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ミトコンドリア病の調査研究  
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総合研究報告書 1

ミトコンドリア病、レット症候群に関する調査研究  
【ミトコンドリア病】

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**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適切な治療をめざして、診断基準・重症度スケールの改定案提出、診療マニュアル改定版の作成、患者レジストリー拡充等を実施した。アウトリーチ活動については、AMED 実用化班や日本ミトコンドリア学会での患者参加行事を援助した。患者レジストリーについては、個人情報保護法に関連した倫理ガイドライン改正に沿う変更を行うとともに、登録を増加させた。診療ガイドラインの作成は、MINDS 型の診療ガイドライン策定を断念し、「ベストプラクティス」方式の「診療マニュアル 2017」の改定版の作成を目指し、AMED 実用化班と協力して担当者の選定を行い、2023 年 6 月に発刊される。生殖補助医療については、科学技術・学術審議会での検討が行われ、核置換技術を用いた特定胚を用いた基礎研究が容認されたものの、臨床的な「核移植治療」は我が国では継続審議となった。疫学研究においては、過去 2 年間で、DPC データを活用することで、都道府県別の患者分布が実際の登録患者数と相関することを、NDC データを活用することで、本邦における有病者数を高い確度で推定できることを見だし、今後の疫学的課題の抽出を行った。

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#### 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。なかでも、中枢神経や骨格筋の症状を主体とするミトコンドリア病が代表的な病型である。

国内においてミトコンドリア病の患者数の厳密な実態調査は行われていない。その理由は患者が多く診療科に分散していること、診断基準が明確ではなかったことなどが挙げられる。そのもっとも大きな要因は確定診断に必要な病理、生化学、遺伝子検査の専門性が高いことにある。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、難病行政に対応した診断基準の改訂を必要としている。

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。

本研究班では、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、本疾患に関する情報提供手段の整備等を行い、臨床病型、重症度、合併症、主な治療の内容などの標準化をめざす。特に、患者レジストリーを進め、具体的な治療に関する臨床研究や治験を進めるコーディネーター役を担うこと、また主に小児のミトコンドリア病を対象としている AMED 難治性疾患実用化研究事業の村山班と連携して診療ガイドラインを作成するとともに、市民公開講座や難病情報センター等を活用し、広報活動を行うことを目的とする。

## B. 方法

### 1) 診断治療体制の構築【後藤、大竹、村山】

ミトコンドリア病の確定診断におけるミトコンドリアDNA検査、核遺伝子パネル検査の保険収載を目指し、令和2年12月に厚労省へ提出した診断基準において、遺伝学的検査の重要性を明確化した。令和4年4月に「ミトコンドリア病の遺伝学的検査の保険収載」がなされ、これまで大学・研究所で行ってきた遺伝学的検査が厳格な精度管理を必要とする臨床検査への急激な変更が必要になった。また、指定難病と小児慢性特定疾患における病型分類、診断基準等の考え方に開きがある。この点については、小児例の成人移行の問題として検討を継続する。医療イノベーションで計画されている都道府県単位の「新・難病医療拠点病院及び難病医療地域基幹病院構想」に沿って「ミトコンドリア病に詳しい医師のネットワーク」の構築を目指す。

### 2) 「患者ケア標準書」の作成【全員】

平成28年12月に刊行した「ミトコンドリア病診療マニュアル2017」においては、現在のミトコンドリア病の診療状況をまとめた。その改訂を見据えつつ、各診療科のエキスパートの意見をまとめた「患者ケア標準書」の作成を目指す。循環器、耳鼻科などのミトコンドリア病で認められる多臓器症状に関しては、関連学会などに協力を呼びかけながら、本研究班が中心となり作成する。

### 3) 患者レジストリーの運用拡大【後藤、大竹、三牧】

すでに成人は国立精神・神経医療研究センターにおいてRemudy方式の患者レジストリーシステムを構築しており、それを拡充させる。小児は、MOバンクにおいて患者レジストリーを始めており、Leigh症候群以外は超希少な疾患が多いため、欧米の患者レジストリーとの連携が有用になる。2つのレジストリーの特徴を有効に生かし、国内だけでなく国際的な共同治験等の将来の臨床研究、治験の推進に役立てる。

### 4) ミトコンドリア病に対する生殖補助医療の検討【後藤】

欧米で始まっている胚に対する核移植は、CSTI（総合科学技術・イノベーション会議）での議論を経て本邦では時期尚早という判断になった。一方で、着床前診断や胚を用いた基礎研究への道が開かれ、これらの技術の科学性、効果や安全性、倫理問題については本研究班で議論を継続させる。

### 5) アウトリーチ活動と国際連携【全員】

疾患セミナーの企画・実行や難病情報センターからの情報発信を行う。今年度も患者会との連携、市民公開講座を開催する。

### 6) 疫学研究【後藤、松田、藤野、三牧】

平成30年度から開始した疫学研究では、DPCデータを活用して、全国の医療機関におけるミトコンドリア病の実態（特に登録患者数）を推測できた。今年度はNCDデータも活用して、各種臨床指標が抽出可能かどうかを検討する。

## C. 結果と考察

### 1) 診断治療体制の構築

平成26年10月に策定した「診断基準」では遺伝学的検査が必須になっておらず、それによる保険収載の遅れが明確になった。令和2年度に改訂打診が厚労省難病対策課からあり、遺伝学的検査のみでも確定診断できる基準に変更し、厚生科学審議会疾病対策部会指定難病検討委員会で令和3年11月から審議が継続している。審議会で示されているスケジュールに依ると、正式な告示と通知の改正は令和5年になるとされている。

一方で、乳児期、小児期に発症するミトコンドリア病は重症例が多く、「代謝病」としての性格が前面にでる傾向がある。そのため、小児慢性特定疾患の認定基準は、代謝病として分類を中心とする認定方式をとっている。小児慢性特定疾患の診断基準との整合性や臨床試験のためのミトコンドリア肝症やミトコンドリア心筋症の個別病型の診断基準作成をAMED実用化班と協議を継続した。

### 2) 「診療マニュアル」等の改定

2012年に本研究班が発行した「ミトコンドリア病パンフレット」は、冊子としても、また、電子媒体（難病情報センターの疾患紹介サイトからダウンロード可能）としても、広く一般市民に利用された。

特に治療表の進歩に重点を置いた改訂版を作成したものの、診断基準の改訂の厚労省における承認が令和4年度中には間に合わず、その確定を待って改訂第2版を発行することになっている。

また、AMED 実用化研究班（村山班）と協力して、「診療ガイドライン」の改定を行う作業を実施した。問題はミトコンドリア病にはグローバルな診断基準が明確にされていないこともあって、エビデンスとして採用できる海外論文等の研究成果が少ない。結果的に、Minds 方式のガイドラインの作成は断念し、欧米で作成されている「ベストプラクティス」方式の刊行物を目指すこととして、編集内容と担当者を選定した。そのため多くの執筆者に加わっていただくために、本研究班の協力者になっていた。

### 3) 患者レジストリーの運用拡大

患者レジストリーについては、AMED 難治性疾患実用化研究班（村山班）と連携して行うこととし、村山班では主に先天代謝異常症として小児（成人）患者レジストリーを行っており、2023年3月で約120名である。一方、国立精神・神経医療研究センターでは、筋ジストロフィーの登録事業（Remudy）を敷衍する形態で、神経症状を中心とする成人（小児）患者レジストリーを開始し、2022年3月現在で32名の登録を完了した。

4) ミトコンドリア病に対する生殖補助医療の検討クが、「核移植治療」でミトコンドリアDNA8993変異をもち、リー脳症の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。

①ミトコンドリア病研究を目的とし、ヒト受精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

は、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、容認することが適当。

②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倫理的課題については、生命倫理専門調査会において引き続き検討。

③ミトコンドリア病の病態解明等のため、ヒト胚核移植胚の作成を行う基礎的研究は、クローン技術規制法の目的に直接的に抵触するものではないと考えられることから、「特定胚の取扱いに関する指針」の改正（令和3年6月30日）によりヒト胚核移植胚の作成を可能としつつ、同時に、作成したヒト胚核移植胚の人又は動物胎内への移植を禁止する等のされてきており、米国では禁止された行為であった。

核移植の臨床応用は継続審議となる一方、基礎研究においては核移植を含めた「ヒトは核移植胚研究」が令和3年6月に容認され、さらに、これまで余剰胚でのみ認められていた核置換を用いた研究を新規胚にも適用できるように見直しが検討され、同時に、研究に必要な対照群胚の作成の容認を含めた最終案が令和5年5月のCSTI（総合科学技術・イノベーション会議）で諮られる予定である。

### 5) アウトリーチ活動と国際連携

AMED 村山班と連携して、患者会活動（WEB 勉強会）の支援をおこなった。コロナ禍の中で、国際学会等における海外の研究者や患者との交流は困難であった。

### 6) 疫学研究

平成30年度から産業医科大学の松田晋哉教授、藤野善久教授が分担研究者として加わり、DPC データを活用した全国の医療機関におけるミトコンドリア病の診断数やその経緯、投与されている薬剤等の調査を行うことで、ミトコンドリア病の医療の実態を把握する研究を開始した。都道府県別の患者分布が実際の登録患者数と相関することがわかり、本法の有用性が確認でき、その解析結果を令和2年4月に「厚生指標」に報告した。さらにNBD データを用いた疫学研究を実施し、本邦における有病者数を高い確度で推定できることを見だし、令和3年4月に英文誌に報告した。

令和4年度は、今後の疫学的課題の抽出を行った。

#### 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、診断基準や重症度スケールの改定作業を進めた。生殖補助医療の情報収集と日本での実現可能性について議論し、対照群の胚作成を含めた核置換技術を用いた余剰胚及び新規胚を用いた基礎研究が可能となった。患者レジストリーは、種々の要因で格段の進歩はないが、グローバルな視点でレジストリーやバイオバンク(研究試料)との連動を図りながら、着実に進めてゆく必要がある。

#### E. 健康危険情報

なし

#### F. 研究発表

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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



総合研究報告書 2

ミトコンドリア病、レット症候群に関する調査研究

【レット症候群】

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研究要旨

本研究では、レット症候群（RTT）の臨床調査研究と診療支援を行なった。また、RTT の患者データベースの 5 年毎の追跡調査を行ない、自然暦調査を行った。

RTT の睡眠障害は日常生活に支障をきたすだけでなく、家族や介護者の生活の質にも影響する。そこで、睡眠障害の状況調査を行なった。その結果、睡眠障害は約 65%にみられた。このうち、80%以上で 1 日の睡眠時間は 8 時間以上保たれていたが、夜間の睡眠は短い傾向にあった。夜間覚醒、夜驚、不規則な睡眠が多くを占めていた。これに対して、薬剤の効果は限定的であった。また、RTT の主症状として、てんかん、便秘、心電図異常についてまとめた。

遺伝子検査では、*MECP2* 変異 15 例、*CDKL5* 変異 1 例、*FOXG1* 変異 1 例であり、これらの患者はデータベースへ登録された。患者データベースに登録されている *MECP2* 変異を有する患者の協力を得て、MRI 検査によって脳内の構造的・機能的ネットワークについての検討を行い、本症に特徴的な 2 つのネットワーク異常を同定した。

臨床診断には必須ではないが、遺伝学的検査は極めて有用である。RTT の遺伝学的検査適用を視野に入れ、類縁疾患の遺伝学的検査について検討した。遺伝学的検査の保険適用、次世代シーケンス解析でのバリエーション解釈、マイクロアレイ染色体検査の活用が、これらの疾患群で重要であることが明らかになった。

引き続き、公開シンポジウム等を開催し、患者及び患者家族だけでなく一般社会に向けて情報発信する。

A. 研究目的

本研究では、レット症候群（RTT）患者データベースの運用、および臨床研究を行なった。

RTT の追跡調査を行い、その結果から自然暦、臨床実態を明らかにし、診断基準を検証した。

睡眠の実態についてアンケート調査を行った。また、診断基準に挙がる症状以外に、医学的な管理・観察の必要な症状は複数あり、それらの特徴の中から、てんかん、便秘、心電図異常について、臨床的特徴について後方視的にまとめた。

RTT の遺伝子診断を行なった。さらに、遺伝学的検査の位置づけを検討した。また、遺伝学的検査として、エクソーム解析の実用化とマイクロアレイ染色体検査の有用性について検討した。

RTT の病態理解を深めるために脳内ネットワーク異常について検討した。

関連学会発表および公開シンポジウム開催し、RTT の普及と啓発を行った。

B. 研究方法

①RTTの臨床研究:RTT患者データベース登録されている30名の登録患者の追跡調査を行った。同一患者での

症状の変化を経年的に解析し、自然暦を調べた。日本小児科学会、日本小児神経学会、重症心身障害学会の協力の協力で、患者の有無と診療状況について調査を行い、レットの臨床評価、重症度分類を検討した。カルテより後方視的に、レット症候群患者の症状について検討した。

②RTT の患者データベースの運用:本年度も患者家族会の協力を得て、新規患者登録を行った。遺伝子診断は、当該施設の倫理委員会の承認を得て、患者あるいは保護者への十分な説明と同意を得て行った。

③睡眠実態調査:レット症候群支援機構が運営するインターネット情報サイト「レッコミ」を通じて、アンケート調査を行った。

④特徴的な症状の調査:便秘と心電図異常について、後方視的にカルテ等の情報を抽出して調査した。

⑤遺伝子検査:遺伝子診断用の DNA は、末梢血白血球より抽出した。レット症候群の遺伝子診断は、*MECP2*、*CDKL5*、*FOXG1* 遺伝子について、サンガー法あるいは MLPA 法にて行った。

⑥変異スクリーニング体制とマイクロアレイ染色体検査の検証:次世代シーケンサーを用いた変異スクリーニング体制を確立した。分析は、メンデル遺伝病

疾患パネル解析 TruSight One Sequence Panel (Illumina) を用いて卓上型次世代シーケンサー MiSeq (Illumina) で解析を進めた。マイクロアレイ染色体検査は Agilent 社製マイクロアレイシステムを用いた。さらに、FISH 解析を加えた。検出された copy number variations (CNV) の意義を検討した。

⑦脳内機能解剖学的ネットワーク解析: 9 例の RTT 患者について、定量的 MRI 画像、拡散テンソル画像、安静時機能的 MRI 画像検査を行い、臨床症状の重症度 (clinical severity score) との関連について検討した。

(倫理面への配慮)

すべての本研究および調査は、当該施設の倫理問題等検討委員会等の承認のもと行った。

### C. 結果

①RTT の臨床研究: 疫学調査とその解析: RTT 患者データベースに登録されている 186 例の登録者のうち 5 年を経過した登録患者について、現況調査として再度登録用紙の提供を依頼し、回収、解析を行なった。

②RTT の患者データベースの運用: 研究期間中に 35 名の登録を更新した。臨床遺伝学的解析では、レット症候群の臨床診断を受けた患者について遺伝子診断を行った。

③睡眠実態調査: MECP2 遺伝子異常を有するレット症候群患者 120 名から回答を得た。睡眠障害は約 65% にみられた。このうち、80%以上で 1 日の睡眠時間は 8 時間以上保たれていたが、夜間の睡眠は短い傾向にあった。夜間覚醒、夜驚、不規則な睡眠が多くを占めていた。これに対して、薬剤の効果は限定的であった。

④特徴的な症状の調査: 便秘情報がある患者 18 名中、便秘を有する患者が 13 名 (72%) であった。

心電図情報のある患者は 14 名であった。概ね 1-2 年ごとに調べられていた。QTc の値は、361-435 msec (平均値 400 msec、中央値 398 msec) で、QT 延長と判定される 460 msec 以上の患者はいなかった。

⑤遺伝子検査: MECP2 変異 15 例、CDKL5 変異 1 例、FOXG1 変異 1 例を同定し、患者データベースへの登録を進めた。

⑥変異スクリーニング体制とマイクロアレイ染色体検査の検証: 遺伝学的検査では、102 例をスクリーニングし、1 例に MECP2 遺伝子既報告変異例の診断ができた。CDKL5 遺伝子異常症は 1 例の診断が可能であった。

⑦脳内機能解剖学的ネットワーク解析: MRI を用いた機能的ネットワーク解析では、2 つの脳内ネットワーク (左右半球間の背側注意ネットワーク、視覚ネットワークと小脳ネットワーク) の機能的結合性低下を見出した。

### D. 考察

RTT の臨床研究では、RTT の自然歴調査は、治験を行う上で重要な資料となる。

遺伝子診断システムを確立し、原因遺伝子の発見に貢献した。診療支援体制が確立してきた。しかし、遺伝子診断は高額で高度な技術を要することから、継続的な診断には経済的な支援が必要である。

RTT の睡眠障害は、50~80% と報告されている。今回の調査でも概ね既報告に一致していた。1 日の睡眠時間は確保されているものの、夜間の睡眠にさまざまな問題があることが明らかになった。夜間の睡眠障害は、患者本人だけでなく、介護者、家族の負担が大きく、今後の課題である。

RTT 患者の便秘の有症率は 70-80% と言われている。便秘に対して、緩下剤、経口薬で無効な場合に座薬や浣腸が推奨されている。QT 延長症候群に該当する患者はいなかったが、QT 間隔が延長する傾向にあった。

今後、遺伝学的検査として導入する際には、施設設備、人材、報告書のありかた、バリエーション解釈の手順書、なども必要かもしれない。

また、今回の脳 MRI 研究により本症に特徴的な構造的・機能的ネットワーク異常が同定された

### E. 結論

RTT の臨床研究では、RTT 患者データベース登録の継続と追跡調査を行なった。遺伝子診断体制を確立した。しかし、高額で高度な技術であり、継続的な診断には経済的な支援が必要である。

また、レット症候群の夜間の睡眠障害は、その内容が特殊であり、今後解決していかねばならない課題である。

便秘は、発症率は 70% 程度であった。多くは酸化マグネシウムや浣腸によりコントロールされていた。QT 延長症候群は認められた患者はいなかった。

今後、レット症候群の保険適用が課題と考えられた。また、実際の網羅的解析がレット症候群および類縁疾患の診断に有用であることを確認した。

### F. 研究発表

#### 1. 論文発表

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なし。

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
小坂仁	ミトコンドリア病入門	村山圭 小坂仁 三牧正和	遺伝子医学M OOK 35号 【ミトコンド リアと病気】	メディカ ルドゥ	大坂	2020	348-352
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## 主要な文献

## DPCデータを用いたミトコンドリア病の記述的研究

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**目的** 従来、わが国のミトコンドリア病の疫学調査は、おもに病院アンケート調査によって行われ、全国規模の患者調査は実施困難であった。しかし、近年電子レセプトデータの利用環境が整備・拡充されつつあり、研究目的の利用も推進されている。本研究では、DPCデータを用いてミトコンドリア病の有病者数の推定を行った。また、今回利用したDPCデータの利用可能性および今後の課題について検討した。

**方法** 一般社団法人診断群分類研究支援機構の調査研究に協力している国内のDPC参加病院のデータを用いて、病名検索によりミトコンドリア病患者を抽出した。主要評価項目は、ミトコンドリア病の有病者数とした。また、副次評価項目として、ミトコンドリア病患者の各種集計（疾病グループ、性別、年齢、在院日数、ICD10分類、各種医療行為、入院患者の都道府県別分布、入院中の死亡）を行った。

**結果** ミトコンドリア病の有病者数は1,386人と推定された。

**結論** DPCデータを利用することで、ミトコンドリア病の有病者数は推定可能であることが示唆された。しかし、より悉皆性の高い調査を行うためには、DPCに参加していない病院の入院患者、および外来患者のデータベースを加えて調査する必要がある。

**キーワード** ミトコンドリア病、指定難病、有病者数、DPC、記述疫学

## I 緒 言

ミトコンドリア病は、わが国の施策として、指定難病制度および小児慢性特定疾患治療研究事業を通じた医療費の助成が行われている希少疾患である。ミトコンドリアは細胞内小器官の一つであり、人体における主要な好氣的エネルギー産生のものである。しかし、先天的な核DNAまたはミトコンドリアDNAの変異・欠失などにより、ミトコンドリアの呼吸鎖酵素複合体（電子伝達系とATP合成酵素）の異常が存在すると、ミトコンドリア病を発症する。ミト

コンドリア病は、中枢神経系や筋骨格系・循環器系・消化器系等あらゆる臓器に症状を呈しうるが、その発症年齢や重症度および生命予後は患者によって様々である。ミトコンドリア病を発症した患者の治療として、ミトコンドリアの代謝経路に保護的と考えられる薬剤の補充療法や、栄養療法・生活指導が行われ、一定の成果を挙げているが、残念ながら現時点で根治的な治療法は存在しない<sup>1)</sup>。

従来、わが国でのミトコンドリア病などの希少疾患の疫学調査は、おもに病院アンケート調査によって行われてきたが、全国の患者を対象

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とした大規模な調査は実施困難であった<sup>2)</sup>。しかし、近年電子レセプトデータの利用環境が整備・拡充されつつあり、電子レセプトデータの疫学研究への利活用が積極的に検討されている<sup>3)~6)</sup>。そこで、本研究ではDPCデータに注目した。DPC(Diagnosis Procedure Combination)とは、診断名と医療行為の体系的な分類であり、わが国の急性期入院医療を対象とした包括支払い制度にも利用されている。DPC参加病院の病床数は、わが国の一般病床入院患者情報の約83%をカバーしている大規模なものである<sup>7)</sup>。

本研究では、このDPCデータを用いてミトコンドリア病の患者数、つまり有病者数を推定し、加えてDPCデータから入手できる医療情報の利活用の可能性および限界について検討した。

## Ⅱ 方法

### (1) 調査対象と方法

本研究では、一般社団法人診断群分類研究支援機構が実施する調査研究に協力しているDPC参加病院のデータを用いた。このデータは、病床数ベースで全DPC参加病院のデータの約9割をカバーしていると推定される<sup>8)</sup>。

本研究では、2014年1月から2015年12月までの2年間のデータを調査対象とした。2014年度は1,189施設(788万件)、2015年度は1,262施設(802万件)であった。これらのデータを用いて、記録されている病名(入院契機病名、資源病名、主病名、併存症、合併症のいずれかで、かつ疑い病名も含む)からミトコンドリア病に関連する病名の検索・抽出作業を実施した(図1)。また、本研究では、検索病名をもとにミトコンドリア病患者を5つの疾病グループ(以下、疾病グループ①~⑤)に分類した(表1)。

### (2) 統計手法

主要評価項目は、ミトコンドリア病の有病者数とした。この有病者数は、抽出したミトコンドリア病患者から重複する患者IDを同一患者とみなし、重複を除外したものを集計すること

図1 データ抽出の流れ

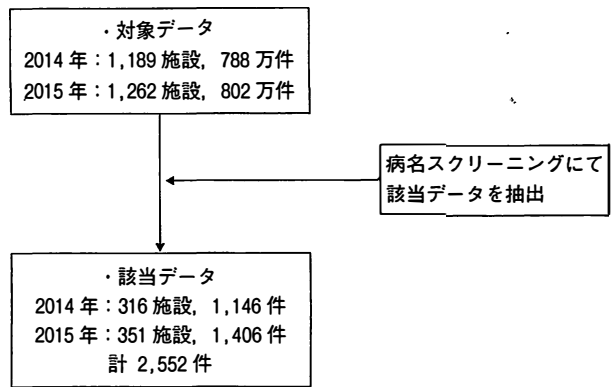


表1 各疾病グループの分類

疾病グループ	病名	病名抽出キーワード
①	MELAS* など†	MELAS, メラス, メラス症候群, 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
②	Leigh脳症	Leigh脳症, Leigh症候群, リー脳症, リー症候群, 亜急性壊死性脳症
③	CPEO/KSS‡	CPEO, 慢性進行性外眼筋麻痺, 慢性進行性外眼筋麻痺症候群, KSS, カーンズ・セイヤ症候群, Kearns-Sayre症候群
④	MERRF§	MERRF, マーフ, 赤色ぼろ線維・ミオクロヌスてんかん症候群
⑤	Leber病	Leber病, レーベル病, レーバー病, レーベル遺伝性視神経症

注 1) \*mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes  
 2) †一部に新生児/乳児ミトコンドリア病も含まれる。  
 3) ‡chronic progressive external ophthalmoplegia/Kearns-Sayre syndrome  
 4) §myoclonus epilepsy associated with ragged-red fibers

によって求めた。

また、副次評価項目としてミトコンドリア病患者の各種集計を行った。患者全体または疾病グループ別の患者数、性別、年齢階級、年齢(中央値)、在院日数(中央値)、ICD10分類別の入院契機病名、各変数(医療行為等)の集計を行った。また、DPCに収載されている患者所在地の郵便番号を用いて、都道府県別の入院患者数を集計した。また、入院中に死亡した患者数を、DPCにおける退院時転帰から求めた。さらに、これらの入院中に死亡した患者の性別、年齢階級、年齢(中央値)、在院日数(中央値)、ICD10分類別の入院契機病名の集計を行った。

統計ソフトは、Stata/IC 15.0 for Windows (StataCorp LLC)を使用した。

(3) 倫理的配慮

本研究は、産業医科大学倫理委員会にて承認を得て実施した(2018年10月10日, 第H30-124号)。

また、本文中の度数表記は、レセプト情報取り扱いにおける最小単位集計の申し合わせに基づいて、患者数10人未満の度数については“<10”の表記とした<sup>9)</sup>(ただし、患者数が0人の項目については、“0”と表記した)。さらに、10人以上であっても、その度数を表記することで合計値から10人未満の項目が推測可能である場合は、次に小さな度数を“<20”などの表記とした。

表2 疾病グループごとの各種集計 (n=1,386)

(単位 人, ( )内%)

	患者全体	疾病グループ				
		① MELASなど	② Leigh脳症	③ CPEO/KSS	④ MERRF	⑤ Leber病
患者数	1 386	1 282(93)	85( 6)	42( 3)	<10	<30
男性	664(48)	614(48)	35(41)	20(48)	<10	<20
女性	722(52)	668(52)	50(59)	22(52)	<10	<10
0歳	64( 5)	62( 5)	<10	0	0	0
1-5	162(12)	141(11)	30(35)	0	0	<10
6-9	78( 6)	67( 5)	15(18)	0	0	0
10-14	94( 7)	81( 6)	15(18)	0	0	<10
15-19	94( 7)	86( 7)	13(15)	<10	0	<10
20-29	139(10)	133(10)	<10	<10	0	<10
30-39	151(11)	142(11)	<10	<10	<10	<10
40-49	176(13)	169(13)	<10	<10	0	<10
50-59	151(11)	142(11)	0	<10	<10	<10
60-69	166(12)	158(12)	0	<10	<10	<10
70-79	89( 6)	82( 6)	0	<10	0	<10
80-	22( 2)	19( 1)	<10	<10	0	0
年齢, 歳: 中央値 (四分位範囲)						
	35(12-55)	35(13-55)	8(4-15)	54.5(41-72)	57(56-59)	35(13-55)
在院日数, 日: 中央値 (四分位範囲)						
	12(6-29)	13(6-29)	6(4-14)	20(5-41)	10(8-29)	19.5(5.5-35)

表3 ICD10分類における入院契機病名別の患者数集計 (n=1,386)

(単位 人, ( )内%)

章	分類ID	分類表記	患者数
1	A00-B99	感染症及び寄生虫症	35( 3)
2	C00-D48	新生物<腫瘍>	14( 1)
3	D50-D89	血液及び造血器の疾患並びに免疫機構の障害	<10
4	E00-E90	内分泌, 栄養及び代謝疾患	300(22)
5	F00-F99	精神及び行動の障害	<10
6	G00-G99	神経系の疾患	439(32)
7	H00-H59	眼及び付属器の疾患	55( 4)
8	H60-H95	耳及び乳様突起の疾患	13( 1)
9	I 00-I 99	循環器系の疾患	134(10)
10	J 00-J 99	呼吸器系の疾患	164(12)
11	K 00-K 99	消化器系の疾患	52( 4)
12	L 00-L 99	皮膚及び皮下組織の疾患	<10
13	M 00-M 99	筋骨格系及び結合組織の疾患	26( 2)
14	N 00-N 99	腎尿路生殖器系の疾患	31( 2)
15	O 00-O 99	妊娠, 分娩及び産じょく<褥>	<10
16	P 00-P 96	周産期に発生した病態	<10
17	Q 00-Q 99	先天奇形, 変形及び染色体異常	<10
18	R 00-R 99	症状, 徴候及び異常臨床所見・異常検査所見で他に分類されないもの	49( 4)
19	S 00-T 98	損傷, 中毒及びその他の外因の影響	32( 2)
20	V 01-Y 98	傷病及び死亡の外因	<10
21	Z 00-Z 99	健康状態に影響を及ぼす要因及び保健サービスの利用	<10
22	U 00-U 99	特殊目的用コード	<10

