.s. no statistical significance.

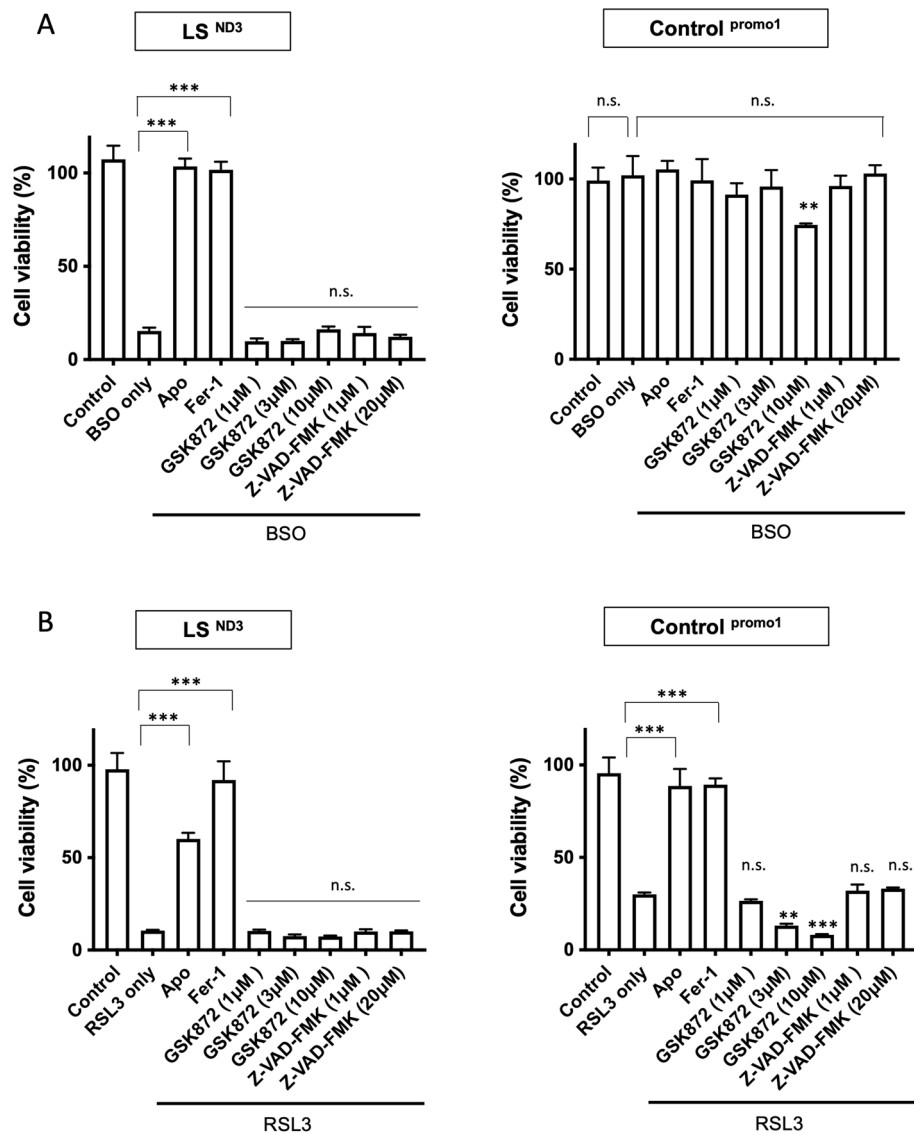


**Figure 2.** The anti-ferroptosis effect of apomorphine. **(A,B)** The cell viability assay of fibroblasts from LS patients (LS<sup>ND3</sup>) (left panel) and control (right panel) in the presence of 100  $\mu$ M BSO and RSL3. **(C)** The cell viability assay of fibroblasts from patients with LS (LS<sup>NDUFA1</sup>), MELAS (MELAS<sup>tRNA-Leu</sup>, MELAS<sup>tRNA-Trp</sup>), mitochondrial cardiomyopathy (MC<sup>ND5</sup>), and KSS (KSS<sup>large deletion</sup>). The cell-protective effects of apomorphine were also shown in comparison to Fer-1, and Lip-1 in the presence of RSL3. Apo apomorphine, Fer-1 Ferrostatin-1. Data (n = 6) are expressed as the mean  $\pm$  SD \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. n.s. indicates no significance.

These findings suggest that apomorphine protects against cell death by ferroptosis, but not by necroptosis or apoptosis, in control fibroblasts and fibroblasts derived from patients with mitochondrial disease.

### Measurement of intracellular ferroptotic metabolites in our cell lines by a BSO cell lipid oxidation assay

Because the accumulation of lipid ROS is a hallmark of ferroptosis, we monitored the lipid ROS accumulation using C11 BODIPY 581/591 by confocal imaging (Fig. 4). The C11 BODIPY 581/591 changes the fluorescence from red to green upon oxidation<sup>29</sup>. Even in the absence of BSO, LS patient-derived fibroblasts showed the oxidized form of C11 BODIPY, which is indicative of lipid ROS (Fig. 4A\_b). Upon BSO treatment, fibroblasts showed an increased lipid ROS accumulation (Fig. 4A\_c). Furthermore, Fer-1, one of the most common inhibitors of ferroptosis, recovered the lipid ROS accumulation. When we examined the effect of apomorphine on the



**Figure 3.** Cell viabilities by apoptosis and necroptosis inhibitors. The effects of an apoptosis inhibitor (GSK872) and a necroptosis inhibitor (Z-VAD-FMK) under conditions of 100  $\mu$ M BSO- (A), and RSL3- (B) induced stress for fibroblast cells for LS patients (LS<sup>ND3</sup>) (left) and controls (right). Apo apomorphine, Fer-1 Ferrostatin-1. Data (n = 3) are expressed as the mean  $\pm$  SD \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001. n.s. indicates no significance.

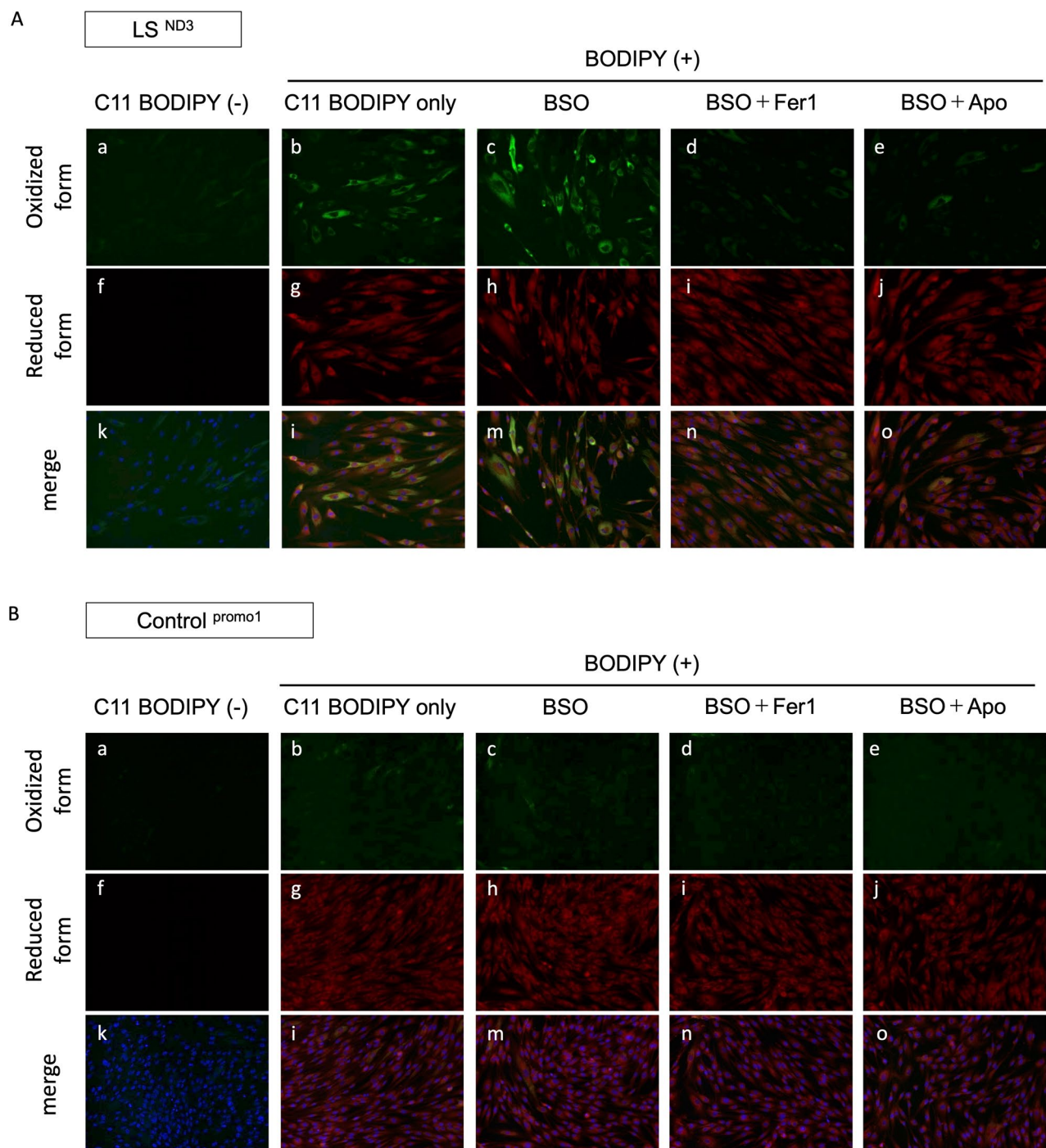
accumulation of lipid ROS in LS patient-derived fibroblasts treated with BSO, apomorphine decreased the lipid ROS accumulation and showed a similar effect to Fer-1 (Fig. 4A\_d,e)<sup>16</sup>. In contrast, control fibroblasts showed almost no change in the degree of the lipid ROS accumulation (Fig. 4B\_a–o).

### The assessment of the expression of genes related to ferroptosis in apomorphine-treated vs. untreated LS fibroblasts by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blotting

We assessed the expression of *prostaglandin endoperoxide synthase 2* (*PTGS2*), a biomarker of ferroptosis that encodes cyclooxygenase-2 (COX-2)<sup>13,17–19</sup> in LS fibroblasts (LS<sup>ND3</sup>). Real-time RT-PCR showed that RSL3 upregulated *PTGS2* expression in LS fibroblasts and that this *PTGS2* upregulation was inhibited by apomorphine and Fer-1 treatment (Fig. 5A). Western blotting for *PTGS2* showed that apomorphine significantly decreased the protein levels of *PTGS2* in LS fibroblasts, and Fer-1 had a similar effect (Fig. 5B). We therefore demonstrated that apomorphine suppressed *PTGS2*, a key factor linking ferroptosis and inflammation, in LS fibroblasts.

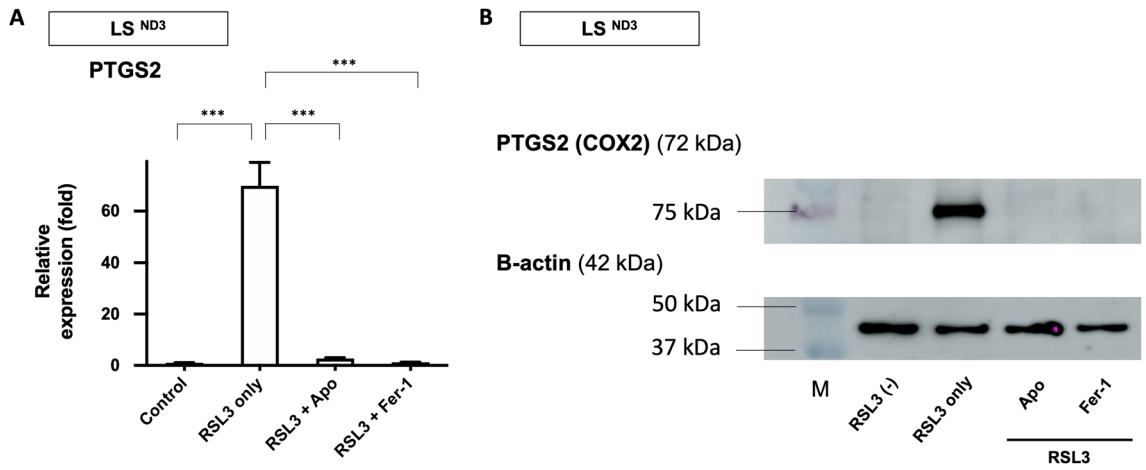
### The analysis of the expression of genes related to ferroptosis in apomorphine-treated vs. untreated LS fibroblasts by real-time RT-PCR

To examine how apomorphine inhibits ferroptosis, we assessed the following key genes related to ferroptosis: *AIFM2* (encoding FSP1), *GPX4*, *ACSL4*, and *SLC7A11* (cystine/glutamate transporter)<sup>13,17–19</sup>. Real-time RT-PCR

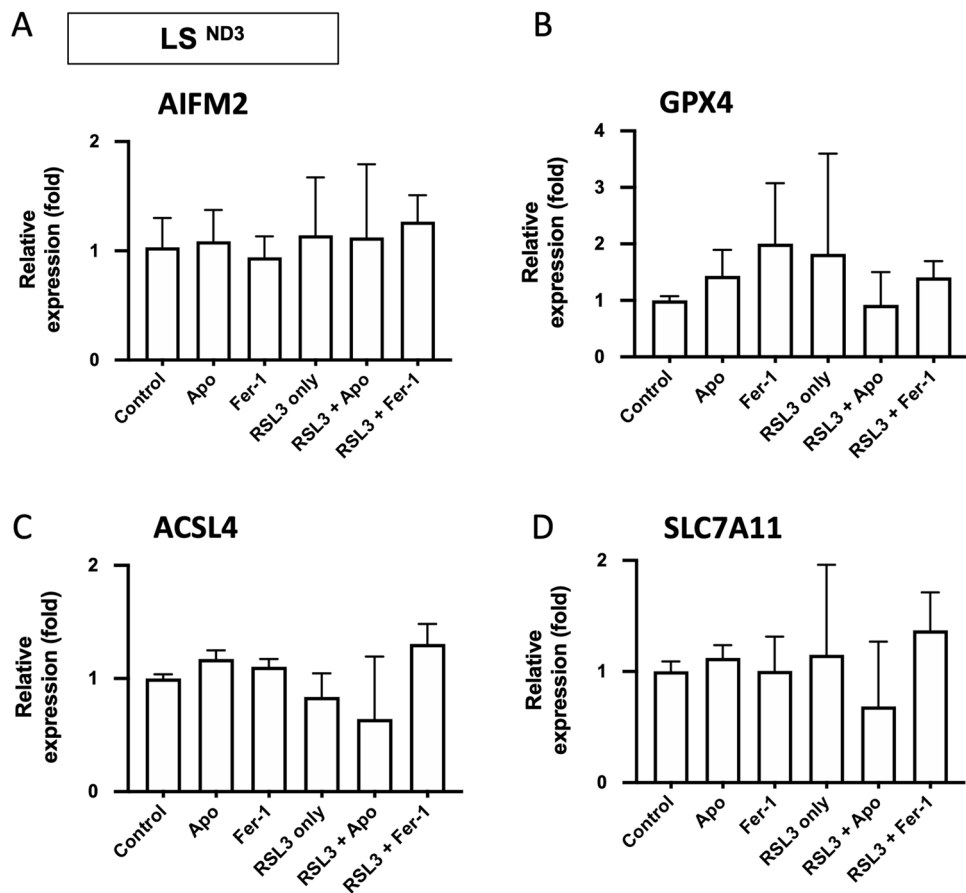


**Figure 4.** Results of a C11 BODIPY581/591 assay in fibroblasts from patients and controls. Measurement of intracellular ferroptotic metabolites by the BSO cell lipid oxidation assay. **(A)** Fibroblasts from LS patients (LS<sup>ND3</sup>) were treated with BSO (100  $\mu$ M) for 24 h in the presence or absence of Apo (1  $\mu$ M) and Fer-1 (1  $\mu$ M). **(B)** Control fibroblasts were treated with BSO (100  $\mu$ M) for 24 h in the presence or absence of Apo (1  $\mu$ M) and Fer-1 (1  $\mu$ M). Lipid peroxidation was assessed by C11 BODIPY581/591 staining. Representative images of C11 BODIPY581/591 staining in LS<sup>ND3</sup> cells and control cells; Oxidized form (**a–e**), Reduced form (**f–j**), and merge (**k–o**). Apo apomorphine, Fer-1 Ferrostatin-1.

showed that apomorphine slightly suppressed the upregulation of *SLC7A11*; however, the differences were not statistically significant. Apomorphine also did not affect the mRNA expression of *AIFM2*, *GPX4*, or *ACSL4* (Fig. 6A–D).



**Figure 5.** An expression analysis of prostaglandin-endoperoxide synthase 2 (PTGS2). **(A)** *PTGS2* mRNA levels in *LS<sup>ND3</sup>* cells treated with RSL3 (100 nM) for 24 h in the presence or absence of Apo (1 μM) and Fer-1 (1 μM) were assessed using real-time RT-PCR. **(B)** *PTGS2* protein levels in *LS<sup>ND3</sup>* cells treated with RSL3 (100 nM) for 24 h in the presence or absence of Apo (1 μM) and Fer-1 (1 μM) were assessed by Western blotting. Full-length gels and blots are included in a Supplementary (Supplemental Fig. 3) as full-length dot blots. *Apo* apomorphine, *Fer-1* Ferrostatin-1. Data are expressed as the mean ± SD; \*\*\**P* < 0.001.



**Figure 6.** The mRNA levels of various ferroptosis marker genes. **(A)** *AIFM2* (encoding FSP1), **(B)** *GPX4*, **(C)** *ACSL4*, and **(D)** *SCL7A11* (Cystine/glutamate transporter) mRNA levels in *LS<sup>ND3</sup>* cells treated with RSL3 (100 nM) for 24 h in the presence or absence of Apo (1 μM) and Fer-1 (1 μM) were assessed by real-time RT-PCR. *Apo* apomorphine, *Fer-1* Ferrostatin-1. Data are expressed as the mean ± SD \**P* < 0.05, \*\* *P* < 0.01, \*\*\**P* < 0.001. n.s. indicates no significance.

## The anti-ferroptosis effect of apomorphine is not related to the dopamine receptor agonist action of apomorphine

Our previous screening did not show the cell protective effects of other dopamine agonists (Ropinirole hydrochloride and Pramipexole dihydrochloride; both specific to D2 receptors) (Supplemental Fig. 2). Therefore, to evaluate whether or not the agonistic action of apomorphine was related to its cell-protective effect, we examined the effect of the addition of agonists and/or antagonists of each dopamine receptor on the cell-protective effect of apomorphine.

Prior to this, the expression of each dopamine receptor in the cells was examined by RT-PCR. The expression of four types of dopamine receptors—D1R, D2R, D4R, and D5R—was confirmed in LS and control fibroblasts, but the expression of D3R was not detected in our fibroblasts (Fig. 7A). Therefore, we examined the action of each dopamine receptor on the protection of apomorphine, except for D3R.

First, we examined the contribution of D4R to the effects of apomorphine using agonists and antagonists of D4R, based on a previous report<sup>10</sup>. As shown in Fig. 7B, the D4R agonists did not enhance the effect of apomorphine, and the antagonists did not inhibit this effect. Similarly, antagonists of D1R and D2R did not affect the effect of apomorphine (Fig. 7C,D). Regarding D5R, as there was no commercially available antagonist for D5R, only the D5R agonist was used, but it did not provide any cell-protective effect (Fig. 7E).

## Discussion

We previously showed that apomorphine protects against BSO-induced stress with an EC<sub>50</sub> of approximately 50 nM and improves the mitochondrial respiratory activity of fibroblasts from patients with LS and MELAS<sup>6</sup>. In the present study, we showed that fibroblasts from mitochondrial diseases including LS and MELAS were vulnerable to ferroptosis, and apomorphine markedly protected fibroblasts from ferroptosis induced by RSL3 in addition to BSO in various types of mitochondrial diseases as well as in normal fibroblasts. The effect of ferroptosis on cell protection was comparable to that of the specific ferroptosis inhibitors Fer-1 and Lip-1. We observed that BSO- or RSL3-induced ferroptosis was not inhibited by inhibitors of apoptosis (Z-VAD-FMK) or necroptosis (GSK-872), and *PTGS2*, which is known to be a marker of ferroptosis, was decreased by the addition of apomorphine. In addition, we confirmed that the effects of apomorphine were not related to its agonistic dopamine action.

Ferroptosis is an iron-dependent form of cell death caused by the accumulation of lipid hydroperoxides and is distinguished from other forms of cell death, such as apoptosis and necroptosis<sup>14,16</sup>. Ferroptosis occurs when GPX4 is directly or indirectly inhibited by GSH depletion, which leads to the accumulation of membrane lipid peroxidation and results in cell death<sup>13,15,16</sup>. RSL3 is commonly used as a specific inducer of ferroptosis and inactivates GPX4, leading to excessive lipid peroxidation, which causes cell death<sup>16</sup>. In contrast, ferroptosis can be suppressed by lipophilic antioxidants, inhibitors of lipid peroxidation, iron chelators, and polyunsaturated fatty acids<sup>16</sup>. Currently, several biomarkers of ferroptosis exist, including protein markers such as *PTGS2*, lipid peroxidation, and ROS<sup>17–20</sup>.

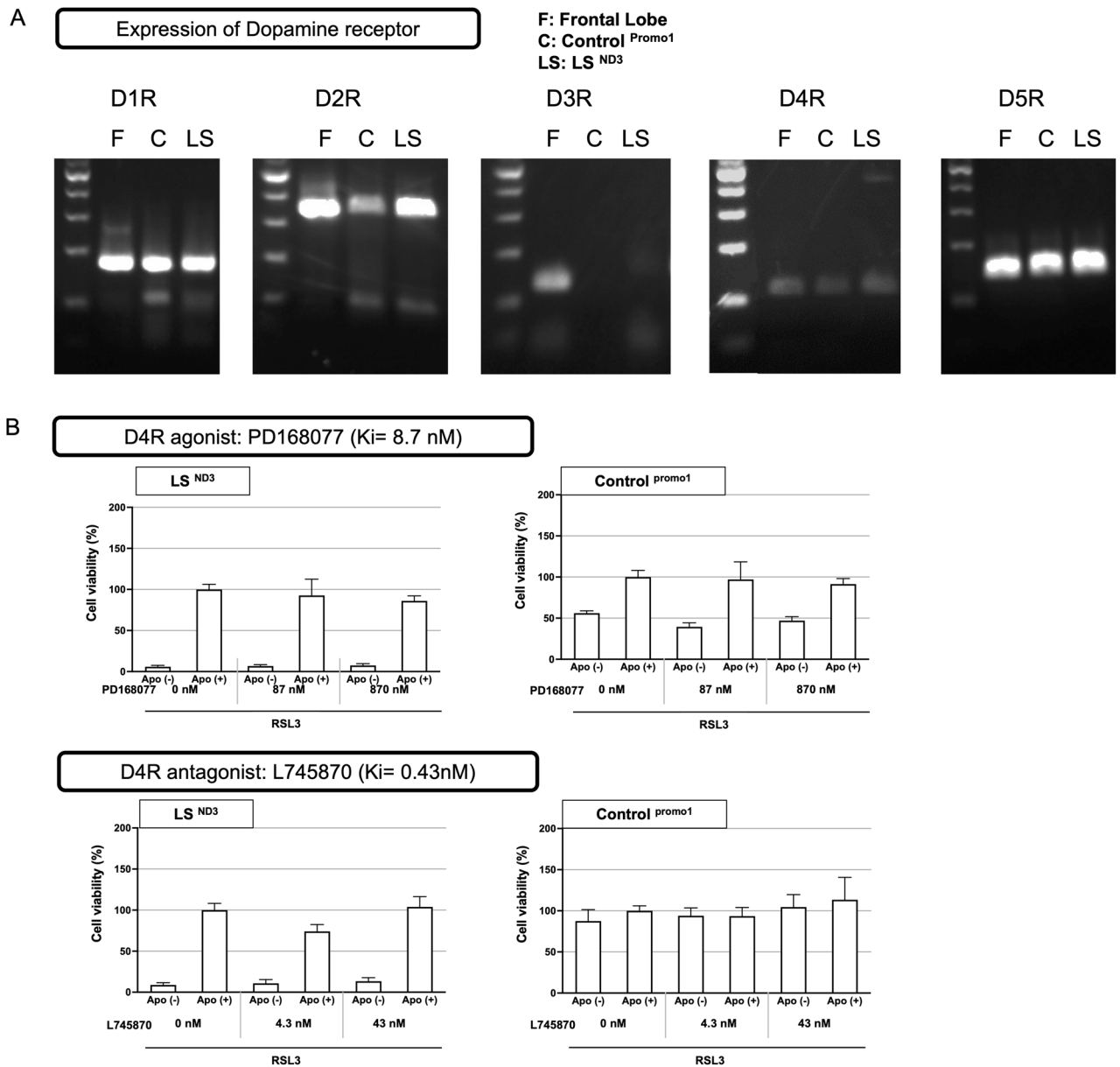
Apomorphine has been administered to patients with advanced PD as a rescue drug for “off” symptoms because of its short half-life. However, the continuous subcutaneous administration of apomorphine reportedly improves both motor symptoms as well as brain glucose metabolism and non-motor symptoms, such as the cognitive function, the executive function, and apathy in PD patients<sup>30,31</sup>. PD is known for the characteristic pathology of progressive dopaminergic neuronal death associated with iron accumulation, and it is suggested to be driven in part by ferroptosis<sup>32,33</sup>. The anti-ferroptosis effects of apomorphine shown in this study may explain these neuroprotective effects. To test this hypothesis, we need to study the effects in cells derived from patients with PD and animal models.

We have already shown that apomorphine suppresses the production of ROS<sup>6</sup>. In the present study, we showed that apomorphine prevented lipid peroxidation and upregulation of the mRNA expression of *PTGS2* (Fig. 5), which are key features of ferroptosis. Studies on cancer cells have indicated that ferroptosis can directly increase the expression of *PTGS2* and cause inflammation by accelerating AA metabolism and promoting the secretion of pro-inflammatory molecules<sup>34,35</sup>. The downregulation of *PTGS2* may be related to the inhibition of various cytokines and chemokines by apomorphine<sup>6</sup>. Recently, ferroptosis has been suggested to be involved in epilepsy with mitochondrial disease<sup>12</sup>. Our study also supports the hypothesis that fibroblasts from various mitochondrial diseases are vulnerable to ferroptosis.

Apomorphine reportedly protects against glutamate-induced oxidative cell death via dopamine receptors, especially D4, in the HT22 cell line<sup>10</sup>. In this assay system, the protective effect was reversed by D4 antagonists but not by D1 or D2 antagonists. A selective D4 agonist also protects neurons from glutamate-induced cell death. However, in our assay system using fibroblasts, the D4 agonist did not exert cell protective effects, and the D4 antagonist did not prevent the cell-protective effect of apomorphine. Furthermore, our data do not support the involvement of subtypes D1, D2, and D5. Therefore, we conclude that the cell-protective effect of apomorphine is not related to dopamine receptors in our system. Dopamine agonistic action causes several common side effects, especially digestive symptoms, such as nausea, vomiting, loss of appetite, and constipation<sup>36</sup>. Our research presents a promising avenue for the potential development of apomorphine derivatives without dopamine agonist activity, which offer cellular protection without the undesirable effects associated with dopamine agonists.

The limitation of the present study was that we were unable to identify the mechanism underlying the protection from ferroptosis, as we only performed an RT-PCR assay of key inhibitory genes for ferroptosis. Elucidation of the binding proteins that explain anti-ferroptosis and their downstream signaling cascades is the next research question that should be explored.

In conclusion, we found that fibroblasts from patients with mitochondrial diseases were vulnerable to ferroptosis, which is inhibited by apomorphine in this study. The cell-protective effect of apomorphine has long been



**Figure 7.** The expression of dopamine receptors (D1R–D5R) and interactions between apomorphine and dopamine receptor agonists or antagonists. **(A)** Agarose gel electrophoresis of the RT-PCR product of dopamine receptors. Results are shown from left to right in the order of D11R(size 184 bp), D2R (size 353 bp), D3R (size 137 bp), D4R (size 115 bp), and D5R (size 149 bp). In each column, the left lane shows the band of the frontal lobe as a positive control, the middle shows the band of control cells, and the right shows the band of LS cells. Original gels are presented in Supplementary Fig. 4. **(B–E)** The results of an RSL3-induced stressed cell viability assay to determine the influence of each dopamine receptor agonist or antagonist on the effects of apomorphine. The cell viability of the Apomorphine-treated group without RSL3 was used as 100% to compare other group’s cell viabilities (n = 3). **(B)** D4R agonist: PD168077 (Ki=8.7 nM) and D4R antagonist: L745870 (Ki=0.43 nM), **(C)** D1R antagonist: SKF683566 (Ki=0.56 nM), **(D)** D2R antagonist: Sulpiride (Ki=0.015 μM), **(E)** D5R agonist: SKF38393 (Ki=0.5 nM).

believed to be a result of D4R agonistic action, but we first revealed that at least part of its anti-ferroptosis effect is not related to the dopamine receptor agonist action. Our study suggests that ferroptosis may be a potential therapeutic target for mitochondrial disease and also provides hope for the creation of new drugs that maintain their cell-protective effect without the dopamine agonist effect, to avoid the adverse effects of dopamine.