病名の検索・抽出作業の結果、2014年は316施設から1,146件、2015年は351施設から1,406件の計2,552件が該当した。この該当した2,552件から同一患者の複数入院を統合すると、有病者数は1,386人であった。

表2では、ミトコンドリア病患者の93%を疾病グループ①(MELASなど)が占め、6%の②(Leigh脳症)と3%の③(CPEO)がそれに続いた。性別は、疾病グループ②は女性が多く(男性35人, 女性50人), 性差が認められたが、他の疾病グループはほぼ均等に分布していた。年齢階級は、疾病グループ①は幅広い階級に分布していたが、②は比較的若年の階級(1歳から19歳まで)に分布する傾向が認められた。全患者の年齢は、中央値が35歳であった。また、疾病グループ②の年齢中央値は8歳であり、他の疾病グループと比較して若年であった。全患者の在院日数は、中央値が12日であった。また、疾病グループ②の在院日数の中央値は6日であり、ほかの疾病グループと比較して短い傾向があった。

全患者のうち、ICD10の分類G(神経系の疾患)が32%, 分類E(内分泌, 栄養及び代謝疾患)が22%と大きな割合を占めた(表3)。また、分類J(呼吸器系の疾患)が12%, 分類I(循環器系の疾患)が10%であり、それらに続いた。

表4 各変数における患者数集計 (n=1,386)

(単位 人, ( ) 内%)

変数名	患者数
生検(皮膚・筋)	95(7)
標本作成	157(11)
染色	26(2)
頭部MRI	0
MRIすべて	657(47)
ピルビン酸	561(40)
乳酸	678(49)
難病外来指導管理料	0
人工呼吸器導入時相談支援加算(難病外来指導管理料)	0
食事(指定難病)	0
在宅酸素	21(2)
在宅経管栄養	48(3)
経管栄養・薬剤投与用カテーテル交換法	27(2)
間歇的経管栄養法加算	0
鼻腔栄養	260(19)
在宅中心静脈栄養法指導管理料	<10
中心静脈設置	127(9)
在宅人工呼吸指導管理料	55(4)
準重症児	22(2)
重症児	23(2)
超重症児	45(3)
胃瘻造設術	25(2)
胃瘻より流動食点滴注入	67(5)
抗でんかん薬	436(31)
精神神経薬	332(24)
強心剤	524(38)
利尿剤	225(16)
血圧降下剤	245(18)
血管収縮剤	49(4)
重曹	86(6)
ウラリット	32(2)
アルギニン	76(5)
カルニチン	186(13)
タウリン	33(2)
ビタミン内服薬, 日	
内服なし	729(53)
1-4日	259(19)
5-9日	147(11)
10日以上	251(18)
ビタミン注射薬, 日	
注射なし	1 032(74)
1-4日	133(10)
5-9日	97(7)
10日以上	124(9)
輸血, 日	
輸血なし	1 320(95)
1-4日	57(4)
5日以上	<10
酸素吸入, 日	
吸入なし	1 066(77)
1-6日	215(16)
7-13日	54(4)
14日以上	51(4)
人工呼吸器, 日	
装着なし	1 166(84)
1-6日	89(6)
7-13日	44(3)
14日以上	87(6)
ICU入室, 日	
入室なし	1 332(96)
1-2日	23(2)
3-6日	12(1)
7日以上	19(1)

表5 患者の都道府県別分布 (n=1,386)

(単位 人, ( ) 内%)

	難病 <sup>1)</sup>	患者数
合計	1 481	1 386
北海道	46	63(5)
青森	18	11(1)
岩手	19	24(2)
宮城	22	23(2)
秋田	6	<10
山形	11	10(1)
福島	17	13(1)
茨城	35	36(3)
栃木	16	14(1)
群馬	22	19(1)
埼玉県	72	51(4)
千葉県	64	67(5)
東京都	166	120(9)
神奈川県	97	68(5)
新潟	30	17(1)
富山	18	<10
石川	14	14(1)
福井	16	10(1)
山梨	3	<10
長野	24	20(1)
岐阜	16	15(1)
静岡県	30	29(2)
愛知	66	68(5)
三重	14	15(1)
滋賀	25	18(1)
京都	36	34(2)
大阪	108	141(10)
兵庫県	62	52(4)
奈良	20	23(2)
和歌山	16	<10
鳥取	5	12(1)
島根	11	11(1)
岡山	15	26(2)
広島	36	43(3)
山口	14	10(1)
徳島	10	<10
香川	9	<10
愛媛	19	18(1)
高知	4	12(1)
福岡	61	60(4)
佐賀	8	18(1)
長崎	22	16(1)
熊本	19	31(2)
大宮	24	21(2)
分崎	27	16(1)
鹿児島	60	57(4)
沖縄	28	32(2)

注 1) 特定疾患(難病)医療受給者証所持者数(2015年度末, 文献10より)。参考のため記載。

続いて, 表4では高頻度に認められたものとして, MRI検査すべて(47%), ピルビン酸の

測定(40%), 乳酸の測定(49%)があった。また, 相対度数が10%を超えたものとして, 標

本作成 (11%), 鼻腔栄養 (19%), 抗てんかん薬 (31%), 精神神経薬 (24%), 強心剤 (38%), 利尿剤 (16%), 血圧降下剤 (18%), カルニチン (13%), ビタミン内服薬の1-4日内服 (19%) および5-9日内服 (11%) および10日以上内服 (18%), 酸素吸入の1-6日吸入 (16%) があった。

都道府県別の入院患者数は (表5), 頻度が高い順から大阪府 (141人), 東京都 (120人), 愛知県 (68人), 神奈川県 (68人), 千葉県 (67人) などであり, 大都市圏を中心に患者数が多い傾向が認められた<sup>10)</sup>。

入院中に死亡した患者数は83人であった (表6)。2014年の死亡患者数は43人であり, 2015年の死亡患者数は40人であった。死亡患者の性別は, やや女性が多く (男性36人, 女性47人), 性差が認められた。死亡患者の年齢階級は, 60-69歳が最も頻度が高く (24%), 50-59歳 (13%) と70-79歳 (13%) がそれに続いた。死亡患者の年齢中央値は, 52歳であった。死亡患者の在院日数中央値は, 28日であった。死亡患者のうち, ICD10分類G (神経系の疾患) が25%と最も大きな割合を占めていた。また, 分類I (循環器系の疾患) の22%と分類J (呼吸器系の疾患) の22%がそれに続いた。

#### IV 考 察

本研究では, ミトコンドリア病の有病者数は1,386人と推定された。参考として, 2014年末の特定疾患 (難病) 受給者証所持者数は1,439人<sup>11)</sup>, 小児慢性特定疾病登録者数は251人 (ミトコンドリアDNA変異による糖尿病 (3人), ミトコンドリア病 (37人), ミトコンドリア脳筋症 (211人) の合計)<sup>12)</sup>であり, 両者の合計である1,690人が, 難病等における申請書類集計によって推定される有病者数である。本研究の推定値の方が小さい (1,386 vs. 1,690) 原因の1つとして考えられるのは, 本研究に利用したデータのカバー率である (実質的には全病床数の約7割)。もう1つの原因は, 比較的重症度が軽度または病状が安定しているなどで, 外来

表6 死亡患者に関する各種集計 (n=83)

		(単位 人, ( ) 内%)	
患者数: 2014年		43	(52)
2015		40	(48)
男性		36	(43)
女性		47	(57)
0歳		<10	
1-5		<10	
6-9		<10	
10-14		<10	
15-19		<10	
20-29		<10	
30-39		<10	
40-49		<10	
50-59		11	(13)
60-69		20	(24)
70-79		11	(13)
80-		<10	
年齢, 歳: 中央値 (四分位範囲)		52	(21-67)
在院日数, 日: 中央値 (四分位範囲)		28	(11-67)
ICD分類別入院契機病名 <sup>1)</sup>			
章	分類ID	分類表記	
4	E00-E90	内分泌, 栄養及び代謝疾患	11 (13)
6	G00-G99	神経系の疾患	21 (25)
9	I00-I99	循環器系の疾患	18 (22)
10	J00-J99	呼吸器系の疾患	18 (22)

注 1) 患者数10人以上の項目のみを記載した。

受診のみでの加療を受けている患者を, 本研究のデータでは把握ができないためと考えられる。この2点において, 本研究の手法ではミトコンドリア病の有病者数が過小評価され得るという限界が示唆される。これらの問題への解決策として, NDB (National Database) を用いることが考えられる。NDBには外来患者の情報も記録されており, より悉皆性の高いミトコンドリア病の調査が可能であると考えられる。

また, 本研究では, 副次評価項目としてミトコンドリア病の入院患者の背景を探索した。DPCデータを用いれば, 疾病グループをはじめとした様々なカテゴリーを設定することで, 比較的容易に患者背景を分析することが可能であることが示唆された。これは, 臓器横断的な症状を呈し, 疾患特異的な検査所見や治療法が存在しないミトコンドリア病に関わらず, 他の多臓器症状を示す希少疾患の調査にも応用可能であると考えられる<sup>13)</sup>。

しかし, DPCデータに記録されている患者ID (正しくはデータ識別番号と呼ばれる) は, 医療機関の変更や転職・退職等における保険者の変更によって変化するため, 患者IDを厳密

な意味での同一個人識別に用いることは現時点では不可能であり、研究利用において大きな制約となっている。例えば、新生児／乳児ミトコンドリア病は、発症時期によって定義されるミトコンドリア病の分類である<sup>14)</sup>が、DPCの横断的なデータ特性では正確な有病者数の推定が困難である。今後の課題として、個人情報に十分配慮した上で、同一患者を長期間追跡することのできる不変の患者IDを割り付けるなどの対策が、研究への利活用の上で重要であると考えられる。

また、2015年1月における「難病の患者に対する医療等に関する法律（難病法）」の施行に伴い、指定難病の対象疾患数は、施行前は56疾患であったものが、2017年度末では330疾患にも増加している<sup>15)</sup>。緒言でも述べたが、全国規模の病院アンケート調査を疾患ごとに一から行うのは、人的・時間的・費用的に今後いっそう負担の大きいものとなっていくと予想される。DPCデータ等のビッグデータの利用体制の構築が推進されれば、これらを代替研究として用いるだけでなく、事前研究に用いてより正確な研究コストを予測し、高いコストが要求される介入研究などの実施可能性を検討することも可能になると考えられる。

## V 結 語

DPCデータを用いて、ミトコンドリア病の有病者数は推定可能であることが示唆された。また、DPCデータに記録されている患者の属性および各種医療行為を分析することで、一部ではあるがミトコンドリア病における医療の現状把握が可能であることが示唆された。

最後に、現状においてDPCデータをはじめとした電子レセプトデータの研究利用は、アクセスの容易さやデータの適切な運用において様々な制約や課題があるものの、今後も積極的に利活用を行うことは、患者・医療者双方に資するものであると考えられる。

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# Estimation of the Number of Patients With Mitochondrial Diseases: A Descriptive Study Using a Nationwide Database in Japan

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## ABSTRACT

**Background:** To provide a better healthcare system for patients with mitochondrial diseases, it is important to understand the basic epidemiology of these conditions, including the number of patients affected. However, little information about them has appeared in Japan to date.**Methods:** To gather data of patients with mitochondrial diseases, we estimated the number of patients with mitochondrial diseases from April 2018 through March 2019 using a national Japanese health care claims database, the National Database (NDB). Further, we calculated the prevalence of patients, and sex ratio, age class, and geographical distribution.**Results:** From April 2018 through March 2019, the number of patients with mitochondrial diseases was 3,629, and the prevalence was 2.9 (95% confidence interval [CI], 2.8–3.0) per 100,000 general population. The ratio of females and males was 53 to 47, and the most frequent age class was 40–49 years old. Tokyo had the greatest number of patients with mitochondrial diseases, at 477, whereas Yamanashi had the fewest, at 13. Kagoshima had the highest prevalence of patients with mitochondrial diseases, 8.4 (95% CI, 7.1–10.0) per 100,000 population, whereas Yamanashi had the lowest, 1.6 (95% CI, 0.8–2.7).**Conclusion:** The number of patients with mitochondrial diseases estimated by this study, 3,269, was more than double that indicated by the Japanese government. This result may imply that about half of all patients are overlooked for reasons such as low severity of illness, suggesting that the Japanese healthcare system needs to provide additional support for these patients.**Key words:** insurance claim review; Japan; medical records; mitochondrial diseases; prevalence

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## INTRODUCTION

Mitochondrial diseases are caused by nuclear or mitochondrial DNA mutations, and patients vary in age of onset, sex, race, affected organ, severity, and prognosis.<sup>1–3</sup> While mitochondrial diseases were long thought to be rare, recent reports<sup>4–19</sup> have suggested that the prevalence of patients in the general population is higher than originally thought. For example, prevalence is 23 per 100,000 general adult population in the northeast of England<sup>6</sup> and 5.7 per 100,000 general adult population in Spain.<sup>15</sup> While there are few reports of mitochondrial diseases in Japan,<sup>17,18</sup> a questionnaire study reported a prevalence of 0.18 per 100,000 general population.<sup>18</sup> However, this study was limited to mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), which comprise only a proportion of mitochondrial diseases. Thus, the prevalence of overall mitochondrial diseases in Japan remains unclear.

In Japan, mitochondrial disease was recognized as an intractable disease under the designation of the Japanese Ministry of Health, Labor, and Welfare (MHLW) in 2009, which brought

about various improvements in the treatment of these conditions. However, several studies have identified issues related to the treatment of mitochondrial diseases.<sup>20,21</sup> First, diagnosis requires a high degree of expertise. Definitive diagnosis requires an integrated approach comprising imaging, pathology, electrophysiology, genetics, and biochemical examinations, in addition to the main clinical manifestation, and the number of well-trained clinicians and hospitals equipped to perform these tests currently appears insufficient for patient needs. Second, recent developments in treatment have increased the survival of patients; in particular, the establishment of a system for transitional care from childhood to adulthood is suggested to be a major factor.<sup>21</sup> Third, the prevalence of some DNA mutations that cause mitochondrial diseases differs by geography.<sup>19</sup> This can make it difficult for patients to gain equal access to medical care, albeit that no reports of geography-related problems have appeared in Japan to date.

Accordingly, to ensure that policy makers make informed decisions and patients and their caregivers receive the best possible care, it is essential to gather basic epidemiological information on mitochondrial diseases, including the number of

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patients and their distribution by age, sex, and geographic location.

Here, we used a nationwide Japanese health claims database to estimate the number and other epidemiological parameters of patients with mitochondrial diseases.

## METHODS

### Data source and patient selection

We used the National Database (NDB), run by the Japanese MHLW,<sup>22,23</sup> to extract data on patients with mitochondrial diseases. The NDB contains information on almost all healthcare claims made from April 2009 in Japan, including patient sex; age class; numerical diagnosis code,<sup>24</sup> which is compatible with the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10); length of stay; costs; procedures; and prefecture where the hospital is located, among others. Data for both in- and outpatients were extracted from April 2009 through March 2019.

The MHLW permitted our use of the NDB. The study was approved by the Ethics Committee of Medical Research, University of Occupational and Environmental Health, Japan (approval number: H30-124).

### Definition of mitochondrial diseases

The following diagnoses were defined as mitochondrial diseases: Pearson syndrome (compatible ICD-10 code: D640); pyruvate dehydrogenase complex (PDHC) deficiency (E744); mitochondrial disorders (E888); MELAS (E888); myoclonus epilepsy associated with ragged-red fibers (MERRF) (E888); mitochondrial neurogastrointestinal encephalopathy (MNGIE) (E888);

mitochondrial cardiomyopathy (E888); mitochondrial hepatopathy (E888); mitochondrial diabetes (E888); Leigh syndrome (LS) (G318); Alpers' syndrome (G318); mitochondrial encephalomyopathy (G713); mitochondrial myopathy (G713); Leber's hereditary optic neuropathy (LHON) (H472); chronic progressive external ophthalmoplegia (CPEO) (H494); and Kearns-Sayre syndrome (KSS) (H498).

These definitions were determined by a well-trained physician and a researcher specializing in mitochondrial diseases. In sampling for this study, patients who had primary or other diagnostic positions were included, while suspected cases were excluded.

### Estimation of the number of patients

The primary outcome of this study was the number of patients with mitochondrial diseases from April 2018 through March 2019. We identified affected individuals using one of the unique identifiers generated by the MHLW and assigned to individuals in NDB. This identifier consists of the health insurance number, date of birth, and sex.

From this data, we also estimated the prevalence of mitochondrial diseases. As the severity of mitochondrial diseases varies widely among patients, and some patients undergo outpatient examination only, we limited our analysis to patients who experienced at least one episode of inpatient care as representative of standard cases requiring clinical intervention above a certain level.

We calculated the prevalence of mitochondrial diseases in Japan by dividing the number of patients with mitochondrial diseases from April 2018 through March 2019 by the total population of Japan as of October 1, 2018<sup>25</sup> as follows:

$$\text{prevalence (per 100,000 general population)} = \frac{(\text{number of patients from April 2018 to March 2019}) \times 100,000}{\text{general population on October 1, 2018}}$$

We calculated the prevalence of mitochondrial diseases in each prefecture in a similar manner. Further, 95% confidence intervals (CIs) of prevalence were calculated using the Wald method. We also used the Wald method to calculate the 95% CI of the ratio of female patients in Japan.

Additionally, we estimated the standardized prevalence ratio (SPR) of patients in each prefecture using indirect standardization. SPR is defined as follows:

$$SPR_i = \frac{O_i}{E_i} \times 100$$

$O_i$ : Observed number of patients in  $i$  prefecture,

$E_i$ : Expected number of patients in  $i$  prefecture

$$= \sum \left\{ \begin{array}{l} (\text{prevalence of patients in Japan by age class}) \\ \times (\text{population by age class in } i \text{ prefecture}) \end{array} \right\}$$

The SPR of  $i$  prefecture is obtained by dividing  $O_i$ , the observed number of patients, by  $E_i$ , the expected number of patients. To calculate  $E_i$ , we used the prevalence in Japan as a reference population, and adjusted for age categorized into three age classes (0–14, 15–64, or  $\geq 65$  years old). The 95% CIs of SPRs were estimated using Fisher's exact CI. For example, a prefecture with an SPR of 100 has the same prevalence as Japan overall, while one with an SPR smaller than 100 has a smaller prevalence than Japan, and vice versa.

We also calculated the empirical Bayes estimator of standardized prevalence ratio (EBSPR) of each prefecture. EBSPR is defined as follows:

$$EBSPR_i = \frac{O_i + \beta}{E_i + \alpha} \times 100$$

$\alpha, \beta$ : estimator

We estimated the EBSPR using a Poisson-Gamma model.<sup>26</sup> We expect that use of EBSPR should smooth out the influence of different population sizes in each prefecture on SPR.

Data analyses were performed using Stata 16.0 (StataCorp, College Station, TX, USA) and EB estimator for Poisson-Gamma model Version 2.1.<sup>27</sup>

## RESULTS

### Number of patients and prevalence of mitochondrial diseases

Within the study period, there were fewer male patients with mitochondrial diseases than female patients (47 vs 53; 95% CI for female ratio, 0.51–0.54), and the majority of patients fell within the age class 0–9 years old (Table 1). A total of 3,629 patients were diagnosed with mitochondrial diseases from April 2018 through March 2019, at a prevalence of 2.9 (95% CI, 2.8–3.0) per 100,000 general population.

**Table 1.** Patients' background ( $n = 3,629$ )<sup>a</sup>

	<i>n</i>	%
Sex, male, <i>n</i> (%)	1,712	47
Age class, years, <i>n</i> (%)		
0–4	315	9
5–9	333	9
10–14	233	6
15–19	244	7
20–29	352	10
30–39	432	12
40–49	496	14
50–59	431	12
60–69	377	10
70–79	302	8
80 over	114	3

<sup>a</sup>This table shows the number of patients with mitochondrial diseases from April 2018 to March 2019 identified in this study. Median age class was 30–39 years (interquartile range: 15–19, 50–59 years).

Table 2 lists the diagnosis codes and the corresponding number of patients. The majority of patients had diagnosis codes for mitochondrial encephalomyopathy and mitochondrial disorders (1,786 and 1,370, respectively).

We also compared the number of patients identified as having mitochondrial diseases in this study with the number using the Japanese medical expense subsidy system, as reported by the

government.<sup>28</sup> The number of patients identified in this study was more than two times greater than the number using the medical expense subsidy system (3,629 vs 1,504).

### Number of patients and prevalence of mitochondrial diseases in each prefecture

Table 3 and Figure 1 show the number of patients and prevalence of mitochondrial diseases in each prefecture from April 2018 through March 2019 in Japan. The prefecture with the greatest number of patients with mitochondrial diseases was Tokyo ( $n = 477/3,629$ , approx. 13%) while Yamanashi had the fewest ( $n = 13/3,629$ , approx. 1%). The prevalence of mitochondrial diseases was highest in Kagoshima (8.4/100,000) and lowest in Yamanashi (1.6/100,000).

We also compared the number of patients identified as having mitochondrial diseases in each prefecture in this study with the number using the Japanese medical expense subsidy system, as reported by the government.<sup>28</sup> The number of patients identified in this study was greater than the number using the medical expense subsidy system in all prefectures in Japan.

### SPR and EBSPR of patients with mitochondrial diseases in each prefecture

Table 4 shows the SPRs and EBSPRs of patients with mitochondrial diseases in each prefecture. Similar to the prevalence shown in Table 3, Kagoshima and Okinawa had the highest SPRs of all prefectures, at 294 and 152.3, respectively. In contrast, Yamanashi and Saitama had the lowest SPRs of all

**Table 2.** Number of patients with mitochondrial diseases with each diagnosis<sup>a</sup>

ICD-10	Diagnosis code	Diagnosis	Total		Female	
			<i>n</i>	%	<i>n</i>	%
D640	8846217	Pearson syndrome	—	—	—	—
E744	8848412	PDHC deficiency	81	2	56	69
E888	8845613	Mitochondrial disorders	1,370	38	750	55
E888	8846079	MELAS	284	8	159	56
E888	8846080	MERRF	15	1	—	—
E888	8846084	MNGIE	—	—	—	—
E888	8846224	Mitochondrial cardiomyopathy	174	5	86	49
E888	8846972	Mitochondrial hepatopathy	32	1	15	47
E888	8849469	Mitochondrial diabetes	62	2	39	63
E888	8849470	Mitochondrial diabetes with eye problems	—	—	—	—
E888	8849471	Mitochondrial diabetes with ketoacidosis	—	—	—	—
E888	8849472	Mitochondrial diabetes with coma	—	—	—	—
E888	8849473	Mitochondrial diabetes with neurologic symptom	—	—	—	—
E888	8849474	Mitochondrial diabetes with renal complication	—	—	—	—
E888	8849475	Mitochondrial diabetes with multiple diabetic complications	—	—	—	—
E888	8849476	Mitochondrial diabetes without diabetic complication	—	—	—	—
E888	8849477	Mitochondrial diabetes with diabetic complication	—	—	—	—
E888	8849478	Mitochondrial diabetes with peripheral circulatory disorder	—	—	—	—
G318	8840933	Leigh syndrome	212	6	108	51
G318	8842457	Alpers' syndrome	—	—	—	—
G713	8841409	Mitochondrial myopathy	253	7	124	49
G713	8841410	Mitochondrial encephalomyopathy	1,786	49	932	52
H472	8848684	LHON	71	2	17	24
H494	8846059	CPEO	106	3	50	47
H498	8831018	Kearns-Sayre syndrome	19	1	10	53

CPEO, chronic progressive external ophthalmoplegia; ICD-10, International Statistical Classification of Diseases and Related Health Problems 10th Revision; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonus epilepsy associated with ragged-red fibers; MNGIE, mitochondrial neurogastrointestinal encephalopathy; PDHC, pyruvate dehydrogenase complex.

<sup>a</sup>This table shows the number of patients with mitochondrial diseases with each diagnosis categorized by domestic diagnosis codes for healthcare claims in Japan. According to the rules for publication of NDB data, we did not show the number of cases in categories with less than 10 patients (indicated by “—” in the table). The sum of patients is not equal to the total number of patients because some patients are given two or more diagnoses.

**Table 3.** Number of patients and prevalence of mitochondrial diseases in each prefecture in Japan from April 2018 to March 2019<sup>a</sup>

Prefecture	NDB				Government report			
	<i>n</i>	%	Prevalence	95% CI	<i>n</i>	%	Prevalence	95% CI
Hokkaido	169	5	3.2	2.7–3.7	62	4	1.2	0.9–1.5
Aomori	22	1	1.7	1.1–2.6	11	1	0.9	0.4–1.6
Iwate	43	1	3.5	2.5–4.7	18	1	1.5	0.9–2.3
Miyagi	79	2	3.4	2.7–4.3	29	2	1.3	0.8–1.8
Akita	19	1	1.9	1.2–3	8	1	0.8	0.4–1.6
Yamagata	29	1	2.7	1.8–3.8	13	1	1.2	0.6–2.0
Fukushima	33	1	1.8	1.2–2.5	20	1	1.1	0.7–1.7
Ibaraki	87	2	3.0	2.4–3.7	42	3	1.5	1.1–2.0
Tochigi	56	2	2.9	2.2–3.7	21	1	1.1	0.7–1.7
Gumma	54	1	2.8	2.1–3.6	26	2	1.3	0.9–2.0
Saitama	125	3	1.7	1.4–2	74	5	1.0	0.8–1.3
Chiba	162	4	2.6	2.2–3	64	4	1.0	0.8–1.3
Tokyo	477	13	3.5	3.2–3.8	182	12	1.3	1.1–1.5
Kanagawa	218	6	2.4	2.1–2.7	102	7	1.1	0.9–1.4
Niigata	56	2	2.5	1.9–3.2	28	2	1.2	0.8–1.8
Toyama	30	1	2.9	1.9–4.1	14	1	1.3	0.7–2.2
Ishikawa	36	1	3.1	2.2–4.4	15	1	1.3	0.7–2.2
Fukui	21	1	2.7	1.7–4.2	12	1	1.6	0.8–2.7
Yamanashi	13	1	1.6	0.8–2.7	1	1	0.1	0.1–6.8
Nagano	52	1	2.5	1.9–3.3	22	1	1.1	0.7–1.6
Gifu	44	1	2.2	1.6–3	16	1	0.8	0.5–1.3
Shizuoka	86	2	2.4	1.9–2.9	31	2	0.8	0.6–1.2
Aichi	174	5	2.3	2–2.7	52	3	0.7	0.5–0.9
Mie	39	1	2.2	1.6–3	11	1	0.6	0.3–1.1
Shiga	53	1	3.8	2.8–4.9	20	1	1.4	0.9–2.2
Kyoto	86	2	3.3	2.7–4.1	36	2	1.4	1.0–1.9
Osaka	263	7	3.0	2.6–3.4	115	8	1.3	1.1–1.6
Hyogo	162	4	3.0	2.5–3.5	63	4	1.1	0.9–1.5
Nara	44	1	3.3	2.4–4.4	19	1	1.4	0.9–2.2
Wakayama	29	1	3.1	2.1–4.5	15	1	1.6	0.9–2.7
Tottori	15	1	2.7	1.5–4.4	5	1	0.9	0.3–2.1
Shimane	22	1	3.2	2–4.9	13	1	1.9	1.0–3.3
Okayama	55	2	2.9	2.2–3.8	13	1	0.7	0.4–1.2
Hiroshima	73	2	2.6	2–3.3	34	2	1.2	0.8–1.7
Yamaguchi	24	1	1.8	1.1–2.6	13	1	0.9	0.5–1.6
Tokushima	16	1	2.2	1.2–3.5	7	1	1.0	0.4–2.0
Kagawa	19	1	2.0	1.2–3.1	10	1	1.0	0.5–1.9
Ehime	42	1	3.1	2.2–4.2	17	1	1.3	0.7–2.0
Kochi	18	1	2.5	1.5–4	3	1	0.4	0.1–1.2
Fukuoka	183	5	3.6	3.1–4.1	58	4	1.1	0.9–1.5
Saga	23	1	2.8	1.8–4.2	12	1	1.5	0.8–2.6
Nagasaki	50	1	3.7	2.8–4.9	26	2	1.9	1.3–2.8
Kumamoto	45	1	2.6	1.9–3.4	27	2	1.5	1.0–2.2
Oita	39	1	3.4	2.4–4.7	23	2	2.0	1.3–3.0
Miyazaki	40	1	3.7	2.6–5	17	1	1.6	0.9–2.5
Kagoshima	136	4	8.4	7.1–10	59	4	3.7	2.8–4.7
Okinawa	68	2	4.7	3.7–6	25	2	1.7	1.1–2.6
Japan	3,629		2.9	2.8–3	1,504		1.2	1.1–1.3

CI, confidence interval; NDB, National Database.

<sup>a</sup>This table shows the number of patients and the prevalence of mitochondrial diseases from April 2018 to March 2019 identified in this study. The same parameters reported by the government based on the number of patients using the Japanese medical expenses subsidy for intractable diseases including mitochondrial diseases are shown as a reference. Prefectures are listed according to their geographic location from north-east to south-west. % values do not sum to 100% because of rounding. Prevalence is prevalence per 100,000 population in each prefecture.

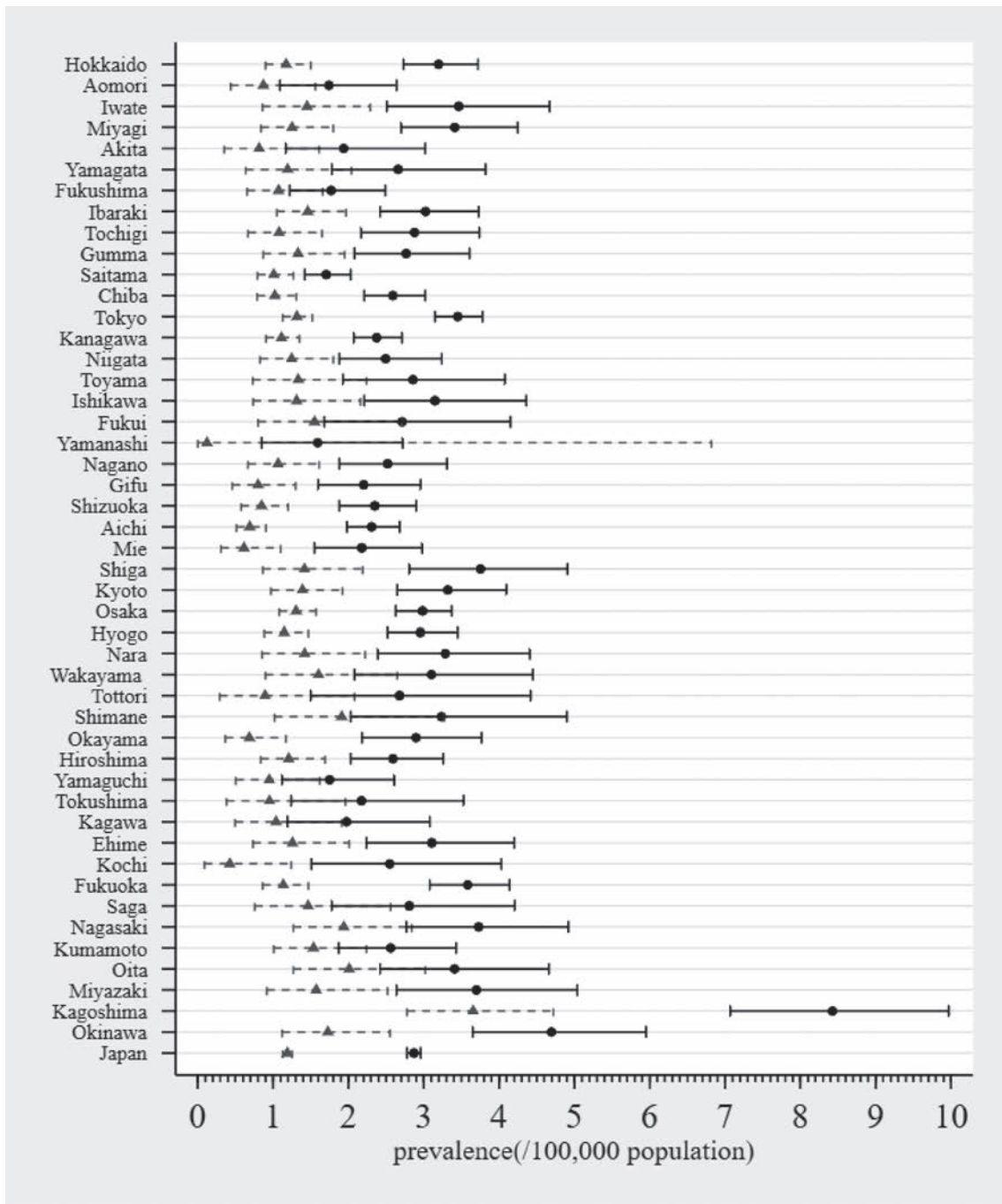
prefectures, at 56 and 59, respectively. Although it is important to consider the effect of population size in each prefecture, in Ishikawa and Okinawa, there were large differences in SPR by sex. The SPRs of female and male patients were 75 and 148.8 in Ishikawa, and 183.6 and 117.7 in Okinawa, respectively.

EBSPRs in each prefecture provided more conservative results than normal SPRs, indicating that values were reaching closer to 100. Similar to results for normal SPRs, Kagoshima and Okinawa

had the highest EBSPRs of all prefectures, at 247.7 and 139.8, respectively. In contrast, Saitama and Fukushima had the lowest EBSPRs of all prefectures, at 61.8 and 71.3, respectively.

## DISCUSSION

Using data from the Japanese NDB, we estimated that the number of patients with mitochondrial diseases from April 2018 through



**Figure 1.** Estimated prevalence of mitochondrial diseases in each prefecture in Japan according to the NDB and government report. Prefectures in this figure are listed according to their geographic location from north-east to south-west. Black points represent the prevalence estimated by this study; solid black lines with caps on both ends represent 95% confidence intervals of the prevalence estimated by this study; grey triangles represent the prevalence indicated by the Japanese government; grey dashed lines with caps on both ends represent 95% confidence intervals of the prevalence indicated by the Japanese government.

March 2019 was 3,629, with a prevalence of 2.9 per 100,000 general population. This study is the first to comprehensively estimate the number of patients with mitochondrial diseases in Japan, along with the distribution of patients by sex, age, and geographic characteristics using health care claims data from the past 10 years.

The Japanese government has established a medical expense subsidy system for patients with intractable diseases, including mitochondrial diseases. According to government statistics from

2018,<sup>28</sup> 1,504 patients with mitochondrial diseases used this system. This number is less than half the number of patients with mitochondrial diseases identified in this study ( $n = 3,629$ ). Similarly, the number and prevalence of patients in each prefecture identified as having mitochondrial diseases in this study were also greater than those using the subsidy system. However, it may not be appropriate to compare the number of patients identified in the present study with that in the government report. This is because, while certification for the government

**Table 4.** SPRs and EBSPRs of mitochondrial diseases in each prefecture in Japan from April 2018 to March 2019<sup>a</sup>

Prefecture	Total				Female				Male			
	<i>n</i>	SPR	95% CI	EBSPR	<i>n</i>	SPR	95% CI	EBSPR	<i>n</i>	SPR	95% CI	EBSPR
Hokkaido	169	114.3	97.7–132.9	113.2	96	119.7	96.9–146.1	116.9	73	107.6	84.3–135.3	106.6
Aomori	22	62.6	39.3–94.8	74.2	—	—	—	—	—	—	—	—
Iwate	43	124.1	89.8–167.1	117.4	23	125.1	79.3–187.8	114.7	20	122.8	75–189.6	112.5
Miyagi	79	119	94.3–148.4	115.9	43	123.0	89–165.6	116.6	36	114.5	80.2–158.5	110.3
Akita	19	71.4	43–111.5	82.2	—	—	—	—	—	—	—	—
Yamagata	29	95	63.6–136.4	97.2	15	92.4	51.7–152.4	96.5	14	97.7	53.4–163.9	100.1
Fukushima	33	62.7	43.2–88.1	71.3	18	66.0	39.1–104.2	78.0	15	59.4	33.3–98	76.1
Ibaraki	87	105.7	84.7–130.4	105.1	39	91.7	65.2–125.3	94.0	48	121.1	89.3–160.5	115.6
Tochigi	56	100.1	75.6–130	100.4	29	100.1	67–143.8	100.4	27	100.3	66.1–145.9	101
Gumma	54	96.9	72.8–126.5	97.9	28	96.6	64.2–139.6	98.1	26	97.6	63.8–143	99.3
Saitama	125	59	49.1–70.3	61.8	67	61.1	47.4–77.6	65.7	58	57	43.3–73.6	63.2
Chiba	162	90.2	76.9–105.3	91.1	76	81.4	64.1–101.8	84.0	86	100.1	80–123.6	100.4
Tokyo	477	119	108.6–130.2	118.4	258	122.7	108.2–138.6	121.3	219	115.1	100.3–131.4	114.1
Kanagawa	218	81.9	71.4–93.5	82.9	115	83.4	68.8–100	85.0	103	80.4	65.7–97.6	82.9
Niigata	56	88.7	67–115.2	91.2	31	93.0	63.2–132	95.4	25	84	54.3–124	90.4
Toyama	30	101.6	68.5–145	101.7	13	83.5	44.5–142.9	91.9	17	121.8	70.9–194.9	111.3
Ishikawa	36	109.8	76.9–152	107.3	13	75.0	39.9–128.2	86.7	23	148.8	94.3–223.3	125
Fukui	21	94.8	58.6–144.8	97.6	11	94.1	47–168.4	97.9	10	95.5	45.8–175.6	99.6
Yamanashi	13	56	29.8–95.8	73.8	—	—	—	—	—	—	—	—
Nagano	52	88.8	66.3–116.5	91.4	35	113.7	79.2–158.2	109.8	17	61.3	35.7–98.2	76.4
Gifu	44	76.9	55.9–103.3	82.0	25	82.6	53.4–121.9	88.5	19	70.6	42.5–110.2	82.4
Shizuoka	86	82.1	65.7–101.4	84.5	48	87.8	64.7–116.4	90.5	38	76	53.8–104.3	82.4
Aichi	174	78.6	67.3–91.2	80.0	97	84.8	68.8–103.5	86.6	77	72	56.8–90	76
Mie	39	76.1	54.1–104	81.8	19	70.4	42.4–109.9	81.0	20	82.5	50.4–127.5	90.4
Shiga	53	127.3	95.4–166.5	120.7	28	128.8	85.6–186.1	117.8	25	125.8	81.4–185.7	115.2
Kyoto	86	116.5	93.2–143.9	114.1	40	101.0	72.2–137.5	101.0	46	134.2	98.2–179	124
Osaka	263	103.9	91.7–117.3	103.8	136	100.4	84.2–118.8	100.5	127	107.8	89.9–128.3	107.1
Hyogo	162	102.9	87.7–120	102.8	77	90.9	71.8–113.6	92.4	85	116.6	93.1–144.1	114
Nara	44	116.1	84.4–155.9	112.1	19	92.0	55.4–143.6	95.7	25	144	93.2–212.5	123.9
Wakayama	29	110.6	74.1–158.9	107.5	15	105.6	59.1–174.2	103.4	14	116.3	63.6–195.2	108.2
Tottori	15	94	52.6–155	97.7	—	—	—	—	—	—	—	—
Shimane	22	115	72.1–174.1	109.3	10	98.9	47.5–182	100.2	12	133.5	69–233.2	113.4
Okayama	55	101.3	76.3–131.8	101.4	33	114.2	78.6–160.4	109.9	22	86.5	54.2–130.9	92.6
Hiroshima	73	89.9	70.5–113.1	91.7	34	79.2	54.8–110.7	84.6	39	101.9	72.4–139.3	102
Yamaguchi	24	62.7	40.2–93.3	73.5	11	53.4	26.7–95.6	72.9	13	73.2	39–125.1	87
Tokushima	16	77.9	44.6–126.6	87.9	—	—	—	—	—	—	—	—
Kagawa	19	69.6	41.9–108.7	80.8	—	—	—	—	—	—	—	—
Ehime	42	110.4	79.6–149.2	108.0	23	112.2	71.1–168.3	107.7	19	108.3	65.2–169	105.4
Kochi	18	92.1	54.6–145.6	96.3	—	—	—	—	—	—	—	—
Fukuoka	183	123.3	106.1–142.5	121.4	99	123.5	100.4–150.4	120.2	84	122.7	97.9–151.9	118.8
Saga	23	96.9	61.4–145.4	98.8	12	94.2	48.7–164.5	97.8	11	100.6	50.2–180	101.6
Nagasaki	50	131.2	97.4–172.9	123.0	25	121.2	78.5–179	113.0	25	142.5	92.2–210.3	123.2
Kumamoto	45	89	64.9–119.1	91.9	20	73.2	44.7–113	82.7	25	107.4	69.5–158.5	105.3
Oita	39	120.7	85.8–164.9	114.8	25	143.6	92.9–212	124.5	14	93.6	51.2–157.2	98.1
Miyazaki	40	129.1	92.2–175.8	120.3	19	113.2	68.2–176.8	107.7	21	147.5	91.3–225.4	123.4
Kagoshima	136	294	246.6–347.7	247.7	79	314.2	248.8–391.6	237.3	57	268	203–347.3	196.5
Okinawa	68	152.3	118.3–193.1	139.8	43	183.6	132.9–247.3	152.4	25	117.7	76.2–173.8	111
Japan	3,629	100	96.8–103.3	100	1,917	100.0	95.6–104.6	100	1,712	100	95.3–104.9	100

CI, confidence interval of SPR; EBSPR, empirical Bayes estimator of standardized prevalence ratio; SPR, standardized prevalence ratio.

<sup>a</sup>This table shows SPRs and EBSPRs of patients with mitochondrial diseases in each prefecture from April 2018 to March 2019 identified in this study. SPRs and EBSPRs were calculated for total, female and male patients. According to the rules for publication of NDB data, we did not show the number of cases in categories with less than 10 patients (indicated by “—” in the table). Values were also concealed when the number of either of males or females was less than 10.

subsidy system is typically based on Japanese clinical criteria,<sup>29</sup> some patients with relatively mild disease severity may not use this system, whose main purpose is to provide treatment-related financial support to patients. Therefore, it may be that the government-reported number of patients will inevitably be an underestimate compared to that identified in the present study.

A previous study in Japan reported that 233 patients had MELAS, with a prevalence of 0.18 (95% CI, 0.17–0.19) per

100,000 general population.<sup>18</sup> In this study, we identified 284 patients with MELAS, with a prevalence of 0.22 (95% CI, 0.20–0.25) per 100,000 general population. Therefore, the number of patients and prevalence of MELAS identified in this study are comparable to those of the previous study. We expect that this epidemiological information will contribute to improving the health care system for patients with mitochondrial diseases in Japan.

We found that prevalence of mitochondrial diseases differed among prefectures. While the reasons for this are unclear, previous studies from other countries suggest that prevalence may differ by geography.<sup>4,6,11,19</sup> For example, a study conducted in the northeast of England reported that patients showing clinical manifestations had a prevalence of 9.6 per 100,000 adult general population,<sup>6</sup> which is about three times higher than that found in this study (2.9 per 100,000 general population). Moreover, a study in Finland suggested that geographical and cultural isolation may cause differences in prevalence.<sup>11,19</sup> Furthermore, a study in Australia showed that the prevalence of mitochondrial diseases among children whose mothers were born in Lebanon is much higher than that for mothers born in other countries.<sup>12</sup> However, we were careful when comparing results obtained using different methods for two main reasons. First, prevalence estimated by a single expert clinical and laboratory referral center can be a greater overestimation than that determined by a study using national health database with large samples. Second, patients extracted from NDB differ in diagnosis period. This difference may affect the number of patients between studies because clinical criteria and the diagnostic methods applied depend on the diagnosis period.<sup>30</sup>

To our knowledge, however, no meaningful report from Asian countries has appeared to date. Further studies are needed to examine the prevalence of mitochondrial diseases in Asia and to compare the results obtained in this study with those in other Asian countries.

We found differences in the prevalence of mitochondrial diseases among prefectures, despite the fact that the genetic background of the Japanese population is thought to be relatively uniform across the country. In addition to genetic factors, the variability in prevalence may be related to differences in health care infrastructure among prefectures in Japan. That is, there may be a concentration of patients in specific medical facilities that are well equipped for diagnosis and treatment. For example, within the Tokyo metropolitan area, the number of patients and prevalence of mitochondrial diseases in Saitama (125, 1.7/100,000), a densely populated prefecture with a population of seven million, was lower than in other large prefectures like Chiba (162, 2.6/100,000) and Kanagawa (218, 2.4/100,000), which are also adjacent to Tokyo. We speculate that there are two main reasons for this. First, there are fewer physicians and specialists per prefecture population in Saitama than Chiba and Kanagawa. Additionally, there are few large hospitals with the ability to provide care for patients with mitochondrial diseases. Second, residents of Saitama have easy access to large hospitals in Tokyo. A similar phenomenon is thought to be occurring in local cities outside the metropolitan area. To improve the provision of health care for patients with mitochondrial diseases, we suggest that, in addition to training healthcare workers and improving clinical guidelines, there is a need to increase the number of medical facilities with the competency to provide care for patients with mitochondrial diseases. Additionally, geographic factors may also be important. We found that patients in Kagoshima and Okinawa had higher prevalence, SPRs, and EBSPRs than all other prefectures. While the reason for the higher values is unclear, these findings suggest that there may be geographic effects because Kagoshima and Okinawa are located in the southernmost part of Japan.

This study has several limitations. First, it is possible that some patients included in this study did not actually have mitochondrial

diseases. While a definitive diagnosis of mitochondrial diseases requires an integrated approach, including genetic testing, some of these tests are not covered by the current health insurance system in Japan, and the number of medical facilities with the capacity to perform them is limited. Therefore, it is difficult to determine whether the diagnoses used in this study are definitive of mitochondrial diseases. However, we expect that few patients would have been misdiagnosed because, unlike common or frequent diseases, mitochondrial diseases are carefully and strictly diagnosed by clinicians. Second, due to the nature of the NDB, patients can be duplicated if they or their caregivers change their health insurance scheme due to a job change, unemployment, or employment, thus leading to a potential overestimation of cases. A previous study using the NDB examined the effects of changing health insurance schemes on the estimation of prevalence.<sup>31</sup> Using the method described in this previous study,<sup>31</sup> we calculated the maximum impact of changing health insurance schemes to be about 6.6% by summing the proportion of patients who were newly unemployed within a year (1.7%) and the proportion who underwent a job change (4.9%) in 2018.<sup>32</sup> Therefore, the effect of changing health insurance schemes is likely relatively small. Third, information on patients who receive public assistance is not included in the NDB. The proportion of people who received public assistance in Japan was about 1.65% in February 2019.<sup>33</sup> Thus, this is expected to have caused only a small underestimation. Despite the above-mentioned limitations, our study provides a valid estimation of the number of patients and prevalence of mitochondrial diseases in Japan.

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## Article

# Long-Term Progression and Rapid Decline in Hearing Loss in Patients with a Point Mutation at Nucleotide 3243 of the Mitochondrial DNA

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**Abstract:** Patients with m.3243A>G mutation of mitochondrial DNA develop bilaterally symmetric sensorineural hearing loss. However, it is unclear how fast their hearing loss progresses over time, and whether they experience rapid progression of hearing loss. In the present study, we conducted a long-term hearing evaluation in patients with MELAS or MIDD who harbored the m.3243A>G mutation of mitochondrial DNA. A retrospective chart review was performed on 15 patients with this mutation who underwent pure-tone audiometry at least once a year for more than two years. The mean follow-up period was 12.8 years. The mean progression rate of hearing loss was 5.5 dB per year. Hearing loss progressed rapidly to be profoundly deaf in seven patients during the observation period. Heteroplasmy and age-corrected heteroplasmy levels correlated with the age of onset of hearing loss. These results indicate that patients with m.3243A>G mutation have a gradual progression of hearing loss in the early stages and rapid decline in hearing to be profoundly deaf in approximately half of the patients. Although it is possible to predict the age of onset of hearing loss from heteroplasmy and age-corrected heteroplasmy levels, it is difficult to predict whether and when the rapid hearing loss will occur.

**Keywords:** hearing loss; dizziness; disequilibrium; diabetes; mitochondrial gene mutations



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## 1. Introduction

Sensorineural hearing loss is frequently associated with mitochondrial disease; it is observed in approximately half of the patients with three main syndromes—chronic progressive external ophthalmoplegia (CPEO); myoclonus epilepsy associated with ragged-red fibers (MERRF); mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) [1]. Hearing loss mainly involves the cochlea in mitochondrial diseases, but it sometimes accompanies central auditory abnormalities [1–5].

Mitochondrial diseases are categorized into two groups: those characterized by ragged-red fibers (RRF), including MELAS, MERRF, and CPEO, and those caused by mutations in protein-coding genes, such as pure encephalopathy without RRF. RRF is a characteristic microscopic appearance of the muscle stained with Gomori trichrome, which is due to the accumulation of abnormal mitochondria below the plasma membrane of the muscle fiber. Hearing loss is highly prevalent in the first group but rarely seen in the second group. Oxidative phosphorylation is impaired in both types of encephalopathy; however, mitochondrial protein synthesis is impaired only in the first group, suggesting that hearing loss is caused by impairment of protein synthesis similar to RRF.

An A-to-g transition mutation at nucleotide pair (np) 3243 in mitochondrial DNA (mtDNA) has been documented in most patients with MELAS and a few patients with CPEO. This mutation has been identified in pedigrees with maternal transmission of diabetes and deafness among families of different racial backgrounds [6–10]. It has been reported that patients with higher heteroplasmy levels of this point mutation exhibit MELAS [11,12], while those with low heteroplasmy levels mainly develop hearing loss and diabetes; those with a low heteroplasmy level have maternally inherited diabetes and deafness (MIDD) [9].

In patients with MELAS and MIDD, hearing is generally normal at birth, and hearing loss occurs eventually, appearing as early as in the teens or twenties and as late as the fifties. Hearing loss, in general, is bilateral and symmetrical; a pure-tone audiogram shows a flat type or falling type of sensorineural loss bilaterally in most cases [6,9]. Hearing loss mainly involves the cochlea [6,13,14]; however, when the disease progresses, retro-labyrinthine and central auditory pathways are also affected [15,16]. In addition to hearing loss, the vestibular system is also involved, and patients eventually suffer from impairment of balance and gait [6,17–19].

We previously reported that, when present for several years, patients with np3243 point mutation showed a slightly progressive decline in their hearing; the progression rate of hearing loss ranged from 1.5 to 7.9 dB per year [7]. However, it is unknown how rapidly their hearing loss progresses during the long-term period, and whether there is a rapid progression of hearing loss. Therefore, in the present study, we evaluated their hearing in the long-term period in patients with MELAS or MIDD who harbored m.3243A>G mutation of mtDNA. We also investigated the relationship between heteroplasmy and age-corrected heteroplasmy levels and the age of onset of hearing loss and the progression rate of hearing loss, as well as the relationship between the age of onset of hearing loss, diabetes, and balance disorder.

## 2. Materials and Methods

### 2.1. Patients

In total, 27 patients with hearing loss and an A-to-g transition at np 3243 in the mtDNA visited the Department of Otolaryngology and Head and Neck Surgery, at the University of Tokyo Hospital, between 1989 and 2021. Among them, 15 patients who underwent audiological examinations for more than two years or until they became completely deaf were enrolled. The patients consisted of four males and eleven females, and their ages ranged from 22 to 66 years (mean: 40 years) at their first visit to our clinic. All patients had hearing loss, with a pure-tone average (PTA) value being greater than 25 dB HL (hearing level) on the initial audiological examination. They were interviewed regarding the onset of hearing loss, balance–gait disorder, diabetes mellitus, and the presence of other signs and symptoms. The presence of diabetes mellitus was confirmed by doctors in the Department of Nutrition and Metabolism of our hospital or their affiliated hospital, and the blood glucose and HbA1c levels were periodically measured in all patients. All procedures were in accordance with the Helsinki declaration and were approved by the University of Tokyo Human Ethics Committee (No. 2487). All patients gave informed consent for the use of their clinical data.

### 2.2. Audiological and Neuro-Otological Evaluation

The patients were evaluated using pure-tone audiometry, in general, every three months, to assess the longitudinal changes in their hearing thresholds. The pure-tone average (PTA) values were calculated as the mean air conduction threshold at 0.5, 1, 2, and 3 kHz. The pure-tone threshold at 3 kHz was obtained from the mean of 2 and 4 kHz, as it is not routinely measured in Japan. For calculating the PTA values, the hearing thresholds at the frequencies showing off-scale were calculated as 5 dB over the maximum sound level generated by an audiometer. The patients were also evaluated using speech

recognition test, tympanometry, acoustic reflex threshold test, auditory brainstem response, and distortion-product otoacoustic emissions (DPOAEs), when necessary.

The patients also underwent a battery of neuro-otological evaluations consisting of a physical examination, neurological examination, and neuro-otological examinations, including caloric and cervical vestibular evoked myogenic potential (cVEMP) testing.

Caloric testing was performed with 2 mL ice water irrigation of the external auditory canal for 20 s. This caloric stimulation method is easier to perform than bithermal irrigation and has high sensitivity and specificity for detecting canal paresis [20]. Electronystagmography was used to record the induced nystagmus while the subject lay supine with their head raised at an angle of 30 degrees. The percentage of canal paresis was calculated as  $100 \times |(MSEV_r - MSEV_l)/(MSEV_r + MSEV_l)|$ , where  $MSEV_r$  is the maximum slow-phase eye velocity of the right side, and  $MSEV_l$  is that of the left side. A value of canal paresis >20% was regarded as abnormally reduced on the affected side [21]. When  $MSEV_r$  and  $MSEV_l$  were <10 degrees/s, it was regarded as a reduced response on both sides [22].

In the role of a stimulus for cVEMP, short tone bursts of 500 Hz (95 dB normal hearing level; 135 dB SPL (peak value); rise/fall time, 1 ms; plateau time, 2 ms) were presented through headphones (type DR-531; Elega Acoustic Ltd., Tokyo, Japan). Surface electromyographic activity was recorded in supine patients from symmetrical sites over the upper half of each sternocleidomastoid muscle (SCM), with a reference electrode on the lateral end of the upper sternum. During recording, the patients were instructed to raise their heads slightly to continuously contract the SCM. Background EMG was monitored during recording to confirm that subjects maintained SCM activity at a sufficient level (150  $\mu$ V). We analyzed the first biphasic wave (p13-n23) from the ipsilateral SCM to the stimulated side. For the evaluation of amplitude. The percentage of cVEMP asymmetry ratio (cVEMP AR) was calculated as  $100|(Ar - Al)/(Ar + Al)|$ , where  $Ar$  is the amplitude of p13-n23 on the right side, and  $Al$  is the amplitude of the p13-n23 on the left side. On the basis of results from normal subjects, the upper limit of cVEMP AR was set to 34.0 [23]. We regarded it as an “absent” response when no reproducible p13-n23 was present in two runs. We regarded it as a “reduced” response when a reproducible p13-n23 was present, and the AR was greater than the predefined upper limit for normal subjects. We regarded it as bilaterally abnormal responses when the response was absent on both sides.

### 2.3. DNA Studies

The invader assay contract by BML (Tokyo, Japan) was applied for the screening of mitochondrial tRNA (Leu). Briefly, mtDNA isolated from the peripheral leukocytes of patients and 1.2  $\mu$ L of primary probe/invader oligonucleotides mixture (containing 0.5  $\mu$ mol/L wild-type primary probes, 0.5  $\mu$ mol/L mutant primary probes, 0.05  $\mu$ mol/L invader oligonucleotide, and 10 mmol/L 3-(N-morpholino) propanesulfonic acid) were poured into each well of the plates. Fluorescent resonance energy transfer (FRET)/Cleavase mixture (Hologic, Marlborough, MA, USA) was added to the probe/invader oligonucleotide-containing plates. Subsequently, 3  $\mu$ L of 5–100 mol/L synthetic target oligonucleotides (positive control), 10  $\mu$ g/mL yeast tRNA (no target control), and denatured genomic DNA samples (>15 ng/ $\mu$ L) were added. Next, 6  $\mu$ L of mineral oil (Sigma, St. Louis, MO, USA) was overlaid into all reaction wells and incubated at 63 °C for 4 h. After incubation, the fluorescence was measured using a Cyto Fluor 4000 fluorescent microplate reader. The heteroplasmy rate for mitochondrial mutations was quantified by the detection of fluorescently labeled and digested PCR products through a fluorescence imaging system [24,25]. The age-corrected heteroplasmy level in leucocytes was calculated using the following formula: (leucocyte heteroplasmy)/ $0.977^{(\text{age}+12)}$ . This correction was previously published by Grady et al. [26]. The current paper refers to this value as the age-corrected heteroplasmy level in leucocytes.

#### 2.4. Statistical Analysis

The correlation coefficients were calculated to assess the relationship between the heteroplasmy and age-corrected heteroplasmy levels and the progression rate of hearing loss and between the heteroplasmy and age-corrected heteroplasmy levels and the onset of hearing loss, balance or gait disorder, and diabetes mellitus, using JMP 16 (SAS Institute, Cary, NC, USA). The age of onset of hearing loss, diabetes, and balance disorder was compared against each other using the Tukey–Kramer test. We performed the Shapiro–Wilk W test to value the normality of the sample. The relationship between the two continuous variables was analyzed by single regression analysis. R<sup>2</sup> represented the coefficient of determination, and the significance of the slope indicated the *p*-value. *p* < 0.05 was considered statistically significant.