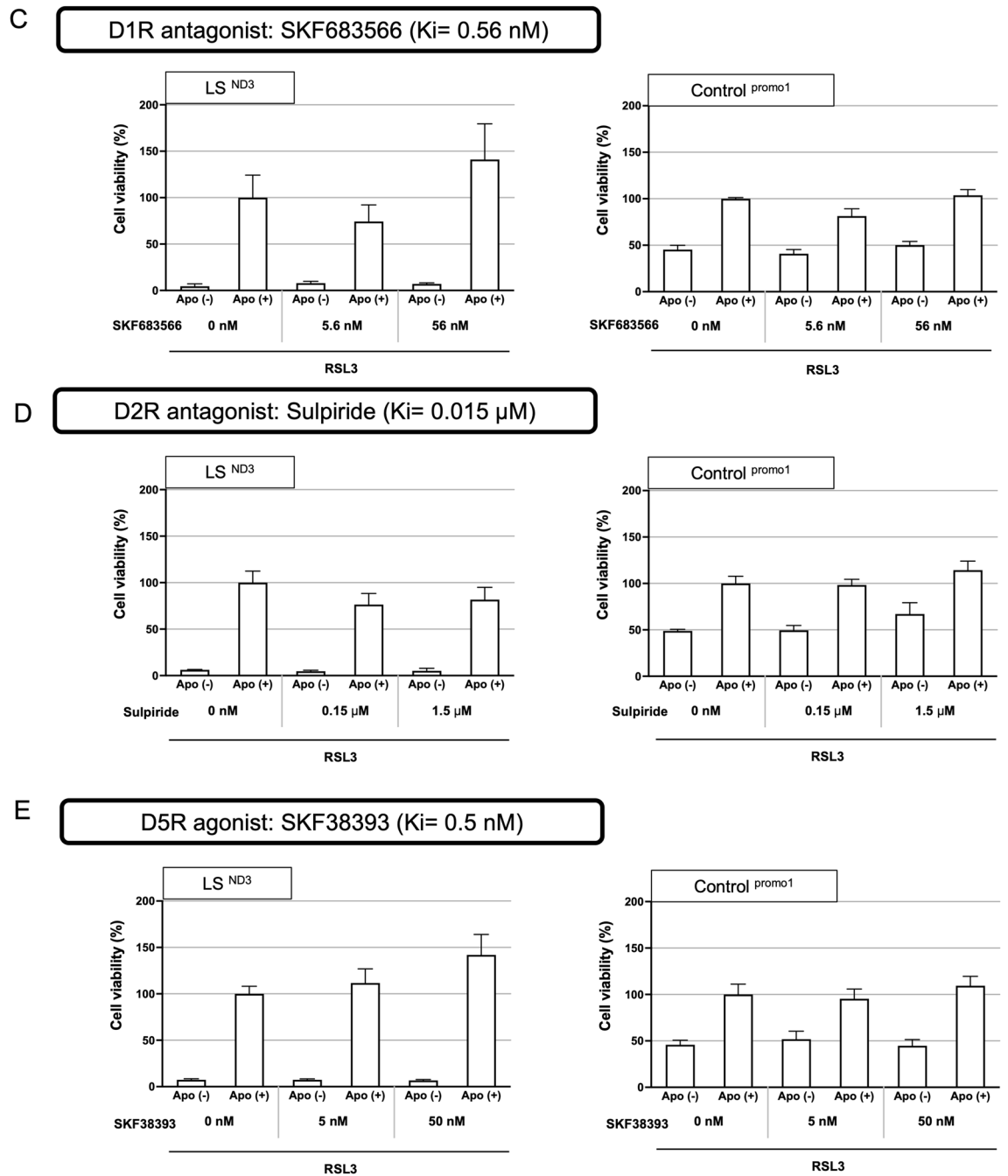


Figure 7. (continued)

## Materials and methods

### Subjects

This study was approved by the Jichi Medical University Clinical Research Ethics Committee (Approval Number: J21-014) and all methods of this study were performed in accordance with the relevant guidelines and regulations. Fibroblasts were obtained from six patients at Jichi Medical University, Kanagawa Children's Medical Center, and Saitama Medical University. The collection was conducted under the approval of the Jichi Medical University Clinical Research Ethics Committee, the Kanagawa Children's Medical Center Review Board and Ethics Committee, and the Ethics Committee of Saitama Medical Center, Saitama Medical University, with approval numbers J21-014, H2021-094, and 11303-06, respectively. Written informed consent was obtained from the parents of each patient. And we used fibroblasts from healthy individuals as controls (normal human dermal fibroblasts purchased from PromoCell Company [#C-12300; Heidelberg, Germany]). Our four patients were diagnosed with LS and MELAS, as previously described<sup>6</sup>. Two of the patients had genetically identified cases of LS, including one with an m.10158 T>C, p(S34P) mutation in *MT-ND3* (Case 1; LS<sup>ND3</sup>) and one with a c.55 C>T, p(P19S) mutation in *NDUFA1* (Case 2; LS<sup>NDUFA1</sup>)<sup>37,38</sup>. Both ND3 and NDUFA1 are subunits of Complex I in the mitochondrial respiratory chain. Fibroblasts were also obtained from two patients with MELAS, including one

with an m.3243 A > G mutation in tRNA-Leu (Case 3; MELAS<sup>tRNA-Leu</sup>) and one with an m.5541 C > T mutation in tRNA-Trp (Case 4; MELAS<sup>tRNA-Trp</sup>)<sup>39</sup>. We additionally obtained fibroblasts from patients with two other types of mitochondrial diseases: mitochondrial cardiomyopathy (Case 5; MC<sup>ND5</sup>) and Kearns-Sayer syndrome (KSS) (Case 6; KSS<sup>large deletion</sup>). The patient with mitochondrial cardiomyopathy who presented with HCM and died at 4 months old had an m.13513G > A mutation in the ND5 subunit of complex I (*MT-ND5* m.13513G > A), with 78.87% heteroplasmy. The patient with KSS was genetically identified as having a large deletion (m.8290-13802) of mitochondrial DNA (Table 1). Fibroblasts with fewer than 20 passages from patients and controls were used in the experiments. The heteroplasmic rate was analyzed by deep sequencing of mutated regions.

### Cell culture and growth conditions

Fibroblasts from patients were cultured in 1.0 g/L low-glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 °C in 5% CO<sub>2</sub>.

### Reagents

L-Butionine (S, R)-sulfoximine (BSO, No. B690270), a glutathione synthesis inhibitor, was purchased from Wako Pure Chemical Industries (Tokyo, Japan). RSL3 (No. S8155) was purchased from Selleck Chemicals (Houston, TX, USA). Fer-1 (No. SML0583) and Lip-1 (No. SML1414) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Z-VAD-FMK (No. 3188-v) and GSK-872 (No. HY-101872) were purchased from Peptide Institute Inc. (Osaka, Japan) and MedChemExpress (Shanghai, China), respectively. All reagents were dissolved in dimethyl sulfoxide (DMSO).

### Cell viability assays

The BSO assay was performed as previously described<sup>16</sup>. We performed several cell viability assays to investigate ferroptosis. Ferroptotic cell death was induced in fibroblasts by ferroptosis inducers (RSL3 at 50–100 nM). In these cell viability experiments, fibroblasts were cultured to semi-confluence and plated at 5,000 cells per well in a 96-well culture plate in normal medium. After incubation for 24 h, apomorphine and several compounds were added as positive controls (Fer-1, or Lip-1) and cultured in the assay medium. All compounds were applied at a concentration of 1 µM unless otherwise indicated. After incubating the cell plates for 24 h at 37 °C (95% humidity and 5% CO<sub>2</sub>), a cell viability assay was performed using Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan). Fluorescence intensity was measured using a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

### The assessment of lipid peroxidation

Lipid peroxidation was examined using the fluorescent dye C11-BODIPY581/591 (No. D3861; Thermo Fisher Scientific, Waltham, MA, USA)<sup>29</sup>. Fibroblasts were cultured to semi-confluence and then plated at 5,000 cells per well in a 96-well culture plate in normal medium. After incubation for 24 h, BSO and several other compounds (e.g. apomorphine and Fer-1) were added to each well. After incubation for 24 h, the cells were labeled with 5 µM C11-BODIPY581/591 for 30 min in the assay medium. The nuclei were stained with Hoechst 33,342. Representative images were obtained using a Keyence All-in-One Fluorescence BZ-X810 microscope (Keyence Co., Itasca, IL, USA).

### RNA extraction and real-time RT-PCR

Total RNA was extracted from fibroblasts using an RNeasy<sup>®</sup> Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions<sup>40</sup>. Total RNA was reverse transcribed to cDNA, followed by amplification by PCR with a Superscript<sup>®</sup> VILO cDNA synthesis kit at 60 °C (Invitrogen; Thermo Fisher Scientific). Real-time RT-PCR was performed using the SYBR Green system with a primer set that amplified a fragment of the target genes to measure the mRNA expression. The following primers were used: *PTGS2* (Forward primer 5'-GCCTGAATGTGC

Case	Cell ID	Disease	Age (years)	Gene mutation	Protein	Mutation rate (%)
1	LS <sup>ND3</sup>	Leigh	0	m.10158 T > C, p.(S34P)	ND3	90
2	LS <sup>NDUFA1</sup>	Leigh	5	c.55 C > T, p.(P19S)	NDUFA1	Nuclear gene
3	MELAS <sup>tRNA-Leu</sup>	MELAS	14	m.3243 A > G	(tRNA-Leu)	21
4	MELAS <sup>tRNA-Trp</sup>	MELAS	23	m.5541 C > T	(tRNA-Trp)	49
5	MC <sup>ND5</sup>	mitochondrial cardiomyopathy	0	m.13513 G > A p.(D393N)	ND5	79
6	KSS <sup>large deletion</sup>	KSS	1	m.8290-13802del	–	–
	Promo1	Control	0	–	–	–

**Table 1.** Fibroblast cell lines from patients with mitochondrial disease. Respiratory chain activities from fibroblasts: LS<sup>ND3</sup>—Complex I 9.8%, II 93.9%, III 94.5%, IV 47.6%, and CS 100.9%. LS<sup>NDUFA1</sup>—Complex I 27.6%, II 104.3%, III 70.1%, IV 77.7%, CS 77.4%. MELAS<sup>tRNA-Leu</sup>—Not available. MELAS<sup>tRNA-Trp</sup>—Complex I 48%, II 103%, III 65%, IV 18%, MC<sup>ND5</sup>—not available. KSS<sup>large deletion</sup>—not available. *LS* Leigh syndrome, *MELAS* myopathy encephalopathy, lactic acidosis, and stroke-like episodes, *MC* mitochondrial cardiomyopathy, *KSS* Kearns-Sayer syndrome.

CATAA-GACTGAC-3', Reverse primer 5'-AAACCCACAGTG-CTTGACACACA-3'), *AIFM2* (Forward primer 5'-ATGGTTCGGCTGACCAAGAG-3', Reverse primer 5'-GCCACCACATCATTGGCATC-3'), *GPX4* (Forward primer 5'-GCCTTCCCGTGTAAACCAGT-3', Reverse primer 5'-GCGAACTCTTTGATCT-CTTCG-3'), *ACSL4* (Forward primer 5'-CCCTGAAGGATTTGAGATTCACA-3', Reverse primer 5'-CCTTAGGT-CGGCCAGTA GAAC-3'), *SLC7A11* (Forward primer 5'-ATGCAGTGGCAGTGA-CCTTT-3', Reverse primer 5'-GGCAAC AAAGATCG-GAACTG-3'), and *GAPDH* (Forward primer 5'-CTTTGTCAAGCTCATTTCCTGG-3', Reverse primer 5'-TCTTC-CTCTTGTGCTCTTGC-3'). The reactions were performed in triplicate. Gene expression was normalized to that of *GAPDH*, and the data were analyzed in the Excel software program, version 16.75 (Microsoft, Redmond, WA, USA) using the  $\Delta\Delta C_t$  method.

### Western blotting

For whole-cell extracts, cells were lysed in TNE buffer (20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA at pH 7.4). Protein concentrations were determined using the Qubit® Protein Assay Kit (Invitrogen; Thermo Fisher Scientific). Whole-cell lysates were mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer and boiled. Western blotting was performed using 20 µg of total protein, and immunoprecipitation was performed using XV PANTERA GEL (NXV-361HP; DRC Co., Ltd, Tokyo, Japan). Total cell proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane by electrotransfer. The membrane was blocked for 1 h at room temperature (RT) using 5% skimmed milk/phosphate-buffered saline with Tween (PBST) and then incubated with the following primary antibodies: rabbit monoclonal anti-PTGS2 (Cox2(D5H5)XP #12282; Cell Signaling, Danvers, MA, USA) at 1:1000 and mouse monoclonal anti-beta-actin (A1978; Sigma-Aldrich) at 1:5000 in PBST overnight at 4 °C. After washing with PBST 3 times for 10 min each, the membrane was incubated with the following secondary antibodies: anti-rabbit IgG horseradish peroxidase (HRP; Cell Signaling) at 1:3000 and anti-mouse IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:2000 for 1 h at RT. After washing with PBST 3 times for 15 min each, the membrane was incubated with Hyper HRP Substrate (TAKARA BIO INC., Ohtsu, Japan) for 2 min. Finally, chemiluminescence from the membrane was imaged using Amersham Imager 680 (GE Healthcare UK Ltd., Little Chalfont, UK). Protein intensities were measured using the ImageJ software program (<https://imagej.net/ij/>) Relative protein levels or protein abundances were normalized to those in the control group.

### Detection of dopamine gene receptor (DRD1-DRD5) expression on control and patient-derived fibroblasts by RT-PCR

RNA was extracted from isolated control and LS patient fibroblasts (LS<sup>ND3</sup>) using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. Commercial human Adult Normal Tissue: Brain: Frontal Lobe (BioChain, Newark, CA, USA) was used as the Positive Control. Total RNA (1500–2000 ng) was reverse-transcribed into first-strand cDNA using the Superscript VILO cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific). RT-PCR was performed using intron-spanning primers (Supplemental Table 1). The D1R/D2R/D5R cycle conditions were as follows: 94 °C for 2 min for polymerase activation, followed by 45 cycles at 98 °C for 10 s, 62 °C for 30 s, 68 °C for 1 min. The D3R cycle conditions were as follows: 94 °C for 2 min for polymerase activation, followed by 45 cycles of 98 °C for 10 s, 62 °C for 15 s, 72 °C for 1 min. The D4R cycle conditions were as follows: 94 °C for 2 min for polymerase activation, followed by 5 cycles of 98 °C for 10 s, 68 °C for 1 min, followed by 5 cycles of 98 °C for 10 s, 66 °C for 1 min, followed by 5 cycles of 98 °C for 10 s, 64 °C for 1 min, followed by 25 cycles of 98 °C for 10 s, 62 °C for 1 min, 68 °C for 7 min. The products were resolved on a 2% agarose gel containing ethidium bromide and photographed.

### Interactions between apomorphine and dopamine receptor agonists or antagonists: Effects on cell viability

Cell viability assays to examine the influence of dopamine receptor agonists or antagonists on the effects of apomorphine were performed under RSL-induced oxidative stress. SKF 83566 cells (no. HY-103430A; MedChem-Express) was used as the D1R antagonist<sup>41</sup>, sulpiride (No. S4655; Selleck Chemicals) as the D2R antagonist<sup>42</sup>, PD168077 (No. HY-21098A; MedChemExpress) as the D4R agonist<sup>43</sup>, L745870 (No. HY-14325; MedChemExpress) as the D4R antagonist<sup>44</sup>, and SKF38393 (No. S7993; Selleck Chemicals) as the D5R agonist<sup>45</sup>. The concentrations used were determined based on the EC<sub>50</sub> and K<sub>i</sub> values<sup>41–45</sup>.

A cell viability assay was performed as previously described<sup>6</sup>. In brief, fibroblasts were cultured in 1.0 g/L low-glucose DMEM with 10% FBS at 37 °C in 5% CO<sub>2</sub> until they reached semi-confluence. Fibroblasts were seeded at 5,000 cells per well in a 96-well plate. After 24 h of incubation, we divided the cells into 3 groups: a DR agonist- or antagonist-treated group, a DR agonist- or antagonist-treated group at a concentration 10 times the EC<sub>50</sub> or K<sub>i</sub> values, and a DR agonist- or antagonist-treated group at a concentration 100 times the EC<sub>50</sub> or K<sub>i</sub> values. Apomorphine was added at a final concentration of 1 µM. After 24 h of incubation, the cell survival rate was checked using Cell Count Reagent SF (Nacalai Tesque). The cell viability of Apomorphin-treated group without RSL3 was used as 100% to compare other groups' cell viabilities.

### Statistical analyses

The results are expressed as the mean ± standard deviation. Comparisons between multiple-group means were performed using a one-way analysis of variance with Bonferroni's post-hoc test. Statistical significance was set at P < 0.05. Statistical analyses were performed using the GraphPad Prism software program version 9.3.1 (GraphPad Software Inc., La Jolla, CA, USA).

## Data availability

The datasets and raw data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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### Author contributions

A.M., T.Y., and H.O. developed the strategy for the research; methodology, C.W., E.F.J., and N.Y.; performed the research, A.M., A.N., M.K., and N.O.; analyzed the data, A.M. and S.A.; resources, N.Y., Y.K., A.O., M.T.; writing—original draft preparation, A.M.; writing—review and editing, N.O. and H.O.

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### Competing interests

The authors declare no competing interests.

### Additional information

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特集 小児科医が知っておくべき筋疾患診療：遺伝学的理解と治療の最新事情

代表的筋疾患

## ミトコンドリア病は多様性の疾患 —病因，病態，治療の現在

後藤雄一<sup>\*,\*\*</sup>

はじめに

ミトコンドリア病の特徴は「多様性」である。遺伝学的には、核 DNA 上の遺伝子（300 個以上）とミトコンドリア DNA (mtDNA) が原因となる。よって遺伝形式は、メンデル遺伝、母系遺伝、突然変異例など多様である。病態としては、エネルギー産生低下とそれ以外の多様なミトコンドリア機能が障害される。臨床的には、広い発症年齢、ほぼすべての臓器の症状、急性・慢性進行性や症状が改善する臨床経過などがあり、きわめて多様な臨床像を呈する。このようなミトコンドリア病を理解し診療を行うには、遺伝学・病理学・生化学を基盤にした診断方法を知り、眼前の患者で起きている病態をできる限り論理的に洞察し、それら多様な症状に対する対処法を実践することが必要になる。

### 疾患概念

ミトコンドリア病とは、ミトコンドリア機能の低下を主因とする疾患の総称である。かつてミトコンドリアは細胞のエネルギー産生を担う細胞内小器官と考えられてきたが、現在はそれ以外に、活性酸素排出、アポトーシス、カルシウムイオン貯蔵、感染防御などの機能を有することが明らかになり、単にエネルギー産生低下のみがミトコンドリア病の基盤になっているというわけでない<sup>1,2)</sup>。さらに、ミトコンドリア内膜に局在している電子伝達系酵素複合体の働きがもっともミトコンドリアに特徴的であることから、電子伝達系酵素複合体の構造変化や活性低下を伴う疾患群を primary mitochondrial disease (一次性ミトコンドリア病) とし、それ以外のミトコンドリア機能低下を伴う疾患群を secondary mitochondrial disease (二次性ミトコンドリア病) と称するようになってきている<sup>3)</sup>(図)。

検査と診断

### 検査と診断

#### 1. 臓器症状に応じた検査

ミトコンドリアは成熟赤血球以外の細胞に存在しているので、ほぼすべての細胞が障害を受ける可能性があり、それらの細胞の機能低下や細胞自体の消滅により、組織・臓器の機能が障害され、臓器症状が出現する。表に主な臓器症状を示す。したがって、出現している症状に応じて、臓器特異的な検査を行い、症状の基盤になっている変化を知ることが必要である。ミトコンドリア病で症状が出やすい臓器症状である中枢神経であれば、画像検査、髄液検査、けいれんなどがあれば脳波検査などを行うし、心臓の症状が疑われる場合は心電図や心エコー、各種血液検査を行うことになる。その際、音が聞こえにくい、物が見えにくいなどの症状があるときは、耳鼻科的検査や眼科的検査が必要で、それぞれの専門医にコンサルトすることが必要になることも多い。

#### 2. 確定診断に必要な検査

ミトコンドリアの機能変化によって発症していることを証明することがミトコンドリア病の確定

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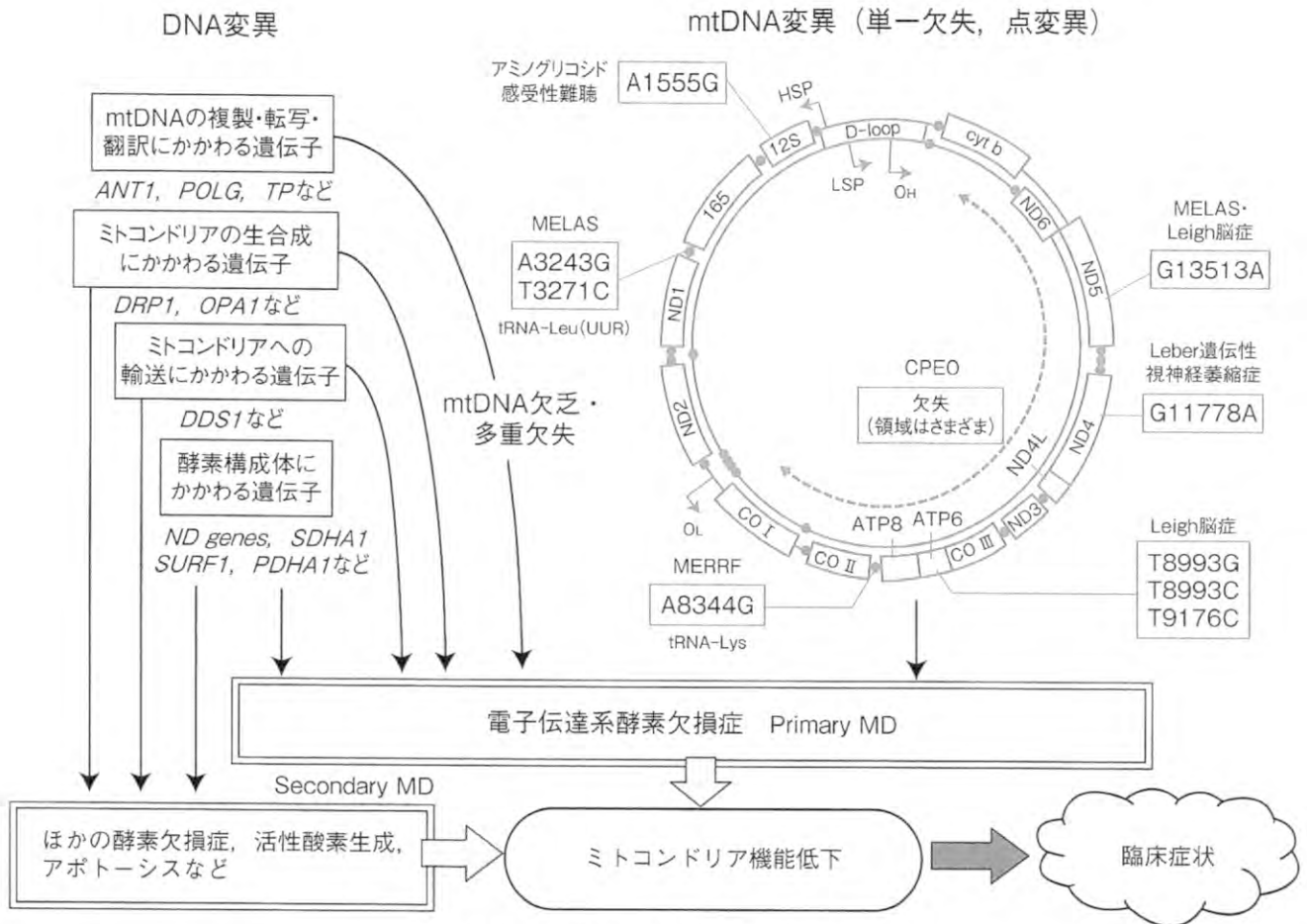


図 ミトコンドリア病の病因の多様性と病態

MD : mitochondrial disease, MELAS : mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes, MERRF : myoclonus epilepsy associated with ragged-red fibers, CPEO : chronic progressive external ophthalmoplegia, LSP : L-strand promoter, HSP : H-strand promoter

表 代表的な臨床症状

中枢神経	けいれん, ミオクローヌス, 失調, 脳卒中様症状, 知能低下, 片頭痛, 精神症状, ジストニア, ミエロパチー
骨格筋	筋力低下, 易疲労性, 高CK血症, ミオパチー
心臓	伝導障害, Wolff-Parkinson-White (WPW) 症候群, 心筋症, 肺高血圧症
眼	視神経萎縮, 外眼筋麻痺, 網膜色素変性
肝	肝機能障害, 肝不全
腎	Fanconi 症候群, 尿細管機能障害, 糸球体病変, ミオグロビン尿
膵	糖尿病, 外分泌不全
血液	鉄芽球性貧血, 汎血球減少症
内耳	感音性難聴
大腸・小腸	下痢, 便秘
皮膚	発汗低下, 多毛
内分泌腺	低身長, 低カルシウム血症, 甲状腺機能低下症

(難病情報センター<sup>6)</sup>)

診断である。

### 1) 遺伝学的検査

すでに 300 以上の核 DNA 上の原因遺伝子が同定されている<sup>3)</sup>。図には代表的な遺伝子のみを示している。これだけ多くの原因遺伝子があると、パネル検査や NGS (next generation sequencing) を用いた検査が必要になってきた。しかし、このような網羅的検査を最初から行うよりは、頻度の高い原因遺伝子に絞って検査を行い、その後に網羅的検査を行うのが一般的である<sup>4,5)</sup>。日本においては、網羅的遺伝学的検査を行えるところが限られており、体制整備が徐々に進んでいるところである。

一方、わずか 1.6 kb の小さい環状 DNA である mtDNA は、一細胞内に数千コピーで存在する(マルチコピー性)、突然変異の頻度が高い(易変異性)、卵からしか遺伝しない(母系遺伝)という特徴をもっている。これらの特徴は、突然変異や母系遺伝という遺伝学的特徴や細胞内に変異型と野生型が混在する状態(ヘテロプラスミー)や変異蓄積などの状態の基盤となっている。変異型が細胞内に存在する場合は、変異率は 0~100% まであり得るが、ヘテロプラスミーの状態では、ある一定の比率以上にならないと細胞障害が起きないことが知られている(閾値効果)。

ヘテロプラスミーで起きる病気の代表であるミトコンドリア脳筋症・乳酸アシドーシス・脳卒中様症候群(MELAS)や慢性進行性外眼筋麻痺症候群(CPEO)などでは、全身の細胞毎で変異率が異なっており、変異率の高い細胞が多い組織や臓器ではその障害が現れるものと理解されている。

### 2) 病理学的検査

ミトコンドリア病における筋生検は、遺伝学的検査で確定できないときには必須の検査であることに変わりはない。欠失した mtDNA (図)は、罹患臓器である骨格筋でしか捉えられない場合があり、血液を用いた検査では不十分のこともある。ミトコンドリア病に特徴的な赤色ぼろ線維(ragged-red fiber: RRF)、高コハク酸脱水素酵素活性線維(strongly SDH-reactive blood vessel: SSV)、シトクローム酸化酵素欠損線維(COX-negative fiber)などを特殊染色で同定する。

### 3) 生化学的検査

血中・髄液中の乳酸・ピルビン酸測定以外に、GDF15 (growth differentiation factor 15) 値測定(今後は測定キットが販売される予定)、尿中有機酸分析、血中アミノ酸分析などの代謝スクリーニング検査が有用である。また、電子伝達系酵素の活性測定は直接的に診断が可能になることもあり有用であるものの、骨格筋で酵素欠損や酵素蛋白量の低下が認められるものの線維芽細胞では同定できないこともあり、生化学検査でも生検筋での測定が決定打になることがある。骨格筋生検の際に筋芽細胞や線維芽細胞を樹立しておく、病因追求の貴重な材料になるので、専門施設にコンサルトすることをお勧めする。

## 診断基準

ミトコンドリア病は指定難病になっており、診断基準が公開されている<sup>6)</sup>。現在、改定版が完成しており、2024年4月に正式に移行するとされている。改定版は、最近の遺伝学的検査法を取り入れたものとなっている。

## 治療

### 1. 対症治療

ミトコンドリア病では多彩な臓器症状が出現する。根本的な治療法がない現況では、対症治療に頼らなくてはならないものの、けいれんには抗けいれん薬、精神症状には精神薬、難聴には補聴器や人工内耳など著効するものがあり、臨床的に重要な対処法である。その際、自分の専門外の臓器症状があれば専門医と相談して治療を行うことが必要であり、また小児科であれば他科の診療内容も含めて全人的に診てゆくことが肝要である。

### 2. 特異的治療と根本治療の可能性

#### 1) 薬物治療

ミトコンドリア機能の低下で起きている代謝異常を調整することは全身状態の改善につながる可能性がある。ミトコンドリア内で作用するビタミン類を経口投与するのは理にかなっているように考えられるが、ビタミン剤による厳密な臨床試験は行われていない。効くに違いはないと考えて投与

している場合が多く、食事がしっかり摂れている子に大量のビタミン剤を与えて、食事の量が減ってしまう事態は本末転倒であり、バランスの取れた食事ができることがより重要である。

MELAS の脳卒中様発作の予防にタウリン大量治療が有効であり、2019年2月に保険収載された。MELAS の原因となる mtDNA 内の m.3243A>G 変異があると、ミトコンドリア内のロイシン転移 RNA にタウリン修飾障害が起こり成熟型にならないことが知られており<sup>7)</sup>、それを経口タウリンの大量投与で修飾障害を改善させ脳卒中様発作を減らすことができるとされている<sup>8)</sup>。そのほか、いくつかの薬剤の臨床試験が行われたり、行っていたりしているが、ここでは触れない。

## 2) 生殖補助医療

### ① 出生前遺伝学的診断, 着床前遺伝学的診断

通常、核 DNA 上の病因遺伝子変異で起きる乳幼児に発症する重篤なミトコンドリア病は、その病的変異が同定されていれば次子の出生前遺伝学的検査は可能である。しかし、mtDNA の場合は、ヘテロプラスミーで起きる病態か、ホモプラスミー（すべてがほとんど同じバリエーションをもつ）の病態かでその適用は異なる。ヘテロプラスミーの場合は、診断に用いる羊水細胞や絨毛細胞の変異率が胎児個体の細胞の変異率をきちんと反映しているのか、または発生の過程で胎児の細胞内の変異率が変化してゆく可能性があり、検査結果で将来の発症を予測するのが困難である。そのため、mtDNA の変化で起きる病気の出生前検査は、ホモプラスミーで起きる病気（たとえば、m.8993T>G や m.8993T>C で起きる Leigh 脳症）に限定される。

### ② ミトコンドリア・ドネーション

ミトコンドリア・ドネーションとは、ミトコンドリア置換法ともよばれ、母親の核 DNA を維持しながら、ミトコンドリアのみを女性ドナーのミトコンドリアと入れ替えることをいう。具体的には、ドナーの卵子の核を取り出し、① その卵子に母親の核を入れてから（未受精胚核置換）父親の精子と人工授精させるか、② その卵子に母親の受精卵の核を入れること（受精胚核置換）をいう。②は、作成について「ヒトに関するクローン技術

等の規制に関する法律（クローン技術規制法）」に触れる特定胚となる。

この操作を受けた胚には、核 DNA を提供した父親、母親とミトコンドリア DNA を提供した女性ドナーの3人の親が存在することになり、大きな倫理的問題を含んでいた。また、病気に関係する mtDNA がわずかながらも卵子内に残る問題や、異なる mtDNA が2種類含まれている場合に正常に発生が進むのかどうかなど生物学的、技術的課題が挙げられおり、長期間の議論が行われた。英国では長い検討期間の後、2015年法律を制定して、このミトコンドリア・ドネーションを臨床的に容認した。遅れて2022年3月にオーストラリアが法律を制定してこの方法を法的に認めている<sup>9)</sup>。

一方、日本でも臨床応用を含めた議論が総合科学技術・イノベーション会議（CSTI）で行われ、令和元（2019）年6月に、ミトコンドリア病研究を目的にしたヒト受精胚に核置換を用いる研究を認める旨の見解が示され、その後文部科学省において速やかな指針の整備を進めることになり、結果的に令和4（2022）年6月にクローン技術規制法が改正され、研究での作成に道が開かれたところである。

## おわりに

ミトコンドリア病の特徴は多様性である。ミトコンドリア病は、DNA、ミトコンドリア、細胞、組織、臓器レベルで理解することが必要であり、それぞれのレベルに対応する分子遺伝学、病理学、生化学、電気生理学、そして生殖補助医療を取り扱う臨床遺伝学などの種々のアプローチを必要とする。とくに、若い医師が興味をもってこの分野に参入してもらいたいと考えている。

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## Key Points

- ① 典型的な症状や複数の臓器症状があるときにミトコンドリア病を疑う。
- ② 診断検査の手始めは、血中乳酸・ピルビン酸値やGDF15値（今後は測定キットが販売される予定）などを測定する。
- ③ 確定診断には遺伝学的検査が主体になりつつあるが、罹患臓器（骨格筋など）の病理や生化学検査が必要となることも多い。
- ④ 多彩な症状の出現に応じて、各臓器の専門科へのコンサルトと全人的な医療を行うことが重要である（ほかの診療科への丸投げはしない）。

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# Characteristics of the muscle involvement along the disease progression in a large cohort of oculopharyngodistal myopathy compared to oculopharyngeal muscular dystrophy

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## Abstract

**Background and objectives** Oculopharyngodistal myopathy (OPDM) is an autosomal dominant myopathy clinically characterized by distal muscle weakness. Even though the identification of four causative genes, *LRP12*, *GIPC1*, *NOTCH2NLC* and *RILPL1*, it is unclear whether the myopathy progressed similarly among OPDM subtypes. We aimed to establish diagnostic clues in muscle imaging of OPDM in comparison with clinicopathologically similar oculopharyngeal muscular dystrophy (OPMD).