### 3. Results

The patients' demographic characteristics of the patients are shown in Table 1. The heteroplasmy levels ranged from 3% to 37%, with the mean being 23.9%. In terms of gender, 4 patients were male, and 11 were female. Most patients had bilaterally symmetric hearing loss with a horizontal or sloping type of audiogram. The mean age of onset of hearing loss was 28.6 years, with acquired hearing loss being perceived as early as 10 years of age in the earliest cases and as late as 56 years of age in the latest cases. At their first visit, 13 patients had diabetes mellitus, 3 had cerebellar atrophy or stroke on head MRI, and 1 had a cardiac disease; two patients eventually developed diabetes during the follow-up period. Other than hearing loss and diabetes mellitus, 11 patients did not show any symptoms or signs suggestive of MELAS.

The observation period ranged from 2 to 22 years, with a mean of 12.8 years. Hearing loss progressed in all patients during the observation period (Figures 1 and 2, Supplementary Figures S1 and S2). In seven patients, hearing deteriorated rapidly to complete deafness from 40 to 63 years of age (mean: 50 years). This episode was not associated with the worsening of pre-existing signs such as diabetes or other symptoms or signs. Before the rapid progression of hearing loss, their hearing level ranged from 56 to 80 dB HL. The rapid progression occurred on both ears almost simultaneously in three patients and at different times in two patients, with the periods between the ears being 1 and 7 years, respectively, and only on one ear in two patients (Figure 3 and Supplementary Figure S3). Although all these patients were treated with oral or systemic steroids, their hearing did not show any improvement. The heteroplasmy level ranged from 9% to 30% (mean: 20%) in seven patients, who showed rapid progression of hearing loss, and from 3% to 37% (mean: 23%) in the remaining eight patients, with no significant difference between them. The age-corrected heteroplasmy level ranged from 18.4% to 95.7% (mean: 68.6%) in seven patients, who showed rapid progression of hearing loss, and from 20.2% to 81.6% (mean: 64.4%) in the remaining eight patients, with no significant difference between them.

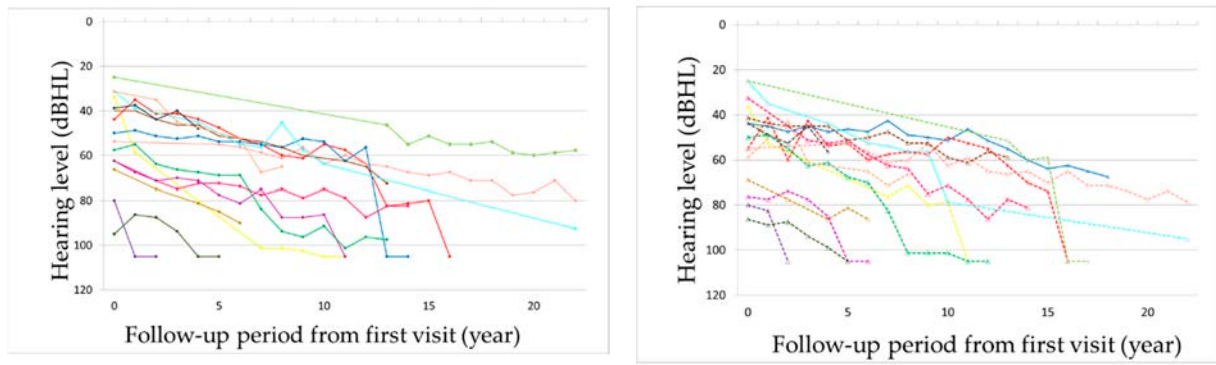
The mean rate of the progression of hearing loss in all 15 patients was 5.5 dB per year. In eight patients, who did not show rapid deterioration of hearing, the progression rate of hearing loss was 3.0 dB per year, while it was 1.9 dB per year before the rapid deterioration in seven patients. When the progression rate of hearing loss prior to the rapid deterioration in the latter was added to the calculation, the mean progression rate of hearing loss was 2.5 dB per year in all patients. When the progression rate of hearing loss prior to the rapid deterioration in the latter was added to the calculation, the mean progression rate of hearing loss was 2.5 dB per year in all 15 patients and did not differ significantly between the patients with and without rapid deterioration in hearing.

Caloric and cVEMP tests were performed in 13 out of 15 patients. In the caloric test, 4 (30%) out of 13 patients showed unilaterally decreased response, and 6 (46%) showed bilaterally decreased response. In cVEMP, 6 (46%) out of 13 patients showed unilateral abnormalities, and 7 (54%) showed bilateral abnormalities.

Table 1. Demographic characteristics of the patients.

Heteroplasmy Level	Age-Corrected Heteroplasmy Level	Gender	Onset of Hearing Loss (y.o.)	Right HL at First Visit (dBHL)	Left HL at First Visit (dBHL)	Progression Rate of Hearing Loss (dB/yr)	Rapid Decline of Hearing	CI	Onset of Balance Disorder (y.o.)	Caloric Test	cVEMP	Onset of Diabetes (y.o.)	MIDD/MELAS
37	81.6	F	15	31.3	58.8	4.2				NE	NE	24	MELAS
36	95.7	M	15	38.8	43.8	2.5		y	31	UNI-H	UNI-N.R.	22	MIDD
32	74.0	F	15	40	41.3	2.5			24	NOR	UNI-N.R.	22	MIDD
30	93.8	F	32	43.8	55	4.8	y	y	54	NOR	UNI-N.R.	39	MIDD
30	81.6	F	30	53.8	55	1.1			33	UNI-H	BI-N.R.	10	MIDD
29	82.6	F	31	33.8	36.3	9.7	y	y	31	UNI-H	UNI-N.R.	21	MIDD
29	71.9	F	22	62.5	32.5	2.1			13	BI-H	BI-N.R.	26	MIDD
25	71.2	F	15	62.5	76.3	6.6	y		30	BI-H	BI-N.R.	21	MELAS
21	81.0	F	45	57.5	50	4	y	y	45	BI-H	BI-N.R.	45	MIDD
15	62.0	F	10	80	80	26.5	y	y		BI-H	BI-N.R.	26	MIDD
14	82.1	M	30	95	86.3	4.5		y	61	UNI-H	UNI-N.R.	33	MELAS
14	43.8	M	37	25	25	2.3	y			NOR	BI-N.R.	31	MELAS
9	35.5	F	30	50	43.8	5	y		47	BI-H	BI-N.R.	26	MIDD
5	20.2	M	46	31.3	25	3.25				NE	NE	20	MIDD
3	18.4	F	56	66.3	68.8	3.95				BI-H	UNI-N.R.	47	MIDD

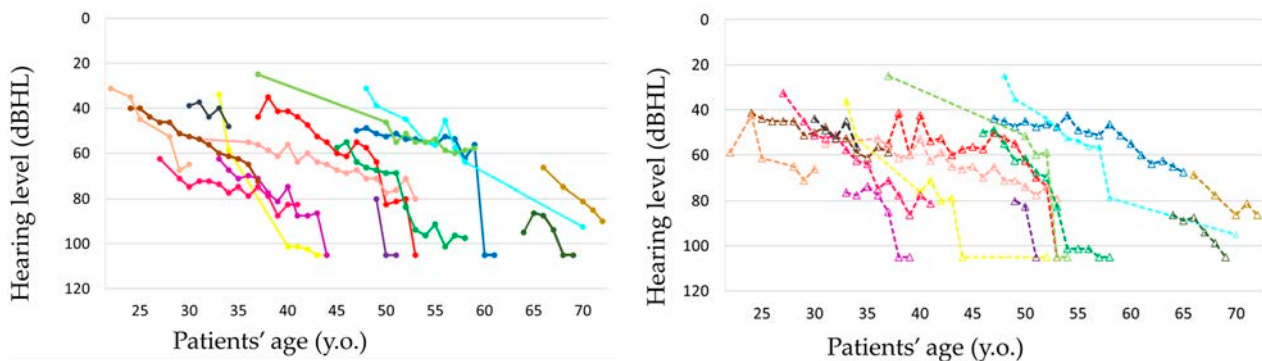
BI-H: bilateral hypoflexia; BI-N.R.: bilateral no-response; CI: cochlear implantation; cVEMP: cervical vestibular-evoked myogenic potential; F: female; HL: hearing level; M: male; MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MIDD: maternally inherited diabetes and deafness; NE: not examined; NOR: normal; UNI-H: unilateral hypoflexia; UNI-N.R.: unilateral hypoflexia; y: yes; y.o.: years old; yr: year.



Right ear

Left ear

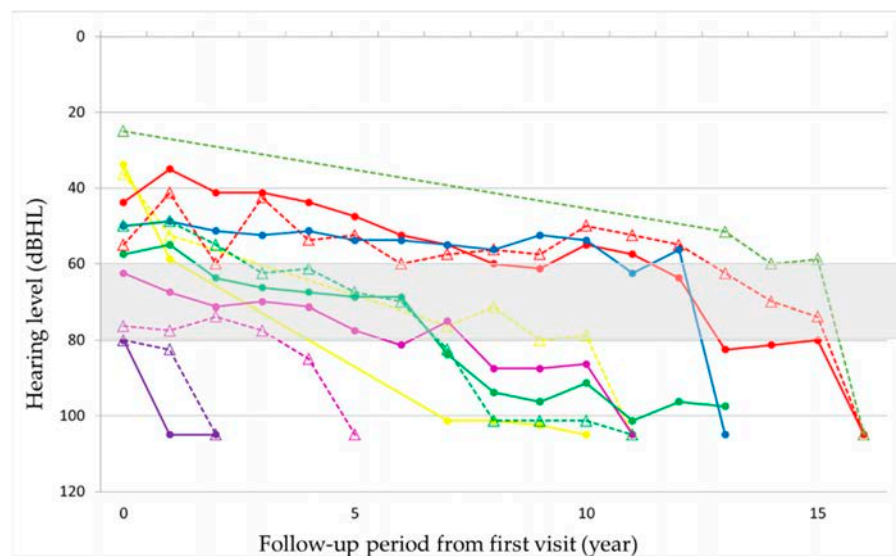
**Figure 1.** Chronological progression of hearing level from the first visit. The different color indicates the different patient, and the same color indicates the same patient.



Right ear

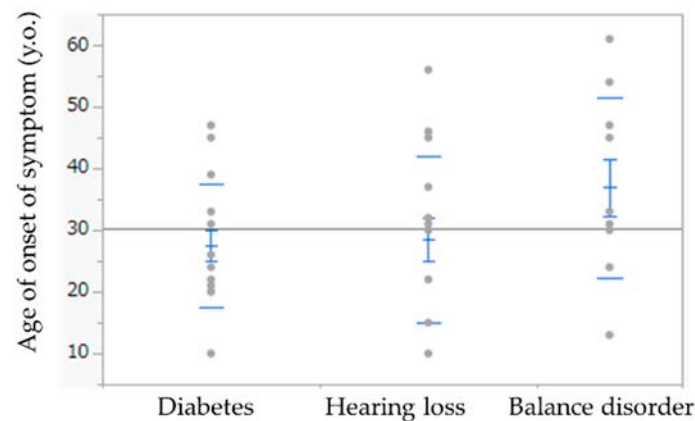
Left ear

**Figure 2.** Relationship between patients' age and progression of their hearing loss. The different color indicates the different patient, and the same color indicates the same patient.



**Figure 3.** Chronological change in hearing level from the first visit in patients who showed a rapid decline in hearing. The different color indicates the different patient, and the same color indicates the same patient. The solid lines with the shaped mark: right ear. The dashed line with the triangle mark: left ear. Two patients (light green and blue) only have one ear shown because the rapid decline was only seen in one ear.

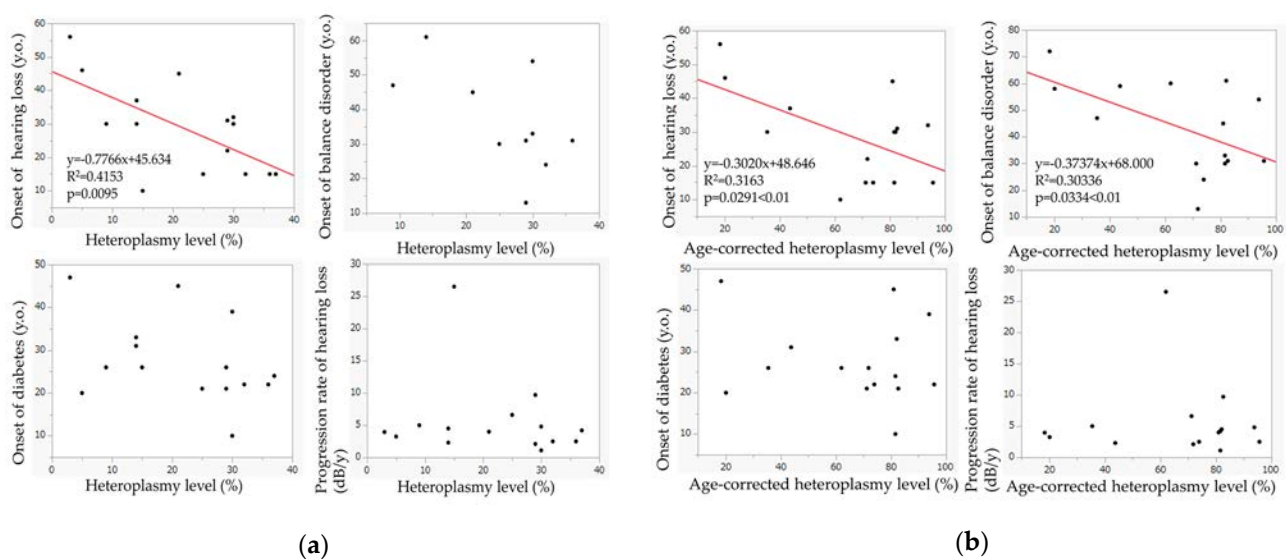
The mean age of onset of hearing loss, diabetes, and balance disorder was 28.6, 27.5, and 36.9 years, respectively (Figure 4). Based on the Shapiro–Wilk test, the  $p$ -values for the age of onset of hearing loss, diabetes, and balance disorder were 0.269, 0.400, and 0.189. The onset of balance disorder was delayed, compared with hearing loss and diabetes, but the difference was not statistically significant. However, balance disorder did not manifest in five patients until the end of the observation period; if we assume that the balance disorder appeared in these five patients one year later than the end of the observation period, the difference would be statistically significant ( $p = 0.0092$  vs. diabetes;  $p = 0.0161$  vs. hearing loss).



**Figure 4.** Age of the onset of diabetes, hearing loss, and balance disorder. Blue upper long bar, upper whisker; blue upper short bar, upper quartile; blue center short bar, median; blue lower short bar, lower quartile; blue lower long bar, lower whisker.

Figure 5a shows the relationship between the heteroplasmy level and the ages of onset of hearing loss, diabetes mellitus, and balance–gait disorder and between the heteroplasmy levels and the progression rate of hearing loss. The heteroplasmy levels showed a significant relationship with the onset of hearing loss ( $p = 0.0095$ ); hearing loss appeared at a younger age in patients with higher heteroplasmy levels. Such a trend was also observed in the relationship between the heteroplasmy levels and the onset of balance disorder. The heteroplasmy levels were not associated with the onset of diabetes or the progression rate of hearing loss.

Figure 5b shows the relationship between the age-corrected heteroplasmy level and the ages of onset of hearing loss, diabetes mellitus, and balance–gait disorder, and between the heteroplasmy level and the progression rate of hearing loss. Age-corrected heteroplasmy levels showed significant correlations with the onset of hearing loss ( $p = 0.0291$ ) and the onset of balance disorder ( $p = 0.0334$ ); balance disorder appeared at a younger age in patients with higher age-corrected heteroplasmy. Age-corrected heteroplasmy level was not associated with the onset of diabetes or the progression rate of hearing loss.



**Figure 5.** (a) Relationship between heteroplasmy level and the onset of hearing loss, balance–gait disorders, and diabetes mellitus and the progression rate of hearing loss; (b) relationship between age-corrected heteroplasmy level and the onset of hearing loss, balance disorders, and diabetes mellitus and the progression rate of hearing loss. The red lines: the regression lines.

#### 4. Discussion

The current study evaluated the hearing in 15 patients with m.3243A>G mutation of mtDNA in the long-term period from 2 to 26 years (mean: 12.8 years) after their first hearing test. The mean age of onset of hearing loss was 28.6 years; hearing loss occurred between 10 and 56 years old. The age of onset of hearing loss was correlated with the heteroplasmy and age-corrected heteroplasmy levels. Initially, from the start of their follow-up, their hearing loss progressed gradually, but the hearing loss progressed rapidly to deafness in seven patients during the observation period. The hearing level ranged from 56 to 80 dB HL before the rapid deterioration of hearing. The progression rate of hearing loss before the rapid deterioration in these patients did not significantly differ from that in the remaining eight patients who did not show rapid hearing deterioration. All these results indicate that it is difficult to predict the rapid hearing decline in patients with m.3243A>G mutation of mtDNA. Oral or systemic steroid treatment was not effective in improving hearing loss.

Unlike m.1555A>G mutation, hearing loss caused by m.3243A>G mutation of mtDNA is progressive. When we first reported the audiological findings of five patients with this mutation in 1996, the progression rate of hearing loss ranged from 1.5 to 7.9 dB per year [8]. Since other studies have not reported the progression rate of hearing loss, it has not been unclear how rapidly the hearing declines in patients with the m.3243A>G mutation. In the current study, the progression rate of hearing loss ranged from 1.1 to 26.5 dB per year, with a mean of 5.5 dB per year. The progression rate of hearing loss was 3.0 dB per year in eight patients without rapid deterioration of hearing and 1.9 dB per year prior to the rapid deterioration in the remaining seven patients. When only the progression rate of hearing loss prior to the rapid deterioration in the latter was added in the calculation, the mean progression rate of hearing loss was 2.5 dB per year in all patients. This progression rate of hearing loss is more rapid than that seen in elderly subjects with age-related hearing loss. Therefore, patients with m.3243A>G mutation should be informed that their hearing loss will progress by an average of 25 dB after 10 years and advised to start wearing hearing aids at an early stage and structure their future living environment.

In the current study, the rapid progression of hearing loss occurred in 7 out of 15 patients, in both ears almost simultaneously in 3 patients, at different times in 2 patients, and only on one ear in 2 patients. This finding is quite interesting since such a rapid decline in hearing has not been reported except in one study. Oshima et al. (1996) reported



the case of a 35-year-old woman with a complaint of right hearing loss and tinnitus in whom the pure-tone audiogram demonstrated 40 dB flat-type sensorineural hearing loss on the left and 85 dB saucer-type sensorineural hearing loss on the right ear [9]. Although the hearing at middle frequencies on the right ear improved after oral administration of steroids, it fluctuated and did not respond to oral administration of steroids or glycerol thereafter. Since hearing did not improve by oral or systemic administration of steroids in any of our patients, the pathophysiology of the fluctuating hearing in the case reported by Oshima et al. [9] is likely different from that of rapid hearing progression in our patients.

In our case series, the rapid decline in hearing occurred after the hearing loss exceeded 55 dB HL. Prior to the rapid progression of hearing loss, their hearing level ranged from 56 to 80 dB HL. It is unclear why such a rapid decrease in hearing occurred. Since the rapid decline in patients' hearing was not associated with worsening of the pre-existing signs and symptoms or other signs and symptoms, it is unlikely that it was caused by a rapid decline in systemic mitochondrial function, at least in the four patients who had rapid hearing loss in both ears at different times or only in one ear. It is possible that the vascular supply to the cochlea was compromised due to diabetes mellitus or the stroke-like episode seen in MELAS. It is also possible that the endolymphatic potential was rapidly reduced due to the acute impairment of energy production in the stria vascularis.

Human temporal bone histopathological studies in patients with MIDD and MELAS showed that the stria vascularis most severely degenerated [27–29]; in a patient with MIDD, there was marked degeneration of the stria vascularis and outer hair cells throughout the cochlea, as well as a reduction in the number of spiral ganglion cells at the base [27]. Severe degeneration of the stria vascularis and degenerative change in the spiral ganglion cells were observed in two patients with MELAS, in whom quantitative DNA studies showed that the proportion of mutant to wild-type mtDNA was similar in both histologically affected and unaffected tissues within the inner ear. Long-term administration of germanium dioxide causes renal failure, emaciation, and muscle weakness in humans [30,31] and body weight loss, myopathy, and nephropathy in rats. The skeletal muscles of rats treated with germanium dioxide showed numerous ragged-red fibers, cytochrome c oxidase-deficient fibers, and the accumulation of electron-dense material in the mitochondria [30,32,33], which resembles the pathological findings observed in patients with mitochondrial encephalomyopathy. We previously reported that guinea pigs fed chows containing 0.5% germanium dioxide for 2 months developed hearing loss, mainly due to the degeneration of the stria vascularis and cochlear supporting cells, and exhibited decreased cytochrome c oxidase activity in the skeletal muscles and kidney [34]. No apparent pathological changes were observed in the utricle, semicircular canal, or the cochlear or vestibular nerve fibers, indicating that germanium dioxide-induced mitochondrial dysfunction mainly affects the stria vascularis and supporting cells in the cochlea, as in the skeletal muscles and kidney, causing hearing impairment in the guinea pigs. This animal study also supported the importance of mitochondrial function in the stria vascularis for the maintenance of hearing function.

In the present study, patients noted balance–gait disorder later, compared with hearing loss, and five patients were not aware of balance or gait disorder. Balance–gait disorder tended to appear later, compared with hearing loss and diabetes mellitus. The use of cVEMP is essential in the diagnosis of saccular dysfunction in patients with moderate-to-profound sensorineural hearing loss [35]. The low metabolic rate of the vestibular apparatus, compared with that of the stria vascularis, may make the vestibule more resistant to mtDNA mutations, leading to a later onset of vestibular dysfunction [21]. However, in 13 patients who underwent caloric and cVEMP tests, 4 (31%) and 6 patients (46%) showed decreased response unilaterally and bilaterally, respectively, in the caloric test, and 6 (46%) and 7 patients (54%) showed abnormalities unilaterally and bilaterally, respectively, in the cVEMP test. The caloric test evaluates the function of the lateral semicircular canal and the superior vestibular nerve, and the cVEMP evaluates the function of the saccule and the inferior vestibular nerve. Therefore, the vestibular systems, semicircular canals, and

otolith organs are also frequently involved in patients with m.3243A>G mutation. Balance and gait disturbances may not become apparent until the vestibular function is severely impaired. It is interesting to note that seven patients with m.3243A>G mutation who had abnormal findings in the caloric and cVEMP tests showed normal responses in the galvanic VEMP test [18], which indicates that the peripheral vestibular end-organs are primarily affected similarly to the auditory system.

Human temporal bone histopathological studies on MIDD and MELAS demonstrated conflicting findings in terms of the degeneration of the vestibular systems. In a patient with MIDD, the vestibular end-organs, including the utricle and semicircular canals, were well preserved [6], whereas two patients with MELAS showed degenerative changes in the vestibular end-organs and Scarpa's ganglions [29]. In one of these MELAS patients, there was a pathological collapse of the membranous wall of the saccule and significant hair cell loss in the saccular macula, utricular maculae, and the cristae of all three semicircular canals [29]. In another patient with MELAS, the hair cells in both cristae and maculae were reduced, and the numbers of Scarpa ganglion cells were reduced to approximately 70% of the mean counts of age-matched control samples [29].

It has been reported that the disease severity and the expression pattern of the impairment across organs and tissues may vary in mitochondrial diseases, depending on the heteroplasmy levels. In general, patients with a higher heteroplasmy level exhibit more severe phenotypes, although the correlation may be weak [26,36–38]. Since the heteroplasmy level in the inner ear cannot be assessed clinically, we adopted the heteroplasmy level in peripheral leucocytes and found that the heteroplasmy level correlated with the onset of hearing loss; hearing loss developed at a younger age in patients with higher heteroplasmy. Previous studies also demonstrated that hearing loss developed earlier in patients with higher heteroplasmy levels [7,11]. The onset of balance–gait disorder correlated weakly with heteroplasmy level but significantly with age-corrected heteroplasmy level. The heteroplasmy level and age-corrected heteroplasmy level did not correlate with the onset of diabetes mellitus. Iwasaki et al. (2011) reported no correlation between heteroplasmy and onset of balance or gait disorder in 13 unrelated patients with m.3243A>G mutation [18]. Other studies showed a negative correlation between the onset of diabetes and heteroplasmy [11,39]. These discrepancies may be due to the small sample sizes, different phenotypes, and different stages of disease among reports. For example, in the current study, only patients with complaints of hearing loss were enrolled; therefore, there were two patients who did not have diabetes mellitus at the time of initial examination. Such bias in patient selection may have resulted in different correlations between the heteroplasmy levels and the onset of diabetes mellitus or balance–gait disorder.

It is worthy to note that the heteroplasmy and age-corrected heteroplasmy levels did not correlate with the progression rate of hearing loss or the presence of a rapid decline in hearing in our cases. It is unclear whether such a trend is also observed in other organs or tissues. De Laat et al. [36] scored disease severity using the Newcastle Mitochondrial Disease Adult Scale (NMDAS), including SF-36 quality of life (QoL) scores, and measured heteroplasmy levels in urinary epithelial cells, leucocytes, and saliva in 151 carriers of m.3243A>G mutation of mtDNA; their results indicate a yearly increase in NMDAS score of 0.47 point in the total group and that heteroplasmy levels in both leucocytes and urinary epithelial cells were only weakly correlated with disease severity. They also observed that physical QoL declined with age and that the most important determinants of QoL decline were hearing loss, speech problems, exercise intolerance, gait instability, psychiatric problems, and gastrointestinal involvement.

No medication is known to prevent or slow the progression of hearing loss associated with m.3243A>G mutation of mtDNA. Since the hearing loss involves mainly the cochlea, hearing aids are effective until the hearing loss becomes severe to profound. When hearing aids become ineffective for oral communication, cochlear implantation, which directly stimulates the auditory nerve, is recommended [40]. In the present study, six patients received a cochlear implant at our department, and all patients except one who developed cerebral

stroke 5 months after the cochlear implant surgery achieved good speech perception. As mentioned above, temporal bone histopathological studies demonstrated that the spiral ganglion cells were relatively well preserved in a patient with MIDD and degenerated in patients with MELAS, whereas the stria vascularis was severely degenerated [26,28]. Since hearing thresholds mainly reflects the functions of the cochlear cells, such as the hair cells and stria vascularis, it is presumed that the retro-cochlear auditory pathways, including the spiral ganglion cells, are still relatively well preserved when patients become profoundly deaf, which explains the high efficacy of cochlear implants. However, during long-term follow-up in the patients who have cochlear implantation with mitochondrial gene mutation, some patients who initially showed good speech perception exhibited deterioration of speech perception [41]. Therefore, patients receiving cochlear implantation should be carefully monitored over the long term.

## 5. Conclusions

This study evaluating the hearing in 15 patients with m.3243A>G mutation in mtDNA in the long-term period demonstrated that hearing loss occurred from 10 to 56 years of age, that the age of onset of hearing loss was correlated with heteroplasmy and age-corrected heteroplasmy levels, that the hearing loss progressed gradually initially, and that the rapid decline in hearing loss to profound deafness occurred in approximately half of the patients during the observation period. The hearing level prior to the rapid decline ranged from 56 to 80 dB HL, and the progression rate of hearing loss prior to the rapid decline was not significantly different from that in the remaining patients, who did not experience a rapid decline in hearing. Heteroplasmy and age-corrected heteroplasmy levels did not correlate with the presence of rapid progression of hearing loss or with the progression rate of hearing loss. These results indicate that it is difficult to predict the rapid decline in hearing in patients with m.3243A>G mutation of mtDNA. Neither oral nor systemic steroid treatment was effective in improving hearing loss.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/life12040543/s1>, Figure S1: Chronological progression of hearing level from the first visit, Figure S2: Relationship between patients' age and progression of their hearing loss, Figure S3: Chronological change in hearing level from the first visit in patients who showed rapid decline in hearing.

**Author Contributions:** Conceptualization, A.S., A.K. and T.Y.; methodology, A.S., A.K. and T.Y.; formal analysis, A.S., A.K. and T.Y.; investigation, A.S., A.K., H.K., T.U., S.I., C.F., M.K. and T.Y.; writing—original draft preparation, A.S., A.K. and T.Y.; writing—review and editing, A.S., A.K., H.K., C.F. and T.Y.; supervision, T.Y.; project administration, T.Y. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** This study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Ethical Standards Committee of the Faculty of Medicine at the University of Tokyo (Application Number 2487).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.


**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; in the decision to publish the results.

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# Cochlear Implantation in Patients with Mitochondrial Gene Mutation: Decline in Speech Perception in Retrospective Long-Term Follow-Up Study

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**Abstract:** Clinical evidence of the effectiveness of cochlear implantation for hearing loss with mitochondrial DNA mutation is limited. Most reports have only described short-term postoperative speech perception, which may not reflect the limitations of cochlear implantation caused by progressive retrocochlear dysfunction. The present study aimed to investigate long-term speech perception after cochlear implantation in patients with severe to profound hearing loss associated with mitochondrial DNA mutation. A retrospective chart review was performed on patients with mitochondrial DNA mutation who had undergone cochlear implantation at the Department of Otolaryngology and Head and Neck Surgery at the University of Tokyo Hospital. We extracted data on causative mutations, clinical types, clinical course, perioperative complications, and short-term and long-term postoperative speech perception. Nine patients with mitochondrial DNA mutation underwent cochlear implantation. The mean observation period was  $5.5 \pm 4.2$  years (range, 1–13 years), and seven patients were followed for more than 3 years. Two of the seven patients who initially showed good speech perception exhibited deterioration during long-term follow-up. The absence of an acute progression of cognitive decline in patients, showing a gradual decrease in speech perception, suggests that the deterioration of speech perception was caused by progressive retrocochlear degeneration. Although most patients with mitochondrial DNA mutation maintained good speech perception for more than 3 years after cochlear implantation, retrocochlear degeneration could cause the deterioration of speech perception during long-term follow-up.

**Keywords:** cochlear implantation; retrocochlear dysfunction; mitochondrial gene mutations

## 1. Introduction

Mitochondria play an important role in intracellular adenosine triphosphate production by oxidative phosphorylation, an essential energy source in nucleated cells. Mutations in mitochondrial DNA (mtDNA) cause dysfunction, especially in tissues with high metabolic demands. In patients with mtDNA mutations, organs that rely on aerobic energy production, such as the visual pathway, heart, central nervous system, and skeletal muscle, are primarily affected. The auditory pathway, including the cochlea, also has large energy demand; therefore, the auditory pathway is an organ that can be profoundly affected by mitochondrial disorders [1–3].

More than half of the patients with mtDNA mutations are affected by a hearing impairment at some time during the disease course [4–6]. Pathological mutations of the mtDNA have been commonly found at the transfer RNAs (tRNAs). To date, more than 90 point mutations in 21 of the 22 mitochondrial tRNA genes have been reported [7,8]. Most of these mutations result in a decreased rate of mitochondrial protein synthesis, causing a deficiency in the energy metabolism of the cell [9]. Approximately 50 mutations of the tRNA genes have been associated with deafness [10]. Associated features of hearing loss include encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS syndrome), diabetes (maternally inherited diabetes and deafness, or MIDD, syndrome), ophthalmoplegia (chronic progressive external ophthalmoplegia, or CPEO, syndrome), cardiac conduction abnormalities with retinopathy and ophthalmoplegia (Kearns–Sayre syndrome), myoclonus epilepsy (myoclonus epilepsy associated with ragged-red fiber, or MERRF, syndrome), ptosis, ophthalmoplegia, gastrointestinal dysmotility, cachexia, peripheral neuropathy, and leukoencephalopathy (mitochondrial neurogastrointestinal encephalopathy, or MNGIE, syndrome). Hearing loss is usually gradual at onset, initially occurs at high frequencies, is predominantly bilaterally symmetrical, and progresses to profound. Hearing loss in patients with mitochondrial diseases is mainly attributed to cochlear dysfunction [11–15], but mitochondrial disorders can also affect the central nervous system, including the central auditory pathway, and can cause psychomotor regression [4,16–19]. Roesch et al. [20] conducted a systematic review of knowledge on hearing loss in genetically proven mitochondrial disease in children. A total of 75 patients from 23 studies were included in the analysis. Retrocochlear hearing loss was found more often (33 out of 75 patients) than expected. Affected genes included OPA1 in 14 patients, FDXR in seven patients, and MT-TL1 in six patients. The clinical courses of these patients, including the age of onset and disease severity, showed diverse characteristics. Takahashi et al. [21] reported histopathological examinations of human temporal bones in MELAS patients and found severe degeneration of the stria vascularis and the spiral ganglion cells. There was severe atrophy of the stria vascularis in all turns of the cochlea, and the remaining stria cells showed vacuole formation and the presence of small, dark-staining, round, and ovoid cells. Both the outer and inner hair cells were generally present, with scattered losses in the lower basal turns. In addition to these findings, the total number of spiral ganglion cells was reduced when compared with the mean values of normal newborn and age-matched control samples, representing a mild neuronal loss. Many spiral ganglion cells showed varying degrees of degenerative change, as evidenced by faint staining of the cytoplasm, loss of cell membrane outline, and loss of nuclear definition. Other histopathological examinations of the human temporal bone also demonstrated that mtDNA A3243G mutation can involve not only the stria vascularis and hair cells but also the spiral ganglion cells [22,23].

A defect in the inner hair cells, the auditory nerve, the connection between them, or the connection between the nerve and brain can lead to auditory neuropathy spectrum disorder (ANSD). ANSD has been reported to be associated with head injury; infections due to various viruses such as measles, mumps, and cytomegalovirus; and high fever and is also caused by specific gene mutations, such as OTOF. ANSD is characteristic of relatively mild hearing impairment with abnormal ABR response and poor speech recognition score, while distortion product otoacoustic emission (DPOAE) is normal [24,25]. In a report from Leruez et al. [26], 8 out of 19 patients with OPA1 gene mutation were reported to have suspected ANSD. Sakai et al. [27] reported a patient with normal DPOAE who had fluctuation of hearing threshold measured by ABR; because the peak latency of wave I and wave V and the intervals of waves I–V were markedly delayed, the existence of a retrocochlear problem was speculated to be a cause of hearing loss.

Cochlear implantation (CI) for patients with severe to profound hearing loss associated with mtDNA mutations has been reported [19,21–23,28–35]. Howes et al. [36] reported a case of MIDD with a speech score of 67% at one-month follow-up. Yasumura et al. [37] reported a case of MELAS with a speech score of 72% at 3-month follow-up. Li et al. [34] reported a case of MNGIE with a speech score of 56% at 3-month follow-up. All of these

reports emphasized that CI is generally effective for patients with mtDNA mutations, but most of them only described speech perception in the short-term postoperative period. Therefore, it is unclear whether the effectiveness of CI is limited by the progression of retrocochlear dysfunction and/or cognitive decline associated with mitochondrial disorder. In fact, a patient has been reported to show poor postoperative speech perception associated with cognitive problems in relatively long-term follow-up [38].

In the present study, we investigated not only short-term but also long-term speech perception after CI in patients with profound hearing loss associated with mtDNA mutation.

## 2. Materials and Methods

A retrospective chart review was performed on patients who had undergone CI at the Department of Otolaryngology and Head and Neck Surgery at the University of Tokyo Hospital from 1991 to 2019. Nine patients were diagnosed with mtDNA mutations via genetic testing, and the additional information extracted included the causative mutations, clinical types, clinical course, perioperative complications, and postoperative speech perception. The Fukuda version of the monosyllabic speech perception test was used to evaluate speech perception before and after CI. Speech performance in noise was evaluated in four patients, including two patients examined twice, using a CI-2004 Japanese open-set sentence test. Tests were performed in quiet, SN20 and SN10. A DPOAE test and a promontory stimulation test were performed to differentiate between retrocochlear and cochlear hearing loss. Cases with obvious decline in attention, executive function, learning/memory, language, perceptual/motor functions, and social cognitive functions during the examination or as reported by family members were considered to have cognitive deterioration. The present study was approved by the Regional Ethical Standards Committee of the Faculty of Medicine at the University of Tokyo (application number 2487) and was conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from the patients for publication of this study.

## 3. Results

### 3.1. Patient Characteristics

The characteristics of the nine patients with mtDNA mutations who underwent CI are shown in Table 1. The mean age at CI was  $45.0 \pm 11.5$  years (range, 22–64 years), and the mean observation period was  $5.5 \pm 4.2$  years (range, 1–13 years). A3243G mutation was identified in seven patients and RRM2B mutation and A8296G mutation were each identified in one patient. Of the seven patients with A3243G mutation, six patients were diagnosed with MIDD, and one was diagnosed with MELAS. A patient with the RRM2B mutation was diagnosed with CPEO, and a patient with the A8296G mutation only had hearing loss. Among the subjects, there were no suspicious findings of cognitive decline preoperatively. No patients showed any response in DPOAE tests, indicating that hearing loss involved the cochlea. All patients except one (patient 7) showed good response in promontory stimulation tests, which indicates that the retrocochlear auditory pathway was markedly involved in patient 7 but not in others.



**Table 1.** Summary of patients.

Patient	Disease	Causative Mutations	Age of Onset of Hearing Loss (years)	Age of Becoming Deaf (years)	Age at CI (years)	Observation Period (years)	Associated Symptoms
1	MIDD	A3243G	30	53	53	3.4	diabetes
2	MIDD	A3243G	32	46	46	13	diabetes
3	MIDD	A3243G	38	44	44	12.2	diabetes
4	MIDD	A3243G	27	64	64	1.2	diabetes
5	MIDD	A3243G	10	50	51	6.2	diabetes
6	MIDD	A3243G	14	36	37	3.1	diabetes
7	MELAS	A3243G	10	44	46	4.0	myopathy, lactic acidosis, stroke-like episode
8	–	A8296G	7	21	22	4.5	-
9	CPEO	RRM2Bs	5	43	43	2.2	mild external ophthalmoplegia

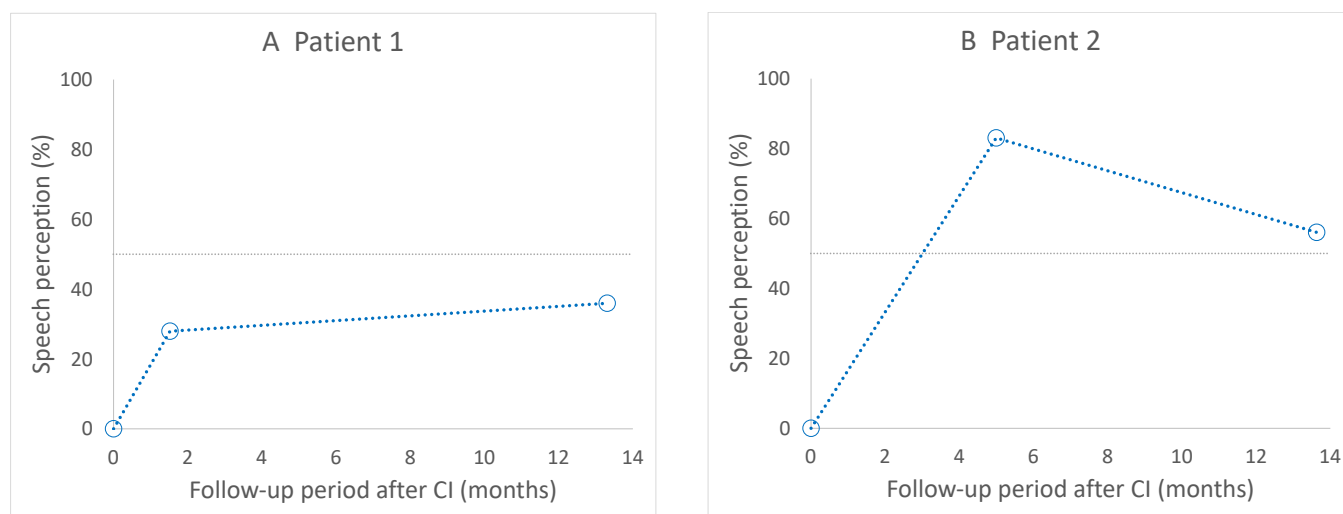
Abbreviations: MIDD, maternally inherited diabetes with deafness; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; CPEO, chronic progressive external ophthalmoplegia; CI, cochlear implantation.

**3.2. Surgical Findings**

No complications were observed during the surgery in any patient. A CI24M (Cochlear®, Lane Cove, Australia) electrode was used in patients 1, 2, and 3; a CI24RE (Cochlear®) electrode in patients 4, 5, 6, 7, and 9; and a CI422 (Cochlear®) electrode in patient 8. As there were no malformed cochlear cases in this series, we chose the latest electrode at surgery. All patients received CI only in the unilateral ear. Full insertion of CI electrodes was achieved in all patients. Electrically evoked compound action potentials were detected in all electrodes in all patients.

**3.3. Postoperative Speech Perception**

Postoperative speech perception results within 14 months after CI are shown in Figure 1. Seven patients achieved scores of  $\geq 50\%$  in the Fukuda version of the mono-syllabic speech perception test after CI, whereas two patients achieved scores of  $<50\%$  (patients 1 and 3).



**Figure 1.** Cont.

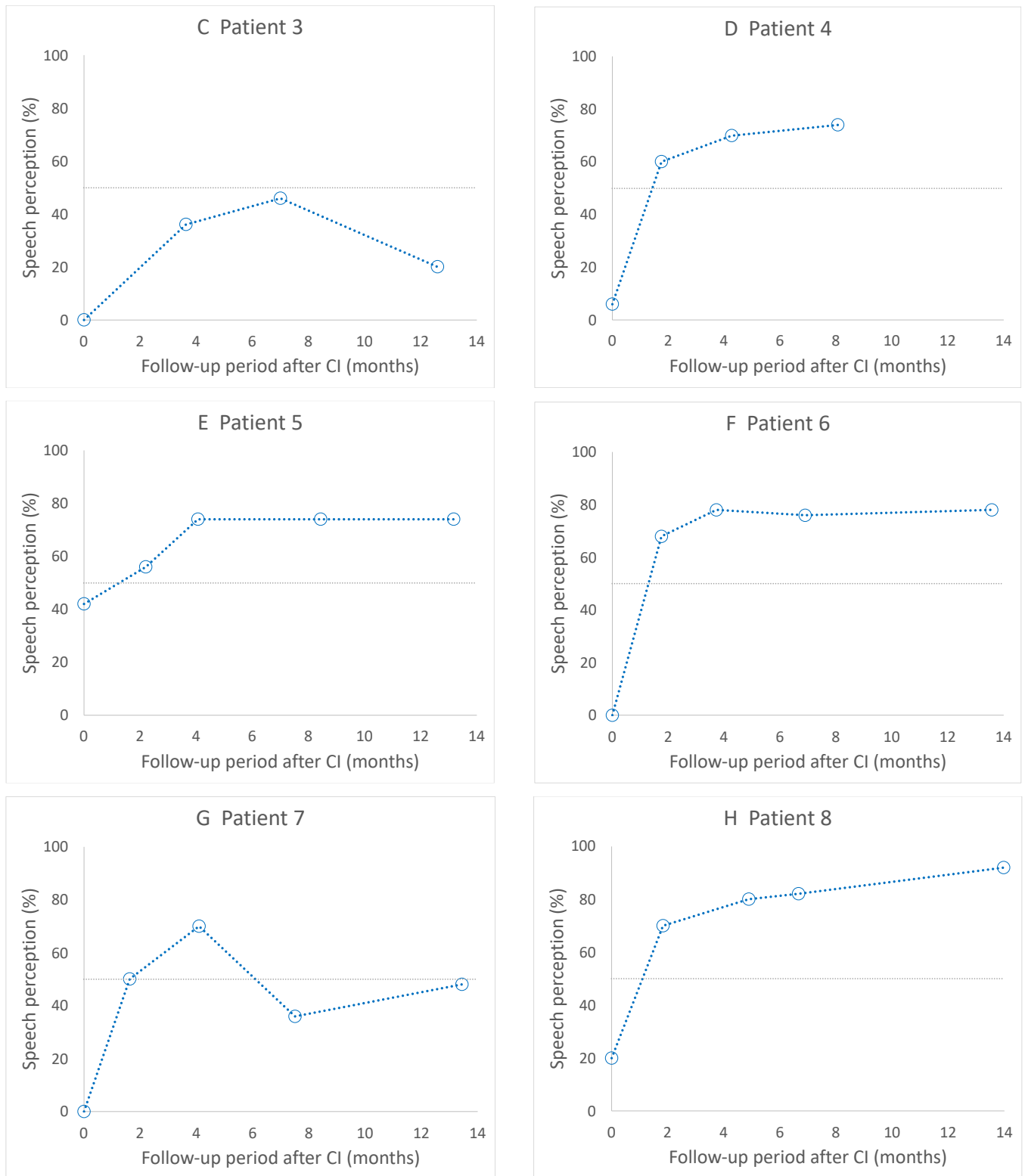
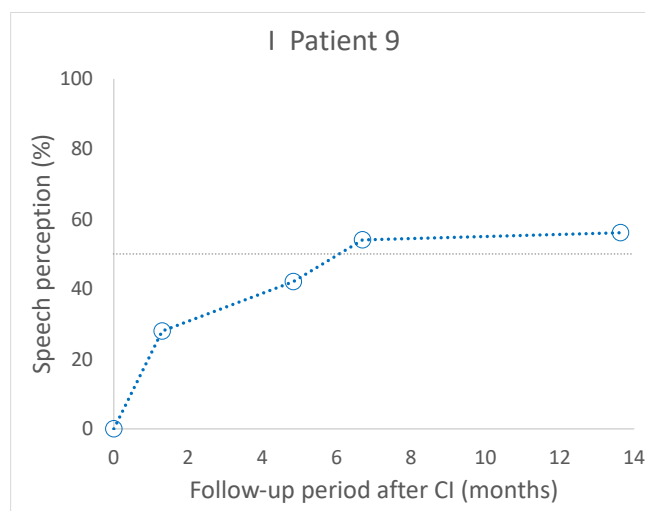


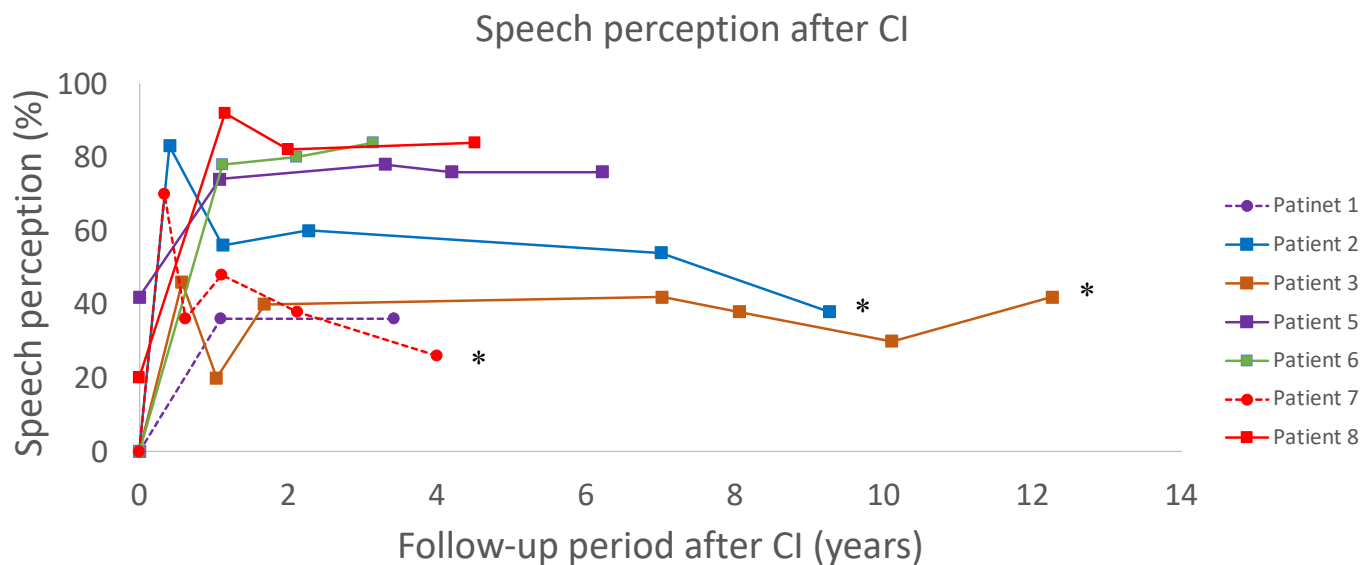
Figure 1. Cont.



**Figure 1.** Short-term results of postoperative speech perception. Seven patients (B,D–I) achieved scores  $\geq 50\%$  after CI, and two patients (A,C) achieved poorer outcomes. CI, cochlear implantation.

The results of long-term postoperative speech perception are shown in Figure 2. Of the seven patients who were followed for more than 3 years, three patients (patients 2, 3, and 5) were followed for more than 5 years. Three patients (patients 2, 3, and 7) showed a decrease in postoperative speech perception of 20% or more. Patient 2 had no identifiable reasons for an acute deterioration in the first year and a gradual deterioration during the long-term follow-up. There was no sign of device failure, such as increasing impedances, an increase in clinical threshold level, or a reduced number of available electrodes in the course of deterioration of speech perception. In patient 3, a temporal shift in speech perception improved after mapping modification, and thereafter, no changes were observed during the long-term follow-up period. In patient 7, an acute deterioration in the first year was attributed to high-order brain dysfunction caused by cerebral infarction, but this episode did not cause the limited usage of the implant or make it difficult to conduct a speech perception test. After this episode, she showed a progressive decline in speech perception, despite the absence of an additional central episode or cognitive decline. There was no sign of device failure, such as increasing impedances, increases in threshold level by NRT, increases in clinical threshold level, or a reduced number of available electrodes in the course of deterioration of speech perception.

The results of sentence recognition tests in noise in four patients are shown in Table 2. Noise significantly influenced speech perception in one patient (patient 4), showing a poor score even in quiet conditions; this patient showed a progressive decline in the monosyllabic speech perception test. The other three patients maintained good scores under noise exposure, and two of them, who were examined twice using a sentence recognition test in noise, showed stable performance for more than three years; these patients also showed stable performance in long-term monosyllabic speech perception tests.



**Figure 2.** Long-term results of postoperative speech perception. Postoperative speech perception for seven patients. \* Three patients with speech perception reduced by 20% or more.

**Table 2.** Results of speech in noise test.

Patient	Tested Year (Years after CI)	In Quiet (%)	S/N20 (%)	S/N10 (%)
4	5.2	45	20	-
5	1.3	95	95	58
	3.9	100	95	78
6	1.5	98	97	73
8	1.5	98	100	57
	3	100	92	-

A CI 2004 speech test was conducted in four patients at some point after CI. CI, cochlear implantation.

#### 4. Discussion

In the present study, we investigated the short-term and long-term postoperative speech perception in nine patients who underwent CI for profound hearing loss with mtDNA mutation. Seven patients exhibited a good score of  $\geq 50\%$  in the Fukuda version of the monosyllabic speech perception test during the first postoperative year, but two of the seven patients showed deterioration during the long-term follow-up period. The short-term results in the current study agree with those in previous reports. Sinnathuray et al. [6] compared the results of postoperative speech perception in 12 patients with mtDNA mutations from 1997 to 2002 and reported good results irrespective of disease type, severity, and duration of hearing loss. Nawal et al. [39] conducted a systematic review of cochlear implantation outcomes in patients with mitochondrial hearing loss. In that study, 13 patients from 11 studies performed speech perception tests, and 10 out of 11 patients scored more than 50% in either speech, word, or phoneme recognition tests.

Deafness associated with mtDNA abnormalities is mainly attributed to dysfunction of the inner ear [11–15], but the retrocochlear auditory pathway may also be involved [4,16,17,21–23]. Short-term improvements in speech perception after CI may wane during long-term follow-up due to the degeneration of the spiral ganglion cells or cognitive decline due to progressive mitochondrial disorder. In a recently reported retrospective case series of five patients with mitochondrial diseases, including MELAS and MIDD, speech perception was preserved during the long-term follow-up period in four patients, but one patient could only use implants for several hours per day and could not conduct the speech perception test within 2 years of surgery [38]. In that report, the authors

speculated that cognitive decline from the disease made the patient unable to recognize the importance of using the implant for the establishment of speech perception.

In the current study, two patients who initially achieved good speech perception exhibited a decrease in speech perception during the long-term follow-up period. In patient 2, neither cognitive decline nor deterioration of the device itself, such as a decrease in the number of available electrodes or an increase in the impedance of electrodes, were observed; therefore, progressive retrocochlear dysfunction was considered as the cause of the deterioration in speech perception. In another patient (patient 7), the initial decline in speech perception was associated with cerebral infarction, but the absence of additional central episodes, cognitive decline, or deterioration of the device itself thereafter suggests that the decline in speech perception during the long-term follow-up after cerebral infarction may be associated with progressive retrocochlear impairment associated with mtDNA mutation.

Previous studies [22–37,40,41] and short-term observations in the present study indicate that patients with mtDNA mutations are good candidates for CI. Notably, however, the long-term observations in the present study also suggest that retrocochlear dysfunction may be responsible for the long-term deterioration of speech perception after CI in patients with mtDNA mutations. Several reports [42–44] have investigated long-term speech performance in patients receiving CI and have observed no decline in speech perception performance. For example, Hilly et al. [42] examined 87 cochlear implant recipients, including 22 patients over 70 years of age, with a mean follow-up of 6.8 years, and found that most patients had a stable outcome during the follow-up period. Even in patients who are older, 13.6 percent improved and none had a reduction in score of more than 20 percent. Dillon et al. [43] followed 14 cochlear implant recipients aged 65 years and older for at least 10 years and found that consonant–nucleus–consonant word scores were stable between 6 months and 1 year of listening experience, improved significantly between 1 year and 5 years, and were stable between 5 years and 10 years. Hearing in Noise Test sentence scores in quiet and in noise showed a similar pattern, with stability in performance between the 6-month to 1-year and 5-year to 10-year follow-up intervals, and significantly improved performance between the 1-year and 5-year follow-up intervals. Therefore, diagnosis of mitochondrial diseases will have an impact on long-term performance of CI as well as future progression of hearing loss. Although CI has the potential to improve the quality of life in these patients, surgeons need to provide information about the possibility of gradual deterioration of speech perception in the long term after CI, so that patients and their families can prepare their future living environments and support. At our institution, we evaluate for mitochondrial genetic abnormalities at the time of initial consultation in patients with symptoms and signs suggestive of maternal inheritance.

Because no objective assessment data were available to confirm the progression of retrocochlear dysfunction, there were no clear predictors of the deterioration of speech perception during the long-term follow-up period. Preoperative diagnosis of retrocochlear involvement may have some impact on long-term performance of CI. All patients in our case series showed an absence of DPOAE response, indicating cochlear involvement. Absent or poor response in promontory stimulation tests indicates retrocochlear dysfunction. In the present study, two patients showed deterioration of speech performance during follow-up; one of them (patient 7) showed poor response in the promontory stimulation test, but the other (patient 2) showed good response. Therefore, it is unclear if preoperative retrocochlear involvement can predict the decline in speech perception in the long-term period. Breneman et al. [45] reported a long-term outcome of cochlear implantation in patients with auditory neuropathy spectrum disorder (ANSD). In that study, 35 patients with a follow-up period of more than six years on average showed as good a response as children with non-ANSD SNHL, which suggests that diagnosis of retrocochlear disease is not sufficient to predict the long-term benefit of CI. Superficial hemosiderosis is also known to present retrocochlear deafness. In a systematic review by Chaudhry et al. [46], 31 out of 44 patients showed improved hearing outcomes following CI, and 22 implants had sustained benefit at the last follow-up. They concluded that longevity of benefit was diffi-

cult to predict because of the progressive nature of the disease and a lack of preoperative prognosticators. Pijl et al. [41] used electrically evoked auditory brainstem response (EABR) and middle latency response (MLR) data derived from two patients with Kearns–Sayre syndrome and found that EABR and MLR were useful for distinguishing between cochlear and retrocochlear hearing loss and for predicting outcomes after CI. Rosenthal et al. [40] reported EABR and MLR data derived from a patient with MELAS syndrome who had significant central nervous system deficits, and proposed prioritizing MLR testing rather than EABR to evaluate the integrity of the auditory pathway. Introduction of these examinations may be useful for the prediction of future performance.

There have been several reports that older adult cochlear implant users have poorer performance of speech in noise compared to younger adults. This may be due to the fact that listening in noise is more susceptible to retrocochlear auditory pathway damage [47,48]. Although the present study could not provide sufficient data, it may be possible to predict the deterioration of speech performance with progression of retrocochlear dysfunction by repeated evaluation under noise conditions.

Generally, central nervous system symptoms, such as stroke-like episodes in MELAS patients, progress slowly [49,50]. Therefore, retrocochlear dysfunction after CI is also likely to progress slowly. Long-term observation may reveal deterioration of speech perception in our patients followed for less than 5 years.