**Methods** Axial muscle CT and/or T1-weighted MRI data from 54 genetically confirmed patients with OPDM (OPDM\_LRP12; n = 43, OPDM\_GIPC1; n = 6, OPDM\_NOTCH2NLC; n = 5) and 57 with OPMD were evaluated. We scored the degree of fat infiltration in each muscle by modified Mercuri score and performed hierarchical clustering analyses to classify the patients and infer the pattern of involvement on progression.

**Results** All OPDM subtypes showed a similar pattern of distribution in the affected muscles; soleus and medial gastrocnemius involved in the early stage, followed by tibialis anterior and extensor digitorum longus. For differentiating OPDM and OPMD, severely affected gluteus medius/minimus and adductor magnus was indicative of OPMD.

**Discussion** We identified a diagnostic muscle involvement pattern in OPDM reflecting its natural history. The results of this study will help in the appropriate intervention based on the diagnosis of OPDM, including its stage.

**Keywords** Oculopharyngodistal myopathy · Oculopharyngeal muscular dystrophy · Skeletal muscle imaging

## Introduction

Oculopharyngodistal myopathy (OPDM) is an autosomal dominant myopathy that is characterized, clinically, by slowly progressive ptosis, ophthalmoplegia, dysphagia, and predominantly distal limb muscle weakness; and, pathologically, by the presence of rimmed vacuoles and small angular fibers. To date, the cause of OPDM has been identified as CGG repeat expansion in the 5'UTR of *LRP12*, *GIPC1*, *NOTCH2NLC*, and *RILPL1* genes [1–5]. However, it is curious that the clinicopathological phenotype is believed to be similar despite the different causative genes [6].

Oculopharyngeal muscular dystrophy (OPMD), on the other hand, is caused by the expansion of the alanine stretch in N-terminal of a protein encoded by *PABPN1* gene, and is clinicopathologically similar to OPDM in terms of oculopharyngeal muscle involvement; however, showing predominantly proximal limb weakness [7]. Interestingly, it has been

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The list of “the OPDM/OPMD Image Study Group” members were listed in Acknowledgements section.

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reported that some OPDM patients can show predominantly proximal muscle weakness, mimicking OPMD [8]. These situations naturally raise the following three questions: (1) Is OPDM a heterogeneous disease? (2) Do the subtypes of OPDM with different causative genes represent different clinical phenotypes? (3) Is the distribution of affected muscles different between OPDM and OPMD?

Skeletal muscle computed tomography (CT) and magnetic resonance imaging (MRI) have been widely used in the diagnosis of neuromuscular diseases. The selective fatty replacement patterns on muscle imaging sometimes show disease-specificity and have been used for diagnosis [9, 10]. Therefore, it may be possible to answer the three questions by using skeletal muscle imaging data, which show the specific patterns of affected muscles in OPDM and OPMD. However, there have been only a few studies of muscle imaging on OPDM [11–16], and in particular, no studies have discussed the differences in skeletal muscle imaging for each OPDM harboring different causative genes. In this study, using imaging data from a large cohort of genetically confirmed patients with OPDM, we aimed to identify the characteristic distribution of muscle involvement reflecting its natural history and to provide helpful information that can be translated into clinical practice.

## Methods

### Subjects

Among the patients with muscles and/or blood samples sent for diagnostic purposes to the National Center of Neurology and Psychiatry (NCNP), a nationwide referral center for neuromuscular diseases in Japan, from January 1, 1978 to December 31, 2021, we searched for the patients with muscle images who were genetically diagnosed to have CGG repeat expansion in *LRP12*, *GIPCI*, *NOTCH2NLC*, and GCN repeat expansion or reported missense mutation in *PABPN1* [17–19]. Images taken at multiple time points in the same patient were treated as independent image series.

### Clinical data

The demographic data of the patients with OPDM and OPMD was obtained from the records maintained in the NCNP muscle repository. The clinical information collected on the patients included the gender, age at muscle imaging, disease duration, genetic information, initial symptoms, and symptoms at examination such as ptosis, ophthalmoplegia, dysphagia, and weakness of limb and neck muscles. We determined the distribution of muscle weakness by comparing between the sum of the Medical Research Council scale in the proximal (shoulder abduction, elbow flexion and

extension, hip flexion and extension, knee flexion and extension) and that of the distal (wrist flexion and extension, finger flexion and extension, ankle flexion and extension) limb.

### Muscle imaging

We reviewed axial muscle imaging data of CT or T1-weighted MRI in OPDM and OPMD patients, including the muscles of the trunk (cervical paraspinal; C-PS, sternocleidomastoid; SCM, levator scapulae; LS, splenius capitis; SC, pectoralis major; PM, deltoid; DEL, infraspinatus; InfS, subscapularis; SubS, trapezius; TPZ, rhomboid; RH, serratus anterior; SeA, thoracic paraspinal; Th-PS, iliocostalis lumborum; ICL, longissimus: LO, multifidus; MF, rectus abdominis; RA), pelvis (gluteus maximus/medius/minimus; GMA/GME/GMI), thigh (rectus femoris; RF, sartorius; SA, gracilis; GR, vastus lateralis/medialis/intermedius; VL/VM/VIM, adductor longus/magnus; AL/AM, semimembranosus; SM, semitendinosus; ST, the short/long head of biceps femoris; SBF/LBF) and calf (tibialis anterior/posterior; TA/TP, extensor digitorum longus; EDL, fibularis longus/brevis; FL/FB, soleus; SOL, medial/lateral gastrocnemius; MGC/LGC). Each muscle was evaluated on both sides and scored at the level of the maximum area. The degree of fat infiltration was determined according to the modified Mercuri score (mMS); stage 0: normal appearance; stage 1: mildly decreased signal density (CT) or increased signal intensity (T1-weighted MRI); stage 2: moderately decreased signal density (CT) or increased signal intensity (MRI) with the confluence in less than 50% of the muscle; stage 3: severely decreased signal density (CT) or increased signal intensity (MRI) in more than 50% of the muscle; and stage 4: the whole muscle is replaced by lower density (CT) or high signal intensity (MRI) [10, 20]. Asymmetry was defined as mMS differing by two or more points on the left and right sides [21]. The MRI and CT images were scored independently by two neurologists who were blinded for the clinical information of the patients.

### Statistical analysis

We used Student's *t* test to compare the quantitative variables in two groups and one-way ANOVA with Tukey's multiple comparisons test for more than two groups. To investigate the correlation between the clinical and muscle imaging findings, Spearman's rank correlation was used. The data was shown as mean  $\pm$  SD and considered statistically significant at  $p < 0.05$ . Statistical analyses, including principal component (PC) analysis, were performed by using GraphPad Prism version 9.4.1 for Windows (GraphPad Software, San Diego, California, USA). The hierarchical analysis with graphical representation as a heatmap was performed using R software version 4.2.1.

## Standard protocol approvals, registrations, and patient consents

This study was approved by the ethical committees of the NCNP (A2022-045).

All of the participants provided a written informed consent for using their data for research.

## Data availability

The data supporting the findings in this study are available from the corresponding author upon request.

## Results

### Clinical findings

A total of 111 patients composed of 43 OPDM\_LRP12, 6 OPDM\_GIPC1, 5 OPDM\_NOTCH2NLC, and 57 OPMD patients were enrolled. The clinical features were shown in Table 1. In OPDM, the average age at onset of symptoms in OPDM\_NOTCH2NLC was significantly younger than that of OPDM\_GIPC1 ( $p = 0.02$ ). Regarding the initial symptoms, ptosis and muscle weakness were common in OPDM\_LRP12 and OPDM\_GIPC1. In contrast, OPDM\_NOTCH2NLC had no ptosis but had neuropsychiatric symptoms as an initial symptom. In all of the OPDM subtypes,

most of the patients showed predominantly distal muscle weakness, while a proportion of patients also showed weakness in the proximal muscles equally, and some showed mainly proximal muscle weakness. In OPMD, on the other hand, the average age at symptom onset was significantly older than those in the OPDM subtype ( $p < 0.0001$ ). Ptosis was the most common symptom at onset, which was followed by muscle weakness and dysphagia. It is important to note that 9.1% of the patients showed non-proximal muscle weakness. Neck muscle weakness was observed in all of the subtypes of OPDM and OPMD, but was particularly frequent in OPDM\_NOTCH2NLC.

### Muscle imaging

We evaluated 39 muscles among all the enrolled patients. One OPDM\_NOTCH2NLC patient, along with 3 OPDM\_LRP12 and 2 OPDM\_GIPC1 patients, had multiple image series at different time points (7, 5, 2, 2, 3, 2 series, respectively). As a result, we reviewed a total of 4914 muscle images of 126 series in 111 patients (Supplemental Table). To analyze the CT and MRI data together in same platform, we evaluated the data from these imaging modalities in the same individuals. Among the enrolled patients, both CT and MRI data were obtained in 178 muscles from 15 patients. The mMS by CT and MRI in these muscles correlated well ( $mMS_{MRI} = 0.9 \times mMS_{CT} + 0.3$ ,  $r^2 = 0.84$ ) as shown in the Supplemental Figure. Using this formula, CT scores were

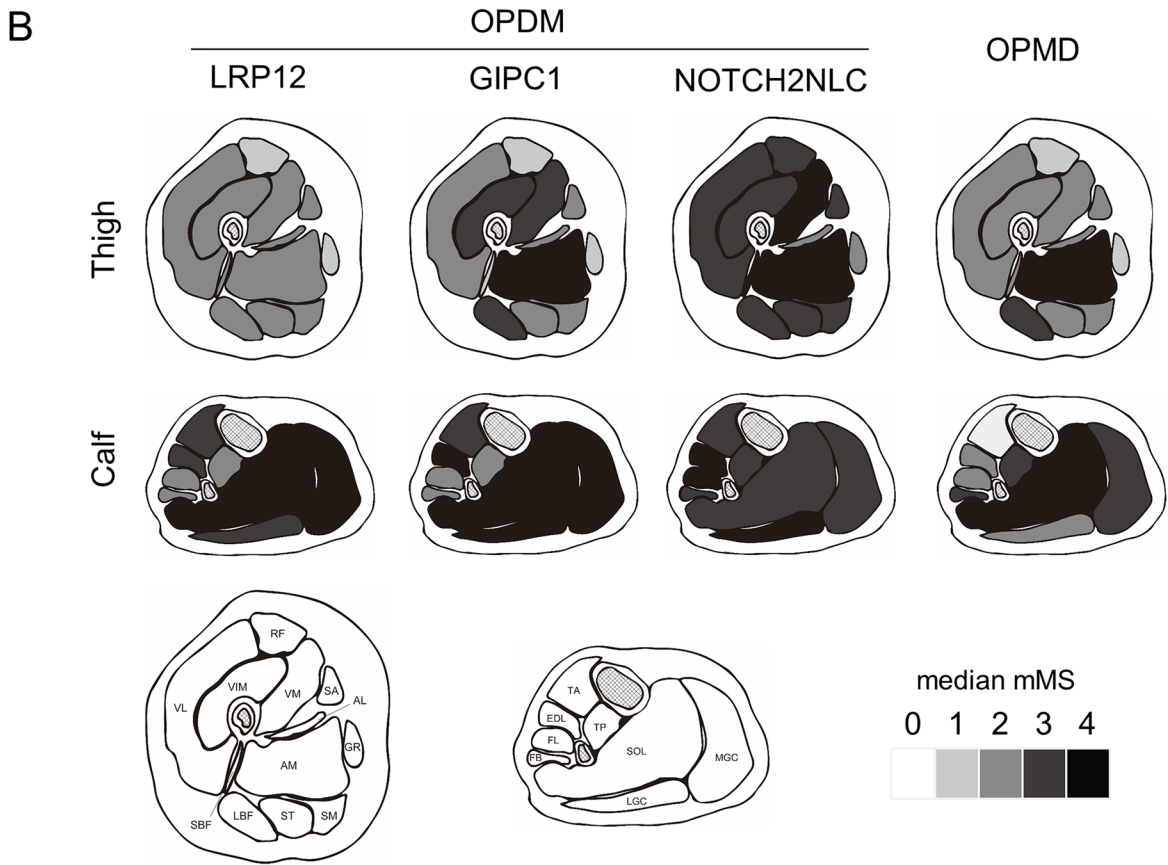
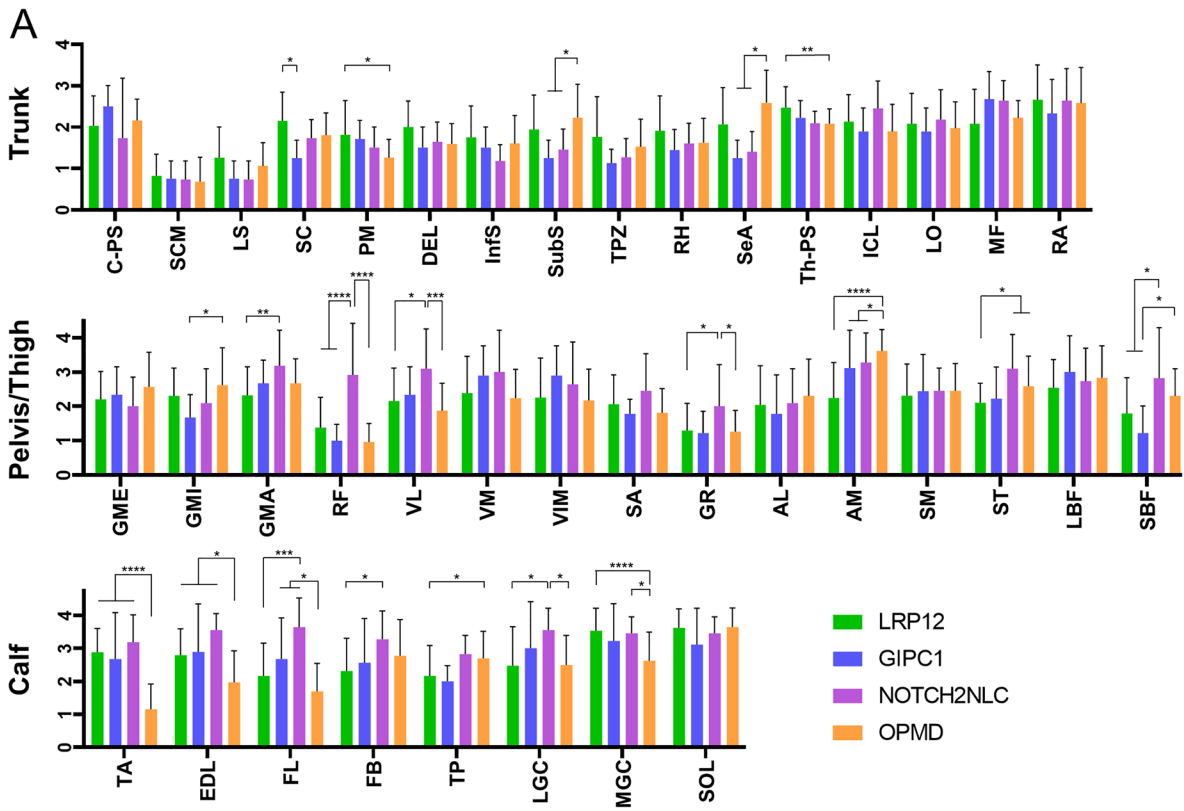
**Table 1** Clinical demographics of OPDM and OPMD

	OPDM_LRP12 (n=43)	OPDM_GIPC1 (n=6)	OPDM_NOTCH2NLC (n=5)	OPMD_PABPN1 (n=57)
Age at onset (y, mean $\pm$ SD)	41 $\pm$ 9.4	46 $\pm$ 10	30 $\pm$ 11*	55.4 $\pm$ 10.0****
Male (%)	58	33	40	56
Initial symptom (%)				
Ptosis	37	50	0	58
Dysphagia	14	0	0	21
Muscle weakness	40	33	40	26
Neuropsychiatric	0	0	60	0
Symptoms at diagnosis (%)				
Ptosis	100	100	60	96
Dysphagia	95	100	60	96
Muscle weakness	95	100	100	91
Proximal > distal	0	17	0	80
Proximal = distal	14	33	40	5.5
Proximal < distal	77	50	60	3.6
Neck	51	40	80	38
CK (IU/L, mean $\pm$ SD)	484 $\pm$ 339	279 $\pm$ 276	474 $\pm$ 514	423 $\pm$ 459

OPDM oculopharyngodistal myopathy, OPMD oculopharyngeal muscular dystrophy, CK creatine kinase

\*\*\*\* $p < 0.0001$  (vs OPDM\_LRP12, OPDM\_GIPC1, OPDM\_NOTCH2NLC),

\* $p < 0.05$  (vs OPDM\_GIPC1)



**Fig. 1** The mMSs of trunk and limb muscles in OPDM and OPMD. (A) The average mMSs of each muscle for three subtypes of OPDM and OPMD are shown in bar plots. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . (B) A graphical summary of the degree of fat replacement in the thigh and calf muscles. *OPDM* oculopharyngodistal myopathy, *OPMD* oculopharyngeal muscular dystrophy, *mMS* modified Mercuri score, *C-PS* cervical paraspinial, *SCM* sternocleidomastoid, *LS* levator scapulae, *SC* splenius capitis, *PM* pectoralis major, *DEL* deltoid, *InfS* infraspinatus, *SubS* subscapularis, *TPZ* trapezius, *RH* rhomboid, *SeA* serratus anterior, *Th-PS* thoracic paraspinial, *ICL* iliocostalis lumborum, *LO* longissimus, *MF* multifidus, *RA* rectus abdominis, *GMA* gluteus maximus, *GME* gluteus medius, *GMI* gluteus minimus, *RF* rectus femoris, *SA* sartorius, *GR* gracilis, *VL* vastus lateralis, *VM* vastus medialis, *VIM* vastus intermedius, *AL* adductor longus, *AM* adductor magnus, *SM* semimembranosus, *ST* semitendinosus, *SBF* the short head of biceps femoris, *LBF* the long head of biceps femoris, *TA* tibialis anterior, *EDL* extensor digitorum longus, *FL* fibularis longus, *FB* fibularis brevis, *TP* tibialis posterior, *LGC* lateral head of gastrocnemius, *MGC* medial head of gastrocnemius, *SOL* soleus

converted to MRI scores and the converted MRI scores were used for analyses after being rounded off. The two scores were equal in all data. The average mMS of each muscle was shown in Fig. 1A and a graphical summary of the thigh and calf muscles using the median mMS was shown in Fig. 1B.

In OPDM, calf muscles were more extensively replaced by fat compared with the thigh muscles ( $mMS = 2.89 \pm 0.41$  vs  $2.20 \pm 0.38$ ,  $p = 0.002$ ). In particular, TA, EDL, MGC, and SOL were severely affected in all subtypes of OPDM. Among the subtypes of OPDM, OPDM\_NOTCH2NLC had more severely affected muscles in comparison with the other subtypes (GMA, RF, VL, GR, AM, SBF, FL, FB, LGC). As for OPMD, there was no significant difference in the degree of fat replacement between thigh and calf muscles ( $mMS = 2.16 \pm 0.66$  vs  $2.36 \pm 0.71$ ,  $p = 0.55$ ). AM and SOL were the most severely affected, while TA, RF, and GR were relatively spared. Comparing OPDM and OPMD, TA and EDL were significantly more severely affected in OPDM than in OPMD, while AM was more severely affected in OPMD. These results suggest that these muscles would be informative in differentiating between the two diseases. In the truncal muscles, SeA and SubS were more severely affected in OPMD than OPDM\_GIPC1 or OPDM\_NOTCH2NLC. Additionally, asymmetric muscle involvement was observed in 15 of 43 OPDM\_LRP12 (25.6%), 1 of 9 OPDM\_GIPC1 (11.1%), 2 of 11 OPDM\_NOTCH2NLC (22.2%), and 6 of 57 OPMD (10.5%) image series.

To visualize the pattern of muscle involvement, we performed a hierarchical analysis and drew the heatmap for the patients with all 28 muscles available in the pelvis, thigh, calf, and thoracic/lumbar paraspinial (OPDM\_LRP12: 32 patients [38 image series], OPDM\_GIPC1: 6 patients [9 image series], OPDM\_NOTCH2NLC: 5 patients [11 image series], OPMD: 36 patients [36 image series]). In the heatmap of OPDM (Fig. 2A), 58 image

series were classified into two large groups, the right group with a significantly longer disease duration than the left ( $17.9 \pm 9.5$  vs  $10.0 \pm 8.8$  years,  $p = 0.002$ ). Also, the dendrogram showed that 28 muscles were classified into four clusters according to the pattern of muscle involvement. Together with disease duration, these clusters may indicate the following implications; MGC, SOL: severely affected from the earliest stage of the disease; FL, FB, LGC, TA, EDL: severely affected in the later stage and can be in early stage; AL, VIM, VM, VL, GMA, AM, SM, LBF, MF, Th-PS, GMI, GME, RA, TP, ST, SA, LO, ICL: affected in the later stage; GR, RF, SBF: relatively spared in the later stage. On the other hand, hierarchical analysis did not clearly divide the patients into each OPDM subtype. Similarly, a PC analysis using each mMS of 28 muscles also did not reveal differences between the OPDM subtypes (Fig. 2B). This may indicate that it is difficult to differentiate OPDM subtypes based on the image findings of these 28 muscles.

We also performed a hierarchical analysis on OPMD ( $n = 36$ ) and the heatmap was shown in Fig. 3. The patients were classified into two large clusters, the right had an older age at muscle imaging than the left ( $70.3 \pm 6.5$  vs  $65.3 \pm 10.0$  years,  $p = 0.104$ ). Muscles were classified into four large clusters, indicating that; AM, SOL: affected in the earliest stage of the disease; FB, GME, GMI, SBF, RA, TP, MGC, LBF, LGC, GMA, ST: severely affected in the later stage; AL, VL, SA, VM, VIM, FL, EDL, ICL, LO, SM, Th-PS, MF: affected in the later stage; GR, RF, TA: spared even in the advanced stage. The patients were not divided by the GCN repeat size.

Next, we investigated the correlation between mMS and clinical information, such as age at muscle imaging (Age) and disease duration (Duration). Based on the heatmap, we selected only the muscles whose severity correlated with disease progression; excluding GR, RF, SBF, MGC, and SOL for OPDM and GR, RF, TA, AM, and SOL for OPMD, and summed the mMS of these muscles. The scores were set as “sum\_OPDM” and “sum\_OPMD”, respectively. In OPDM, we observed a significant positive correlation between Age and sum\_OPDM only in OPDM\_NOTCH2NLC ( $p < 0.0001$ ) (Fig. 4, left) while the Duration and mMS sum\_OPDM were significantly correlated in OPDM\_LRP12 and OPDM\_NOTCH2NLC ( $p < 0.05$ ) (Fig. 4, right). Similarly, in OPMD, sum\_OPMD were significantly correlated with both Age and Duration.

Then, to examine the image-based separation of OPDM and OPMD patients, we performed a hierarchical analysis on the combined OPDM and OPMD images (79 patients, 94 image series) (Fig. 5A). The patients were classified into subclusters according to each disease. Interestingly, even the patients with atypical muscle weakness, i.e., equal weakness in proximal and distal muscles, or distal muscle



**Fig. 2** Hierarchical and PC analysis of OPDM. (A) The heatmap shows the fat replacement in the thigh, calf, lumbar paraspinal, and thoracic paraspinal muscles (row) in each patient (column). The muscles and patients are clustered based on the degree of fat infiltration, OPDM subtype, duration of the disease, and age. The degree of fat infiltration is indicated by the red scale color as shown in the right upper corner. At the top of the heatmap, the duration of the disease is shown in grayscale and the OPDM subtype of each patient is color-coded. (B) PC analysis did not show a distinct separation between OPDM subtypes

predominance in OPMD or proximal muscle predominance in OPDM, were correctly included in the disease subclusters.

Lastly, to identify the informative muscles differentiating OPDM and OPMD, we performed PC analysis on the data of mMS of 28 muscles from combined OPMD and OPDM patients. The plot showed that OPDM and OPMD are clearly separated and with a relative difference in distribution (Fig. 5B). The loading plot showed that AM, GME, GMI, and TP have a strongly negative influence on PC2 (more likely to be affected in OPMD), while TA, EDL, MGC, Th-PS, and FL have a strongly positive influence (preferentially affected in OPDM), indicating that these muscles are important for distinguishing between OPDM and OPMD (Fig. 5C). In our study, patients whose mMS for TA score was greater than or equal to that for AM would correspond to the diagnosis of OPDM with a sensitivity of 86.4% and a specificity of 100%.

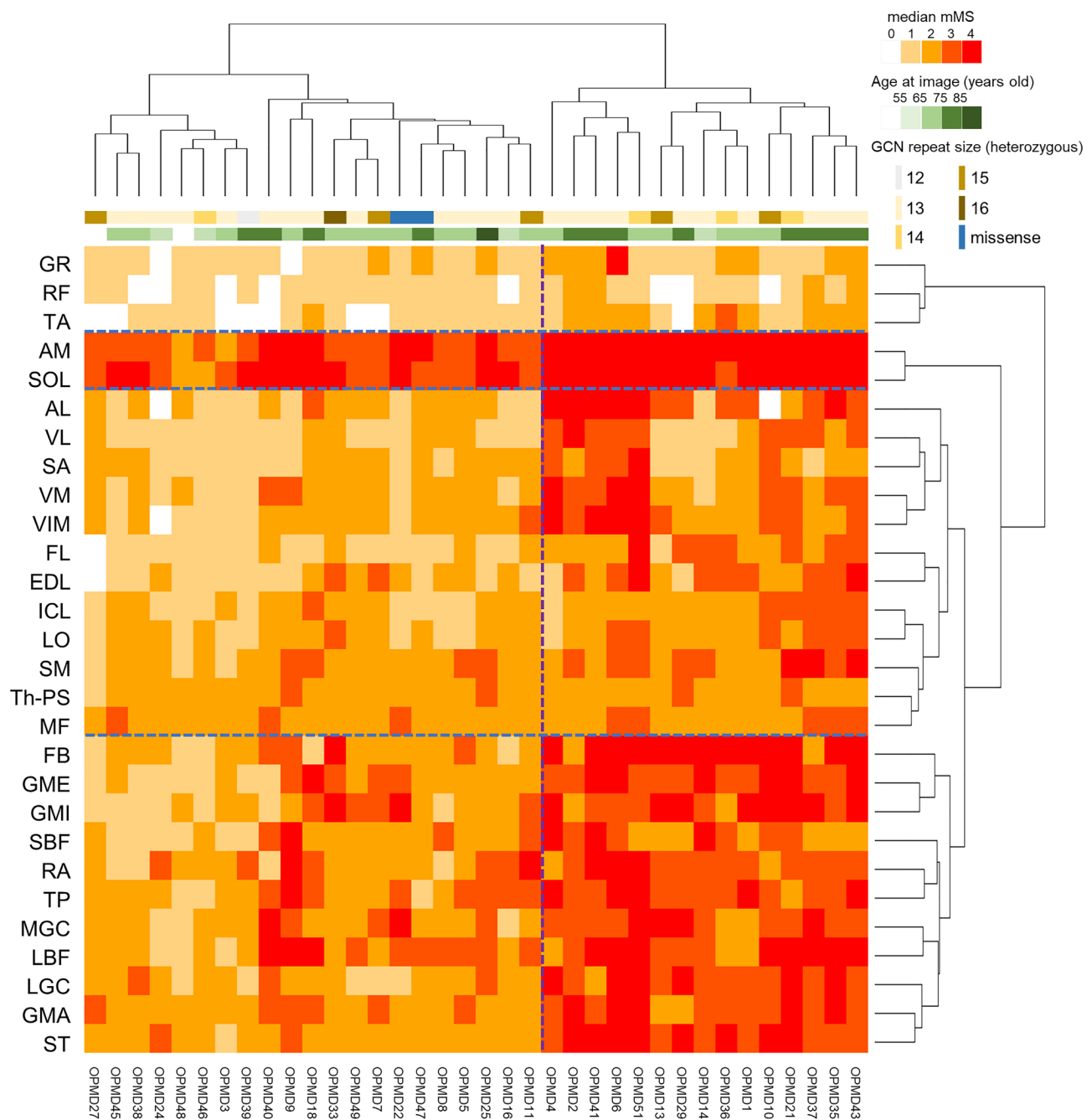
## Discussion

Our large cohort study of the muscle imaging findings of genetically confirmed patients with OPDM and OPMD illustrated the pattern of characteristic muscle involvement that can differentiate the two diseases. By hierarchical clustering and PC analysis, we obtained three major results: (1) In OPDM, SOL and MGC were the most severely affected from the earliest stage, followed by FL, FB, LGC, TA, and EDL, while GR, RF, SBF were spared even in the advanced stage; (2) Imaging findings could not significantly differentiate the three subtypes of OPDM; (3) For differentiation between OPDM and OPMD, severe involvement in TA, EDL, and MGC suggests OPDM, whereas if in AM, GMI, GME indicates OPMD.