It should be noted that heterogeneity of the presented samples may affect the interpretation of the results because of the relatively small number of cases. Although the time from deafness to surgery was around one year in most cases, there was diversity in the onset of hearing loss and the age of surgery. Heteroplasmy is also known to affect disease severity and the expression pattern of the impairment across organs and tissues [51,52], but was not analyzed in this study.

## 5. Conclusions

We retrospectively reviewed short-term and long-term speech perception after CI in nine patients with deafness associated with mtDNA mutations. Two of the seven patients who initially achieved good speech perception scores exhibited a deterioration in speech perception during the long-term follow-up. The absence of acute progression of cognitive decline in conjunction with the gradual decline in speech perception suggests that retrocochlear dysfunction associated with mitochondrial disorder could be responsible for the deterioration of speech perception.

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Original article

# Meaningful word acquisition is associated with walking ability over 10 years in Rett syndrome

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## Abstract

**Purpose:** To investigate walking ability in Japanese patients with Rett syndrome (RTT).

**Methods:** Walking ability was assessed in 100 female Japanese patients with RTT using univariate and multivariate analysis in all age groups, and in patients over 10 years of age. We analyzed walking ability and confounding factors including prenatal-perinatal histories, developmental milestones, somatic and head growth, anthropometric data, body mass index, age of loss of purposeful hand use, age at onset of stereotypic hand movement, history of autistic behavior, age at regression, presence or absence of seizures, and the results of *MECP2* genetic examination from the Japanese Rett syndrome database.

**Results:** Univariate analysis revealed that acquisition of walking in all age groups was significantly correlated with the acquisition of meaningful words, microcephaly, and crawling ( $P < 0.0001$ ,  $P = 0.005$ ,  $P < 0.0001$ , respectively). Univariate analysis revealed that walking ability over 10 years of age was significantly correlated with acquisition of meaningful words, microcephaly, and body mass index ( $P < 0.0001$ ,  $P = 0.005$ ,  $P = 0.0018$ , respectively). *MECP2* mutations R306C, R133C, and R294X were significantly associated with different acquisition of crawling ( $P = 0.004$ ) and walking ( $P = 0.01$ ). Multivariate analysis revealed that only acquisition of meaningful words was significantly correlated with walking ability over 10 years of age. This trend excluded the genetic effects of R306C, R133C, and R294X.

**Conclusions:** Meaningful word acquisition was robustly associated with walking ability over 10 years. Prognosis of walking ability may be predicted by the acquisition of meaningful words. This information is potentially useful for early intervention and the planning of comprehensive treatment for young children with RTT.

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**Keywords:** Rett syndrome; Univariate analysis; Multivariate analysis; Motor function; Ambulation; Methyl-CpG binding protein 2 mutation; Japanese database

## 1. Introduction

Rett syndrome (RTT) (OMIM #312750) is a neurodevelopmental disorder primarily affecting females. Most cases of RTT are caused by de novo mutations in the gene encoding methyl-CpG binding protein 2 (MECP2) [1]. Approximately 90%–95% of typical RTT cases exhibit loss-of-function mutations in the *MECP2* gene of the X-chromosome [2].

Clinical manifestations include microcephaly, loss of psychomotor abilities, intellectual disability (ID), autistic behaviors, and hand stereotypies. Recent large cross-sectional studies revealed substantial clinical variability in *MECP2* mutations [2]. It has been reported that girls and women with the mutations p.Arg270\* (R270X) or p.Arg255\* (R255X) present with more severe motor disability, whereas those with mutations p.Arg306Cys\*, p.Arg133Cys\*, and p.Arg294\* (R306C, R133C, R294X, respectively), and C-terminal deletions exhibit a milder phenotype, and, in most cases, acquire the ability to walk [3–7]. However, it is difficult to estimate genotype-phenotype correlations because of the role of X inactivation. In the present study, we utilized the Japanese Rett syndrome database (JRSD), which was established in October 2012 and contains clinical data from over 102 RTT patients. Walking ability is important for family counselling and planning for the provision of care for young children with RTT. In the current study, we investigated factors related to ambulation ability in young children over 10 years of age with or without genetic effects, based on the JRSD.

## 2. Patients and methods

### 2.1. Japanese Rett syndrome database and subjects

The JRSD is operated by management officers consisting of child neurologists and RTT family associations, and is supported by the Japanese government. The registration document is completed with a combination of parents' questionnaire responses and doctor's examination data after children's diagnosis with RTT with clinical criteria and/or genetic abnormality [2]. A total of 102 female patients with RTT were registered from October 2012 to December 2015. For the current study, we obtained informed consent from all RTT parents and ethical approval from each institution. The JRSD registration document contains prenatal/perinatal history, developmental milestones, somatic and head growth, anthropometric data, growth status (body mass

index [BMI]), age at regression, age at development of stereotypic hand movement, loss of purposeful hand function, history and age of onset of autistic behavior, eating and swallowing ability, vocalization/verbalization, periodic breathing, hand and foot temperature, dystonia, tremor, seizures, scoliosis, muscle tone, dental and oral problems, and genetic examinations.

### 2.2. Gene testing

Total genomic DNA was prepared from peripheral blood leukocytes according to standard procedures. Participants in this study underwent complete *MECP2* mutation analysis, including exon 1 and evaluation for large DNA rearrangement, by Southern blotting or by multiple ligation-dependent probe amplification (MLPA) analysis. Whole exome sequencing (WES) was performed in some patients, as previously described [8].

### 2.3. Sentinel surveillance and statistical analyses

The Kaplan–Meier method was used to estimate survival curves and the log-rank test was used to compare estimated survival curves. In addition, univariate and multivariate logistic regression models were employed to test the relationships between walking ability over 10 years of age and other factors. All variables were visually inspected to assess their distribution. When variables were judged to be skewed and the normality assumption was not tenable, non-parametric tests, Spearman's rank correlations and Mann-Whitney U-tests were employed.  $\chi^2$  test and Fisher's exact test were used to examine the relationships between categorical variables. P-values less than 0.05 were considered to indicate significant differences. JMP Pro 13 (SAS institute, Cary, NC, USA) was used to perform all data analyses. The research protocol was approved by the Ethics Committees of National Center of Neurology and Psychiatry, and the Ethics Committees of each participating institution.

## 3. Results

Two patients were excluded from the study because of incomplete data for RTT diagnosis, according to recent clinical diagnostic criteria [2]. We analyzed registered data from 86 typical and 14 atypical RTT patients. All patients were female, and ages ranged from 1 year to 43 years of age (mean  $\pm$  SD; 14.5  $\pm$  11.2 years of age;

Table 1  
Clinical distribution in Japanese Rett Syndrome Database.

Symptoms	Age at registration					Total
	1–5 yrs	6–10yrs	11–15yrs	16–20yrs	>21yrs	
Number of patients	19	25	18	6	32	100
<b>Main criteria</b>						
Partial/complete loss of acquired purposeful hand skills	19 (100)	23 (92.0)	14 (77.8)	5 (83.3)	25 (78.1)	86 (86)
Partial/complete loss of acquired spoken language	10 (52.6)	13 (52.0)	12 (66.7)	4 (66.7)	21 (68.8)	60 (60)
Gait abnormalities	17 (89.4)	22 (88.0)	17 (94.4)	6 (100)	30 (93.8)	92 (92)
Stereotypic hand movements	19 (100)	24 (96.0)	18 (100)	6 (100)	32 (100)	99 (99)
<b>Supportive criteria</b>						
Breathing disturbances when awake	11 (57.8)	17 (68.0)	13 (72.2)	6 (100)	18 (56.3)	65 (65)
Bruxism when awake	14 (73.6)	20 (80.0)	13 (72.2)	5 (83.3)	18 (56.3)	70 (70)
Impaired sleep pattern	11 (57.8)	14 (56.0)	8 (44.4)	6 (100)	22 (68.8)	61 (61)
Abnormal muscle tone	19 (100)	19 (76.0)	15 (83.3)	6 (100)	29 (90.6)	88 (88)
Peripheral vasomotor disturbances	18 (94.7)	24 (96.0)	18 (100)	6 (100)	28 (87.5)	94 (94)
Scoliosis/kyphosis	3 (15.7)	12 (48.0)	14 (77.8)	6 (100)	29 (90.6)	64 (64)
Inappropriate laughing/screaming spells	18 (94.7)	20 (80.0)	15 (83.3)	6 (100)	20 (62.5)	79 (79)
Autistic behavior with intense eye communication	16 (84.2)	16 (64.0)	13 (72.2)	6 (100)	24 (75.0)	75 (75)
Epilepsy	7 (36.8)	16 (64.0)	13 (83.3)	6 (100)	26 (81.3)	68 (68)

N = number of patients; yrs = years.

Table 2  
Distribution of *MECP2* mutations in Japanese Rett Syndrome Database.

	Number of patients per total
<i>MECP2</i> mutation examination	92/102 (90.2%)
<i>MECP2</i> mutations, identified	88/92 (95%)
Typical	86/102 (84%)
<b>Genotype</b>	
	N(%)
R168X	11 (11.8)
T158M	8 (8.6)
R255X	8 (6.4)
R270X	5 (5.4)
R294X	5 (5.4)
R133C	5 (5.4)
R306C	4 (4.3)
R306W	3 (3.2)

median; 11.4 years of age). Table 1 shows the age distribution and frequency of symptoms and signs. Of 100 RTT patients, 92 (92%) underwent genetic examination by Southern blotting, MLPA, or WES.

Of these patients, 88 (95%) exhibited various *MECP2* mutations (Table 2). *MECP2* mutations included R168X (11 patients) (12.5%), T158M (eight patients) (9%) and R255X (six patients) (6.8%) (Table 2). All patients developed head control (median; 4 months of age). Ninety-seven patients acquired the ability to roll over (median; 6 months), while three were never able to roll over (3%). Seventy-nine patients were able to sit unassisted (median; 8 months) (79%). Fifty-six patients acquired the ability to crawl (median; 11 months) (56%). Forty-nine patients did not acquire the ability to walk without support (median; 18 months) (49%). The age of acquisition of walking ranged from 11 months to 72 months (median: 18 months). Forty

patients did not develop the ability to produce meaningful words (40%). Among the other patients, the age of first meaningful words ranged from 10 months to 72 months of age. Fifty-eight patients exhibited a severely small head circumference (56%).

### 3.1. Correlation of acquisition of walking ability, meaningful words and microcephaly

We hypothesized that walking ability over 10 years of age may be related to a range of factors highlighted in previous studies, as follows: acquisition of meaningful words, presence or absence of microcephaly, dystonia, abnormal muscle tone, breathing abnormalities, age of onset of stereotypic hand movement, age of regression of hand function, and presence or absence of scoliosis [9–12].

First, we analyzed all data related to motor function, then examined acquisition of walking ability in all age groups. The Kaplan–Meier method was used to assess the relationships between acquisition of head control, sitting alone, crawling, meaningful words, microcephaly and acquisition of walking. Fig. 1 shows the estimated mean proportion of patients who acquired walking at all ages, for 100 patients. The proportion of patients who acquired the ability to walk increased until 100 months, then tended to flatten. Patients who acquired meaningful words showed a significantly greater rate of acquiring walking than those who did not acquire meaningful words, for all age groups ( $P < 0.001$ ). Patients with absence of microcephaly exhibited a significantly greater rate of acquiring of walking than those with microcephaly ( $P = 0.005$ ).

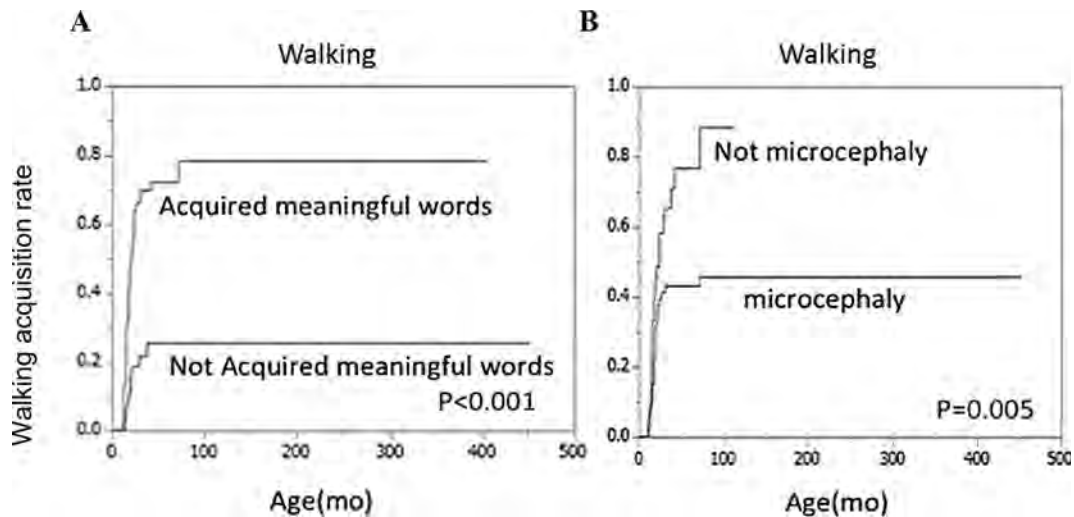


Fig. 1. Relationship between walking ability, acquisition of meaningful speech and microcephaly. Walking ability was significantly different between patients who acquired meaningful words and those who did not acquire meaningful words,  $P < 0.001$  (A). Walking ability was significantly different between patients with and without microcephaly,  $P = 0.005$  (B), Kaplan–Meier method.

### 3.2. Acquisition of walking and effect of *MECP2* mutation type (R306C, R133C, R294X)

Genotype *MECP2* mutation severity was categorized into mild (R306C, R133C, R294X) and other, based on previous reports [13]. To assess acquisition of walking, we also excluded R306C, R133C, R294X gene mutations, because an increasing number of patients with RTT did not undergo *MECP2* gene testing in recent years. Fig. 2 shows an analysis of the acquisition of crawling (Fig. 2A) and walking (Fig. 2B) in relation to gene mutations, including R306C, R133C, R294X and other mutations. The rate of acquisition of crawling

increased with age, until 30 months (Fig. 2A), and the number of patients that acquired walking increased until 40 months of age, then tended to flatten (Fig. 2B). Patients with mild phenotype mutations (R306C, R133C, R294X) exhibited significantly higher rates of acquiring crawling and walking ( $P = 0.004$ ,  $P = 0.01$ , respectively).

### 3.3. Analysis of walking ability over 10 years of age

Age distribution was observed and analyzed in two age categories: over 10 years old, and all ages [12,13]. According to previous studies of motor symptoms

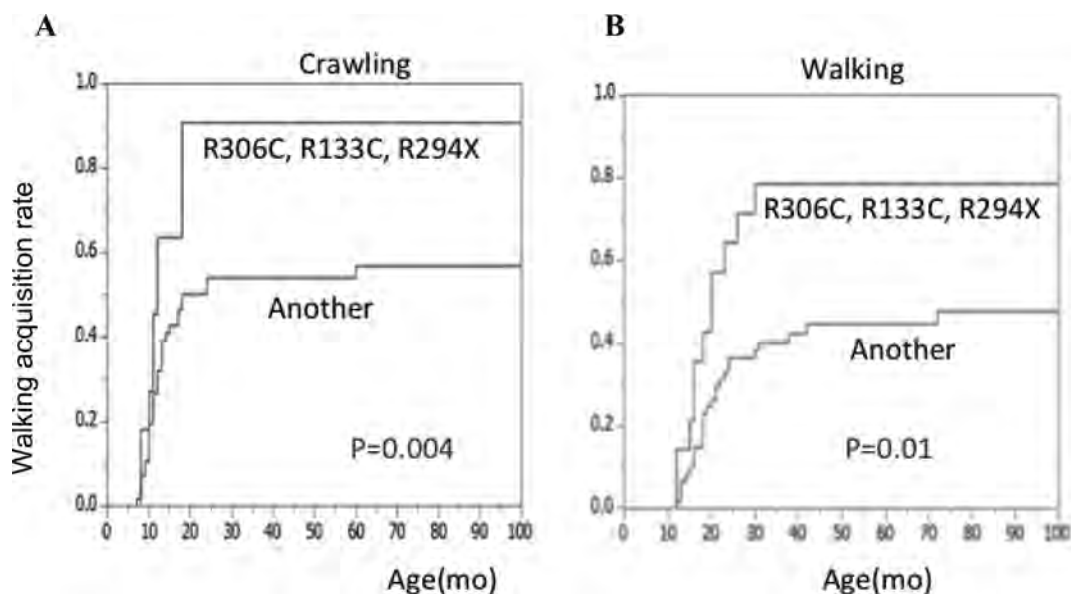


Fig. 2. Crawling and walking ability were significantly different between patients with *MECP2* mutations of R306C, R133C, R294X and other mutations. Crawling ability and walking ability were significantly different between patients with *MECP2* mutations of R306C, R133C, R294X and other mutations.  $P = 0.004$  (A), and  $P = 0.01$  (B), respectively, Kaplan–Meier method.

including walking, we divided patients into four groups, as follows: those currently able to walk, those who were previously able to walk then lost walking ability, those unable to walk at over 10 years of age, and those with unknown walking status (Fig. 3). Of 56 patients over 10 years old, 31 were currently able to walk, 17 patients never learned to walk, six patients had lost the ability to walk, and two patients had unknown walking status. We divided participants into two groups depending on walking prognosis, as follows: participants who were still walking after 10 years of age, and a group of participants who were able to walk previously then lost the ability to walk, or who had never walked (Figs. 3, 4). The Kaplan–Meier method was used to assess the relationships between acquisition of crawling, acquisition of meaningful words, and walking ability over 10 years of age. The results shown in the figures indicate that walking ability was related to the acquisition of crawling (Fig. 4A), and the acquisition of meaningful words (Fig. 4B) over 10 years of age. As shown in Fig. 4, the proportion of patients with the ability to walk over 10 years of age was significantly related to the acquisition of crawling, and the acquisition of meaningful words ( $P < 0.001$ ,  $P < 0.001$ , respectively).

Univariate analysis and multivariate analysis were used to assess the relationships between walking ability and early clinical signs. Univariate analysis revealed that walking ability was significantly correlated with meaningful word acquisition, microcephaly and BMI ( $P < 0.0001$ ,  $P = 0.005$ ,  $P = 0.0018$ , respectively). Lower BMI was negatively correlated with walking ability (Table 3). Walking ability was not related to dystonia, abnormal muscle tone, presence of breathing abnormalities, stereotypic hand movement, regression of hand function, or scoliosis. Multivariate analysis revealed that walking ability was only significantly correlated with

meaningful words ( $P = 0.0003$ ; odds ratio = 15.872). Even when we excluded gene effects, acquisition of meaningful words was the only robustly significant factor associated with walking ability over 10 years of age. Microcephaly, BMI, dystonia, scoliosis, and crawling were not correlated with walking ability over 10 years of age (Table 4).

#### 4. Discussion

RTT, a neurodevelopmental disorder predominantly affecting females, has been characterized by apparently normal initial development followed by frank regression of fine motor and communication skills, typically between 6 and 18 months of age [13]. Our univariate analysis revealed that walking ability was correlated with crawling, meaningful word acquisition, microcephaly, *MECP2* mutation type, and BMI. Multivariate analysis revealed that only meaningful word acquisition was robustly related to walking ability when patients were over 10 years old. A previous study reported that walking was delayed in 30/38 RTT patients (79%); of these, 18 patients could not crawl until 4 years of age [14]. Furthermore, 55% of patients began to walk without having acquired the ability to crawl [14]. Another study reported that patients that had obtained meaningful words began crawling and walking significantly earlier than those without meaningful word acquisition, using univariate analysis [15]. Never acquiring the ability to walk is reported to be related to the early loss of language [11]. The abnormal acquisition of early skills is reported to be in accord with a marked decrease in head size beginning in early postnatal life [16], and the current data support these previous findings. In the current study, acquisition of meaningful words was a robust factor related to various symptoms or functions,

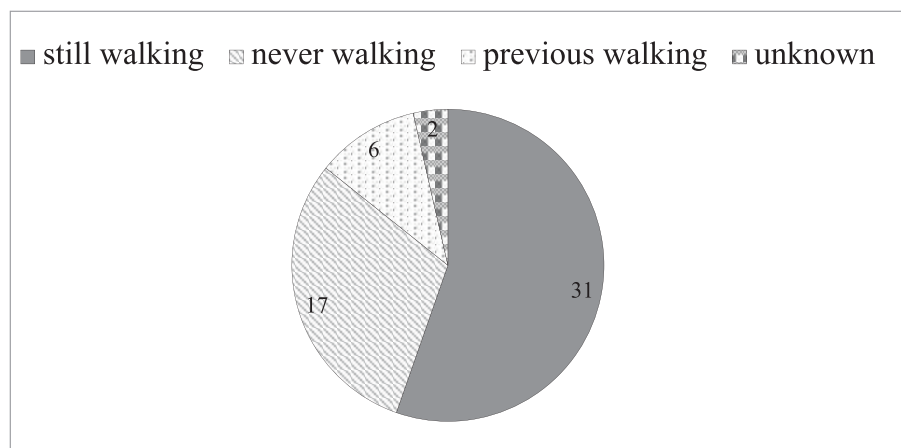


Fig. 3. Division of patients into four groups based on acquired and sustained walking abilities over 10 years of age We divided patients into four groups based on acquired and sustained walking abilities over 10 years of age: currently able to walk, previously able to walk but cannot walk at present, never able to walk, and unknown. Of 56 patients over 10 years old, 31 were currently able to walk, 17 patients never learned to walk, six patients had lost the ability to walk, and two patients had unknown walking status.

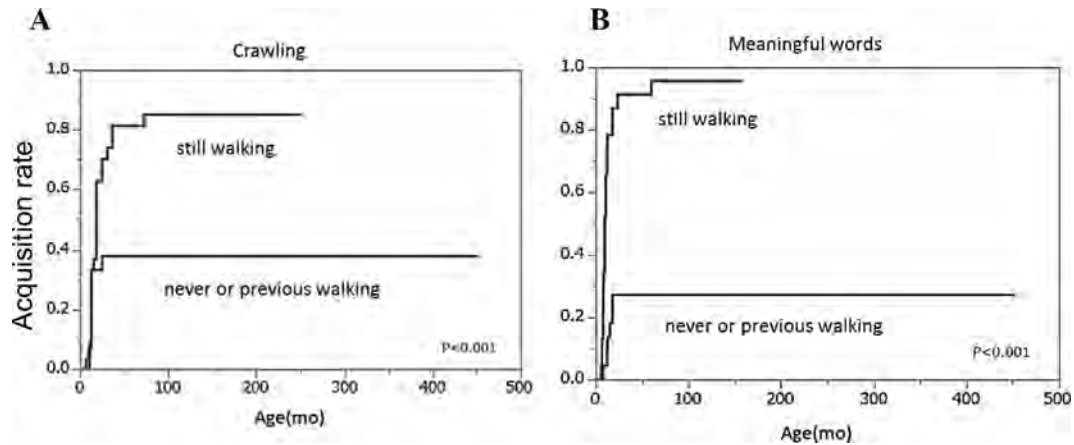


Fig. 4. Relationships between walking ability, crawling ability, and acquisition of meaningful words over 10 years of age. For patients over 10 years old, walking ability was significantly different between those who acquired crawling and those who did not acquire crawling ( $P < 0.001$ ) (A). Walking ability was significantly different between patients who acquired meaningful words and those who did not acquire meaningful words ( $P < 0.001$ ) (B), Kaplan–Meier method. We divided participants into two groups depending on walking prognosis, as follows: participants who were still walking after 10 years of age, and participants who were either able to walk then lost walking ability or who were never able to walk (Fig. 3).

Table 3

Univariate analysis of walking ability and other factors over 10 years of age.

Factors	All patients with RTT		Excluding the patients with R306C, R133C, R294X variants	
	Odds ratio	P-value	Odds ratio	P-value
Meaningful words	7.125	<0.0001	7.959	0.0001
Microcephaly	0.265	0.005	0.261	0.0071
Dystonia	0.833	0.7357	0.831	0.7617
Scoliosis	0.778	0.6572	0.786	0.6905
BMI	1.279	0.0018	1.372	0.0019

Walking ability was significantly correlated with the acquisition of meaningful words, microcephaly and BMI;  $P < 0.0001$ ,  $P = 0.0005$ ,  $P = 0.0018$ , respectively. Lower BMI was negatively correlated with walking ability. BMI: Body Mass Index

Table 4

Multivariate analysis of walking ability and other factors over 10 years of age.

Factor	All patients with RTT		Excluding the patients with R306C, R133C, R294X variants	
	Odds ratio	P-value	Odds ratio	P-value
Meaningful words	15.872	0.0003	16.506	0.0015
Microcephaly	1.366	0.7132	1.706	0.5877
Dystonia	0.526	0.4024	0.685	0.6623
Scoliosis	0.758	0.7357	0.918	0.921
BMI	1.192	0.1186	1.257	0.1452

Walking ability was significantly correlated with the acquisition of meaningful words ( $P = 0.0003$ ; odds ratio = 15.872). Although we excluded the effects of genes, meaningful word acquisition was significantly correlated with walking ability.

including cortical ability (microcephaly), growth/nutrition (BMI), motor ability (crawling), and genotype.

Witt-Engerström et al. reported that, on the basis of acquired and sustained walking ability, patients with RTT aged 22–44 years could be divided into three groups: currently walking, previously walking, and never able to walk [12]. Dystonic signs were most common among patients who were previously able to walk then became unable to walk. Early progression of scoliosis and weakness were most prevalent among patients who were never able to walk [12]. Extrapyramidal signs,

including stereotypic hand movements, gait disturbance, bruxism, bradykinesia, hypomimia, scoliosis, rigidity and dystonia, have been observed in almost all patients with RTT, affecting the daily lives and walking ability of patients [17,18]. We divided patients over 10 years of age into four groups (Fig. 3). Walking ability was related to meaningful words, but dystonic signs and scoliosis were not related to walking ability. The discrepancy between the current findings and the previous report may have arisen because the diagnosis of dystonia was not reported in our study [12]. Data regarding dystonia were

collected in 67 of 100 cases (67%). Among these 67 cases, the presence of dystonia in patients with RTT was reported in 20 cases (29.9%). Over 10 years, data regarding dystonia were collected in 42 of 56 cases (75%). Among these 42 cases, dystonia was reported in 14 cases (33.3%). It is possible that the diagnosis of dystonia is difficult in some cases, and our database did not include the detail of the neurological examination of extrapyramidal signs. Unfortunately, we also did not evaluate the severity of scoliosis in the current study.

Our study confirmed the importance of genotypes associated with severe and mild phenotypes [3–5]. Tarquine et al. reported that typical RTT was associated with more severely affected growth (height, weight, head circumference, and BMI) than atypical RTT. Decreased growth, including body weight, height and microcephaly, was associated with more impaired development, higher disease severity, and specific MECP2 mutations (pre-C-terminal truncation, large deletion, T158M, R168X, R255X, and R270X) [16]. In previous reports, the mutations T158M, R255X and R168X, and R270X have generally been associated with more severe phenotypes, while R306C, R133C, R294X and 3' truncations have been associated with less severe disease [3–5]. Patients with R306C, R133C, R294X and 3' truncations are reported to acquire more gross motor skills and lose fewer skills, particularly in fine motor and expressive language abilities [6,7]. However specific mutations may not be the only determinant of severity within specific individuals due to the existence of other factors, such as X-chromosome inactivation, genetic background (the interplay of other genetic variations), and distribution of abnormal genes in specific brain regions [3,5,19,20]. Mutation type has some effect on the phenotypic manifestation of RTT, and the pattern of X inactivation is thought to determine phenotypic severity [21]. MECP2 interacts with a wide variety of cofactors. The intrinsically disordered nature of MECP2 permits a high degree of structural flexibility, allowing MECP2 to interact with many diverse protein partners. MECP2 utilizes a variety of mechanisms to regulate gene expression, which is dependent on the proteins with which it interacts at any given time. The clinical variability of these mutation suggests that it plays a major role in the function of MECP2 protein [22].

The database used in this study included information provided by parents and caregivers of RTT patients, with confirmation by a pediatric neurologist. Furthermore, 92 of 100 (92%) RTT participants underwent gene testing. Using a Japanese database, our results revealed that acquisition of meaningful words was the only factor that was robustly and significantly correlated with walking ability over 10 years. However, microcephaly, dystonia, scoliosis and BMI were not correlated with walking abilities in the multivariate analysis.

## 5. Limitations of this study

Several limitations of this study may should be considered in interpreting the results. The principal limitation was the relatively small sample size of the study, limiting the generalizability of the results. Our study also used a cross-sectional design. However, our study also had several unique features, including collaborative study of parents or caregivers, and direct examination by a pediatric neurologist. In most previous studies, data were derived from questionnaires without direct assessment of participants by clinicians experienced in the diagnosis of RTT. In addition, we performed multivariate analysis, because many factors may be tightly linked with other factors.