For all subtypes of OPDM and OPMD except for OPDM\_GIPC1, disease duration correlated with the severity of fat infiltration. However, the age at muscle imaging did not correlate with mMS in OPDM\_LRP12 and OPDM\_GIPC1. This may reflect the possibility that age by itself does not necessarily correlate with disease progression, since disease onset depends on the repeat length in these subtypes of OPDM [6, 11, 22].

To date, only limited case series have reported the imaging features of OPDM with selective involvement in the posterior calf muscles, such as SOL and MGC/LGC, regardless of the causative gene, in addition to AM and LBF in OPDM\_NOTCH2NLC [2–5, 11, 20, 22, 23]. And one report by Yu et al. described that the pattern of muscle involvement with disease progression is similar among OPDM subtypes caused by *LRP12*, *GIPC1*, and *NOTCH2NLC* mutation [4]. In our study, we found that OPDM\_NOTCH2NLC tended to exhibit a more extensive and severely affected muscle distribution at thigh level than the other subtypes; however, hierarchical and PC analysis did not differentiate the muscle involvement pattern among OPDM subtypes. This may be due to the small number of cases in OPDM\_GIPC1 and OPDM\_NOTCH2NLC. Therefore, further accumulation of the cases and investigation on image analysis is needed. In OPMD, on the other hand, the early involvement in AM and SOL has been reported [12–14], which was consistent with our findings. In the truncal muscles, selective fat replacement of the serratus anterior and subscapularis muscles has been reported [12], but in our study, there was no significant difference between OPDM\_LRP12 and OPMD in these muscles. In addition, GNE myopathy and Miyoshi type myopathy are also important differentials of OPDM as distal myopathy. In the former, the quadriceps femoris muscles are relatively spared, while TA, thigh flexors, and adductor muscles are selectively affected [24]. Miyoshi myopathy has recently been reported to have equal degree of fat replacement in distal and proximal muscles regardless of the stage of the disease, as is thought to be the same disease as limb girdle muscular dystrophy R2 [25, 26]. Although these findings appear to be similar in part to those of OPDM, we may clarify the differences in the pattern of muscle involvement in these “distal myopathies” by using hierarchical and principal component analysis which we used in this study and will contribute to make the differential diagnosis of these myopathies by imaging findings in future.

The strength of our study is that we identified affected muscles with natural disease courses as a group rather than a single one using a hierarchical analysis. Interestingly, even cases with a distribution of atypical muscle weakness were classified exactly as OPDM or OPMD. This provides more robust clues for diagnosis and clarifies where we should focus our attention on physical examinations. For example, observing how the patient walks and measuring the strength of the GME and GMI may help us get closer to a diagnosis even without the skeletal muscle imaging. Furthermore, our findings would provide insight into the patient's stage of disease and prognosis of muscle symptoms. This could serve as indices when clinical trials are conducted in the future and be useful in guiding patients in their daily lives, rehabilitation, and orthotic adjustments.

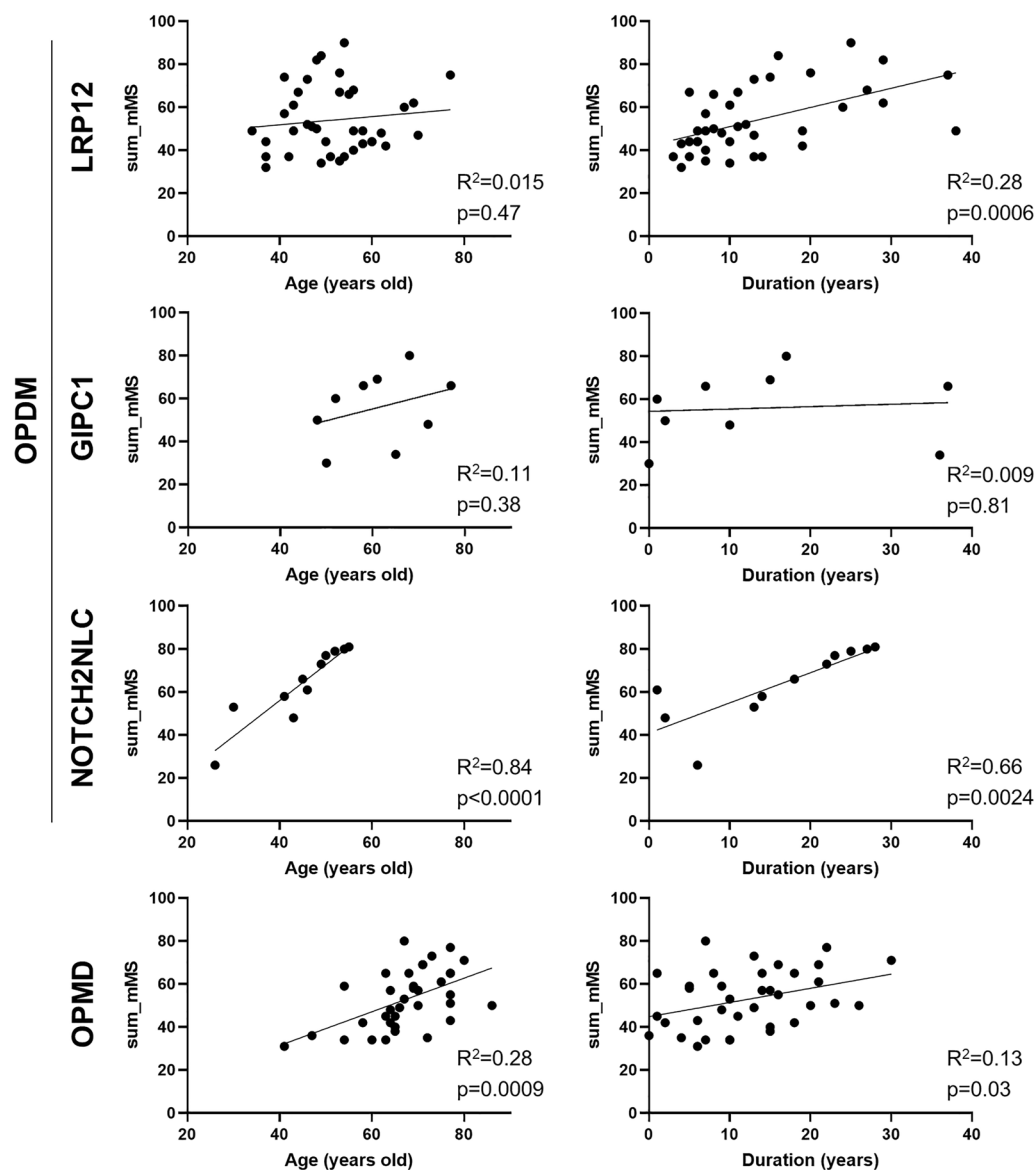


**Fig. 3** Hierarchical analysis of OPMD. The involved muscles in the thigh, calf, lumbar paraspinal, and thoracic paraspinal muscle on each patient are shown. The muscles and patients are clustered based on

the degree of fat infiltration and age. The degree of fat infiltration is indicated by the red scale color. The age at muscle imaging is shown in green scale, and genotype is shown in yellow scale

This study has several limitations. First, the number of patients harboring CGG repeat expansion in *GIPC1* or *NOTCH2NLC* was small compared to patients with *LRP12*. In addition, patients with *RILPL1* mutation were reported only from China and not included in our cohort [27]. Second, we regarded the repeated scans of patients as individual imaging series for analysis. This can lead to biases in which individual characteristics influence group ones.

For example, we used 7 image series of the same patient in the OPDM\_NOCTH2NLC group ( $n = 11$ ), and it is possible that the pattern of muscle involvement of this patient affected that of this group. However, we did not detect any statistical difference in the distribution of affected muscles in OPDM\_NOCTH2NLC from OPDM due to other causative genes, therefore, this type of bias may not be significant in our study. Third, this study was retrospective and we



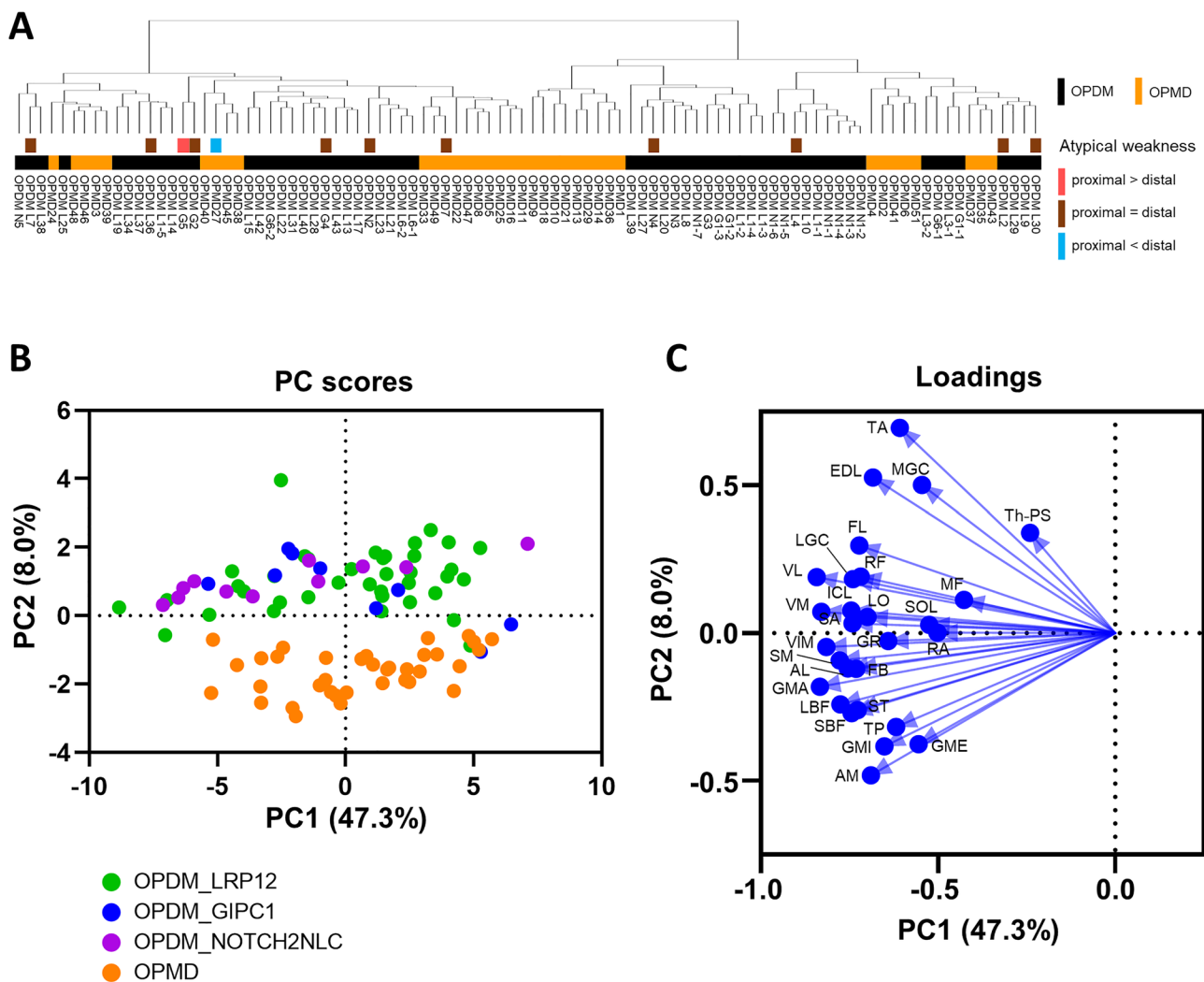
**Fig. 4** Correlation of total mMS with clinical features. On linear regression analyses, a significant positive correlation between age at muscle imaging (Age) and sum of mMS was seen in OPDM\_ NOTCH2NLC ( $p < 0.0001$ ), while the disease duration (Duration)

and sum of mMS were significantly correlated in all subtypes of OPDM ( $p < 0.05$ ). In OPMD, sum of mMS was significantly correlated with both Age and Duration

only analyzed 28 muscles due to incomplete data. To better characterize skeletal muscle imaging in OPDM, and to confirm the results from our study, a prospective cohort with whole-body muscle imaging data will be needed. However, we demonstrated that these 28 muscles could explicitly characterize the distribution of affected muscles in OPDM and were at least adequate in differentiating OPDM from OPMD.

This study provided the following answers to the questions raised in Introduction: (1) Is OPDM a heterogeneous disease?—Yes, OPDM is genetically and clinically heterogeneous. (2) Do the subtypes of OPDM with different causative genes represent different clinical phenotypes?—No, the

subtypes of OPDM with different causative genes represent similar clinical patterns of affected muscles on disease progression. (3) Is the distribution of affected muscles different between OPDM and OPMD?—Yes, the distribution is different between OPDM and OPMD. This large cohort study has statistically defined the disease stage-dependent muscle involvement pattern in OPDM caused by CGG repeat expansion in *LRP12*, *GIPC1*, and *NOTCH2NLC*. This is not only useful for the diagnosis of OPDM including its disease stage, but also for more efficient genetic testing by highlighting the differences from OPMD, which is often difficult to differentiate.



**Fig. 5** Hierarchical and PC analysis of OPDM and OPMD. **(A)** The patients were classified based on the hierarchical clustering indicated by the subclusters according to each disease. The patients with atypical muscle weakness were also correctly included in the disease sub-

clusters. **(B)** PC analysis showed that OPDM and OPMD are clearly separated. **(C)** The loadings on the PC analysis revealed muscles with positive/negative influence on PC2, indicating which is more likely to be affected in OPDM/OPMD, respectively

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00415-023-11906-9>.

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**Author's contributions** NE and IN designed the study; NE, SN, M-Og, TK, SH, IN, TM, MMY, MN, M-Oc, MS, KS, SY and TY acquired and analyzed the data; NE, SN, SH and IN wrote and edited the manuscript; and IN supervised the project. All authors read and approved the final manuscript.

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## Declarations

**Conflicts of interest** The authors report no competing interests.

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## Anti-mitochondrial antibody-mediated myopathy with cardiac involvement: reply

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We would like to thank Finsterer<sup>1</sup> for his interest in our article.<sup>2</sup> In his letter,<sup>1</sup> the importance of differential diagnosis including the other muscular disorders was emphasized.

In our case, presence of myopathy was evidenced by pathological changes in muscle biopsy samples.<sup>1</sup> Of note, we routinely perform a full battery of histochemistry for all muscle biopsy samples, including succinate dehydrogenase (SDH), cytochrome c oxidase (COX), SDH/COX double stain, as well as more than 20 different antigens for immunohistochemistry, including a variety of proteins deficient in muscular dystrophies. In our case, there were no findings suggestive of mitochondrial abnormalities, such as ragged red fibres, COX-negative fibres, and strongly SDH-reactive blood vessels, and all muscular dystrophy-associated proteins were normally expressed. Further, there were no findings of fatty replacement on muscle imaging analysis, which is one of the features suggesting hereditary myopathy.<sup>3</sup> Blood test showed positive anti-mitochondrial M2 antibody (AMA) and normal lactate level (0.9 mmol/L). There were no findings indicating dysfunction of the other organs based on physical examination, blood tests, and imaging studies. The information on detailed family history showed no histories of muscular or cardiac disorders, pacemaker implantation, or sudden death, except for atrial fibrillation in her mother. These findings ensured an adequate diagnosis of AMA-associated myopathy, and there was no clear indication for genetic testing.

We are afraid that his suggestion that mitochondrial myopathy should be ruled out seems to be irrelevant for the reasons as follows. First, as mentioned above, there were no clinicopathological features suggesting mitochondrial disease. Second, AMA has never been associated with mitochondrial myopathy.<sup>4</sup> Although he showed recent meta-analysis,<sup>5</sup> it merely evaluated the diagnostic value of AMA for cardiac involvement in idiopathic inflammatory myopathies (IIMs), not its pathogenicity.

Among a variety of myositis-specific antibodies (MSAs) reported in IIMs, anti-nuclear antibody was positive (×80), but ribonucleoprotein, Sjögren's syndrome - A, Sjögren's syndrome - B, and Jo-1 antibodies were all negative in our case. Remarkably, MSAs have the particularity of being mutually exclusive, the co-existence of two or more in the same patient being exceedingly rare (reportedly 0.18%).<sup>6</sup>

We are sure that the other cardiac diseases were adequately ruled out. There were no findings indicating arrhythmogenic right ventricular (RV) cardiomyopathy including electrocardiogram changes, regional RV wall motion abnormalities, or pathological changes in endomyocardial biopsy samples. Amyloidosis was ruled out since Congo red staining was negative both in skeletal muscle and myocardial tissues. Fluor-deoxy-glucose (FDG)-positron emission tomography was performed for suspected sarcoidosis. Although that showed diffuse uptake of FDG in cardiac muscles indicating false positive finding, there were no other lesions (i.e. lymph nodes) with FDG uptake. Biomarkers and gallium scintigraphy demonstrated negative findings.

There was no evidence of epithelioid cell granulomas in endomyocardial biopsy samples. From these findings, sarcoidosis was deemed highly unlikely.<sup>1</sup>

In summary, in our case, the diagnosis of AMA-related myopathy was made, and the other cardiac and muscular disorders were ruled out with certainty. The pathogenicity of AMA and mitochondrial derangement in AMA-associated myopathy remain to be elucidated and need further investigation.

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### Data availability

Data that are referenced are publicly available.

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# Clinical Classification of Variants in the Valosin-Containing Protein Gene Associated With Multisystem Proteinopathy

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## Abstract

### Background and Objectives

Pathogenic variants in the valosin-containing protein (*VCP*) gene cause a phenotypically heterogeneous disorder that includes myopathy, motor neuron disease, Paget disease of the bone, frontotemporal dementia, and parkinsonism termed multisystem proteinopathy. This hallmark pleiotropy makes the classification of novel *VCP* variants challenging. This retrospective study describes and assesses the effect of 19 novel or nonpreviously clinically characterized *VCP* variants identified in 28 patients (26 unrelated families) in the retrospective *VCP* International Multicenter Study.

### Methods

A 6-item clinical score was developed to evaluate the phenotypic level of evidence to support the pathogenicity of the novel variants. Each item is allocated a value, a score ranging from 0.5 to 5.5 points. A receiver-operating characteristic curve was used to identify a cutoff value of 3 to consider a variant as high likelihood disease associated. The scoring system results were confronted with results of *in vitro* ATPase activity assays and with *in silico* analysis.

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## Glossary

**ALA** = age at last assessment; **ALS** = amyotrophic lateral sclerosis; **ACMG** = American College of Medical and Genomic Genetics; **AOO** = age of onset; **CADD score** = combined annotation dependent depletion; **CM** = cardiomyopathy; **D** = dementia; **Dys** = dysautonomia; **ED** = extrapyramidal disorder; **FTD** = fronto temporal dementia; **IBMPFD** = Hereditary Inclusion-Body Myopathy with Paget's Disease of the Bone and Frontotemporal Dementia; **LL** = lower limbs; **LMN** = lower motor neuron signs; **LP** = likely pathogenic; **MND** = motor neuron disease; **MSP** = multisystem proteinopathy; **Myo** = myopathic pattern; **Neu** = neurogenic pattern; **NGS** = next generation sequencing; **PDB** = Paget disease of the bone; **RA** = regional atrophy; **RV** = rimmed vacuoles; **SIFT** = sorting intolerant from tolerant; **SW** = scapular winging; **UL** = upper limbs; **UMN** = upper motor neuron signs; **VCP** = Valosin-containing protein; **VUS** = variant of unknown significance.

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## Results

All variants were missense, except for one small deletion-insertion, 18 led to amino acid changes within the N and D1 domains, and 13 increased the enzymatic activity. The clinical score coincided with the functional studies in 17 of 19 variants and with the *in silico* analysis in 12 of 19. For 12 variants, the 3 predictive tools agreed, and for 7 variants, the predictive tools disagreed. The pooled data supported the pathogenicity of 13 of 19 novel VCP variants identified in the study.

## Discussion

This study provides data to support pathogenicity of 14 of 19 novel VCP variants and provides guidance for clinicians in the evaluation of novel variants in the VCP gene.

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## Introduction

Valosin-containing protein (VCP), or p97, is a hexameric protein from the AAA+ (ATPases Associated with diverse cellular Activities) family involved in the remodeling of molecules using the energy of ATP hydrolysis.<sup>1</sup> VCP is encoded by the VCP gene, a 17-exon gene on chromosome 9.<sup>2</sup> Variants in the VCP gene were initially described in patients with inclusion body myopathy, Paget disease of the bone, and frontotemporal dementia (IBMPFD).<sup>2</sup> However, this acronym is insufficient to capture the expanding phenotypic spectrum of VCP patients, and currently, this disease is more accurately considered a member of a group of conditions known as multisystem proteinopathy (MSP).<sup>3,4</sup> In fact, only 3–12% of patients with VCP-MSP show the typical triad of IBMPFD<sup>5</sup>; there is heterogeneous phenotypes within families<sup>6–8</sup>; and variants in VCP can lead to a plethora of clinical presentations making the diagnosis a complicated task.<sup>6,9–19</sup>

To date, only missense variants have been described in patients with VCP-MSP.<sup>3,7,20</sup> In all cases, the pattern of inheritance is dominant. The mechanism of VCP dysfunction is likely context-dependent because assays studying VCP mutant function *in vitro* and *in vivo* support a gain and loss of function mechanism. *In vitro*, most pathogenic variants have an increase in ATPase activity, what reflects an induced structural change allowing for an increase in ATP accessibility and ADP release. By contrast, cells and animals expressing VCP-MSP variants behave similarly to VCP chemical or genetic inhibition suggesting that VCP pathogenic variants are dysfunctional.<sup>21</sup> How the apparent increase in ATPase activity *in vitro* correlates with a loss of VCP function *in vivo* remains to be explored but is likely due to the complex interactions of adaptor proteins mediated by the ATPase cycle.

Challenges in asserting the pathogenicity to novel VCP variants are due to the diverse phenotypic presentations, the possible varied gene penetrance, and the fact that ancillary tests may support the diagnosis but do not show pathognomonic features. In addition, patients could be seen by clinicians with different backgrounds; neurologists specialized in dementia, movement disorders, or muscle diseases; endocrinologists; or rheumatologists, which could lead to fragmented and siloed care making difficult the recognition that there is a monogenic disease segregating in the family.

Current strategies to evaluate the pathogenicity of novel variants often rest solely on genetic evidence such as variant rarity in population databases or segregation of the variant with phenotype in families. This approach does not consider that in multisystem diseases, such as in VCP-MSP, sometimes only limited phenotypic information is available for the ordering clinician. In addition, there is no clear consensus on functional assays that may help define pathogenic function of VCP variants.

In this study, we report 19 novel variants in the VCP gene identified in the collaborative VCP International Multicenter Study.<sup>8</sup> We have developed a scoring system to help in the assessment of the potential pathogenicity of novel VCP variants and have confronted the results of our score with results of functional testing.

## Methods

### Patients

Patients included in this report are part of the descriptive retrospective VCP International Multicenter Study that collected

**Table 1** Scoring System

Item	Description	Points allocated
Item 1	Presence of a VCP-MSP core phenotype in the patient: myopathy, dementia, <sup>a</sup> PDB, motor neuron disease, and/or extrapyramidal disorder	1
Item 2	Positive family segregation following an autosomal dominant pattern of inheritance	1
Item 3	First-degree relative showing any of the VCP-MSP core phenotypes (even without genetic confirmation)	1
Item 4	Novel variant reported in an unrelated individual (from an unrelated family) with a VCP-MSP core phenotype <sup>b</sup>	1
Item 5	Muscle biopsy of the patient showing rimmed vacuoles or protein inclusions/aggregates	1
Item 6	Presence of “fat pockets” on axial T1-w MRI of the thigh and/or legs.	0.5

Abbreviations: MSP = multisystem proteinopathy; PDB = Paget disease of the bone.

The scoring system aids in evaluating the phenotypic level of evidence supporting the pathogenicity of a novel and/or nonpreviously clinically characterized variant in the VCP gene using clinical, family history, and ancillary test data available on clinical practice. For each variant, the points of the items present are added, and based on the total score, the variant is classified as follows:

- High likelihood disease associated variant: >3 points.
- Probable disease associated variant: 2–3 points.
- Variant with undetermined association: 0.5–1.5 points.

This scoring system is suggested to be applied after a novel/VUS variant in the VCP gene was identified and other genetic diagnosis are less likely to explain the clinical phenotype of the patient.

This scoring should be reapplied on each regular patient's clinical follow-up to build up the phenotypic level of evidence longitudinally. This may require collecting additional data on sign/symptoms, family history, ancillary test results, and published literature evidence.

<sup>a</sup> Preferably, but not exclusively, frontotemporal dementia.

<sup>b</sup> This is the case for variant p.Ile216Met, p.Ile369Thr, p.Ile241Ser, or p.Met158Thr in this study.

clinical, genetic, and ancillary test data from 255 patients seen in 52 centers from 24 countries.<sup>8</sup> “To standardize variant interpretation, which was performed differently within individual countries, all genetic variants were centrally reviewed by an experienced geneticist from the John Walton Muscular Dystrophy Centre using the criteria suggested by the American College of Medical and Genomic Genetics (ACMG) as a guide.<sup>22</sup> All variants were analyzed using larger databases including 1,000 genomes, dbSNP, ExAC and Exome Variant Server. Variant nomenclature was based on transcript reference NM\_007216.3. Inclusion criteria for the present report were adapted from the one used in the VCP International Multicenter Study and included: (1) patients  $\geq 18$  years old heterozygous for a novel or a previously reported but not thoroughly clinically characterized as pathogenic (P) or Likely Pathogenic (LP) variant in the VCP gene and (2) enough data available in the clinical notes to answer questions about age of disease onset, symptoms and clinical signs at onset and/or during disease's progression, family segregation analysis, and ancillary test results.<sup>8</sup>” Two additional patients with novel pathogenic variants who were not part of the VCP International Multicenter Study were also included in this study.

### Standard Protocol Approvals, Registrations, and Patient Consents

The VCP International Multicenter Study obtained Caldicott approval from The Newcastle upon Tyne Hospitals Register Audit (project number 10833, Caldicott Approval: 7918) and institutional review board approvals from the LMU Klinikum at Ludwig-Maximilians University in Munich (project 21-0071); Washington University School

of Medicine Institutional Review Board, USA (no 201103416); and the Johns Hopkins Hospital Institutional Review Board, Baltimore, USA (no 00288171). The novel variants in the VCP gene associated with the VCP-MSP article was approved by the human studies review committee at Washington University School of Medicine in St. Louis (201903027). These ethics committees cataloged this study as an audit because it collected deidentified retrospective data of patients with VCP. This study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

### Development of a Pathogenicity Score

To evaluate the phenotypic level of evidence to support the pathogenicity of the novel and/or nonpreviously clinically characterized variants, a score was developed (Table 1). The score consists of 6 items assessing information that can be obtained on daily clinical practice. These items were chosen based on the authors' clinical experience and because they are commonly used to support a diagnosis of VCP-MSP in the literature. Item 6, which refers to muscle MRI, requires the presence of the so-called fat pockets in the skeletal muscle of the patients. This is a feature that can be found in patients with VCP-MSP as previously reported<sup>23</sup> and as shown in the muscle MRI images in eAppendix 1 (links.lww.com/NXG/A625). Each item is allocated a value to support their contribution to the variant association with the clinical phenotype. The sum of all the items leads to a total score, with a minimum of 0.5 and maximum of 5.5 points. We considered a variant to be high likelihood disease associated if its total score was greater than 3 points, probably associated if the total score was between 2 and 3 points, and undetermined association if the score was less than 2 points.

**Table 2** Classification of the Novel Variants Based on the Scoring System

DNA m (Protein m)	Highest score in the variant
<b>Scoring system applied to the 4 most frequent variants identified in the VCP International Multicenter Study</b>	
c.464G>A, (p.Arg155His)	5.5
c.463C>T (p.Arg155Cys)	5.5
c.476G>A (p.Arg159His)	5.5
c.277C>T (p.Arg93Cys)	5.5
<b>High likelihood disease associated variant</b>	
c.648A>G (p.Ile216Met)	5.5
c.722T>G (p.Ile241Ser)	5
c.1105A>T (p.Ile369Phe)	4.5
c.431_432delGAinsAC (p.Arg144His)	4.5
c.473T>C (p.Met158Thr)	4.5
c.1106T>C (p.Ile369Thr)	4.5
<b>Probable disease associated variant</b>	
c.268A>G (p.Asn90Asp)	3
c.367G>A (p.Val123Met)	3
c.196G>A (p.Glu66Lys)	2.5
c.463C>G (p.Arg155Gly)	2
c.490A>C (p.Lys164Gln)	2
c.286C>G (p.Leu96Val)	2
c.625T>G (p.Cys209Gly)	2
<b>Variant with undetermined association</b>	
c.80T>C (p.Ile27Thr)	1.5
c.1988A>G (p.Lys663Arg)	1.5
c.265 C>G (p.Arg89Gly)	1
c.1057A>G (p.Ile353Val)	1
c.697A>G (p.Ile233Val)	1
c.335A>G (p.Lys112Arg)	1

For a detailed description of the clinical scoring system applied to each novel variants, please refer to eAppendix 2 ([links.lww.com/NXG/A626](https://links.lww.com/NXG/A626)).

To assess the validity of the scoring system, we first tested the score using several patients harboring the 4 most frequent pathogenic variants included in the VCP International Multicenter Study: p.Arg155His, p.Arg155Cys, p.Arg159His, and p.Arg93Cys, and then, we scored the novel nonpreviously clinically characterized variants and all the variants included in the VCP International Multicenter Study (eAppendix 2, [links.lww.com/NXG/A626](https://links.lww.com/NXG/A626), 58 variants in total).

## In Silico Analysis

The deleteriousness of the novel variants was predicted by 3 independent in silico tools commonly used in daily routine: Mutation Taster ([mutationtaster.org/](https://mutationtaster.org/)),<sup>24</sup> SIFT (sorting intolerant from tolerant, [sift.bii.a-star.edu.sg/](https://sift.bii.a-star.edu.sg/)),<sup>25</sup> and CADD (combined annotation-dependent depletion, [cadd.gs.washington.edu/](https://cadd.gs.washington.edu/)).<sup>26</sup> These tools use predictive algorithms, based on different variables such as sequence homology, physical properties of amino acids, or evolutionary conservation of the protein sequence to establish whether an amino acidic change could affect protein function and, therefore, its potential pathogenicity.

## In vitro ATPase Assay

Human VCP plasmid (TCB197) was subjected to site-directed mutagenesis with primers containing mutations to create each of the indicated variants. Proteins were purified as described.<sup>27</sup> Purified VCP (12.5  $\mu$ L of 50  $\mu$ M; final concentration in the reaction was 25 nM) was diluted in 20 mL of assay buffer [5 mL of 5 $\times$  assay buffer A (1 $\times$  = 50 mM Tris pH 7.4, 20 mM MgCl<sub>2</sub>, 1 mM EDTA) mixed with 15 mL of water and 25  $\mu$ L 0.5M TCEP, 25  $\mu$ L 10% Triton] to make the enzyme solution. 40  $\mu$ L of the enzyme solution was dispensed into each well of a 96-well plate. The ATPase assay was prepared by adding 10  $\mu$ L of 1,000  $\mu$ M ATP (Roche, pH 7.5) to each well and by incubating the reaction at room temperature for 25 minutes. Reactions were stopped by adding 50  $\mu$ L of BIOMOL Green reagent (Enzo Life Sciences). Absorbance at 635 nm was measured after 4 minutes on the Synergy Neo Microplate Reader (Bio-Tek). All assays were performed in triplicate, and the activity was averaged from independent experiments.

## Statistical Analysis

Data were expressed as number and percentage for categorical variables and as mean  $\pm$  SD for quantitative ones. For VCP ATPase assays, statistical significance was defined using a 2-way analysis of variance across all samples compared with the wild-type control.

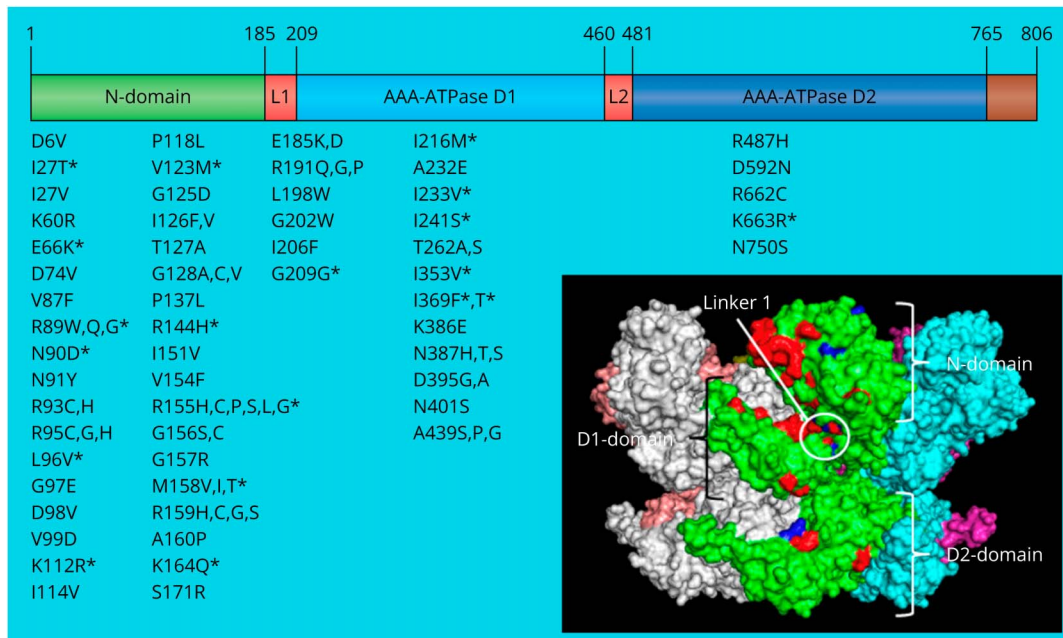
Mean difference in the 6-item score between pathogenic/likely pathogenic variants and variants of unknown significance, of the 58 variants included in the VCP International Study, was explored using independent sample T tests. To identify which score predicted with the highest sensitivity and specificity whether a variant was pathogenic/likely pathogenic based on the ACGM criteria, a receiver-operating characteristic curve was performed (eAppendix 3, [links.lww.com/NXG/A627](https://links.lww.com/NXG/A627)) and its area under the curve and the optimal cutoff point (Youden index) were calculated.

A level of significance of 0.05 was used for hypothesis testing. Statistical analysis was performed using the program IBM SPSS statistics, version 28.

## Data Availability

The clinical, genetic, and enzymatic test data reported in this study are available on reasonable request to the corresponding authors.

**Figure 1** Location of the Novel Mutations in the VCP Gene and Protein



Scheme of the location of all the variants described in the VCP gene and protein structure. The VCP protein contains 806 amino acids and is constituted by an N-terminal domain involved in the cofactor and ubiquitin-binding function, a D1 domain involved in the assembly of VCP homohexamer, a D2 domain responsible for the major ATPase activity, and the C-terminal domain involved in nuclear localization by interacting with other proteins. The N-domain and D1 domain are connected by N-D1 linker (L1), and the D1 and D2 domains are connected by flexible D1-D2 linker (L2). All variants are listed underneath the domain affected; those with an asterisk are considered novel. The black square contains a 3D render of a VCP hexamer. Each subunit is independently colored. The positions of previously reported pathogenic residues are denoted in red and the novel reported variant residues in blue within a single green monomer. VCP = valosin-containing protein.

## Results

We identified 19 previously uncharacterized variants in the VCP gene from 28 patients with presumed VCP-MSP from 26 unrelated families (Table 2, eAppendix 2, [links.lww.com/NXG/A626](https://links.lww.com/NXG/A626), and eAppendix 4, [links.lww.com/NXG/A628](https://links.lww.com/NXG/A628)). Eighteen of the 19 variants had not been previously reported in GNOMAD. One variant, p.Ile233Val, had been reported twice, and GNOMAD provided with a minor allele frequency of 7.95E-06. Six of the 19 variants had been reported in ClinVar, and one of these 6 were additionally reported in LOVD. These 7 previously reported variants were not thoroughly clinically characterized in the literature though. Figure 1 shows the localization of the novel variants in the VCP gene.

### Phenotypic Scoring

As shown in eAppendix 2 ([links.lww.com/NXG/A626](https://links.lww.com/NXG/A626)), at least one patient carrying the novel variants on the VCP gene manifested with one or more of the VCP-MSP core phenotypes (e.g., myopathy, PDB, FTD, ALS or parkinsonism), except for one patient (Family 26 III-1), harboring the c.335A>G (p.Lys112Arg) variant, who presented with signs of upper motor neuron involvement without muscle weakness. In all cases, pathogenic variants in other genes were excluded by exome or panel-based sequencing. Notably, muscle weakness was reported in 85.7% (24/28) of the cases,

FTD in 21.4% (6/28), and PDB in 18.0% (5/28). No patient showed the classical IBMPFD triad. Fifteen of the 28 patients had a first-degree relative with an VCP-MSP core phenotype, dementia being the most common symptom in the relatives (10/28).

All patients were heterozygous for their VCP variant, compatible with a dominant inheritance. All variants were missense, except for one small deletion-insertion that created the missense variant p.Arg144His. Eighteen of the 19 variants led to amino acid changes within the N and D1 domains, where 64 of the 68 previously reported VCP-MSP variants were located (Figure 1).

To establish the validity of our scoring system, we first evaluated the score obtained by the 4 most common VCP variants—p.Arg155His, p.Arg155Cys, p.Arg159His, and p.Arg93Cys. These 4 variants achieved the highest score possible of 5.5 points. Then, we applied the scoring system to the 28 patients with the 19 novel variants described here, obtaining a range of scores from 0.5 to 5.5 (Table 2). Examples of the classical clinical and ancillary test findings seen in patients with VCP-MSP found in our cohort are described in eAppendix 1 ([links.lww.com/NXG/A625](https://links.lww.com/NXG/A625)).

### In Silico Analysis

The in silico predictions on the 4 most common pathogenic mutations predicted them to be probably deleterious. Table 3

**Table 3** In Silico Analysis

DNA change	Protein change	CADD score	Mutation taster	POLYPHEN-2
<b>More prevalent pathogenic variants</b>				
c.464G>A	p.Arg155His	24.5	DC	PrD 0.989
c.463C>T	p.Arg155Cys	33	DC	PrD 1
c.476G>A	p.Arg159His	24.1	DC	PsD 0.517
c.277C>T	p.Arg93Cys	31	DC	PrD 0.998
<b>Novel variants</b>				
c.648A>G	p.Ile216Met	23.6	DC	PrD 1
c.722T>G	p.Ile241Ser	29.9	DC	PrD 1
c.1105A>T	p.Ile369Phe	27.2	DC	PrD 1
c.431_432delGAinsAC	p.Arg144His	n/a	DC	PrD 1
c.473T>C	p.Met158Thr	24.2	DC	PB 0.988
c.1106T>C	p.Ile369Thr	26.7	DC	PrD 1
c.268A>G	p.Asn90Asp	23.1	DC	PsD 0.602
c.367G>A	p.Val123Met	26.9	DC	PsD 0.696
c.196G>A	p.Glu66Lys	23.9	DC	B 0.002
c.463C>G	p.Arg155Gly	25.8	DC	PrD 1
c.490A>C	p.Lys164Gln	25.2	DC	PrD 1
c.286C>G	p.Leu96Val	23.2	DC	B 0.028
c.625T>G	p.Cys209Gly	24.1	DC	PsD 0.704
c.80T>C	p.Ile27Thr	23	DC	PrD 0.968
c.1988A>G	p.Lys663Arg	23.7	DC	B 0.005
c.335A>G	p.Lys112Arg	22.9	DC	B 0.066
c.265C>G	p.Arg89Gly	23.2	DC	PrD 1
c.1057A>G	p.Ile353Val	20.3	DC	B 0.008
c.697A>G	p.Ile233Val	22.6	DC	PsD 0.810

Abbreviations: B = benign; DC = disease-causing; PB = possible benign; PrD = probable deleterious; PrD = probable deleterious; PsD possible deleterious. In silico scores for the 4 prevalent variants identified in the VCP International Multicenter Study and the 19 variants described in this study. CADD scores >20: top 1% deleterious variants in the genome.

shows the results of the predictive algorithms for each of the 19 novel variants that ranged from benign to probably deleterious.

### In Vitro ATPase Assays

To further understand whether the variants identified lead to a functional change in the VCP protein, we purified a recombinant wild-type VCP, the 4 most common pathogenic VCP mutants (VCP-Arg155His, VCP-Arg155Cys, VCP-Arg159His, and VCP-Arg93Cys), a previously reported benign variant (VCP-Ile27Val), and the new 19 VCP variants.

VCP hydrolyzes ATP, and the rate of hydrolysis is enhanced by most of the previously described VCP pathogenic variants. VCP-WT ATPase activity was arbitrarily set to 100%. The common 4 pathogenic variants demonstrated a 4 to 5-fold increase in ATPase activity consistent with previous studies, whereas the VCP- p.Ile27Val benign variant had only a 1.6-fold increase in ATPase activity. Using these data points, we selected a 3-fold increase as being consistent with a dysfunctional variant. Thirteen of the novel variants met this threshold (Figure 2).

A summary of the evidence obtained for each variant using the scoring system, in silico analysis, and enzymatic activity is provided in Table 4. For 12 variants, there was agreement between the 3 predictive tools, including 9 variants predicted to be deleterious and 3 variants predicted to be nondeleterious.

For 7 variants, the predictive tools disagreed. For 3 of them (p.Met158Thr, p.Glu66Lys and p.Leu96Val), clinical score and enzymatic activity supported pathogenicity while in silico studies did not. Conversely, in other 2 variants (p.Ile27Thr and p.Ile233Val), clinical score and enzymatic activity supported nonpathogenicity while in silico studies did.

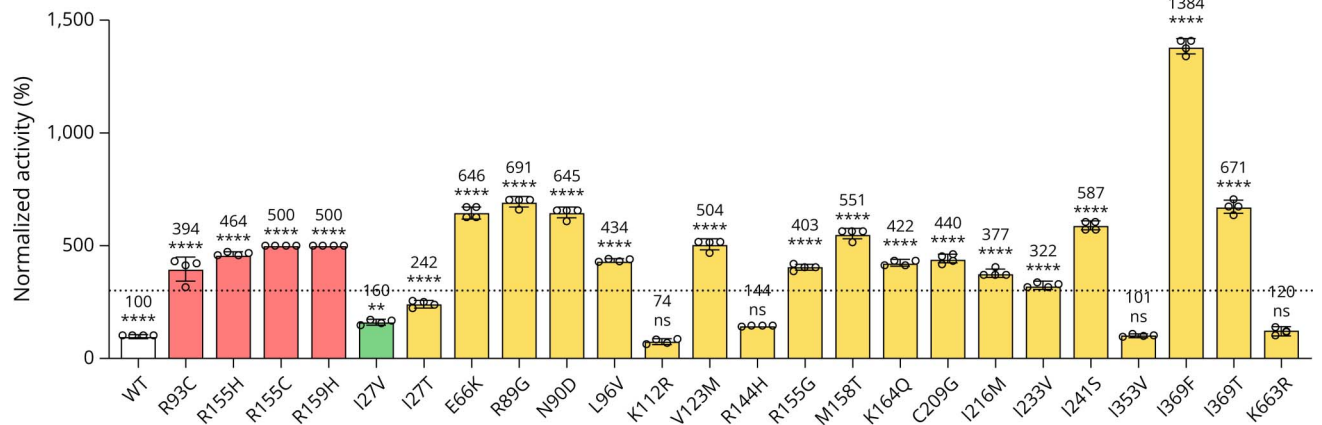
For the variant p.Arg144His, both the scoring system and the in silico algorithms predicted pathogenicity, but the enzymatic activity did not. This variant was found in a patient with isolated muscle disease with a muscle biopsy showing rimmed vacuoles and a MRI showing patchy fat replacement in the quadriceps and who had several relatives with dementia, ALS, and muscle disease. The variant obtained 4.5 of 5.5 points in the scoring system, but the enzymatic activity was normal.

For the variant p.Arg89Gly, the in silico studies and enzymatic activity suggested pathogenicity, but the clinical score was very low (1 point). This variant was found in a patient with isolated muscle weakness, with no relevant family history, who had just fiber atrophy in the muscle biopsy but not rimmed vacuoles or protein aggregates and who did not have an MRI.

## Discussion

The increasing availability of next-generation sequencing panels and whole-exome/genome sequencing is leading to the identification of new variants in the VCP gene.<sup>28</sup> The evaluation of these novel variants is challenging because of the intrafamilial and interfamilial phenotype variability<sup>7</sup> and because of the progressive nature of the condition, implying that patients might not show the whole clinical spectrum when the results of the genetic tests are obtained. As a result, VCP-MSP diagnosis can be missed in early stages of the disease or clinical features could be attributed to other diagnosis.<sup>20</sup> This situation can be even more complex if thorough family history or segregation studies are not obtained. Moreover, patients present with a variety of clinical, ancillary tests or family history findings and allocating them a hierarchical weight in

**Figure 2** Enzymatic in Vitro Test



Basal ATPase activity was determined from purified recombinant VCP protein. Values are normalized to VCP-wild type activity and presented as percent change from VCP-wild type. Red bars represent known pathogenic variants. Green bars represent known benign variants. Yellow bars represent the novel variants investigated. The dotted line represents the 3-fold increase threshold in ATPase activity consistent with a dysfunctional variant. ns: nonsignificant difference. \*\* $p < 0.05$ ; \*\*\*\* $p < 0.0001$ . VCP = valosin-containing protein.

the analysis of the potential pathogenicity of a new variant is challenging. It is crucial to estimate adequately the pathogenicity of new variants to avoid overestimating variants that could lead to a wrong diagnosis and stop the surveillance of other etiologies behind the patients' phenotype. In addition, when it comes to interpreting novel variants, information about its rarity based on their frequency in population databases is not enough to confirm pathogenicity. Even if a variant is labelled as VUS following the ACGM criteria, clinicians should consider other information such as patient phenotype, family history, segregation in the family, and ancillary test results to support the potential pathogenicity of the variant.

The scoring system proposed here intends to be a tool to support clinicians when facing a novel variant in the VCP gene in clinical practice. The current standard of care in VCP suggests that symptomatic patients should be assessed every 6–12 months or more frequently if required.<sup>29</sup> The evolving nature of this disease implies that a novel variant that scored low early in disease progression can end up having a higher score. This could be the case of the p.Arg89Gly variant, for which the in silico and enzymatic studies suggested pathogenicity, but it was found in a young patient with isolated weakness, therefore, leading to a low clinical score. Updating the scoring system on each clinical visit is encouraged in these cases because new symptoms can appear later in disease progression further supporting the diagnosis of the disease. The clinical score coincided with the functional studies in 17 of 19 new variants and with the in silico prediction in 12 of 19 new variants, suggesting that functional studies, which assess enzymatic function, may provide a more reliable prediction of the potential pathogenicity of variants.

VCP has 2 ATPase domains, D1 and D2, which are organized as 2 stacked rings with a central channel, whereas its

regulatory N domain is situated at the periphery of the D1 ring.<sup>30</sup> Reported pathogenic variants do not seem to alter VCP oligomerization but increase basal ATP hydrolysis activity, which is mediated through the D2 domain.<sup>30</sup> Pathogenic variant residues are commonly found in the interface between the N and D1 domains, suggesting that communication between these 2 regions is important for disease pathogenesis.<sup>1</sup> Other variants might affect the association of VCP with cofactors.<sup>31</sup> All the novel mutations identified in this study change an amino acid in the N terminal or D1 domain, except for the variant p.Lys663Arg located in the D2 domain. Being an ATPase enzyme, VCP hydrolyzes ATP releasing an inorganic phosphate. We have performed an enzymatic test comparing the intrinsic ATPase activity of VCP-wild type with several previously reported MSP mutations and the novel mutations, as reported in other studies.<sup>13</sup> Most of the novel variants reported here increased ATP activity compared with VCP-wild type. For those variants in which the ATPase activity did not differ from the VCP-wild type, clinical score and/or in silico analysis suggested nonpathogenicity. The only exception to this is the p.Arg144His variant that was suggested as pathogenic by the in silico studies and found in a patient with clinical data highly suggestive of the disease, but with a normal enzymatic activity. An elevation in basal ATPase activity has been described for most VCP variants, with the exception of 2 previously reported variants. Specifically, the p.Glu185Lys variant described in a large family with axonal CMT and p.Asp395Gly in 2 families with a pathologically distinct dementia (vacuolar tauopathy) had unchanged and reduced ATPase activity, respectively.<sup>32,33</sup> These variants suggest that VCP mutations could alter VCP functionality by an ATPase-independent manner. Thus, a reliance exclusively on in vitro ATPase assays of VCP function may be misleading.

**Table 4** Summary of the Study Results

Variant	Scoring system	In silico analysis	Enzymatic analysis	Interpretation
c.648A>G; p.Ile216Met	5.5	Deleterious	+	Agreement in the 3 tools, deleterious
c.722T>G; p.Ile241Ser	5	Deleterious	+	Agreement in the 3 tools, deleterious
c.1105A>T; p.Ile369Phe	4.5	Deleterious	+	Agreement in the 3 tools, deleterious
c.431_432delGAinsAC; p.Arg144His	4.5	Deleterious	-	Enzymatic analysis disagrees
c.473T>C; p.Met158Thr	4.5	Inconclusive	+	In silico analysis disagrees, Polyphen-2 PB
c.1106T>C; p.Ile369Thr	4.5	Deleterious	+	Agreement in the 3 tools, deleterious
c.268A>G; p.Asn90Asp	3	Deleterious	+	Agreement in the 3 tools, deleterious
c.367G>A; p.Val123Met	3	Deleterious	+	Agreement in the 3 tools, deleterious
c.196G>A; p.Glu66Lys	2.5	Inconclusive	+	In silico analysis disagrees, Polyphen-2 B
c.463C>G; p.Arg155Gly	2	Deleterious	+	Agreement in the 3 tools, deleterious
c.490A>C; p.Lys164Gln	2	Deleterious	+	Agreement in the 3 tools, deleterious
c.286C>G; p.Leu96Val	2	Inconclusive	+	In silico analysis disagrees, Polyphen-2 B
c.625T>G; p.Cys209Gly	2	Deleterious	+	Agreement in the 3 tools, deleterious
c.80T>C; p.Ile27Thr	1.5	Deleterious	-	In silico analysis disagrees, Polyphen-2 PrD
c.1988A>G; p.Lys663Arg	1.5	Inconclusive	-	Agreement in the 3 tools, nondeleterious
c.335A>G; p.Lys112Arg	1	Inconclusive	-	Agreement in the 3 tools, nondeleterious
c.265 C>G; p.Arg89Gly	1	Deleterious	+	Scoring system disagrees
c.1057A>G; p.Ile353Val	1	Inconclusive	-	Agreement in the 3 tools, nondeleterious
c.697A>G; p.Ile233Val	1	Deleterious	-	In silico analysis disagrees, Polyphen-2 PrD

The table shows the evidence obtained for each variant using the scoring system, the in silico analysis, and the in vitro ATPase assay (enzymatic activity) and the variant effect interpretation based on the agreement of the 3 tools.

The results of muscle biopsy and MRI in patients with VCP-MSP are not specific<sup>34</sup> and can overlap with conditions such as myofibrillar myopathies, other multisystem proteinopathies, or myopathies with rimmed vacuoles.<sup>4</sup> In this context, an enzymatic test that measures the effect of a variant in the ATPase activity can aid in diagnosis.<sup>13,35</sup> However, assessing the intrinsic ATPase enzymatic activity in VCP requires purified recombinant protein. Moreover, there is not an easier assay that uses blood, muscle, or even cerebrospinal fluid samples. Other blood biomarkers, such as tumor necrosis factor alpha and epidermal growth factor, have been suggested to differentiate patients with VCP-MSP from healthy controls,<sup>36</sup> but these biomarkers are not disease-specific and can be found in other inflammatory conditions.<sup>37,38</sup>

Three of the variants described here affected amino acidic residues that have been previously identified in patients with VCP-MSP. One variant affected the hot spot amino acid position Arginine 155 codified by exon 5, creating the p.Arg155Gly variant. A second variant was identified at amino acid residue Arginine at position 89, creating a p.Arg89Gly missense variant. Notably, p.Arg89Gln and p.Arg89Trp variants have been

reported in a patient with sporadic ALS and distal myopathy with cognitive impairment without family history, respectively.<sup>39,40</sup> Another variant created a p.Ile27Thr missense change which affected the same amino acid that the already reported p.Ile27Val variant. Although this later variant has been found in cohorts of sporadic inclusion body myositis and Parkinson disease, it has a MAF of 0.005 to 0.0006 depending on population ethnicity and was equally represented in healthy controls, suggesting that it may be benign.<sup>29,41</sup>

Limitations of this study include the retrospective nature of the VCP International study, from which the novel variants were obtained. Missing data could affect the score system result leading to underestimation of the variant pathogenicity. The sensitivity of the clinical scoring system to label the variants can progress over time because the clinical, muscle MRI and biopsy results can be modified along disease progression, reinforcing the idea that the scoring system should be applied on a regular basis. A greater than 3-fold increase in VCP-variant ATPase activity is helpful to support MSP-VCP mutant pathogenicity but could miss potential pathogenic variants because VCP mutation dysfunction may occur

independent of ATPase activity. Other assays of VCP function may need to be used. These could include assays of cellular autophagic or proteosomal activity.

In conclusion, we have developed a clinical score able to predict pathogenicity of new variants in the *VCP* gene with high accuracy that highly correlates with the results of in silico and enzymatic activity studies. This score could be of great help in the diagnostic process of patients with novel variants in daily clinics. This study provides data to support pathogenicity of 13 of 19 new *VCP* variants based on the fact that (1) patients affected had clinical and family history data highly suggestive of the VCP-MSP; (2) most of the new variants reported resulted in an increase in enzymatic activity in the in vitro assays; (3) novel variants are not present in public databases of controls (dbSNP); (4) these variants affected highly conserved residues across species in the N-terminal and D1 domain, in which most pathogenic VCP pathogenic variants have been reported; (5) no variants were found in other genes which were reported in the patients with MSP, FTD, or ALS previously; and (6) these variants were predicted to be deleterious by 3 prediction in silico assays.

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## Disclosure

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## Introduction

CHRONIC KIDNEY DISEASE (CKD) is a global public health concern.<sup>1</sup> In Japan, the number of CKD in the adult population increased to 14.8 million in 2015, which was 14.6% of the adult population.<sup>2</sup> CKD potentially progresses to end-stage kidney disease (ESKD), requiring renal replacement therapy (RRT) and is a recognized risk factor for cardiovascular disease (CVD) and premature death.<sup>3,4</sup>

The Frontier of Renal Outcome Modifications in Japan (FROM-J) is a prospective, stratified, cluster-randomized trial that evaluates the effectiveness of a practice facilitation program involving multidisciplinary care for CKD management by primary care physicians (PCPs). Since April 1, 2008, to October 19, 2008, 2,379 patients with CKD aged between 40 and 74 years have enrolled from 49 local medical associations in 15 different prefectures and were followed up in the FROM-J study. Participants were assigned to two intervention groups as follows: standard intervention, standard treatment according to the CKD clinical guideline<sup>5</sup>; and advanced intervention based on a practice facilitation program. The practice facilitation program comprised patient education by dietitians at PCPs' clinics, newsletters to patients, patient encouragement to visit PCPs during interruptions, alerts to PCPs to inform the timing of referral to nephrologists, and support for PCPs to control patient data.<sup>6</sup> Recently, additional data collection and analyses, including patient-reported outcome measures, were conducted to investigate the long-term outcomes of the intervention in the 10th-year after the start of the FROM-J study (FROM-J 10 study). A total of 1,473 participants had continued clinical visits to PCPs, who became older people aged  $\geq 70$  years, while there were dropouts, withdrawals, and deaths. Among 2,379 patients with CKD, 7.5% initiated RRT, 8.5% developed CVD, 12.4% had a 50% decline in estimated glomerular filtration (eGFR), and 8.9% died, as shown in Figure 1. A certain

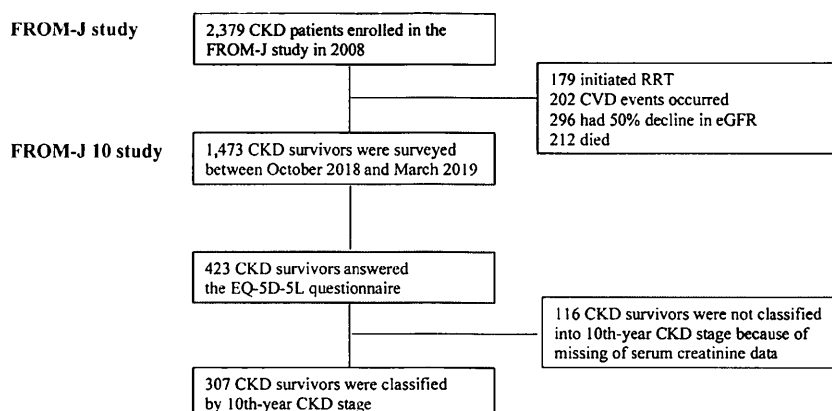
percentage of long-term CKD survivors lived with serious complications, including ESKD and CVD.<sup>7</sup>

An increasing number of clinical studies on CKD have used patient-reported outcome measures, including questionnaires measuring health-related quality of life (HRQOL).<sup>8-13</sup> EuroQol 5 Dimension 5 Level (EQ-5D-5L) is one of the most frequently used generic preference-based HRQOL measures for CKD.<sup>14</sup> The quality of life (QOL) score is known to decrease in patients with advanced CKD.<sup>8-11</sup> CKD survivors have lived with the disease and its complications, such as anemia, malnutrition, ESKD, and CVD,<sup>7,15,16</sup> which might affect their QOL scores. However, there are few studies on QOL scores in long-term survivors of CKD.<sup>10</sup> Therefore, we aimed to measure QOL scores in 10 years long-term survivors with CKD using EQ-5D-5L and examine the predictors and determinants of the clinical indices for the measured QOL scores.

## Methods

### Instrument for HRQOL

EQ-5D-5L, a generic preference-based measure of HRQOL that is standardized and validated for use in Japan, was used in this study.<sup>17,18</sup> It is administered to patients who are asked to grade five dimensions (mobility, self-care, usual activities, pain/discomfort, and anxiety/depression) of their health state at one of five levels ("no problem," "slight problems," "moderate problems," "severe problems," and "extreme problems"). The QOL score, with ranges of 1 for perfect health (no problem in any dimension), 0 for death, and  $-0.025$  for severe problems in all dimensions, is determined by the five-dimension health state profile according to a tariff reflecting the preference of the population. Moreover, it is a scalar quantity and is easy to use for statistical analysis compared with multidimensional QOL measurements. A higher score indicates better HRQOL, whereas a smaller or negative score indicates worse HRQOL.



**Figure 1.** Flow diagram of the study. Among 1,473 CKD survivors enrolled in the FROM-J study, 423 CKD survivors (38.2%) responded to the EQ-5D-5L. A total of 307 CKD survivors were classified into 10th-year CKD stages. A total of 116 participants were not classified into the 10th-year CKD stage because of missing serum creatinine data.

**Table 1.** Patients' Characteristics (n = 423)

Variables	Baseline data (2008)		10th-year data (2018)	
	n	% or mean (SD)	n	% or mean (SD)
Male	299	70.7%	299	70.7%
Mean age (years)	423	61.6 (7.9)	423	71.6 (7.9)
Body mass index	367	25.9 (3.9)	401	24.4 (4.0)
Systolic blood pressure (mmHg)	419	135 (13)	399	130 (13)
Diastolic blood pressure (mmHg)	419	78 (9)	398	74 (10)
Mean albumin (g/dL)	315	4.3 (0.3)	144	4.1 (0.3)
Mean hemoglobin (g/dL)	357	13.9 (1.6)	267	13.5 (2.0)
Comorbidities				
Hypertension	376	89.1%	376	89.1%
Diabetes	219	51.8%	219	51.8%
Hyperlipidemia	292	69.2%	292	69.2%
Hyperuricemia	171	40.5%	171	40.5%
Renal function				
Mean serum creatine(mg/dL)*	387	1.04 (0.45)	275	1.55 (1.28)
Mean eGFR (mL/min/1.73 m <sup>2</sup> )*	387	60.3 (20.0)	275	45.2 (20.5)
CKD stages†				
G1	35	8.3%	4	0.9%
G2	163	38.5%	57	13.5%
G3a	148	35.0%	79	18.7%
G3b	47	11.1%	70	16.6%
G4	28	6.6%	45	10.6%
G5	2	0.5%	20	4.7%
G5D	–	–	32	7.6%
stage unknown	–	–	116	27.4%
Clinical events				
Initiation of RRT	–	–	32	7.6%
Occurrence of CVD	–	–	23	5.4%
50% decline in eGFR‡	–	–	47	11.1%
Intervention groups				
Standard intervention	206	48.7%	206	48.7%
Advanced intervention	217	51.3%	217	51.3%

CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; RRT, renal replacement therapy; SD, standard deviation.