In conclusion, our findings may be useful for informing the development of early intervention methods, and the planning of comprehensive treatment for young infants with RTT. The acquisition of meaningful words was only significantly correlated with walking ability over 10 years of age among patients with RTT.

## Author contributions

All authors have been involved in drafting or revising the manuscript, have given final approval, and agree to be accountable for all aspects of the work involved. Each author's individual participation is outlined below. TS, Shin N, ST, TM, and MI did the conceptualization and design of the study and acquisition, analysis, and interpretation of the data. MK and TK did the statistical analysis of the data. Tetsu T, KY, SN, TT, YY, YK, and CH performed the follow-up examinations and interpretation of the data.

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## Conflict of interest

None of the authors have conflicts of interest to declare.



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## CLINICAL REPORT

# MeCP2\_e2 partially compensates for lack of MeCP2\_e1: A male case of Rett syndrome

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Email: takeguchi5p@asahikawa-med.ac.jp**Abstract****Background:** Rett syndrome (RTT) is a neurodevelopmental disorder that predominantly affects girls. Its causative gene is the X-linked *MECP2* encoding the methyl-CpG-binding protein 2 (MeCP2). The gene comprises four exons and generates two isoforms, namely *MECP2\_e1* and *MECP2\_e2*. However, it remains unclear whether both MeCP2 isoforms have similar function in the brain.**Methods:** We report a case of a boy with typical RTT. Male cases with *MECP2* variants have been considered inviable, but somatic mosaicism of the variants can cause RTT in males. Whole-exome sequencing was performed to search for the genetic background.**Results:** A novel nonsense and mosaic variant was identified in exon 1 of *MECP2*, and the variant allele fraction (VAF) was 28%. Our patient had the same level of VAF as that in reported male cases with mosaic variants in *MECP2* exon 3 or 4, but manifested RTT symptoms that were milder in severity compared to those in these patients.**Conclusion:** This is probably because the variants in *MECP2* exon 3 or 4 disrupt both isoforms of MeCP2, whereas the variant in exon 1, as presented in this study, disrupts only MeCP2\_e1 but not MeCP2\_e2. Therefore, our findings indicate that MeCP2\_e2 may partially compensate for a deficiency in MeCP2\_e1.**KEYWORDS**

male with Rett syndrome, MeCP2 isoform, MeCP2\_e1, MeCP2\_e2, somatic mosaicism

## 1 | INTRODUCTION

Rett syndrome (RTT) is a neurodevelopmental disorder primarily occurring in girls. It is caused by a loss-of-function variant in one copy of the X-linked gene *MECP2* (OMIM #300005) that encodes methyl-CpG-binding protein 2 (MeCP2). In females with typical RTT due to random

X-chromosome inactivation (XCI), approximately 50% of the cells express the variant *MECP2* and the other half express the wild-type *MECP2*. Males manifesting the symptoms of typical RTT also have an *MECP2* variant that is found in females with typical RTT. These males have either an extra X-chromosome (Klinefelter syndrome) or somatic mosaicism of the variant (Kleefstra et al., 2004; Schonewolf-Greulich

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et al., 2019; Schwartzman, Bernardino, Nishimura, Gomes, & Zatz, 2001; Topcu et al., 2002; Villard, 2007; Zhang et al., 2019). Evidence can be obtained from male RTT patients with somatic mosaicism of the *MECP2* variant to better understand the relationship between variant allele fractions (VAFs) and the clinical severity of RTT, because the effect of XCI on the phenotype does not need to be considered in male cases.

*MECP2* comprises four exons and generates two isoforms: *MECP2\_e1* and *MECP2\_e2* as a result of the alternative splicing of exon 2. MeCP2\_e1 is translated from a start site in exon 1, and exon 2 is skipped through alternative splicing, whereas MeCP2\_e2 is translated from a start site in exon 2 (Mnatzakanian et al., 2004). In this study, we sought to investigate the case of a male RTT patient mosaic for nonsense variant in exon 1 of *MECP2* that disrupts only MeCP2\_e1 but not MeCP2\_e2. To examine whether MeCP2\_e2 can ameliorate some neurological symptoms due to the affected MeCP2\_e1 functions, we compared the clinical severity of the present case with those of other reported male cases carrying the mosaic variants that affect both MeCP2\_e1 and MeCP2\_e2.

## 2 | MATERIALS AND METHODS

### 2.1 | Patient background and informed consent

The patient was a young boy with typical RTT phenotype who fulfilled the diagnostic criteria for the disorder (Neul et al., 2010). He and his parents gave informed consent to participate in this study. The experimental protocols were approved by the Committee for Ethical issues at Asahikawa Medical University.

### 2.2 | Mutation analysis of the *MECP2*

For Sanger sequencing, their DNA was used as the template for polymerase chain reaction (PCR). Appropriate primers were used to yield DNA fragments spanning the entire *MECP2* coding region and the intron–exon boundaries (Takahashi et al., 2008). The PCR fragments were analyzed using automated sequencing. Whole-exome sequencing of the DNA was performed on a HiSeq2000 sequencer (Illumina) with 101 bp paired end reads and 6 bp index reads. Exome data processing, variant calling, and variant annotation were performed as previously described (Itoh et al., 2018). To confirm the variant identified in the Sanger and whole-exome sequencing, the DNA fragment encompassing the variation site was amplified by PCR using the primers 5'-CATCACAGCCAATGACGGGC-3' (forward) and

5'-CATCCGCCAGCCGTGTCGTC-3' (reverse), and it was subsequently digested with restriction endonuclease Dde I. The reaction products were then visualized through ethidium bromide staining after electrophoresis on a 2% agarose gel.

### 2.3 | RNA isolation and RT-PCR

To examine the expression levels of *MECP2\_e1* and *MECP2\_e2* isoforms, total RNA was extracted from the peripheral blood cells using the PAXgene Blood RNA Kit (QIAGEN GmbH). Reverse transcription (RT) was performed using the SuperScript First-Strand Synthesis System (Invitrogen Corporation) for generation of cDNA using 1 µg of total RNA in a 20 µl reaction. Primers were designed for simultaneous amplification of both isoforms: a forward primer in exon 1 (exon1F, 5'-GAGAGGGCTGTGGTAAAAGC-3') and a reverse primer in exon 3 (exon3R, 5'-GATGGAGCGCCGCTGTTTGG-3'), which generated a 328-bp product for *MECP2\_e1* and a 452-bp product for *MECP2\_e2*. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as described (Itoh et al., 2012). The PCR products were visualized by ethidium bromide staining, following electrophoresis on 2% agarose gels. The optical densities of the bands were quantified using an image analysis system and ImageJ software (National Institutes of Health; Bethesda, MD). The obtained PCR products were purified from an agarose gel and directly sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems).

## 3 | RESULTS

### 3.1 | Case report

The 9-year-old male patient was born after 39 weeks of uneventful pregnancy without asphyxia. His birth weight and head circumference were 2,865 g (−0.7 SD) and 33.0 cm (−0.4 SD), respectively. He acquired head control at 3 months of age, walked alone and spoke meaningful words at 9 months, and had no apparent developmental delay until he was about 2 years old. Thereafter, his development stagnated, and it was followed by a period of regression. He became less interested in his toys and hardly had any speech. At 3 years and 6 months, he was diagnosed with autism spectrum disorder and intellectual disability. At 4 years, his purposeful hand skills began to regress, and stereotypic movements such as hand wringing appeared. At 6 years, he lost his minimal spoken language, his gait became unsteady and wide based, and his head circumference was only 47.8 cm (−2.2 SD), making his postnatal microcephaly more evident. He developed epilepsy at 7 years, but his seizures were eventually controlled following treatment with carbamazepine and topiramate. The chromosomal

analysis revealed normal 46,XY karyotype. No abnormal findings were observed in brain MRI and various tests related to congenital metabolic disorders.

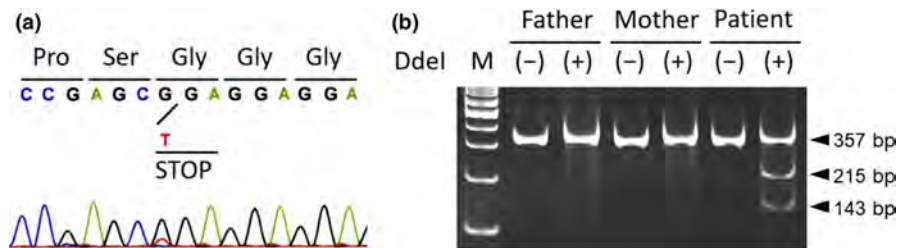
### 3.2 | Molecular studies

We performed the Sanger sequencing of *MECP2* for genetic diagnosis, but the initial analysis failed to identify the pathogenic variant of this gene. Subsequently, we performed whole-exome sequencing and identified a novel nonsense and mosaic variant in exon 1 of *MECP2*, NM\_001110792.1: c.31G>T; p.(Gly11\*). Deep sequencing confirmed the presence of the T allele uniquely in the patient sample at a ratio of 25 mutant T allele reads to 63 G allele reads, a VAF of 28%. The reexamination of the *MECP2* variant using Sanger sequencing confirmed the wild-type sequence, with only a small amount of the variant allele, suggesting somatic mosaicism (Figure 1a). This nonsense variant created a new Dde I restriction site. Consequently, PCR-restriction digestion analysis of the DNA obtained from the patient and his parents revealed novel fragments in addition to the estimated wild-type fragment only in the patient sample, further confirming that the mosaic variant occurred de novo (Figure 1b). RT-PCR results revealed that the variant did not

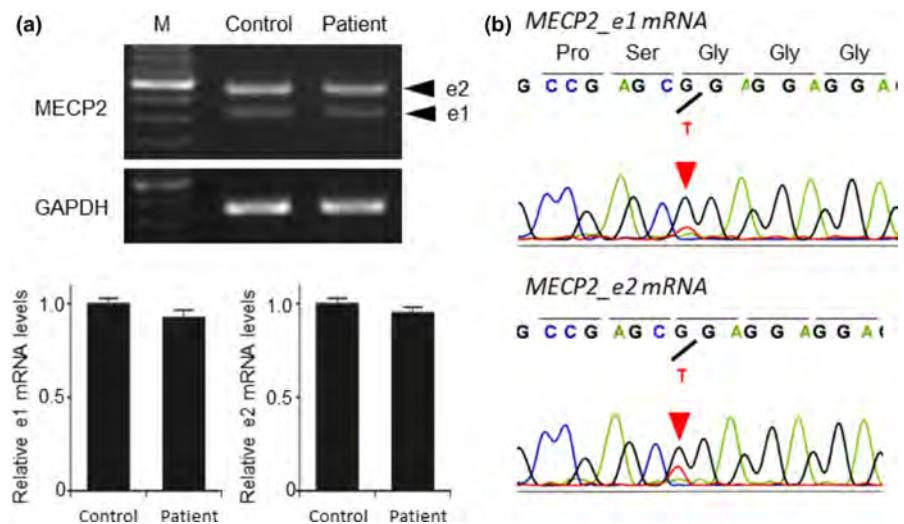
affect the expression levels of both *MECP2\_e1* and *MECP2\_e2* (Figure 2a). Analysis of cDNA showed the presence of an abnormal transcript with the nonsense variant of exon 1 in a mosaic state (Figure 2b).

### 3.3 | Compensatory role of MeCP2\_e2 for lack of MeCP2\_e1

The nonsense variant identified in the present case affected the coding sequence of *MECP2\_e1* but not of *MECP2\_e2*. Based on this finding, we further examined whether MeCP2\_e2 is able to ameliorate the affected MeCP2\_e1 function by comparing the clinical severity of the present case with those of six previously reported RTT males carrying *MECP2* mosaic variants that affect both MeCP2\_e1 and MeCP2\_e2 (Table 1). This comparison revealed that the median VAFs and the age at onset of regression of the reported male patients were 25% (range 9–36) and 13 months (range 8–18), respectively, whereas in the present case with 28% VAF, the developmental problems were not noticed until the patient was 2 years old. At 9 years, he was already able to walk independently, although his balance was poor. Among the six previously reported cases, only three were able to walk with or without support.



**FIGURE 1** A novel mosaic variant of *MECP2* in a male patient with typical Rett syndrome. Electropherogram shows the nonsense variant in exon 1 of *MECP2* [NM\_001110792.1: c.31G>T; p.(Gly11\*)] in mosaicism (a). Dde I digestion of the PCR product encompassing the variation site shows additional fragments (143 and 215 bp), which resulted from the G-to-T transition creating a new Dde I restriction site in the patient but not in his parents (b). These additional fragments are observed together with the 358 bp wild-type fragment, confirming the mosaic variant in the patient



**FIGURE 2** Equal amounts of *MECP2\_e1* and *MECP2\_e2* mRNA and abnormal transcript with the nonsense variant in a mosaic state. RT-PCR results reveal that both *MECP2\_e1* and *MECP2\_e2* mRNA amounts are unaffected in the patient. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control (a). Sequencing analysis of the obtained PCR products shows the presence of abnormal transcript with the nonsense variant in a mosaic state (b)

**TABLE 1** Relationship between variant allele fractions and the clinical severity in males with typical Rett syndrome associated with mosaic *MECP2* variants

Age at evaluation (Authors)	Variants			Onset of regression	Hand skills	Language	Gait	Stereotypy
	Nucleotide change	Predicted effect on protein sequence	VAFs <sup>a</sup>					
9 years (Present case)	c.31G>T	p.(Gly11*)	28%	24 months	Poor	Lost	Poor	Present
12 years (Topcu et al., 2002)	c.808C>T	p.(Arg270*)	36%	11 months	Lost	Never	Never	Present
11 years (Kleefstra et al., 2004)	c.473G>T	p.(Thr158Met)	25%	13 months	Lost	Lost	Lost	Present
2 years (Zhang et al., 2019)	c.316C>T	p.(Arg106Trp)	26%	18 months	Never	Never	Never	Present
2 years (Zhang et al., 2019)	c.353G>A	p.(Gly118Val)	20%	13 months	Poor	Lost	Poor	Present
8 years (Schonewolf-Greulich et al., 2019)	c.1308dupT	p.(Gln437Serfs*50)	9% (15%)	18 months	Poor	Lost	Poor	Present
9 years (Schonewolf-Greulich et al., 2019)	c.808C>T	p.(Arg270*)	36% (45%)	8 months	Lost	Never	Poor	Present

<sup>a</sup>VAFs, variant allele fractions in blood lymphocytes (in fibroblasts).

## 4 | DISCUSSION

We present clinical and molecular findings in a male RTT mosaic for a novel nonsense variant of *MECP2*. Deep sequencing with a next-generation sequencing method revealed the small amount of the variant allele c.31G>T; p.(Gly11\*) with 28% VAF, which the initial Sanger sequencing failed to identify. The PCR-restriction digestion analysis further confirmed the somatic mosaicism of the variant. RT-PCR results revealed that both *MECP2\_e1* and *MECP2\_e2* mRNA amounts were unaffected in the patient, suggesting that the nonsense variant in exon 1 of *MECP2* likely escapes nonsense-mediated mRNA decay (NMD) and does not affect transcription of *MECP2\_e2*. The level of sensitivity of a premature-termination codon (PTC) - containing mRNA to NMD is multifactorial. It has been shown that mRNAs carrying PTCs in close proximity to the translation initiation AUG codon escape NMD, called the “AUG-proximity effect” (Silva, Ribeiro, Inacio, Liebhaber, & Romao, 2008). Consequently, this variant may lead to translational reinitiation at a downstream AUG codon producing an N-terminally truncated protein functionally distinct from wild-type MeCP2\_e1, but does not affect the translation of *MECP2\_e2*.

The majority of the RTT-associated *MECP2* variants are located in exons 3 and 4, which simultaneously disrupt both the MeCP2\_e1 and MeCP2\_e2 isoforms. RTT associated with exon 1 variants of *MECP2* is rare, and its detection rate is 8.1% in patients with typical or atypical RTT (Saunders, Minassian, Chow, Zhao, & Vincent, 2009). However, exon 2 variants that exclusively affect MeCP2\_e2 have never been identified in RTT, suggesting that MeCP2\_e2 does not have

an essential function in the brain. Supporting this notion, a mouse model with a deletion in *MECP2* exon 2 failed to recapitulate the neurologic symptoms characteristic of RTT (Itoh et al., 2012). In this study, we presented the nonsense variant in exon 1 of *MECP2* that disrupted MeCP2\_e1 but not MeCP2\_e2. Notably, however, we examined the variant using DNA extracted from peripheral blood leukocytes, so that it is uncertain whether the VAF in brain will be equivalent to that observed in the present study. Nonetheless, this study demonstrated that a male carrying an *MECP2* exon 1 mosaic variant and expressing 28% less MeCP2\_e1 in blood than normal individuals exhibited the typical RTT phenotype, indicating that even a mild reduction of MeCP2\_e1 is sufficient to cause RTT.

In a previous study using the isoform-specific knockout mice in which MeCP2\_e1 was lacking while the expression of MeCP2\_e2 was preserved, the neurologic deficits of RTT were recapitulated (Yasui et al., 2014). The study implied that an RTT phenotype may occur even in the presence of MeCP2\_e2, which is unable to compensate for the lack of MeCP2\_e1. Nevertheless, recent animal studies have revealed the partial rescue of Rett-like symptoms in MeCP2-null mice through the reexpression of MeCP2\_e2 (Jugloff et al., 2008; Kerr et al., 2012). However, there had been no clinical evidence whether MeCP2\_e2 is able to ameliorate some neurological symptoms due to the affected MeCP2\_e1 functions. Genotype-phenotype correlations are difficult to make in female RTT patients because of the differences in XCI. Examination of male RTT patients with somatic mosaicism of the *MECP2* variant allowed us to assess the relationship between VAFs and clinical severity. Comparison of the clinical severity of the present case involving the *MECP2\_e1*-specific variant

with those of other reported male cases with mosaic variants that affect both MeCP2\_e1 and MeCP2\_e2 revealed that the present case had a milder phenotype even though the VAFs were almost the same (Table 1). In conclusion, this study is the first to present clinical and molecular evidence that MeCP2\_e2 may partially compensate for the deficiency of MeCP2\_e1, although further functional studies are needed.

## ACKNOWLEDGMENTS

We sincerely thank the patient and his parents, whose help and participation made this work possible.

## CONFLICT OF INTEREST

None of the authors declare any conflict of interest related to this study.

## AUTHOR CONTRIBUTIONS

RT and ST conceived and planned the study, and drafted the manuscript. MI performed whole-exome sequencing. RT, ST, MK, RT, and NS performed genetic analysis. YT, YL, and NS acquired clinical phenotype data. All authors provided important feedback on the analysis and manuscript, and approved the final version.

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# Taurine, Coenzyme Q<sub>10</sub>, and Hydrogen Water Prevents Germanium Dioxide-Induced Mitochondrial Dysfunction and Associated Sensorineural Hearing Loss in mouse <sup>☆</sup>

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## ABSTRACT

Mitochondrial dysfunction has been implicated in numerous common diseases as well as aging and plays an important role in the pathogenesis of sensorineural hearing loss (SNHL). In the current study, we showed that supplementation with germanium dioxide (GeO<sub>2</sub>) in CBA/J mice resulted in SNHL due to the degeneration of the stria vascularis and spiral ganglion, which were associated with down-regulation of mitochondrial respiratory chain associated genes and up-regulation in apoptosis associated genes in the cochlea. Supplementation with taurine, coenzyme Q10, or hydrogen-rich water, attenuated the cochlear degeneration and associated SNHL induced by GeO<sub>2</sub>. These results suggest that daily supplements or consumption of antioxidants, such as taurine, coenzyme Q10, and hydrogen-rich water, may be a promising intervention to slow SNHL associated with mitochondrial dysfunction.

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## 1. Introduction

Mitochondria are the primary organ generating cellular adenosine triphosphate (ATP) and play a central role in a variety of cellular processes, including calcium signaling, reactive oxygen species (ROS) generation, and apoptosis (Yamasoba et al., 2007). Based on these important roles, impairment of mitochondrial function has been implicated in numerous common diseases and conditions, such as cardiovascular disease, neurodegenerative diseases, metabolic disorders, and even in normal aging (Wang et al., 2016).

Mitochondrial dysfunction is typically associated with sensorineural hearing loss (SNHL). For example, in humans, several mutations and deletion in mitochondrial DNA (mtDNA) have been reported to cause both syndromic and non-syndromic forms of SNHL. Further, patients with age-related hearing loss (ARHL) have a significant load of acquired mtDNA mutations in their auditory tissues (Gopinath et al., 2009). In animal models, accumulation of mtDNA mutations by the mutator allele of the mitochondrial

Polγ DNA polymerase has shown ARHL acceleration (Kujoth et al., 2005). Even in acute SNHL, such as noise- and aminoglycoside-induced hearing loss, impairment of mitochondrial function has been shown to play an important role (Böttger and Schacht, 2013; Fujimoto and Yamasoba, 2019). Considering these findings, establishing a good animal model presenting mitochondrial dysfunction with hearing loss and discovering the methods for preventing the symptoms occurring in these animals seems to play an important role in overcoming many SNHL diseases as well as many other systemic diseases.

It has been demonstrated that chronic intake of germanium dioxide (GeO<sub>2</sub>) both in humans and animal models causes symptoms and pathological findings similar to those in patients with mitochondrial encephalomyopathy, which is known as mtDNA mutation disease (Higuchi et al., 1989; Takeuchi et al., 1992; Asaka et al., 1995; Kim et al., 1998; Higuchi et al., 1991; Sanai et al., 1990; Wu et al., 1992; Li et al., 2001; Lin et al., 2006). For example, the skeletal muscles from rats treated with GeO<sub>2</sub> for 23 weeks contained numerous ragged-red fibers and cytochrome-c oxidase (COX)-deficient fibers and showed reduced enzyme activities in the mitochondrial respiratory chain, such as rotenone-sensitive NADH-cytochrome-c reductase and COX (Higuchi et al., 1991). These results suggest that GeO<sub>2</sub> administration can reproduce several pathological conditions caused by mitochondrial dysfunction.

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tion, which may be useful in elucidating diseases associated with mitochondrial dysfunction and their treatment.

Moreover, we have previously reported that diet supplemented with 0.5% GeO<sub>2</sub> caused profound SNHL associated with degeneration of the stria vascularis and supporting cells in guinea pigs (Yamasoba et al., 2006). This result indicated that GeO<sub>2</sub> application would be also used to create SNHL animal with mitochondrial dysfunction. Although we speculated that cochlear degeneration caused by GeO<sub>2</sub> intake was associated with mitochondrial damage, mitochondrial function was not investigated in this previous study.

ROS are known to be closely related to mitochondrial dysfunction. ROS are continuously produced via normal metabolism by the electron transport chain in mitochondria. In normal status, cells effectively remove ROS by their innate ROS defense systems, such as superoxide dismutase (SOD), catalase, and glutathione (Finkel and Holbrook, 2000; Raha and Robinson, 2000; Dereköy et al., 2004). However, uncontrolled leakage of ROS by irregular respiratory chain and/or decrease in the defense systems can lead to cellular dysfunction. It has been shown that the lack of SOD1 or glutathione peroxidase resulted in severe hearing loss or higher susceptibility to noise exposure, which causes excessive ROS production and induces damage to the outer and inner hair cells (Ohlemiller et al., 2000). These reports suggest that daily dietary intake of ROS scavengers may augment the defense system against ROS and thereby prevent cellular damage caused by mitochondrial damage or dysfunction.

In the current study, we investigated whether chronic intake of GeO<sub>2</sub> results in cochlear mitochondrial impairment and associated SNHL in CBA/J mice. Next, we investigated the effects of ROS scavengers, taurine, coenzyme Q10 (CoQ10), or hydrogen-rich water (Huxtable, 1992; Erdem et al., 2000; Qiao et al., 2015; Koh et al., 2014; Das et al., 2009; Manna et al., 2009; Alam and Hafiz, 2011; Roy and Sil, 2012; M Sikorska et al., 2014; M Sikorska et al., 2014; Sohet et al., 2009; Someya et al., 2009; Yamada et al., 2015; Ohsawa et al., 2008; Sato et al., 2008; Ohsawa et al., 2007; Hayashida et al., 2008; Yoshida et al., 2012; Fukuda et al., 2007; Nakashima-Kamimura et al., 2009; Lin et al., 2011; Fransson et al., 2021), on cochlear degeneration and SNHL induced by GeO<sub>2</sub>.

## 2. Material and methods

Female CBA/J mice were purchased from CLEA Japan (Tokyo, Japan). The experimental protocol was approved by the Committee for the Use and Care of Animals at the University of Tokyo and conformed to the NIH Guidelines for the Care and Use of Laboratory Animals.

### 2.1. Experimental protocols

#### 2.1.1. Experiment 1: development and analysis of mouse model of progressive hearing loss by chronic oral intake of GeO<sub>2</sub>

Ten 2-month-old CBA/J mice were used. Five of them were given chow containing 0.15% GeO<sub>2</sub> for 4 months. The amount of GeO<sub>2</sub> was determined from a previous report using rats (Wu et al., 1992). The remaining five animals were given the normal chow serving as control. In the preliminary experiment, auditory brainstem response (ABR) thresholds were measured at 0, 2, 3 and 4 months at 2, 4, 8, and 16 kHz (Supplemental Figure 1). Animals given GeO<sub>2</sub> showed increase of ABR thresholds and became profoundly deaf at 4 months. Therefore, histological changes and gene expression were evaluated 4 months after the start of germanium administration. The left cochlea, muscle, and kidney was fixed with 2% PFA and 2.5% glutaraldehyde, the cochlea was additionally decalcified, and embedded in epoxy resin. Ultrathin sections were examined under transmission electron microscope. The

right cochleae were used for gene transcriptional analysis of the cochlea by DNA micro array.

Another 10 two-month-old CBA/J mice were used to confirm gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Five animals were given chow containing 0.15% GeO<sub>2</sub> and the remaining five animals were given the normal chow for 4 months. The cochleae were dissected and RNA was extracted.

#### 2.1.2. Experiment 2: prevention of GeO<sub>2</sub> –induced cochlear damage by oral intake of ROS scavengers

Two-month-old CBA/J mice that showed auditory brainstem response thresholds within the normal laboratory range were used. Forty animals were given chow containing 0.15% GeO<sub>2</sub> for 3 months and assigned to one of the four groups (*n* = 10 each) according to the content of their drinking water: 1) water without antioxidant; 2) water containing 0.3% taurine (Wako Inc., Osaka, Japan); 3) water containing 150 μM water-soluble CoQ10 (Aqua Q10L10, Nisshin Pharma Inc., Tokyo, Japan); and 4) hydrogen water (Blue Mercury, Tokyo, Japan). The hydrogen water was placed in a closed glass vessel and changed every other day, which minimized the leakage of hydrogen from the water and maintained the concentration to be greater than 0.4 mM 1 day later (Ohsawa et al., 2007), and the remaining 10 animals were fed with normal chow and water as a control. Amounts of chow that each group ate were measured to confirm that there was no difference of the eating amounts among groups. Body weight of each animal was also measured before they were euthanized.

The 3-month ABR measurement took longer than usual to obtain the ABR threshold, and several animals died during the ABR measurement due to additional anesthesia. As a result, the final numbers analyzed were 10, 7, 9, 6, and 7 animals for the control, GeO<sub>2</sub>±normal water, GeO<sub>2</sub>±taurine, GeO<sub>2</sub>±hydrogen, and GeO<sub>2</sub>±CoQ10 groups, respectively. In this experiment, the significant protective effect of the ROS scavenger on GeO<sub>2</sub> was confirmed by 3 months, so we decided to euthanize the animals at 3 months instead of 4 months to avoid further discomfort to the animals, to prevent further sample loss, and to reduce the expense of the agents.

### 2.2. Assessment of hearing function

Detailed protocols for ABR measurements have been described elsewhere (Kinoshita et al., 2013). Briefly, two examiners who were blinded to the experiment and measured ABRs with a tone burst stimulus (2, 4, 8, 16, and 32 kHz) using an ABR recording system (Neuropack Σ MEB5504, Nihon Kohden, Tokyo, Japan). Mice were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg, i.m.) and ketamine hydrochloride (40 mg/kg, i.m.). Needle electrodes were placed subcutaneously at the vertex (active electrode), beneath the left pinna (reference electrode), and beneath the right ear (ground). The sound stimulus consisted of a 15-ms tone burst, with a rise-fall time of 1 ms at frequencies of 2, 4, 8, 16, and 32 kHz. The sound intensity varied in 5-dB intervals near threshold. To obtain a waveform, 1024 tone presentations given at the rate of 17/s were averaged with the Neuropack MEB-2208 evoked potential measuring system (Nihon Kohden, Tokyo, Japan). The threshold was defined as the lowest intensity level at which a clear reproducible wave V could be observed in the trace. When an ABR waveform could not be evoked, the threshold was determined to be 110 dB SPL (5 dB greater than the maximum intensity (105 dB SPL) produced by the system). ABR thresholds were measured at 2 and 6 months of age in experiment 1 and 2 and 5 months of age in experiment 2.