\*Participants with RRT were not included.

†The baseline CKD stage (2008) was estimated from serum creatinine values either at the enrollment or in October 2008.

‡50% decrease in eGFR was defined as a 50% or greater decrease in eGFR at 10th-year from October 2008 or April 2009.

## Study Design and Participants

We conducted a patient questionnaire survey on CKD survivors enrolled in the FROM-J study. The 10th-year data collection was performed by either PCPs or participants who filled out questionnaires, including EQ-5D-5L questionnaires, from October 2018 to March 31, 2019. The detailed designs and main results of the

FROM-J and FROM-J 10 studies have already been reported.<sup>6,7</sup> This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Clinical Research at the University of Tsukuba (No.80 and No.1311).

## Study Variables

The baseline and 10th-year demographic and clinical characteristics, laboratory data (serum albumin, hemoglobin, and serum creatinine), and the presence of comorbidities were based on the FROM-J and FROM-J 10 studies, respectively. The prevalence of comorbidities remained the same over the 10th-year follow-up. The eGFR was calculated from serum creatinine, age, and sex using the Japanese equation as follows:  $eGFR (mL/min/1.73 m^2) = 194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$  (for female). The baseline and 10th-year CKD stages were determined in the FROM-J and FROM-J 10 studies, respectively, according to the modified classification for the Japanese people based on the CKD clinical guidelines.<sup>5</sup> RRT was defined as chronic maintenance dialysis (hemodialysis and peritoneal dialysis) and renal transplantation, and the initiation of these therapies was assessed. CVD events were defined as cardiovascular death; and nonfatal myocardial infarction, nonfatal stroke, and heart failure (requiring hospitalization).<sup>6,7</sup> A 50% decline in eGFR was defined as a 50% or greater decrease in eGFR at 10th-year from October 2008 or April 2009.

## Statistical Analysis

Data are summarized using proportions, means, or median values, as appropriate. The differences in QOL scores between the sex and intervention groups were tested using the Mann-Whitney U test. The QOL score differences among age groups and CKD stages were tested using the Jonckheere-Terpstra test for trend and illustrated utilizing box and whisker plots. The Tobit model was applied to identify the clinical indices predicting and determining 10th-year QOL scores, considering that its distribution was censored at 1. All analyses were performed using the Stata SE software version 17 (StataCorp LLC, College Station, TX, USA). Statistical significance was set at  $P < .05$ .

## Results

We collected 423 responses from 1,473 CKD survivors, as shown in Figure 1. The overall response rate was 38.2%. A total of 116 participants could not be classified into the 10th-year CKD stages because of the lack of serum creatinine data. Table 1 shows the baseline and 10th-year characteristics of patients. The respondents included 299 males (70.7%) and 124 females (29.3%). Regarding the 10th-year data, the mean age was 71.6 year old, serum creatinine was 1.55 mg/dL, and eGFR was 45.2 mL/min/1.73 m<sup>2</sup>. Proportions of 10th-year CKD stages were 0.9% for G1, 13.5% for G2, 18.7% for G3a, 16.6% for G3b, 10.6% for G4, 4.7% for G5, 7.6% for G5D, and

**Table 2.** 10th-year QOL Scores by EQ-5D-5L (n = 423)

Characteristics	n	Mean	Median	95% confidence interval	Interquartile range	P value
All	423	0.893	1.000	0.880–0.906	0.826–1.000	
Sex						
Male	299	0.894	1.000	0.877–0.911	0.826–1.000	.370
Female	124	0.891	0.895	0.868–0.914	0.823–1.000	
Age at the 10th-year survey						
<60	31	0.944	1.000	0.906–0.982	0.895–1.000	<.001
60–69	132	0.924	1.000	0.906–0.943	0.871–1.000	
70–79	185	0.895	1.000	0.875–0.915	0.831–1.000	
≥80	75	0.811	0.826	0.770–0.852	0.707–1.000	
Comorbidities						
Hypertension						
presence	376	0.892	1.000	0.878–0.906	0.826–1.000	.448
absence	46	0.902	1.000	0.857–0.946	0.867–1.000	
Diabetes						
presence	219	0.900	0.895	0.858–0.899	0.825–1.000	.077
absence	204	0.908	1.000	0.891–0.926	0.831–1.000	
Hyperlipidemia						
presence	292	0.894	0.948	0.878–0.909	0.825–1.000	.832
absence	130	0.892	1.000	0.866–0.917	0.826–1.000	
Hyperuricemia						
presence	171	0.873	0.895	0.850–0.896	0.825–1.000	.021
absence	251	0.906	1.000	0.890–0.923	0.831–1.000	
CKD stages at the 10th-year survey						
G1+G2	61	0.892	1.000	0.856–0.928	0.825–1.000	.125
G3a	79	0.900	1.000	0.866–0.933	0.831–1.000	
G3b	70	0.948	1.000	0.928–0.969	0.895–1.000	
G4	45	0.851	0.867	0.802–0.900	0.780–1.000	
G5	20	0.909	0.892	0.867–0.951	0.828–1.000	
G5D	32	0.824	0.828	0.767–0.881	0.755–1.000	
Stage unknown	116	0.888	0.948	0.861–0.914	0.825–1.000	
Clinical events						
Initiation of RRT						
presence	32	0.824	0.828	0.767–0.881	0.755–1.000	.002
absence	391	0.899	1.000	0.885–0.912	0.831–1.000	
Occurrence of CVD						
presence	23	0.877	1.000	0.811–0.943	0.753–1.000	.647
absence	400	0.894	1.000	0.880–0.908	0.826–1.000	
50% decline in eGFR*						
presence	47	0.893	0.889	0.860–0.926	0.825–1.000	.118
absence	223	0.906	1.000	0.888–0.925	0.844–1.000	
Intervention groups						
Standard intervention	206	0.890	1.000	0.870–0.911	0.825–1.000	.988
Advanced intervention	217	0.895	1.000	0.878–0.913	0.826–1.000	

CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; QOL, quality of life; RRT, renal replacement therapy.

The differences in QOL scores between the sex, comorbidities, clinical events, and intervention groups were tested using the Mann–Whitney U test.

The QOL score differences among age groups and CKD stages were tested using the Jonckheere–Terpstra test for trend.

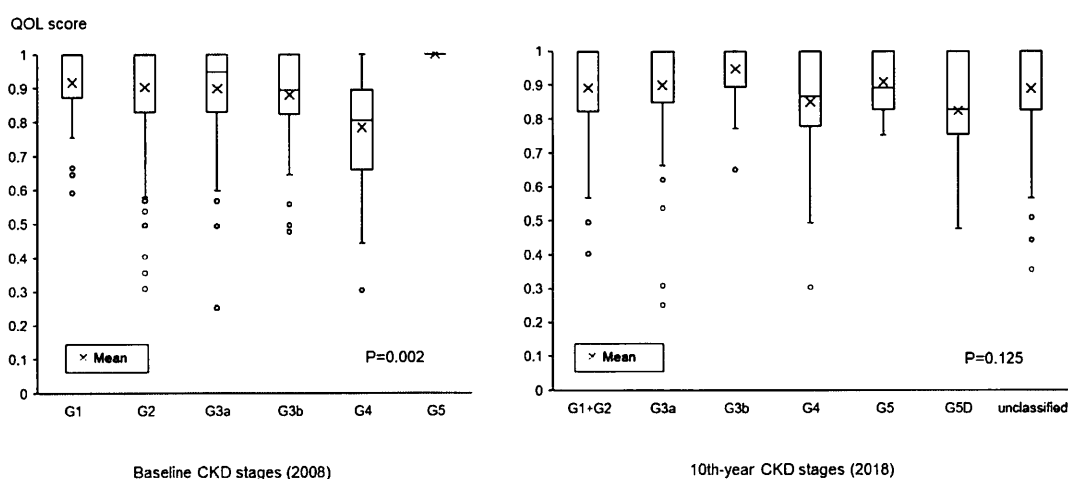
\*Participants receiving RRT were not included.

27.4% for stage unknown. Regarding clinical events, 32 participants initiated RRT, 29 were on hemodialysis, 2 were on peritoneal dialysis, 1 was on kidney transplantation, 23 developed CVD and 47 participants had 50% decline in eGFR during 10 years.

Table S1 shows the health status according to the EQ-5D-5L questionnaire. The proportion of patients

who responded “no problem” was 69.7% for mobility, 94.3% for self-care, 78.5% for usual activities, 66.2% for pain/discomfort, and 86.5% for anxiety/depression.

Table 2 presents the mean and median QOL scores. The mean QOL score of all was 0.893 (95% confidence interval (CI), 0.880–0.906), and the median QOL score was 1.000 (interquartile range (IQR), 0.826–1.000). The mean QOL



**Figure 2.** Box and whisker plots of QOL scores based on the baseline and 10th-year CKD stages. The decrease in QOL scores along with baseline CKD stages was significant according to the Jonckheere–Terpstra test for trend ( $P = .002$ ). The measured QOL scores were not significantly associated with the 10th-year CKD stages according to the Jonckheere–Terpstra test for trend ( $P = .125$ ).

score in participants with RRT was 0.824 (95% CI, 0.767–0.881), and the median was 0.828 (IQR, 0.755–1.000). Limited to hemodialysis patients, the mean QOL score was 0.822 (95% CI, 0.760–0.884), and the median was 0.831 (IQR, 0.757–1.000). The mean QOL score in participants with CVD was 0.877 (95% CI, 0.811–0.943), and the median was 1.000 (IQR, 0.723–1.000). The mean QOL score in participants with 50% decline in eGFR was 0.893 (95% CI, 0.860–0.926), and the median was 0.889 (IQR, 0.825–1.000). Figure 2 illustrates the measured QOL scores based on the baseline and 10th-year CKD stages in a box plot with a mark showing the mean. The decrease in QOL scores along with baseline CKD stages was significant according to the Jonckheere–Terpstra test for trend ( $P = .002$ ).

Table 3 shows the results of the Tobit regression analysis aimed at identifying the clinical predictors of 10th-year QOL scores among the baseline data. We performed three models of regression analysis, selecting hemoglobin (model 1), serum creatinine (model 2), and eGFR (model 3) considering the multicollinearity based on results of simple regression analysis. The analysis revealed that baseline age, systolic blood pressure, and history of hyperuricemia were significant predictors. The intervention groups (standard or advanced intervention) were not associated with the 10th-year QOL scores.

Table 4 shows the results of the Tobit regression analysis aimed at identifying the clinical determinants of 10th-year QOL scores among the 10th-year data. Sex, age, and 10-year diabetes and hyperuricemia were significant factors.

## Discussion

We measured QOL scores using the EQ-5D-5L in 10 years long-term survivors with CKD based on the FROM-J study. Our results demonstrated the QOL scores

of patients surviving CKD under the clinical practices of PCPs. The measured mean QOL scores decreased with age, with values of 0.944 (<60 years), 0.924 (60–69), 0.895 (70–79), and 0.811 ( $\geq 80$  years). Although all our participants had CKD, some had more serious comorbidities than others, and the age-related decline in QOL scores was similar to previous general population norm studies in Japan and overseas.<sup>17–22</sup> The measured mean QOL score in males was slightly higher than that in females, which is also commonly observed in many countries; however, the underlying reason is unclear.<sup>18–22</sup>

Advanced intervention by a practice facilitation program in the FROM-J study has been shown to have long-term effects on patients with CKD by preventing CVD events.<sup>7</sup> The mean QOL score of participants in the advanced intervention was 0.895 (95% CI, 0.878–0.913), which was slightly higher than 0.890 (95% CI, 0.870–0.911) in the standard intervention. However, there was no significant difference, and the practice groups were not predictors of the QOL scores. In our study, the QOL score of participants with CVD events was lower than that of those without CVD events. We considered that the practice facilitation program might indirectly have positive effects on QOL scores in patients with CKD through the reduction of CVD events.

Similar to previous studies, this study showed a significant trend of a decline in QOL scores along with the baseline CKD stage.<sup>8–11</sup> The renal function was found to be neither a clinical predictor nor determinant of 10th-year QOL scores. Some studies have reported that renal function estimated from serum creatinine may not adequately reflect the renal function of older people because serum creatinine levels are affected by muscle mass, sex, and age.<sup>23,24</sup> We considered that 10th-year renal function in our participants might be affected by their older ages and serious complications.

**Table 3.** Tobit Regression Analysis for Clinical Predictors of 10th-year QOL Scores Among Baseline Data (2008)

Variables	Simple regression analysis		Multiple regression analysis					
	Coefficients	P value	Model 1 (hemoglobin)		Model 2 (creatinine)		Model 3 (eGFR)	
			Coefficients	P value	Coefficients	P value	Coefficients	P value
Sex (male = 0, female = 1)	-0.0175	.549	-0.0262	.482	-0.0562	.084	-0.0551	.085
Age	-0.0096	<.001	-0.0085	<.001	-0.0093	<.001	-0.0097	<.001
Body mass index	-0.0034	.345	-0.0036	.342	-0.0035	.341	-0.0030	.412
Systolic blood pressure (mmHg)	-0.0022	.029	-0.0023	.051	-0.0024	.037	-0.0024	.036
Diastolic blood pressure (mmHg)	0.0018	.024						
Mean albumin (g/dL)	0.0796	.096						
Mean hemoglobin (g/dL)	0.0225	.009	0.0108	.300				
Comorbidities								
Hypertension	-0.0275	.531						
Diabetes	-0.0551	.040	-0.0393	.195	-0.0559	.069	-0.0470	.124
Hyperlipidemia	-0.0048	.870	-0.0005	.988	0.0054	.862	0.0066	.833
Hyperuricemia	-0.0703	.010	-0.0867	.005	-0.0840	.008	-0.0977	.003
Renal function								
Serum creatine (mg/dL)	-0.0511	.081			-0.0173	.623		
eGFR (mL/min/1.73 m <sup>2</sup> )	0.0012	.068					-0.0005	.555
CKD stage*								
G2	-0.0312	.545						
G3a	-0.0480	.355						
G3b	-0.0779	.199						
G4	-0.2286	.001						
G5	1.1614	.985						
Intervention groups (standard intervention = 0, advanced intervention = 1)	0.0056	.835	-0.0013	.964	0.0103	.717	0.0073	.797
Constant			1.8590	<.001	2.1138	<.001	2.1402	<.001
Number of observations			316		337		337	
Pseudo R-squared			0.1591		0.1747		0.1752	

CKD, chronic kidney disease; QOL, quality of life; eGFR, estimated glomerular filtration rate.

We performed three models of regression analysis by considering the multicollinearity.

\*Compared with CKD stage G1

The mean QOL scores in participants receiving RRT and hemodialysis were 0.824 and 0.822, respectively. These scores were lower than 0.899 for non-RRT patients with CKD. This result is consistent with previous studies showing that the QOL scores of hemodialysis patients were lower than those of nondialysis patients with CKD.<sup>8,25</sup> There is one report about QOL scores measured by EQ-5D-5L for 717 Japanese older patients aged  $72.9 \pm 6.5$  years who received maintenance hemodialysis for more than 5 years. Their QOL score was 0.738 (95% CI, 0.723–0.754),<sup>26</sup> which was lower than that of our study. They reported that hemodialysis duration was a determinant of lower QOL scores. We also consider that difference in QOL scores

might be due to the duration of hemodialysis, which was  $15.4 \pm 8.8$  years compared with our  $4.4 \pm 0.5$  years.

The significance of a history of hyperuricemia as both a predictor and determinant of QOL scores is a new finding of this study. A previous study reported that gout reduces physical HRQOL, regardless of serum uric acid levels or urate-lowering therapy.<sup>27</sup> We considered that there might be some participants with gout in the presence of hyperuricemia because hyperuricemia is the most important risk factor for the development of gout.<sup>28</sup> The baseline systolic blood pressure was negatively associated with the QOL scores. This result is consistent with a previous study<sup>9</sup> and a Chinese study on QOL scores measured using the

**Table 4.** Tobit Regression Analysis for Clinical Determinants of 10th-year QOL Scores Among 10th-year Data (2018)

Variables	Simple regression analysis		Multiple regression analysis					
	Coefficients	P value	Model 1 (hemoglobin)		Model 2 (creatinine)		Model 3 (eGFR)	
			Coefficients	P value	Coefficients	P value	Coefficients	P value
Sex (male = 0, female = 1)	-0.0175	.549	-0.0769	.058	-0.0888	.006	-0.0938	.040
Age	-0.0096	<.001	-0.0108	<.001	-0.0110	<.001	-0.0113	<.001
Body mass index	0.0015	.653	-0.0004	.926	-0.0036	.378	-0.0034	.399
Systolic blood pressure (mmHg)	-0.0023	.028	-0.0012	.346	-0.0010	.360	-0.0011	.295
Diastolic blood pressure (mmHg)	0.0003	.814						
Mean albumin (g/dL)	0.1529	.029						
Mean hemoglobin (g/dL)	0.0170	.054	-0.0009	.928				
Comorbidities								
10-year hypertension	-0.0275	.531						
10-year diabetes	-0.0551	.040	-0.0712	.053	-0.0992	.001	-0.0939	.002
10-year hyperlipidemia	-0.0048	.870	-0.0131	.729	-0.0018	.953	-0.0017	.956
10-year hyperuricemia	-0.0703	.010	-0.0885	.016	-0.0931	.002	-0.1043	.001
Renal function								
Serum creatine(mg/dL)	-0.0016	.901			0.0043	.713		
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.0002	.771					-0.0012	.131
CKD stage*								
G3a	0.0145	.749						
G3b	0.1194	.015						
G4	-0.0702	.170						
G5	0.0074	.913						
G5D	-0.1157	.039						
stage unknown	-0.0071	.866						
Clinical events								
Initiation of RRT	-1.2874	.007	-0.0582	.521	-0.1365	.203	-0.1495	.010
Occurrence of CVD	-0.0169	.775	-0.0608	.374	0.0398	.552	0.0285	.670
50% decline in eGFR†	-0.0443	.319						
Intervention groups (standard intervention = 0, advanced intervention = 1)	0.0056	.835	0.0203	.552	0.0131	.651	0.0125	.665
Constant			2.1441	<.001	2.1988	<.001	2.307	<.001
Number of observations			249		292		292	
Pseudo R-squared			0.1720		0.2897		0.2999	

CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; QOL, quality of life.

We performed three models of regression analysis by considering the multicollinearity.

\*Compared with CKD stage G1+G2.

†Participants receiving RRT were not included.

EQ-5D-3L in patients with hypertension.<sup>29</sup> The management of hypertension, diabetes and hyperuricemia, shown as significant predictors or determinants of 10th-year QOL scores, is recommended in the CKD guidelines.<sup>5</sup> We suggest the importance of CKD guideline-based practices from the perspective of HRQOL improvement.

This study had some limitations. First, the sample size was small owing to excessive missing 10-year values. Moreover, 27.4% of participants with CKD were not categorized based on their 10-year CKD stages, which might affect the association between QOL scores and renal function. These missing values also affected the selection of

the variables in the multiple regression analysis. Second, because of the self-reported questionnaire surveys by mailing, participants who responded to the questionnaires tended to be healthier than those who could not. Sicker patients, especially those with CKD stage G5, may have already initiated RRT or were lost to death before the survey. Third, we could not evaluate clinical events other than the initiation of RRT, CVD or 50% decline in eGFR and could not examine changes in the clinical indices or drug treatments. These unevaluated factors that might contribute to QOL scores in patients with CKD should be examined in further studies.

In conclusion, our results suggest that baseline age, a history of hyperuricemia and systolic blood pressure are important clinical predictors of QOL scores in long-term CKD survivors. CKD guideline-based practices,<sup>5</sup> prevention of ESKD/CVD and management of hypertension, diabetes and hyperuricemia, might contribute to future HRQOL in patients with CKD.

### Practical Application

This study shows that CKD complications, such as ESKD and CVD, negatively affect the QOL scores in 10 years long-term survivors with CKD. The baseline age, a history of hyperuricemia and systolic blood pressure are important clinical predictors of the 10th-year QOL scores in older survivors aged  $\geq 70$  years with CKD. Therefore, our study suggests that CKD guideline-based practice is important for patients with CKD from the perspective of improving HRQOL.

### Credit Authorship Contribution Statement

**Reiko Okubo:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization. **Masahide Kondo:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – review & editing, Supervision. **Toshiyuki Imasawa:** Project administration, Conceptualization, Writing – review & editing. **Chie Saito:** Resources, Project administration. **Hirayasu Kai:** Resources, Project administration. **Ryoya Tsunoda:** Project administration, Resources. **Junichi Hoshino:** Conceptualization, Writing – review & editing. **Tsuyoshi Watanabe:** Resources, Writing – review & editing. **Ichiei Narita:** Resources, Writing – review & editing. **Seiichi Matsuo:** Resources, Writing – review & editing. **Hirofumi Makino:** Resources, Writing – review & editing. **Akira Hishida:** Resources, Writing – review & editing. **Kunihiro Yamagata:** Conceptualization, Writing – review & editing, Supervision, Project administration.

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### Supplementary Data

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1053/j.jrn.2023.10.001>.

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## Case report



# Changes in histopathology and heteroplasmy rates over 8 years and effectiveness of taurine supplementation in a patient with mitochondrial nephropathy caused by *MT-TL1* mutation: A case report

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## ABSTRACT

The m.3243A > G mutation in the *mitochondrially encoded tRNA leucine 1 (MT-TL1)* gene is known to cause mitochondrial nephropathy. However, its long-term effects of the m.3243A > G mutation on renal histopathology or heteroplasmy rates remain unknown. Here we present the case of a female patient who underwent renal biopsy at 34 years of age to investigate the reason for a low estimated glomerular filtration rate (eGFR) of 47.9 mL/min/1.73 m<sup>2</sup>. Light microscopy revealed nephrosclerosis with granular swollen epithelial cells (GSECs) in the renal tubules. Genetic testing revealed the m.3243A > G mutation in the *MT-TL1* gene. Over a follow-up period of 8 years, the eGFR declined at a rate of 1.50 mL/min/1.73 m<sup>2</sup>/year. A second renal biopsy was performed at the age of 42 years; the patient's glomerular sclerosis rate had increased from 45.5% to 63.2%, and the frequency of GSECs in the collecting ducts had increased from 5.8% to 20.8%. Furthermore, the heteroplasmy rate in blood cells and urinary sediment cells increased from 9% to 20% and 20% to 53%, respectively. Taurine therapy was initiated just after the second kidney biopsy. To date, after approximately 3 years of taurine administration, the rate of eGFR decline has markedly decreased to 0.26 mL/min/1.73 m<sup>2</sup>/year. This experience suggests that an increased heteroplasmy rate may be associated with the progression of mitochondrial nephropathy caused by *MT-TL1* mutation. Furthermore, our case is the first to suggest the effectiveness of taurine for mitochondrial nephropathy caused by the m.3243A > G mutation in the *MT-TL1* gene.

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## 1. Introduction

Several reports have shown that focal segmental glomerulosclerosis (FSGS) is a characteristic pathological change in mitochondrial nephropathies caused by mitochondrial DNA (mtDNA) mutations, such as those in the *mitochondrial tRNA leucine 1 (MT-TL1)* gene [1–3]. In such cases, the mitochondria in glomerular podocytes lose their normal function, and the resulting podocyte damage has been thought to be involved in the etiopathogenesis of FSGS lesions [4]. However, our prior analysis of 63 renal biopsy cases with the m.3243A > G mutation in *MT-TL1* showed that 11.4% of these cases were diagnosed via histopathology as nephrosclerosis without FSGS lesions [3]. Although the lack of FSGS lesions in renal biopsy sections may be due to sampling errors, whether this error alone can explain the lack of FSGS lesions is debatable. The clinical manifestations and pathological changes of mitochondrial disease caused by mtDNA mutations are highly variable and are impacted by the heteroplasmy rate that differs among organs and cells [5].

The long-term effects of the m.3243A > G mutation on renal histopathology or heteroplasmy rates are unknown. In addition, although taurine is reportedly effective at treating mitochondrial diseases with *MT-TL1* mutations that were already proven to cause a taurine modification defect in tRNA<sup>Leu</sup> (UUR), as in m.3243A > G [6], its effect on mitochondrial nephropathy is unclear. In this context, we present a patient with an m.3243A > G mutation who underwent two renal biopsies performed 8 years apart, necessitated by a gradual decrease in estimated glomerular filtration rate (eGFR). We investigated the factors contributing to the progression of nephropathy in this patient by analyzing the changes in histopathology and heteroplasmy rates of mtDNA. Furthermore, this study is the first report verifying the effectiveness of taurine in such patients.

## 2. Case presentation

Herein, we describe the case of a 45-year-old Japanese woman with no significant medical history prior to the second pregnancy at 32 years of age. The patient was initially found to have proteinuria and a decreased renal function in week 27 of the second pregnancy and gave birth to the second child at 28 weeks of gestation due to an imminent risk of premature birth. One day after delivery, proteinuria was 1.2 g/day and eGFR was 48.3 mL/min/1.73 m<sup>2</sup>, as calculated using the serum creatinine value and the Japanese equation (eGFR [mL/min/1.73 m<sup>2</sup>] = 194 × Age<sup>-0.287</sup> × Cre<sup>-1.094</sup> [x 0.739 if female]) [7]. Although the proteinuria disappeared 3 months after delivery, the eGFR was persistently <50 mL/min/1.73 m<sup>2</sup>. At 34 years of age, 15 months after the second delivery, the patient was referred to our hospital by her primary doctor for decreased renal function. At the first visit to our hospital, the serum creatinine level was 1.12 mg/dL (eGFR: 46.0 mL/min/1.73 m<sup>2</sup>), without proteinuria. Three months after the first visit to us, the patient was admitted for a kidney biopsy to investigate the reason for persistent renal dysfunction.

Physical examination at admission showed the following: height 160.0 cm, body weight 45.5 kg, body mass index 17.8, blood pressure 116/73 mmHg, and a regular heart rate of 67 bpm. Neurological examination results were normal. The patient had no headaches, edema, or skin lesions. The serum creatinine level was 1.08 mg/dL (eGFR: 47.9 mL/min/1.73 m<sup>2</sup>) (Table 1) without proteinuria (0.03 g/day) or hematuria (0–1/HPF). Except for hyperuricemia (uric acid: 6.5 mg/dL), all blood test results were normal

**Table 1**  
Laboratory tests on admission.

	1st biopsy	2nd biopsy
Age	34	42
<b>Blood cell count</b>		
WBC (/μL)	3900	5400
RBC (/μL)	415 × 10 <sup>4</sup>	458 × 10 <sup>4</sup>
Platelet (/μL)	1,67,000	1,87,000
<b>Blood chemistry</b>		
Albumin (g/dL)	4.9	4.7
Sodium (mEq/L)	139	140
Potassium (mEq/L)	4.1	4.2
Chloride (mEq/L)	101	108
BUN (mg/dL)	15.5	25.1
Cre (mg/dL)	<b>1.08</b>	<b>1.48</b>
eGFR (ml/min/1.73m2)	<b>47.9</b>	<b>31.9</b>
Uric acid (mg/dL)	<b>6.5</b>	<b>7.8</b>
Fasted blood glucose (mg/dL)	88	98
HbA1c (%)	5.7	5.8
Serum lactate (mg/dL)	9.1	12.5
Serum pyruvic acid (mg/dL)	0.46	0.86
<b>Urinarysis</b>		
Glucose (test tape)	(–)	(–)
Protein (g/day)	0.03	0.02
RBC (/HPF)	0–1	0–1
NAG (IU/L)	5.2	4.6
b2-MG (mg/L)	37	75

WBC, white blood cells; RBC, red blood cells; BUN, blood urea nitrogen; Cre, creatinine; eGFR, estimated glomerular filtration rate; UA, uric acid; HbA1c, hemoglobin A1c by NGSP; NAG, N-acetylglutamate; b2-MG, microglobulin; HPF, high-power field. Bold texts indicate abnormal values.

(Table 1), and the serum lactate level was within the normal range (9.1 mg/dL). Blood tests did not suggest the existence of diabetes mellitus, the patient was not a smoker, and was not taking any prescribed medications. Audiometric tests revealed pure tone averages of 25.0 dB in the right and 20.0 dB in the left ears ( $[500 \text{ Hz} + 1000 \text{ Hz} \times 2 + 2000 \text{ Hz}]/4$ ), indicating a normal hearing ability. An echocardiogram revealed a normal heart function. Brain magnetic resonance imaging (MRI) was not performed as neurological symptoms and stroke-like episodes were absent.

The patient reported that her mother had died at 42 years of age due to diabetic nephropathy-induced uremia. Her brother was diagnosed with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) at 7 months of age and died at 37 years of age. The present patient was born after 36 weeks of gestation, with a birth weight of 2720 g. Both of her children are healthy with no symptoms; the first was born prematurely at 29 weeks of gestation, with a birth weight of 1566 g, and the second at 28 weeks of gestation with a birth weight of 1502 g. Neither showed proteinuria or renal dysfunction during health checkups.

The renal pathological findings of the present case are summarized in Table 2. Fig. 1A–C shows the light microscopy images of the first kidney biopsy. Renal pathology showed nephrosclerosis. Five glomeruli among observed eleven glomeruli had global sclerosis (45.5%), and two glomeruli were collapsed (18.2%). All other glomeruli showed almost normal appearance without any sclerotic lesions, tuft adhesions, or endocapillary proliferation. Age-inappropriate disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) were also observed in the intralobular arteries (Fig. 1A) and afferent arterioles (Fig. 1B); arteriolar hyalinosis and intimal thickening were not observed. Furthermore, granular swollen epithelial cells (GSECs) were also apparent in the collecting ducts on Masson Trichrome staining (Fig. 1C). Electron microscopy examination showed an increase in the number of abnormal mitochondria in podocytes and collecting duct cells (Fig. 1D–F).

The presence of GSECs, podocytes with abnormal mitochondria, and a relevant family history prompted us to consider the possibility of mitochondrial nephropathy due to mtDNA mutation. We performed genetic analysis using mtDNA extracted from peripheral blood mononuclear cells and urine sediment cells [8]. The m.3243A > G mtDNA mutation was detected in both blood and urine sediment cells. Heteroplasmy rates of blood and urine sediment cells were 9% and 20%, respectively (Table 2).

After the first kidney biopsy and genetic analysis, the patient was followed up without prescribing any medications as there were no apparent clinical symptoms, and proteinuria was never detected ( $<0.15 \text{ g/g Cre}$ ). Urinary N-Acetylglucosamine and  $\beta$ 2-microglobulin levels were within the normal range. However, after the first kidney biopsy, the patient became aware that her hearing ability was decreasing. In addition, the eGFR slowly but gradually decreased. Therefore, the patient was later admitted for a second kidney biopsy to investigate the cause of the gradually decreasing renal function.

On the 2nd admission, the patient's body weight was 45.5 kg, and blood pressure was 110/61 mmHg. Physical examination did not reveal any abnormalities; the serum creatinine level had increased to 1.48 mg/dL (eGFR:  $31.9 \text{ mL/min/1.73 m}^2$ ) without proteinuria (0.02 g/day). Although the uric acid level was high (6.5 mg/dL), all blood test results were normal and had not changed from the results at the first admission (Table 1). An audiometric test showed pure tone averages of 45.0 dB in the right and 40.0 dB in the left ears ( $[500 \text{ Hz} + 1000 \text{ Hz} \times 2 + 2000 \text{ Hz}]/4$ ), indicating that the patient had experienced hearing loss. The echocardiogram was normal.

The renal pathological findings are summarized in Table 2. Light microscopy of the second biopsy specimen (containing 19 glomeruli) yielded 12 glomeruli with global sclerosis (63.2%) and one collapsing glomerulus; all other glomeruli appeared normal. There were more GSECs in the collecting ducts in this biopsy compared to the first biopsy (53% and 20%, respectively) (Fig. 2A, Table 2). AiDIVs were observed in the intralobular arteries and afferent arterioles, similar to those at the first biopsy (Fig. 2B and C). An electron microscopical analysis revealed accumulation of abnormal mitochondria in podocytes (Fig. 2D). In addition, vascular smooth muscle cells of arterioles were filled with abnormal mitochondria (Fig. 2E and F).

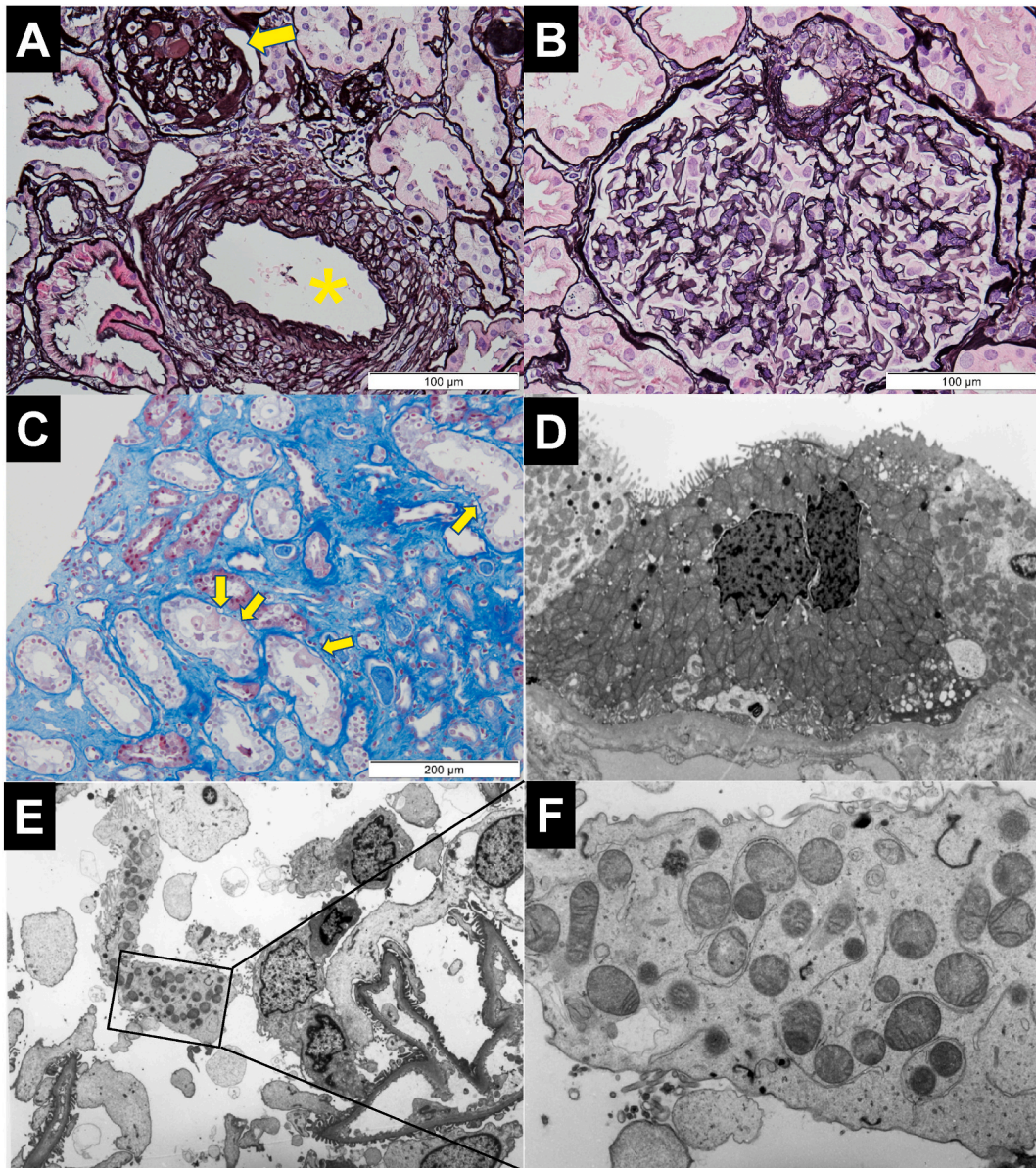
Next, we analyzed heteroplasmy rates of mtDNA with m.3243A > G. The heteroplasmy rates in the peripheral blood mononuclear cells and urinary sediment cells were 20% and 53%, respectively, showing an increase over the 8 years (Table 2). The heteroplasmy rate of kidney tissue was 75%.

Due to the gradually decreasing eGFR and the confirmed renal tissue damage with progressively increasing global glomerular sclerosis, we decided to administer taurine (12 g/day in three divided doses after each meal). The least squares regression line of the eGFR trend using all the values from the first visit date showed that the patient's eGFR was declining at a rate of  $1.50 \text{ mL/min/1.73 m}^2/\text{year}$  prior to taurine treatment (Fig. 3). After a period of 1022 days (about 2.8 years) since taurine was first prescribed, the rate of eGFR decline markedly decreased to  $0.26 \text{ mL/min/1.73 m}^2/\text{year}$ , as determined by linear regression using least square method (Fig. 3). The

**Table 2**  
Comparison of histopathological findings and heteroplasmy rates of mtDNA.

	1st biopsy	2nd biopsy
<b>Renal Pathology</b>		
Global sclerosis	5/11 (45.5%)	12/19 (63.2%)
Segmental sclerosis	0/11	0/19
Podocytes with abnormal mitochondria accumulation	+	+
GSECs in collecting ducts	16/274 (5.8%)	38/183 (20.8%)
<b>Rate of m.3243 A &gt; G heteroplasmy</b>		
Blood cells	9%	20%
Cells in urinary sediments	20%	53%
Kidney tissue	n.d.	75%

mtDNA, mitochondrial DNA; GSEC, granular swollen epithelial cell.  
n.d., not done.

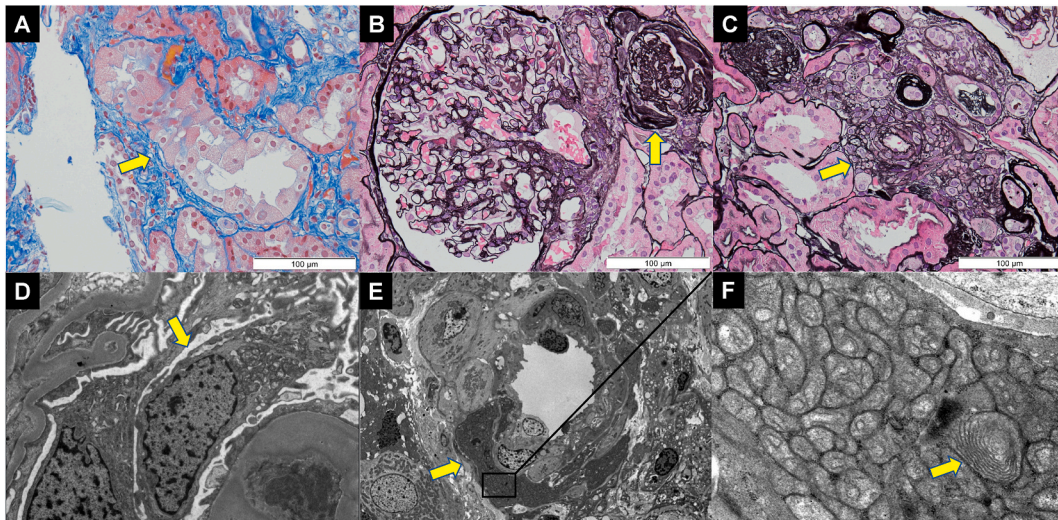


**Fig. 1.** Microscopic histopathological examination of the first kidney biopsy specimen. (A) One glomerulus is globally sclerosed (arrow). Age-inappropriate disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) can be observed in an interlobular artery (asterisk) on periodic acid-methenamine-silver and hematoxylin eosin (PAM-HE) staining. (B) Residual glomeruli show a normal appearance. The afferent arteriole of this glomerulus showed AiDIVs on PAM-HE staining. (C) Granular swollen epithelial cells (GSECs) can be observed among the renal tubules and in the collecting duct (arrows) on Masson Trichrome staining. (D) Mitochondria with abnormal cristae structures accumulate in a collecting duct cell (electron microscopy; magnification 1500 × ). (E) Mitochondria are increased in a podocyte (electron microscopy; magnification 1500 × ). (F) High magnification of the area enclosed in the square shown in (E). The mitochondrial cristae structures exhibit ripping and loss, and the mitochondria are enlarged (electron microscopy; magnification 8000 × ).

only drug prescribed to the patient during this 2.8-year period was taurine. The uric acid level normalized after taurine administration. However, there has been no improvement in hearing impairment.

### 3. Discussion

FSGS cases with the m.3243A > G mutation often show an accumulation of abnormal mitochondria in podocytes upon electron microscopy, which suggests that mitochondrial dysfunction-induced podocytopathy may underline this disease [1,2,4]. However, our prior analysis showed that 11.4% of mitochondrial nephropathy cases with the m.3243A > G mutation were diagnosed as

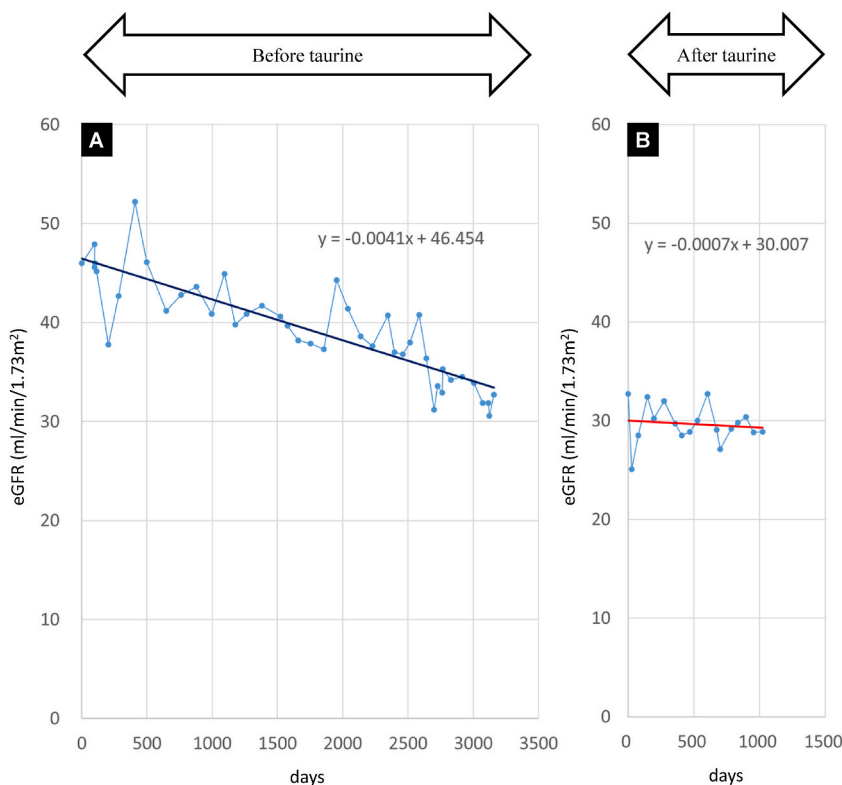


**Fig. 2.** Microscopic histopathological examination after the second kidney biopsy. (A) Most of the cells in the collecting duct (arrow) were classified as GSECs on Masson Trichrome staining. (B) One glomerulus is globally sclerosed (arrow). Another residual glomerulus appears normal while the vascular smooth muscle cells of the afferent arteriole of this glomerulus shows AiDIVs on PAM-HE staining. (C) AiDIVs can be observed in an arteriole (arrow) on PAM-HE staining. (D) Enlarged mitochondria can be observed in a podocyte (arrow; electron microscopy; magnification  $10,000\times$ ). (E) A vascular smooth muscle cell (arrow) of an arteriole is filled with enlarged mitochondria (electron microscopy; magnification  $2500\times$ ). (F) High magnification of the area enclosed in square (E). Cristae structures in accumulated mitochondria are disarranged. The crista structure of one mitochondrion exhibits a spiral shape (arrow; electron microscopy; magnification  $30,000\times$ ).

nephrosclerosis without FSGS lesions [3]. The pathogenesis of why nephrosclerotic lesions without FSGS occur in mitochondrial nephropathy remains unclear. Sampling error may mean that FSGS lesions were not included in the limited renal biopsy specimens. However, we believe that sampling error alone cannot explain our data, as renal biopsy was performed twice in our case. As observed upon electron microscopy, abnormal mitochondria accumulated in the vascular smooth muscle cells (Fig. 2E and F). This finding suggests that mitochondrial dysfunction may occur and cause cellular dysfunction in vascular smooth muscle cells. In fact, despite the absence of hypertension and in spite of the patient's age, the mesenteric vascular smooth muscle cells of the interlobular arteries were unequal in size and arrangement (Figs. 1A, 3B and 3C). Thus, these age-inappropriate disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) were assumed to cause abnormal glomerular hemodynamics, resulting in pathological changes similar to hypertension-induced nephrosclerosis.

In our case, two renal biopsies were performed with an intervening period of over 8 years. One weakness of this report was that the specimens from the first renal biopsy were not available for re-examination, and the mtDNA heteroplasmy rate could not be determined in the kidney. However, the rate of heteroplasmy increased markedly in the blood and urine sediment cells during this 8-year period, and the GSECs also increased between the first and second biopsy. This increase suggests that the rise in the heteroplasmy rate and the reduced mitochondrial function in the renal cells contributed to the progression of nephropathy. A previous report showed a relationship between the elevated m.3243A > G heteroplasmy rate in leukocytes and age-related changes [9], which may explain the age-inappropriate pathological changes in the renal lesions observed in our patient based on the increased heteroplasmy rate. Alternatively, other reports have shown that the heteroplasmy rates of the m.3243A > G pathogenic variant of *MT-TL1* in leukocytes decrease over time [10,11], which differs from the results in our case, where the heteroplasmy rates in leukocytes and urine sediment cells increased over time. Although the reason for this cannot be clearly demonstrated, it has been speculated that the decline in quality control of mtDNA decreases with age and the heteroplasmy rate of mtDNA increases, and this impaired quality control of mtDNA may have been the cause in this case as well [12–14]. In the future, it will be necessary to investigate the change in heteroplasmy rate of mtDNA over time in mitochondrial nephropathy cases with this pathological variant in order to resolve this difference.

Prior to treatment, the eGFR in the present patient declined at a rate of  $1.50\text{ mL/min}/1.73\text{ m}^2$  per year. As the eGFR was  $31.9\text{ mL/min}/1.73\text{ m}^2$  at the time of the second renal biopsy, it was estimated that the patient would reach end-stage kidney disease (ESKD) (under  $10\text{ mL/min}/1.73\text{ m}^2$ ) at approximately 60 years of age. The m.3243A > G mutation reduces the taurine modification of the leucine tRNA anticodon, resulting in the UUG codon not being appropriately recognized, leading to abnormal synthesis of mitochondrial proteins [15,16]. This reduction in tRNA function in cases with the pathogenic variants in *MT-TL1* that were already proven to cause a taurine modification defect in tRNA<sup>Leu</sup> (UUR), as in m.3243A > G, can be improved by taurine supplementation, which restores mitochondrial function [6]. So far, the patient has been taking taurine for approximately three years, during which time the rate of eGFR decline improved markedly to  $0.26\text{ mL/min}$  per year (Fig. 3). Assuming that this rate of eGFR decline could be maintained, we calculated that the patient would reach ESKD (under  $10\text{ mL/min}/1.73\text{ m}^2$ ) at approximately 115 years of age, because the current eGFR is about  $28\text{ mL/min}/1.73\text{ m}^2$ . Although this is a single patient case report, it is the first study to show the long-term effects of taurine treatment on mitochondrial nephropathy caused by impaired taurine modification of mitochondrial tRNAs. It should be



**Fig. 3.** Estimated glomerular filtration rate (eGFR) before (A) and after (B) taurine treatment. The slope is determined by a linear regression line using least square method. The horizontal axis shows the time in days, and the coefficient was multiplied by 365 to obtain the slope per year. The slope of eGFR prior to taurine administration was calculated using all eGFR measurements taken from the date of the first visit to the date of initiation of taurine administration, including the day of administration. The first renal biopsy was performed on day 99 and the second one was on day 3124. The slope of the post-taurine eGFR uses all measurements from the date of taurine administration to the present.

noted that this is an evaluation based on only one case treated with taurine, and the effect cannot be proven until additional cases are analyzed. Ultimately, randomized clinical trials with taurine supplementation are needed to address this research.

#### 4. Conclusion

In conclusion, we believe that this case report provides three important insights into mitochondrial nephropathy. First, our analysis suggests that the etiopathogenesis of nephrosclerosis in mitochondrial nephropathy may be caused by abnormal mitochondrial function in vascular smooth muscle cells. Second, the heteroplasmy rates of mutated mtDNA increase over time, resulting in progression of nephropathy; these changes may be non-invasively captured by the analysis of urine sediment cells. Third, taurine administration may be effective at ameliorating *MT-TL1* mutation-induced mitochondrial nephropathy, although further studies are needed to independently validate our findings.

#### Declarations

##### Author contribution statement

Toshiyuki Imasawa: Conceived and designed the experiments; Wrote the paper.

Hiroshi Kitamura, Yukiko Yatsuka, Yasushi Okazaki: Performed the experiments.

Takehiko Kawaguchi, Kei Murayama: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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### Data availability statement

Data will be made available on request.

### Declaration of interest's statement

The authors declare no conflict of interest.

### Ethics approval and consent to participate

This study was approved by the Ethics Committee of Chiba Children's Hospital [2014-11-05 (on 18th November 2014), 2017-024 (on 23rd August 2017), and 2020-015 (on 9th June 2020)]. The patient provided informed consent for publication of this case report.

### Consent for publication

Written informed consent was obtained from the patient for the publication of this case report and all accompanying images.

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## Case Report

Focal segmental glomerulosclerosis with a mutation in the *mitochondrially encoded NADH dehydrogenase 5* gene: A case report

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## ABSTRACT

NADH dehydrogenase 5 (ND5) is one of 44 subunits composed of Complex I in mitochondrial respiratory chain. Therefore, a *mitochondrially encoded ND5 (MT-ND5)* gene mutation causes mitochondrial oxidative phosphorylation (OXPHOS) disorder, resulting in the development of mitochondrial diseases. Focal segmental glomerulosclerosis (FSGS) which had podocytes filled with abnormal mitochondria is induced by mitochondrial diseases. An *MT-ND5* mutation also causes FSGS. We herein report a Japanese woman who was found to have proteinuria and renal dysfunction in an annual health check-up at 29 years old. Because her proteinuria and renal dysfunction were persistent, she had a kidney biopsy at 33 years of age. The renal histology showed FSGS with podocytes filled with abnormal mitochondria. The podocytes also had foot process effacement and cytoplasmic vacuolization. In addition, the renal pathological findings showed granular swollen epithelial cells (GSECs) in tubular cells, age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs), and red-coloured podocytes (ReCPos) by acidic dye. A genetic analysis using peripheral mononuclear blood cells and urine sediment cells detected the m.13513 G > A variant in the *MT-ND5* gene. Therefore, this patient was diagnosed with FSGS due to an *MT-ND5* gene mutation. Although this is not the first case report to show that an *MT-ND5* gene mutation causes FSGS, this is the first to demonstrate podocyte injuries accompanied with accumulation of abnormal mitochondria in the cytoplasm.

## 1. Introduction

Mitochondrial diseases/disorders are rare and occur every 1 in 5000 births [1]. Mitochondria play a key role in the biosynthesis of adenosine triphosphate (ATP), the main energy source of cells, through oxidative phosphorylation (OXPHOS) using the mitochondrial respiratory chain

(MRC) complex. Given that the genes related to the MRC complex are encoded in mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), mitochondrial diseases can occur in cases of mtDNA or nDNA mutations [2,3]. As ATP is mandatory for all cells that require energy, the clinical phenotypes of mitochondrial diseases are versatile and are expressed as encephalopathy [4], myopathy [5], cardiomyopathy [6], hepatopathy

**Abbreviations:** ND5, NADH dehydrogenase 5; *MT-ND5*, mitochondrially encoded ND5; OXPHOS, oxidative phosphorylation; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; FSGS, focal segmental glomerulosclerosis; GSECs, granular swollen epithelial cells; AiDIVs, age-inappropriately disarranged and irregularly sized vascular smooth muscle cells; ReCPos, red-coloured podocytes; ATP, adenosine triphosphate; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; Cr, creatinine; sCr, serum creatinine; eGFR, estimated glomerular filtration rate; COX IV, cytochrome c oxidase subunit 4.

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[7], deafness [8], and diabetes mellitus [9]. Mitochondrial nephropathy, which occurs because of a mitochondrial disorder in nephrons, has also been reported [10–12].

Focal segmental glomerulosclerosis (FSGS) is a histological term, rather than a specific disease category and diagnosed by the presence of sclerosis in parts of at least one glomerulus in the kidney biopsy specimen [13]. It can be classified into primary, secondary, genetic, and unknown forms of FSGS [14]. Among causative genes of mitochondrial nephropathy, m.3243A > G pathogenic variant in *MT-TL1* gene on mtDNA, which encodes mitochondrial transfer RNA leucine 1, is majority [12]. The m.3243A > G mutation leads to genetic cause of FSGS with accumulation of abnormal mitochondria in podocytes [11,15]. Because podocyte injury plays a key role in the pathogenesis of FSGS [16–18], impaired mitochondrial function in podocytes should induce FSGS [19–22].

The pathogenic variants in *mitochondrially encoded NADH dehydrogenase 5 (MT-ND5)* gene cause mitochondrial diseases such as mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [23–25]. ND5 is one of the 44 subunits of mitochondria respiratory complex I. The pathogenic variants in *MT-ND5* gene also cause FSGS [26–29]. However, in all cases with FSGS lesions presented in the past reports, abnormal mitochondria or their accumulation were not identified in podocytes. In another recent report of three cases with nephropathy due to *MT-ND5* gene mutation, one of the cases showed FSGS lesion. The authors of this report concluded that FSGS lesion of their case was secondary [28]. The pathogenesis of the secondary FSGS is considered to involve intraglomerular hypertension caused by hypertension or obesity, by adaptation to nephron loss due to progression of glomerulosclerosis or by a low nephron number due to low birth weight [30,31]. Therefore, it has not been evident whether genetic FSGS occurs with a pathogenic variant in *MT-ND5* gene. Here, we report the case with an *MT-ND5* mutation with apparent FSGS lesions. In this case, foot process effacement and cytoplasmic vacuolization in podocytes with accumulation of abnormal mitochondria were observed.

## 2. Case presentation

This case occurred in a Japanese woman born at 39 weeks of

gestation with a birth weight of 3210 g. She had no remarkable medical history. Annual health check-ups showed no proteinuria on urinalysis until age 29. At 29 years of age, the annual health check-up revealed proteinuria (1+) for the first time using a urine dipstick and a high serum creatinine (sCr) level of 1.01 mg/dL. Her estimated glomerular filtration rate (eGFR) using the Japanese Eq. [32] was calculated to be 54.0 mL/min/1.73 m<sup>2</sup>. At the same check-up, she was also found to have a slight hearing disturbance. Additionally, she had a headache once a week from 30 years of age and was diagnosed by a neurologist with migraine, uncontrolled with triptan. Her health check-up at 32 years of age again showed proteinuria (1+) and increased sCr (1.06 mg/dL). Hematuria has never been noted before. At 33 years of age, she was referred to the Yamanashi Prefectural Central Hospital by her primary doctor to examine the reason for her proteinuria and decreased eGFR. At the first visit, proteinuria (1.09 g/gCr), elevated sCr (1.28 mg/dL), and elevated uric acid (7.5 mg/dL) were detected. Febuxostat and sodium bicarbonate were prescribed for the treatment of hyperuricemia. Because of the continued presentation of proteinuria and decreased renal function, she was admitted for a kidney biopsy, at 6 months following the initial visit.

Upon admission, her blood pressure was 114/70 mmHg. Physical examination showed a height of 152 cm, body weight of 43.8 kg, and body mass index of 19.0. No crackles or murmurs were detected on chest auscultation. No abnormal neurological findings were observed. Neither skin lesions nor pitting oedema were detected. The laboratory data on admission are summarised in Table 1, which shows decreased eGFR and proteinuria. Hematuria was not observed. In the data we measured, there were no data to suggest the presence of tubular dysfunction such as Fanconi's syndrome or distal tubular acidosis. The long × short axis of the kidneys measured 101 × 38 mm on the left and 104 × 50 mm on the right, indicating no renal atrophy. The electrocardiogram test result was normal, and echocardiography revealed normal cardiac function. The standard pure-tone hearing test showed a right-ear value of 38.8 dB and a left-ear value of 40.0 dB. She was therefore diagnosed with sensorineural hearing loss by an otolaryngologist. No visual field defects were noted. Furthermore, because of hearing loss and headache symptoms, a brain magnetic resonance imaging (MRI) was also performed to examine for brain lesions, but no abnormal findings were found.

Her parents had died because of cancer (mother, breast cancer,

**Table 1**  
Laboratory data on admission.

Blood cell count			Blood chemistry			Immunology			Urinalysis		
WBC	5400	/μL	TP	6.8	g/dL	CH50	54	U/mL	Gravity	1.01	
RBC	373	×10 <sup>4</sup> /μL	Alb	3.9	g/dL	C3	76.1	mg/dL	pH	7.0	
Hb	11.5	g/dL	AST	21	IU/L	C4	19.3	mg/dL	RBC	1–4	/HPF
Hct	35.1	%	ALT	11	IU/L	IgG	1340.3	mg/dL	WBC	<1	/HPF
MCV	94.1	fl	LDH	186	IU/L	IgA	225.7	mg/dL	<u>Protein</u>	<u>2.04</u>	<u>g/gCr</u>
MCHC	32.8	%	ALP	149	IU/L	IgM	180.1	mg/dL	Glucose	(–)	
Plt	25.7	×10 <sup>4</sup> /μL	Tbil	0.56	mg/dL	ANA	(–)		NAG	8.6	U/L
			<u>BUN</u>	<u>21.8</u>	mg/dL	M protein	(–)				
			<u>Cr</u>	<u>1.26</u>	mg/dL	HBs Ag	(–)				
			<u>eGFR</u>	<u>40.8</u>	mL/min/1.73 m <sup>2</sup>	HCV Ab	(–)				
Coagulation			K	5.0	mEq/L						
APTT	34.0	sec	P	3.1	mg/dL						
PT	99.0	%	UA	5.4	mg/dL						
INR	0.97	INR	Tcho	255	mg/dL						
Fib	373.0	mg/dL	CRP	0.01	mg/dL						
			FBS	85	mg/dL						
			HbA1c	5.3	%						

Aberrant values are underlined.

WBC, white blood cells; RBC, red blood cells; Hb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; Plt, platelet; APTT, activated partial thromboplastin time; PT, prothrombin time; INR, international normalized ratio; Fib, fibrinogen; TP, total protein; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; Tbil, total bilirubin; BUN, blood urea nitrogen; Cr, creatinine; eGFR, estimated glomerular filtration rate (by the Japanese equation); K, potassium; P, phosphate; UA, uric acid; Tcho, total cholesterol; CRP, C-reactive protein; FBS, fasting blood sugar; HbA1c, haemoglobin A1c (NGSP); CH50, 50% haemolytic complement activity; C3, complement 3; IgG, immunoglobulin G; ANA, antinuclear antibody; M protein, monoclonal protein; HBs Ag, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; NAG, N-acetyl glucosaminidase; HPF, high power field.

father, colon cancer) without kidney disease or any hearing difficulty. Neither of her two older brothers had any abnormalities in kidney function, urine, or hearing. She had a 7-year-old daughter and a 5-year-old son. Her daughter's birth weight was 2840 g at 41 weeks and 2 days of gestation, and her son's birth weight was 2632 g at 39 weeks of gestation. Her daughter was born using suction delivery, whereas her son was born through normal delivery. None of her children had any urinary abnormalities or hearing difficulties, as examined during medical check-ups. However, the children sometimes complained of headaches that had not yet been diagnosed by any doctor.

A percutaneous kidney biopsy was performed under ultrasound guidance. Light microscopy photographs of renal biopsy specimens are shown in Fig. 1. Nine glomeruli were observed in the specimens. One glomerulus was globally sclerosed. Furthermore, segmental sclerosis was observed at the perihilar area in two glomeruli, compatible with the perihilar variant in the Columbia classification of FSGS (Fig. 1a) [13,30]. Interestingly, red-coloured podocytes (ReCPos), whose cytoplasm was dyed red, were observed using AZAN trichrome staining (Fig. 1a and b). Neither mesangial proliferation nor hypercellularity were detected. Interstitial fibrosis and tubular atrophy were observed in almost 30% of the interstitium. There were no abnormal findings such as thickening or lamellation of the tubular basement membrane. Characteristically, granular swollen epithelial cells (GSECs), reported to be specific to mitochondrial diseases [33,34], were markedly observed in the distal tubular cells using AZAN trichrome staining (Fig. 1c). Such GSECs were emphasised by staining with anti-cytochrome c oxidase subunit 4 (COX IV) antibody [mouse anti-COX IV antibody (20E8C12)] (ab14744; Abcam PLC., Cambridge, UK; Fig. 1d). COX IV staining is recognised as a loading control for mitochondria [34,35]. Furthermore, the sizes of the vascular smooth muscle cells in arterioles were irregular, and their arrangement was disorganized in a manner similar to that seen in older patients, despite the younger age (33 years) of the patient, without any medical history that may have caused arteriosclerosis (Fig. 1e). Such age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) were also noticeable in the interlobular arteries (Fig. 1f). Immunofluorescence analysis revealed IgM, C3 and C1q deposits in the glomeruli (Fig. 1g-i). IgG and IgA were negative in observed glomeruli. Electron microscopy analyses (Fig. 2a–e) also showed podocytes with increased mitochondria that had lost their normal cristae structure. Foot processes of such podocytes with accumulation of abnormal mitochondria were effaced (Fig. 2b and d). A vacuolization was also observed in the cytoplasm in the podocyte with mitochondrial accumulation (Fig. 2b). We also detected cells with increased abnormal mitochondria in the parietal epithelial cells of the Bowman's capsule (Fig. 2f). Although the glomerular basement membrane was partially thin at the part along with the podocytes with abnormal mitochondria, the membranes at other parts showed normal thickness (> 250 nm) without lamination or reticulation. Electron dense deposits were not observed in mesangium or glomerular basement membranes. These renal pathology findings strongly suggest genetic FSGS due to mitochondrial disease.

The serum lactate and pyruvate levels were slightly elevated at 21.0 mg/dL (normal: 3.0–17.0 mg/dL) and 1.34 mg/dL (normal: 0.30–0.94 mg/dL), respectively. The pathological finding of accumulation of abnormal mitochondria in glomerular epithelial cells and tubules, the presence of sensorineural hearing difficulty, and elevated blood lactate led us to perform a comprehensive genetic analysis of mitochondrial diseases.

After obtaining informed consent from the patient according to protocol and permission from our ethics committees, genomic DNA extracted from the patient's peripheral mononuclear blood cells and urine sediment cells was analysed by targeted resequencing coupled with next-generation sequencing, followed by Sanger sequencing. The DNA was subjected to fragmentation and library preparation using the Lotus DNA Library Prep Kit (#10001074; Integrated Device Technology, Inc., San Jose, CA, USA) according to the manufacturer's instructions.

Target enrichment was performed with xGen Human mtDNA Research Panel (#1075705; Integrated Device Technology) targeting the whole mtDNA and with custom xGen® Predesigned Gene Capture Pools (Integrated Device Technology)/xGen Lockdown Probe pool (Integrated Device Technology) targeting the exons of 367 nuclear-encoded genes that cause mitochondrial diseases. The library was then sequenced on the Illumina MiSeq platform using the MiSeq Reagent Kit v2 (MS-102-2002; Illumina, Inc., San Diego, CA, USA). A pathogenic mtDNA variant of m.13513G > A was detected in both the patient's blood cells and urine sediment cells (Fig. 3). The Minor Variant Finder (MVF) software program (Thermo Fisher Scientific, Waltham, MA, USA) revealed that the heteroplasmy rates of the blood cells and urine sediment cells were 10.3% (forward: 11.6%/reverse: 9.0%) and 62.2% (forward: 63.7%/reverse: 60.7%), respectively. Therefore, we diagnosed her with renal dysfunction and proteinuria due to FSGS caused by an *MT-ND5* mutation.

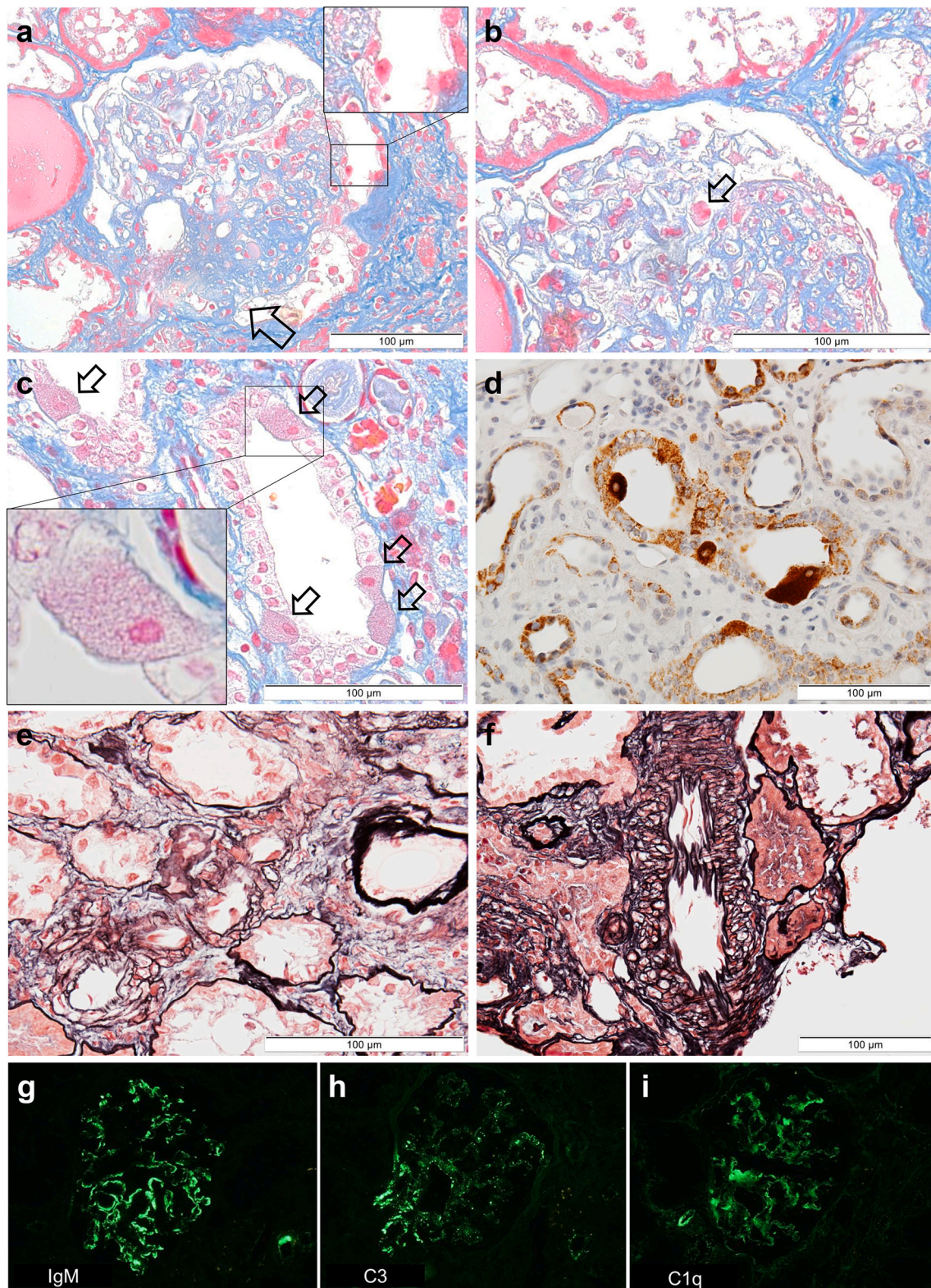
### 3. Discussion and conclusions

*MT-ND5* gene encodes NADH dehydrogenase 5 (ND5), which is a subunit of mitochondria respiratory complex I. Therefore, *MT-ND5* mutations cause mitochondrial diseases such as MELAS [23,36,37], Leigh syndrome [36–39], and Leber hereditary optic neuropathy [37]. Our case with the m.13513G > A variant in the *MT-ND5* gene showed FSGS lesions with injured podocytes that had accumulation of abnormal mitochondria in the cytoplasm. The m.13513G > A has already been confirmed as a pathogenic variant of the mitochondrial disease using MITOMAP, a human mitochondrial genome database (<https://www.mitomap.org/MITOMAP>).

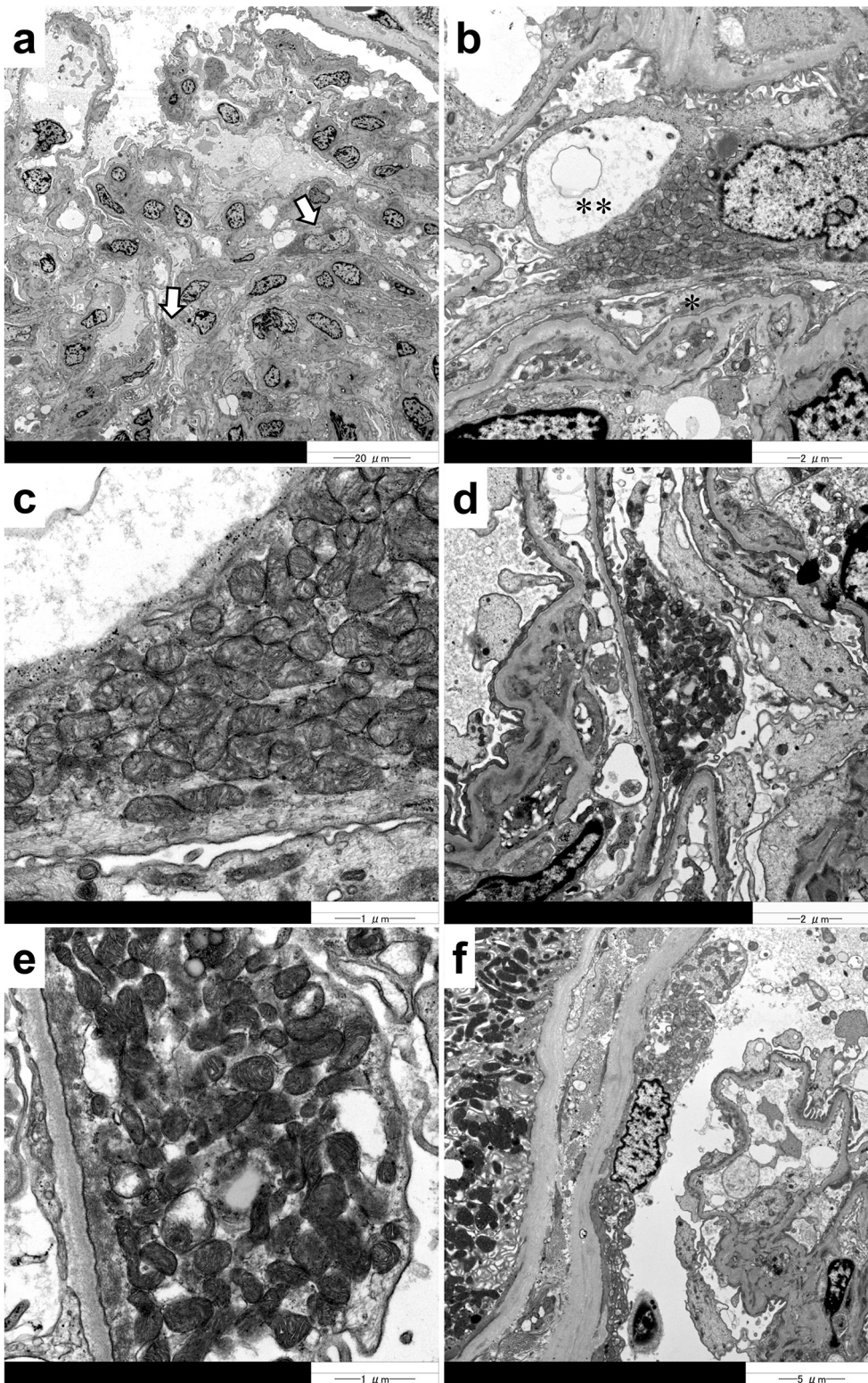
Mitochondrial diseases cause genetic FSGS [11,12,15]. Its etiology is postulated to be caused by genetically disrupted mitochondrial function in podocytes [19,22]. As an indication of this, cases with FSGS due to m.3243A > G mutation had podocytes filled with mitochondria with abnormal cristae structure [10,11]. In the past, several cases of mitochondrial diseases due to *MT-ND5* mutations have been also reported to show FSGS lesions [26–29]. However, in these cases, pathological findings of podocyte injuries with accumulation of abnormal mitochondria were not confirmed, leaving the possibility of secondary FSGS caused by intraglomerular hypertension [14,28,30,31]. In contrast, our present case showed apparent FSGS lesions, and we detected increased abnormal mitochondria in the glomerular podocytes that also had pathological findings indicating their injuries such as foot process effacement and the cytoplasmic vacuolization. Our patient did not have hypertension, obesity, or a history of low birth weight. Furthermore, the diameters of all glomeruli observed under light microscopy in this patient were < 250 μm (mean ± SD; 149 ± 72 μm). Therefore, we deemed the etiology of FSGS, in this case, to be a disorder of the mitochondrial OXPHOS system due to an *MT-ND5* mutation and not due to 'secondary' by intraglomerular hypertension [14,30,31]. The pathological picture of tubulointerstitial nephropathy with GSECs was also observed in this case. This tubulointerstitial damage may be due to the *MT-ND5* mutation, as has been reported in the past [28].

In general, mitochondrial diseases induced by mtDNA mutations vary markedly based on differences in the rates of heteroplasmy by cell type or organ [40,41]. Therefore, while the reason for the differences between the present and the previous cases with the pathogenic variant in *MT-ND5* gene could not be completely ascertained, the heteroplasmy rate of mtDNA with the pathogenic variant in podocytes might have been higher in our case than that in the previous cases.

In this case, IgM, C3, and C1q were positive in glomeruli (Fig. 1g-i). Since neither hematuria, mesangial cell proliferation, nor electron dense deposits were observed, we considered that it was unlikely that IgM nephropathy or C1q nephropathy was complicated [42,43]. On the other hand, glomerular IgM and C3 deposits frequently accompany FSGS [44]. In addition, IgM and C3 were positive in glomeruli of two out of three cases report by Bakis et al., too [28]. Therefore, IgM and C3



**Fig. 1.** Light microscopy and immunofluorescence findings (a): The segmental sclerosis lesion at the perihilar area is indicated by an arrow. A red-coloured podocyte (ReCPo) is surrounded by a square, magnified at the top right (AZAN stain). (b): The ReCPo is indicated by an arrow (AZAN stain). (c): GSECs in the distal tubules are indicated by arrows (AZAN stain). One GSEC is magnified at the lower left. (d): Staining of COX IV emphasizes tubular cells, including many mitochondria, which correspond to GSECs. (e): In the afferent arteriole that connects to the glomerulus of (a), the sizes of the vascular smooth muscle cells are irregular, and their arrangement is disorganized, similar to those seen in older patients (PAM-HE stain). (f): Age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) are observed in an interlobular artery (PAM-HE stain). (g): IgM deposits are detected in glomeruli. (h): IgG deposits are detected in glomeruli. (i): C1q deposits are detected in glomeruli.

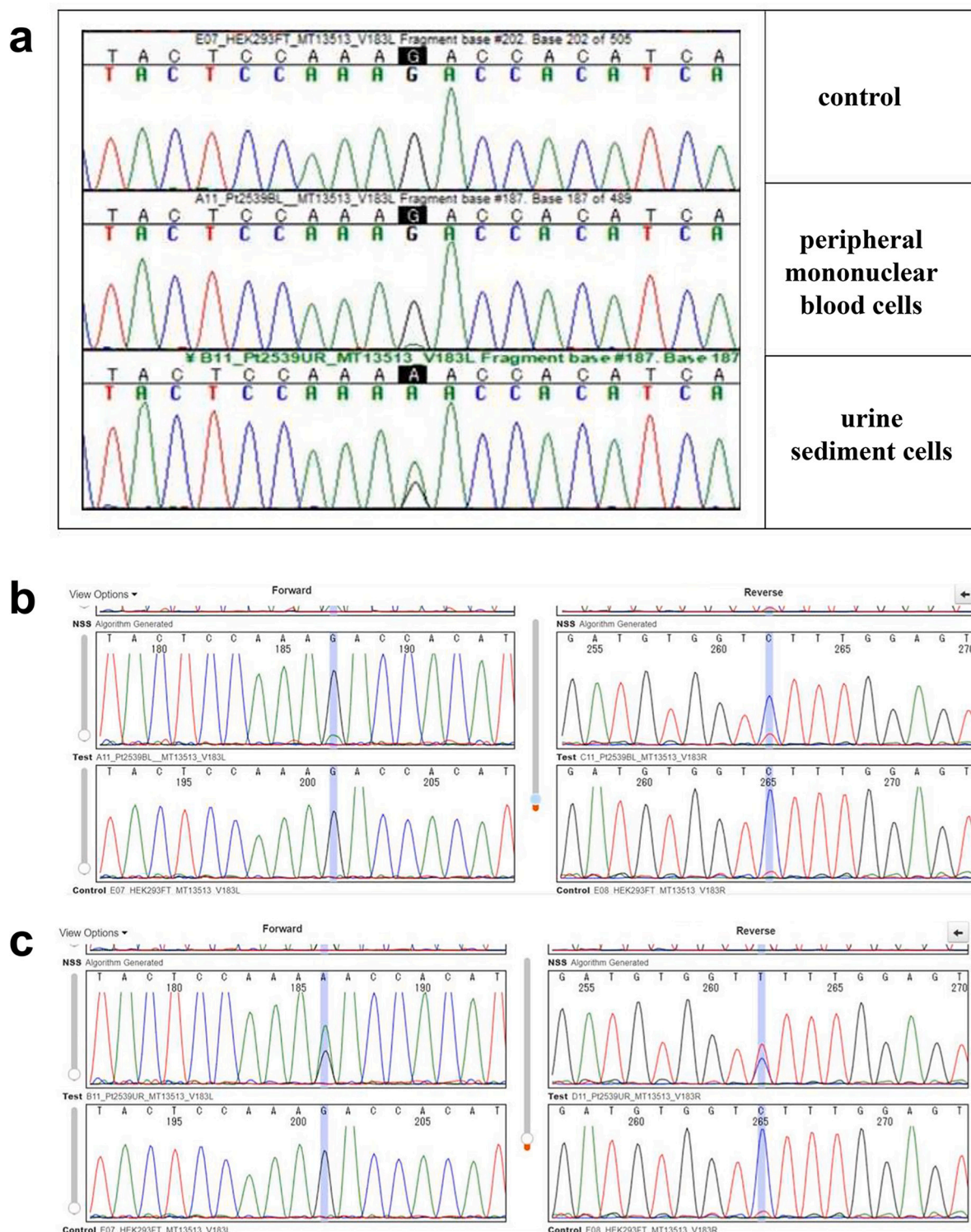


**Fig. 2.** Electron microscopy findings (a): Arrows indicate podocytes filled with increased mitochondria. (b): In a podocyte, abnormally high numbers of mitochondria are seen in the cytoplasm. Foot process effacement (\*) and cytoplasmic vacuolization (\*\*) are observed in this podocyte. (c): Magnification of (b) reveals that the increased mitochondria have lost their organized cristae structure. (d): A podocyte is filled with mitochondria. The foot process of this podocyte is effaced. (e): Magnification of (d) reveals that mitochondria with disorganized cristae increase. (f): The parietal epithelial cells in Bowman's capsule are also filled with abnormal mitochondria.

deposition may be related to a part of the pathogenesis of FSGS caused by *MT-ND5* mutation. Although the reason for C1q deposition in glomeruli is also not clear. Because C1q is reported to bind to mitochondria, it may be associated with the pathogenesis of FSGS by driving oxidative stress [45–47].

This case should be suggestive because it had several characteristic light-microscopical findings of mitochondrial nephropathy. In our case, GSECs with red-coloured cytoplasm were observed with AZAN

trichrome staining. As mitochondria are dyed red by acidic dyes such as AZAN, red-coloured GSECs indicate that they abnormally include many mitochondria [33]. In addition, as COX IV is a subunit of complex IV, COX IV staining can be used to stain mitochondria [35] and may help confirm the presence of abnormally increased numbers of mitochondria [34,48]. Furthermore, age-inappropriately disarranged and irregularly sized vascular smooth muscle cells (AiDIVs) were observed in the arterioles and intralobular arteries in this case. Such findings have also been



**Fig. 3.** Genetic analysis (a): The electropherogram from Sanger sequencing indicates that the patient has the m.13513 G > A variant in her peripheral mononuclear blood and urine sediment cells. (b): The rate of heteroplasmy calculated by the MVF software program is 10.3% (forward: 11.6%/reverse: 9.0%) in peripheral mononuclear blood cells (upper panel: patient, lower panel: control). (c): The rate of heteroplasmy is 62.2% (forward: 63.7%/reverse: 60.7%) in urine sediment cells (upper panel: patient, lower panel: control).

reported in other cases of mitochondrial nephropathy [12,34]. Interestingly, red-coloured podocytes (ReCPo) were detected using AZAN trichrome staining, as we recently reported in a case of mitochondrial nephropathy [49]. Like GSECs, ReCPo abnormally include many mitochondria in their cytoplasm [49]. In summary, in cases of FSGS or glomerulosclerosis with unknown aetiologies, the existence of GSECs,

AiDIVs, and ReCPo should be carefully evaluated. COX IV staining may aid in the detection of abnormally increased mitochondrial counts.

We describe the weaknesses of our report. In this case, because mitochondrial disease was suspected based on the pathological findings of abnormal mitochondria, the presence of hearing loss, and high blood lactate levels, we comprehensively examined the genes associated with

mitochondrial diseases. Therefore, genetic FSGS due to other gene mutation [50] cannot be completely ruled out. In addition, because genetic testing was performed only on the patient but not on other family members, it could not be investigated whether they carried the heteroplasmic mitochondrial variant. The parents were already deceased and the two healthy brothers did not consent to genetic analysis. The two her children were still under 18 years old and the patient did not wish genetic test. Furthermore, measurement of MRC enzyme activity using kidney biopsy specimens is an effective for confirming mitochondrial dysfunction [51,52]. However, it could not be performed due to the paucity of kidney specimens for this analysis.

In conclusion, this is the first case report which indicates that the pathogenic variant an *MT-ND5* gene causes accumulation of abnormal mitochondria in podocytes, which leads to forming FSGS.

### Ethics approval and consent to participate

The original study to perform a genetic analysis for mitochondrial diseases was approved by the Ethics Committee of Chiba Children's Hospital (2014-11-05). In addition, the analysis of this case was also approved by the Ethics Committee of Yamanashi Prefectural Central Hospital (2020-16). The patient provided informed consent and permission to participate in the study.

### Consent for publication

Written informed consent was obtained from the patient for the publication of this case report and accompanying images. A copy of the written consent is available for review by the editor of this journal. All authors consented to the publication of the manuscript in *BMC Nephrology*.

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### Authors' contributions

TN drafted the manuscript. TI was involved in the coordination of the study and draft preparation. IN and MW provided clinical information. KH performed the pathological analysis. YY and YK performed molecular genetic studies. YO performed sequence alignment and data curation. KM was involved in the coordination of the study and data curation. YJ was involved in the coordination of the study and clinical information. All authors read and approved the final manuscript.

### Declaration of Competing Interest

The authors declare that they have no competing interests.

### Data availability

Most of the clinical data used and analysed in this case report are presented in this manuscript. More detailed information is available from the corresponding author upon request.

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## ミトコンドリア病の診療水準やQOL向上を目指した調査研究班

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次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業
2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究
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(氏名・フリガナ) 三牧 正和・ミマキ マサカズ

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	国立精神・神経医療研究センター	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

## その他 (特記事項)

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

## 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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## 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究に係るCOIについての指導・管理の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (有の場合はその内容: )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

令和6年4月5日

国立保健医療科学院長 殿

機関名 国立大学法人福井大学

所属研究機関長 職 名 学長

氏 名 上田 孝典

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業

2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究

3. 研究者名 (所属部署・職名) 学術研究院医学系部門・教授

(氏名・フリガナ) 井川 正道 (イカワ マサミチ)

#### 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

#### その他 (特記事項)

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

#### 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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#### 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (無の場合はその理由: 別紙参照)
当研究に係るCOIについての指導・管理の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (有の場合はその内容: )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

(別紙)

本研究については、全学の利益相反マネジメント規則に基づき、研究者から「本研究に関する経済的な利益関係がない」旨の報告があり、利益相反マネジメント委員会の事務担当者が審査不要である旨を確認している。

国立保健医療科学院長 殿

機関名：埼玉医科大学

所属研究機関長 職 名：学長

氏 名 竹内 勤

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業

2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究

3. 研究者名 (所属部署・職名) ゲノム医療科・小児科・特任教授

(氏名・フリガナ) 大竹 明・オオタケ アキラ

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	埼玉医科大学病院	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称： )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

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その他 (特記事項) なし

(※2) 未審査の場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

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研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

国立保健医療科学院長 殿

機関名 順天堂大学

所属研究機関長 職 名 学長

氏 名 代田 浩之

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

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3. 研究者名 (所属部署・職名) 大学院医学研究科・教授

(氏名・フリガナ) 岡崎 康司・オカザキ ヤスシ

## 4. 倫理審査の状況

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人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	埼玉医科大学	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

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## 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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## 6. 利益相反の管理

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当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
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(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

国立保健医療科学院長 殿

機関名 自治医科大学

所属研究機関長 職 名 学 長

氏 名 永 井 良 三

次の職員の令和 5 年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

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3. 研究者名 (所属部署・職名) 医学部小児科学・教授

(氏名・フリガナ) 小坂 仁 ・ オサカ ヒトシ

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	自治医科大学	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

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## 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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## 6. 利益相反の管理

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国立保健医療科学院長 殿

機関名 国立精神・神経医療研究センター

所属研究機関長 職 名 理事長

氏 名 中込 和幸

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

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3. 研究者名 (所属部署・職名) メディカル・ゲノムセンター・特任研究部長  
(氏名・フリガナ) 後藤雄一・ゴトウユウイチ

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
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人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	国立精神・神経医療研究センター	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

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(留意事項) ・該当する□にチェックを入れること。  
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令和6年3月25日

国立保健医療科学院長 殿

機関名 国立精神・神経医療研究センター

所属研究機関長 職 名 理事長

氏 名 中込 和幸

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業

2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究

3. 研究者名 (所属部署・職名) トランスレーショナル・メディカルセンター・センター長

(氏名・フリガナ) 小牧 宏文・コマキ ヒロフミ

#### 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	国立精神・神経医療研究センター	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

#### その他 (特記事項)

(※2) 未審査の場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

#### 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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#### 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究に係るCOIについての指導・管理の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (有の場合はその内容: )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

国立保健医療科学院長 殿

機関名 国立大学法人東北大学

所属研究機関長 職 名 総長

氏 名 大野 英男

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業

2. 研究課題名 ミトコンドリア病の診療水準や QOL 向上を目指した調査研究

3. 研究者名 大学院医学系研究科周産期医学分野・准教授

立花 眞仁・タチバナ マサヒト

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

## その他 (特記事項)

(※2) 未審査の場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

## 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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## 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究に係るCOIについての指導・管理の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (有の場合はその内容: 研究実施の際の留意点を示した。 )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

令和6年3月25日

国立保健医療科学院長 殿

機関名 国立研究開発法人  
国立精神・神経医療研究センター

所属研究機関長 職名 理事長  
氏名 中込 和幸

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業
2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究
3. 研究者名 (所属部署・職名) 神経研究所 疾病研究第一部・部長  
(氏名・フリガナ) 西野 一三 ・ ニシノ イチゾウ

4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	国立精神・神経医療研究センター	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他 (特記事項)

(※2) 未審査の場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究に係るCOIについての指導・管理の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (有の場合はその内容: )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

令和6年3月22日

国立保健医療科学院長 殿

機関名 産業医科大学

所属研究機関長 職 名 学長

氏 名 上田 陽一

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業

2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究

3. 研究者名 (所属部署・職名) 産業生態科学研究所・教授

(氏名・フリガナ) 藤野 善久・フジノ ヨシヒサ

#### 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	産業医科大学	<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

#### その他 (特記事項)

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(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

#### 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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#### 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究に係るCOIについての指導・管理の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (有の場合はその内容: )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

国立保健医療科学院長 殿

機関名 順天堂大学

所属研究機関長 職 名 学長

氏 名 代田 浩之

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

- 研究事業名 難治性疾患政策研究事業
- 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究
- 研究者名 (所属部署・職名) 難治性疾患診断・治療学 教授  
(氏名・フリガナ) 村山 圭・ムラヤマ ケイ

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

## その他 (特記事項)

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## 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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## 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究に係るCOIについての指導・管理の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (有の場合はその内容: )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

国立保健医療科学院長 殿

機関名 福岡大学

所属研究機関長 職 名 学長

氏 名 永田 潔文

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

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2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究

3. 研究者名 (所属部署・職名) 医学部小児科学講座・講師

(氏名・フリガナ) ハツ賀 秀一・ヤツガ シュウイチ

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

## その他 (特記事項)

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

## 5. 厚生労働分野の研究活動における不正行為への対応について

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## 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
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当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
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(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

国立保健医療科学院長 殿

機関名 独立行政法人  
国立病院機構病院千葉東病院

所属研究機関長 職名 病院長

氏名 西村 元伸

次の職員の令和5年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1. 研究事業名 難治性疾患政策研究事業
2. 研究課題名 ミトコンドリア病の診療水準やQOL向上を目指した調査研究
3. 研究者名 (所属部署・職名) 国立病院機構千葉東病院 診療部・統括診療部長  
(氏名・フリガナ) 今澤 俊之 (イマサワ トシユキ)

## 4. 倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入 (※1)		
	有	無	審査済み	審査した機関	未審査 (※2)
人を対象とする生命科学・医学系研究に関する倫理指針 (※3)	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
遺伝子治療等臨床研究に関する指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>
その他、該当する倫理指針があれば記入すること (指針の名称: )	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>		<input type="checkbox"/>

(※1) 当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

## その他 (特記事項)

(※2) 未審査に場合は、その理由を記載すること。

(※3) 廃止前の「疫学研究に関する倫理指針」、「臨床研究に関する倫理指針」、「ヒトゲノム・遺伝子解析研究に関する倫理指針」、「人を対象とする医学系研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

## 5. 厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 <input checked="" type="checkbox"/> 未受講 <input type="checkbox"/>
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## 6. 利益相反の管理

当研究機関におけるCOIの管理に関する規定の策定	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究機関におけるCOI委員会設置の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合は委託先機関: )
当研究に係るCOIについての報告・審査の有無	有 <input checked="" type="checkbox"/> 無 <input type="checkbox"/> (無の場合はその理由: )
当研究に係るCOIについての指導・管理の有無	有 <input type="checkbox"/> 無 <input checked="" type="checkbox"/> (有の場合はその内容: )

(留意事項) ・該当する□にチェックを入れること。  
・分担研究者の所属する機関の長も作成すること。

国立保健医療科学院長 殿

機関名 北海道大学

所属研究機関長 職名 総長

氏名 寶金 清博

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3. 研究者名 (所属部署・職名) 大学院医学研究院 生殖・発達医学分野小児科学教室・講師  
(氏名・フリガナ) 武田 充人・タケダ アツヒト

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