### 2.3. Transmission electron microscopic observation of the cochlea, kidneys, and soleus muscles in animals given GeO<sub>2</sub>

In experiment 1, animals were euthanized at the age of 6 months after the last ABR measurements. The left cochlea, muscle, and kidney were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde, cochlea was additionally decalcified, and embedded in epoxy resin. Ultrathin sections were examined under transmission electron microscope.

### 2.4. Histological analysis of the cochlea under light microscope

In experiment 2, the cochlear pathology was examined under light microscope. Detailed preparation and examination protocols for determining cochlear pathology have been described previously (Lin et al., 2006; Kinoshita et al., 2013). Briefly, all animals were euthanized under deep anesthesia with xylazine hydrochloride and ketamine hydrochloride at the age of 5 months. The left cochlea was immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline overnight at 4 °C and decalcified in 10% ethylenediaminetetraacetic acid solution. The specimens were then dehydrated through a graded alcohol series and embedded in paraffin. The embedded tissues were cut into 5- $\mu$ m thick sections parallel to the modiolus, and two sequential sections were mounted on glass slides and deparaffinized. Five sections at an interval of three slides (i.e., at an interval of approximately 30- $\mu$ m) were stained with hematoxylin and eosin and observed under a light microscope (Nikon Eclipse E800M, Tokyo, Japan, 40 $\times$  objective) to evaluate spiral ganglion cell (SGC) densities and stria vascularis degeneration in the lower-basal turn.

The number of SGCs and the area of Rosenthal's canal of the lower-basal turn were measured using Photoshop CS4 software, and SGC density (SGC number/mm<sup>2</sup>) was calculated, as previously reported (S Someya et al., 2007). In brief, the number of SGCs in each profile were counted with computer monitors. The area of the Rosenthal's canal profile was determined in each photomicrograph by outlining the margin of bony canal using 'Select' tool. The number of the pixel of the Rosenthal's canal was measured using 'Histogram' tool. The pixels were then converted to the area by calculating the number of pixels per unit area. The density of SGC was calculated for each profile of the ganglion as the number of SGCs divided by the area of Rosenthal's canal (mm<sup>2</sup>).

The area of the stria vascularis of the lower-basal turns was measured in digital photomicrographs using Photoshop CS4 software. The proportions of affected areas were also measured in digital photomicrographs. From these data, degeneration rate was calculated by the vacuolar degenerated area divided by the total area of stria vascularis.

### 2.5. Gene transcriptional analysis of the cochlea by DNA micro array

Detailed protocols for gene expression profiling analysis using Affymetrix microarray analysis have been described (Affymetrix 2004; Lee et al., 1999). Briefly, the right cochleae of the animals were used in this study. The cochleae were placed in a micro centrifuge tube, flash frozen in liquid nitrogen, and stored at -80 °C. Total RNA was extracted from the frozen cochleae by using the TRIzol reagent (Life Technologies, Grand Island, NY). We hybridized each sample to a single Affymetrix MOE 430A Gene Chip (Affymetrix, Santa Clara, CA). Signals in each image were normalized to minimize an overall variability in hybridization intensities by a global scaling method using the Affymetrix software as described in the previous report (S Someya et al., 2007). A gene was considered "expressed" if it displayed a "present" call in at least one GeneChip based on the Affymetrix "present/absent call" algorithms. All genes considered "not expressed" were eliminated

from our analysis. To identify genes whose expression was significantly altered by GeO<sub>2</sub>, each control sample ( $n = 5$ ) was compared to each GeO<sub>2</sub> sample ( $n = 5$ ), generating a total of 25 pairwise comparisons. Gene expression change was considered significant when the P value was <0.05 and the fold change was >1.2. We then used Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Dennis et al., 2003) and Expression Analysis Systematic Explorer (EASE) (Hosack et al., 2003) to assign identified genes to "GO (Gene Ontology): Biological Process" categories of Gene Ontology Consortium ([www.geneontology.org](http://www.geneontology.org)). We also used EASE to determine the total number of identified genes that were assigned to each Biological Process category and the total number of genes on the array in each Biological Process category and to identify "GO: Biological Process" categories statistically associated with AHL-correlated genes by performing Fisher exact tests. The Fisher exact score represents the probability that an overrepresentation of germanium-induced hearing loss-correlated genes in a certain GO: Biological Process category occurs by chance (Hosack et al., 2003). When the Fisher Exact score is < 0.05 for a given GO: Biological Process category, this gene list is considered to be specifically associated (enriched) in the Biological Process category. Gene probe sets were considered "genes" if they had been assigned a "gene symbol" annotation by DAVID.

### 2.6. Quantitative RT-PCR

We used the same mRNA pools for both microarray and quantitative RT-PCR analyses. Detection of mRNA was performed with an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Duplicate reactions for each primer set were run simultaneously in a 96-well plate using the TaqMan EZ RT-PCR kit.  $\beta$ -Actin was used as an internal standard. Oligonucleotide primers and MGB fluorescent probes (TaqMan Gene Expression Assays) were purchased from Applied Biosystems. Detailed protocols for analysis by qRT-PCR have been described (Someya et al., 2008). All data were reported as mean  $\pm$  SEM.

### 2.7. Statistical analysis

Sigma Stat statistical software was used and all data were expressed as mean  $\pm$  SD. ABR thresholds, HC survival rates, SGC densities, and SV thicknesses were compared among groups by one-way analysis of variance, and then pairwise comparisons were performed by using Bonferroni's test.

## 3. Results

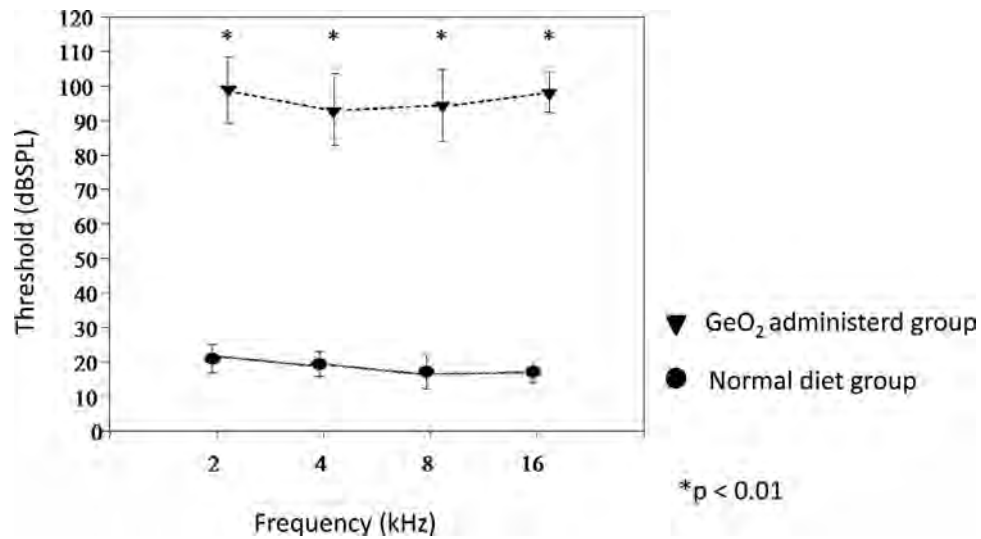
### 3.1. Auditory and histopathological findings of animals given GeO<sub>2</sub>

In experiment 1, CBA/J mice were orally given GeO<sub>2</sub>-containing chows for 4 months from the age of 2 months. The ABRs examined at 2 months of age before experiments were within the normal laboratory range in all 10 animals and did not differ between animals given chows with and without GeO<sub>2</sub>. At the age of 6 months, ABRs showed that animals given GeO<sub>2</sub>-containing chows developed profound hearing loss at all frequencies examined, while those given normal chows maintained normal ABR thresholds (Fig. 1).

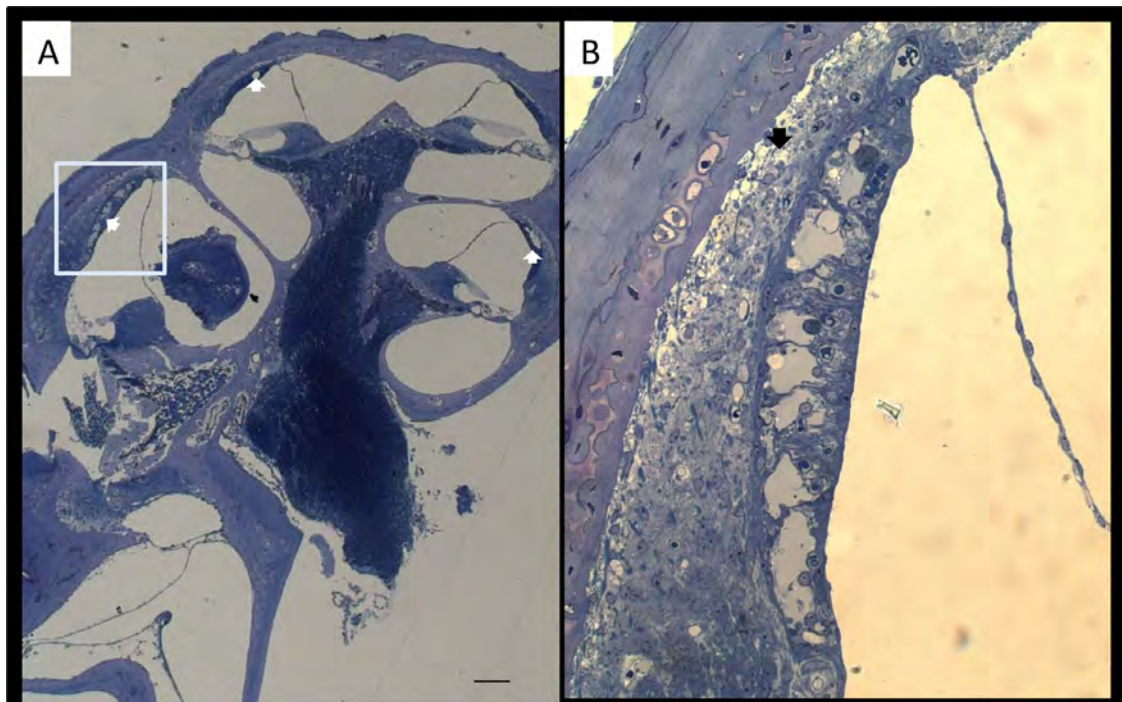
The histopathological examination showed that animals given GeO<sub>2</sub>-containing chows exhibited marked degeneration of the stria vascularis in almost all cochlear turns, more markedly in the lower turn (Fig. 2). Animals given normal chows did not develop any of such pathologies (data not shown).

Transmission electron microscope examination revealed marked vacuolar degeneration in the stria vascularis, where almost all mitochondria contained electron-dense inclusions. Similarly, the distal tubular epithelium of the kidney and the sole muscles showed





**Fig. 1.** Threshold after 4 months of GeO<sub>2</sub> treatment in CBA mice. CBA/J mice treated GeO<sub>2</sub>-containing chows for 4 months showed profound hearing loss in all frequency (triangle), while CBA mice with normal diet showed normal hearing (circle).



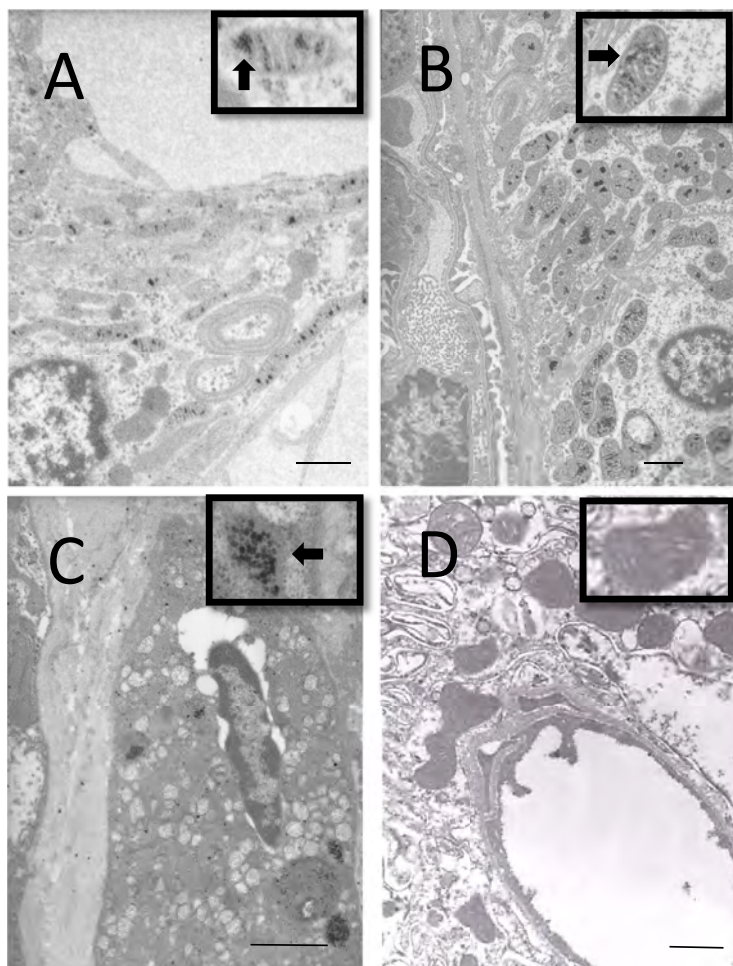
**Fig. 2.** Representative light micrographs of the cochlea after administration of GeO<sub>2</sub> for 4 months. Enlarged image of stria vascularis for captured area (B). Degeneration of the stria vascularis indicated with white arrow is seen in almost all cochlear turns, more markedly in the lower turn (A). Severe vacuolar degeneration of the stria vascularis is found in the lower basal turn (B). Bar = 100 μm in (A), 50 μm in (B).

many electron-dense deposits inside the degenerated mitochondria (Fig. 3).

### 3.2. Overview of microarray analysis

To identify genes and Biological Process categories associated with GeO<sub>2</sub>-induced hearing loss, we conducted genome-wide gene expression analysis using RNA samples isolated from the cochlear tissues of 6-month-old CBA mice (n = 5). Using Affymetrix Gene Chip, we found that 3827 gene probe sets were significantly down-regulated, and 3327 gene probe sets were significantly up-regulated in the cochlear tissues of 6-month-old mice treated with

GeO<sub>2</sub> compared to 6-month-old controls given normal chow. These significantly altered gene probe sets were further assigned to “GO: Biological Process” categories using Database for Annotation, Visualization, and Integrated Discovery (Huang da et al., 2009), which assigned a classification to 3827 of the downregulated and 3327 of the upregulated genes. A summary of the “Gene Ontology (GO): Biological Process” categories associated with germanium-induced hearing loss is shown in Table 1. The complete set of microarray data has been submitted to the GEO (Gene Expression Omnibus) repository (<http://www.ncbi.nlm.nih.gov/geo/>) with GEO Accession number GSE84735. The EASE analysis revealed that 16 Go: Biological Process categories, including “mitochondrion,” “mitochondrial



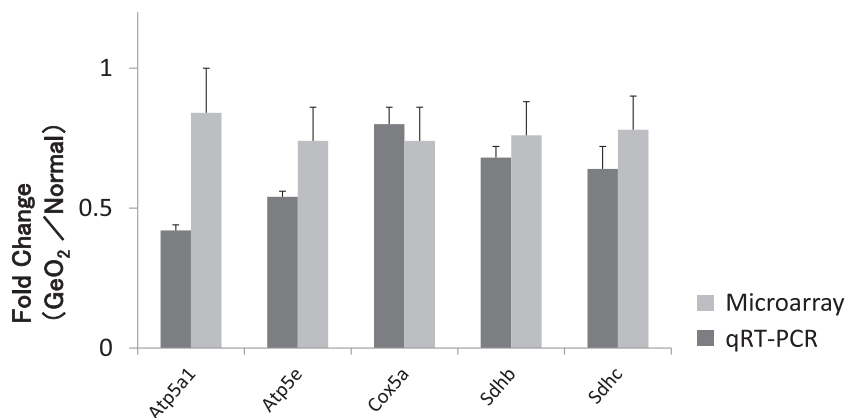
A: Stria vascularis of GeO<sub>2</sub> treated mouse  
 B: Kidney of GeO<sub>2</sub> treated mouse  
 C: Soleus muscle of GeO<sub>2</sub> treated mouse  
 D: Stria vascularis of non-treated mouse

**Fig. 3.** Ultrastructural findings of the intermediate cells of stria vascularis (A), kidney (B), soleus muscle (C) of GeO<sub>2</sub> treated mice. Transmission electron microscope showed vacuolar degeneration of the stria vascularis (A). The arrow head indicates degenerated mitochondria containing electron-dense inclusion. The distal tubular epithelium of the kidney (B) and soleus muscles (C) showed many electron-dense deposits inside the degenerated mitochondria. Ultrastructural findings from a stria vascularis of non-treated mice (D). Inset: high-power view of mitochondria. and Bar = 1 μm.

**Table 1**  
 Summary of the “GO: Biological Process” categories associated with germanium-induced hearing loss.

Biological Process Categories	N	TN	EASE
<b>Down-regulated (3827 classified genes)</b>			
Mitochondrion	225	679	0.000
Mitochondrial membrane	81	226	0.000
Mitochondrial envelope	85	242	0.000
Mitochondrial inner membrane	76	209	0.000
Mitochondrial electron transport chain	14	27	0.000
Mitochondrial ribosome	17	39	0.000
Mitochondrial matrix	21	57	0.001
Mitochondrial lumen	21	57	0.001
NADH dehydrogenase activity	15	35	0.001
NADH dehydrogenase (Quinone) activity	15	35	0.001
NADH dehydrogenase (Ubiquinone) activity	15	35	0.001
Tricarboxylic acid cycle	10	20	0.004
Acetyl-CoA catabolism	10	21	0.005
Oxidative phosphorylation	17	51	0.010
Acetyl-CoA metabolism	12	31	0.012
ATP binding	184	99	0.049
<b>Up-regulated (3327 classified genes)</b>			
Transcription, DNA-dependent	289	1418	0.000
Regulation of transcription, DNA-dependent	283	1397	0.000
Ligase activity	71	293	0.001
Endocytosis	35	1540	0.002
Apoptosis	88	433	0.018
Programmed cell death	88	439	0.025

Column titles: N, the number of identified genes in the category; FC, fold change; TN, the total number of genes in the category on the Gene Chip; EASE, EASE test score.



**Fig. 4.** The qRT-PCR validation of microarray data. The data represent the fold change in gene expression of 6-month-old mice treated with GeO<sub>2</sub> compared to 6-month-old controls given normal chow. The qRT-PCR analyses for *Atp5a1*, *Atp5e*, *Cox5a*, *Sdhb*, and *Sdhc*. The qRT-PCR results were in agreement with the microarray findings (light gray bar) that the expression of these mitochondrial function-associated genes was significantly decreased in the cochleae of germanium-treated mice (dark gray bar).

inner membrane,” “mitochondrial electron transport chain,” “oxidative phosphorylation,” and tricarboxylic acid cycle, were significantly associated with germanium-induced mitochondrial dysfunction genes (Fisher exact score  $p < 0.05$ ), and 818 out of 1863 genes in these categories on the Gene Chip were significantly downregulated in the cochleae of germanium-applied animals (Table 1).

### 3.3. Downregulation of genes associated with germanium-induced mitochondrial dysfunction

Table 2 shows a list of down-regulated genes encoding components of the mitochondrial respiratory chain in the cochlea. Twenty-eight genes encoding components of the mitochondrial respiratory chain were found to be significantly down-regulated ( $P$  value  $< 0.05$ ) (Table 2). Of these, three genes encode for components of the “respiratory chain complex I” (NADH dehydrogenase complex), including *Ndufs2*, *Ndufs7*, and *Ndufv2*; two genes encode for components of the “respiratory chain complex II” (succinate dehydrogenase complex), including *Sdhb* and *Sdhc* genes; two genes encode for components of the “respiratory chain complex III”, including *Cyc1* and *Cyc5*; one gene encode for components of the “respiratory chain complex IV” (cytochrome c oxidase subunits), including *Cox5a*; and 11 genes encode for components of the “respiratory chain complex V” (ATP synthase subunits), including *Atp5k*, *Atp5e*, and *Atp5a1*. The analyses of qRT-PCR were conducted for *Atp5a1*, *Atp5e*, *Cox5a*, *Sdhb*, and *Sdhc*, to validate the microarray results. The qRT-PCR results were in good agreement with the microarray findings that expression of these mitochondrial function-associated genes were significantly decreased in the cochleae of GeO<sub>2</sub>-treated mice (Fig. 4). These results provide the evidence that GeO<sub>2</sub>-induced hearing loss is associated with the down-regulation of genes involved in the mitochondrial respiratory chain complexes in the cochlea of CBA/J mice.

### 3.4. Effect of antioxidants on ABR threshold shifts induced by GeO<sub>2</sub>

The total amount of weekly dietary intake and final body weights for each group is shown in supplementary Table 1.

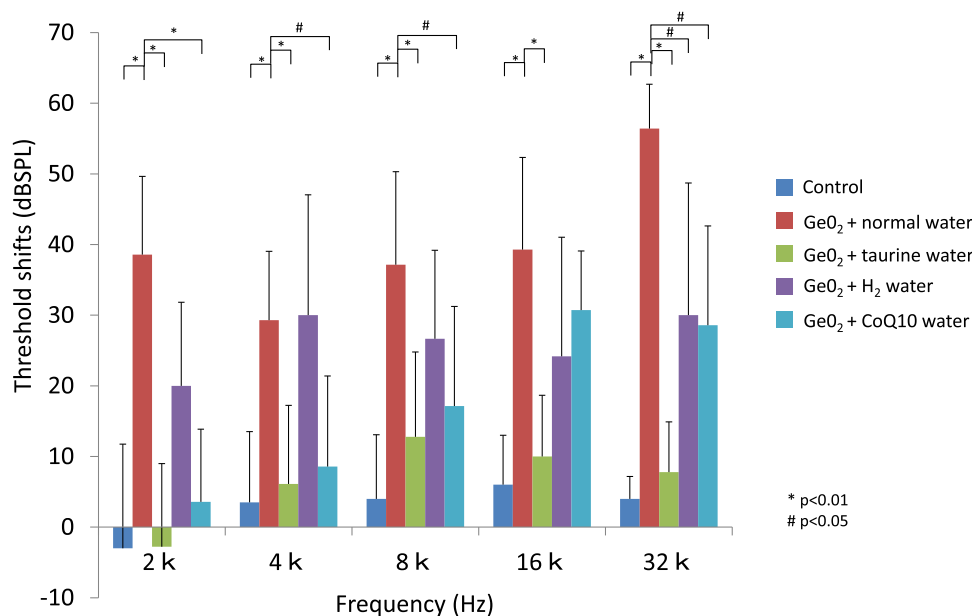
Animals given normal chow and water almost maintained ABR thresholds until 5 months of age, whereas animals given GeO<sub>2</sub>-containing chow and normal water from 2 months of age for 3 months showed approximately 30 to 50 dB threshold shifts. The difference of the threshold shifts was significantly different between controls and animals given GeO<sub>2</sub>+normal water at all frequencies (Fig. 5).

ROS scavengers all provided preventive effect against GeO<sub>2</sub>-induced hearing loss, with taurine showing the strongest effect. Animal given GeO<sub>2</sub>+taurine developed only slight threshold shifts at all frequencies. The threshold shifts in animals given GeO<sub>2</sub>+taurine were significantly different ( $p < 0.01$ ) at all frequencies compared with those given GeO<sub>2</sub>+normal water and were not significantly different at any frequencies compared with controls. CoQ10 prevented GeO<sub>2</sub>-induced threshold shifts predominantly at lower frequencies, with substantial threshold shifts at higher frequencies. Threshold shifts in animals given GeO<sub>2</sub>+CoQ10 were significantly smaller at 2, 4, and 8 kHz ( $p < 0.01$  in 2 kHz,  $p < 0.05$  in 4 and 8 kHz) compared to animals given GeO<sub>2</sub>+normal water. Hydrogen water also provided some preventive effect, but the effect was smallest among the three ROS scavengers. The threshold shifts in animals given GeO<sub>2</sub> and hydrogen water showed approximately 20–30 threshold shifts at all frequencies, which were significantly different from those in animals given GeO<sub>2</sub>+normal water only at 32 kHz ( $p < 0.05$ ). When compared among animals given GeO<sub>2</sub> and ROS scavengers, threshold shifts in animals given taurine were significantly smaller at 2, 4, and 32 kHz ( $p < 0.01$ ) compared to those given hydrogen water and significantly smaller at 16 and 32 kHz ( $p < 0.01$ ) compared with animals given CoQ10. Threshold shifts in animals given CoQ10 were significantly smaller only at 4 kHz ( $p < 0.05$ ) compared to animals given hydrogen water. These data indicated that taurine has the strongest preventive effect, followed by CoQ10 and then hydrogen water.

### 3.5. Effect of antioxidants on degeneration of the spiral ganglion and stria vascularis induced by GeO<sub>2</sub>

The average of total area of stria vascularis at the lower basal turn for all groups are shown in Supplemental Figure 2. There were no significant differences in the average of total areas of stria vascularis among groups. Mice that did not take GeO<sub>2</sub> showed nearly normal appearance of the stria vascularis and SGCs at the age of 5 months (data not shown), whereas mice administered GeO<sub>2</sub>+normal water for 3 months showed vacuolar degeneration of stria vascularis and severe degeneration of the SGCs mainly in the lower-basal and upper-basal turns in the cochlea.

The extent of degeneration in the stria vascularis and SGCs were significantly ameliorated ( $p < 0.01$ ) in animals given GeO<sub>2</sub> and one of the antioxidants when compared to those given GeO<sub>2</sub> + normal water (Figs. 6 and 7), indicative that all the antioxidants protected from GeO<sub>2</sub>-induced cochlear degeneration. The protective effect was most significant in taurine supplementation; there was no significant difference in the extent of degeneration



**Fig. 5.** ABR threshold shifts after 3 months of GeO<sub>2</sub> treatment

The control group showed almost no threshold shifts in all frequency. Animals given GeO<sub>2</sub> and water without antioxidant showed significant threshold shift in all frequency from 2 to 32 kHz. The taurine supplemented group prevented the threshold shift in all frequency in the level that was nearly the same as the control group. CoQ10 group prevented a threshold shift in most of the frequency but there was a substantial threshold shift in higher frequencies compared to control animals. Hydrogen water group had a substantial threshold shift in all frequency compared to the control group but showed some protective effect in limited frequencies compared to animals given GeO<sub>2</sub> + normal water.

either in the stria vascularis or SGCs between animals given GeO<sub>2</sub>+taurine and controls without GeO<sub>2</sub> intake. The extent of degeneration in the stria vascularis and SGCs in animals given GeO<sub>2</sub>+hydrogen water or CoQ10 was significantly smaller ( $p < 0.01$ ) compared to those given GeO<sub>2</sub>+normal water, but significantly greater ( $p < 0.01$ ) compared to controls without GeO<sub>2</sub> intake. When compared among animals given GeO<sub>2</sub> and one of three antioxidants, animals given taurine showed significant protective effect against the degeneration of the stria vascularis and the SGCs when compared to those given hydrogen water or CoQ10 ( $p < 0.001$ ). There was no significant difference in the degeneration of either stria vascularis or the SGCs between animals given GeO<sub>2</sub>+CoQ10 and those given GeO<sub>2</sub>+hydrogen water.

#### 4. Discussion

The current study demonstrated that oral intake of 0.15% GeO<sub>2</sub> for 4 months caused profound hearing loss associated with severe degeneration of the stria vascularis and SGCs in CBA mice. Transmission electron microscopic examination revealed electron-dense inclusions in degenerated mitochondria not only in the cochlea but in the kidney and muscle. Microarray gene expression analysis of the cochlea revealed down-regulation of 16 categories, including the “mitochondrion,” “mitochondrial inner membrane,” “mitochondrial electron transport chain,” and “oxidative phosphorylation.” qRT-PCR confirmed the down-regulation of five representative genes associated with mitochondrial respiratory chain in the cochlea. These findings indicate that dietary oral administration of 0.15% GeO<sub>2</sub> in CBA mice is a promising animal model to investigate SNHL associated with mitochondrial dysfunction. We also observed that GeO<sub>2</sub>-induced SNHL and cochlear degeneration could be ameliorated by dietary intake of water containing taurine, CoQ10, or hydrogen, with taurine providing the strongest protection. These findings suggest that dietary intake of these antioxidants could be used to slow or treat SNHL and other phenotypes associated with

mitochondrial dysfunction such as mitochondrial encephalomyopathy.

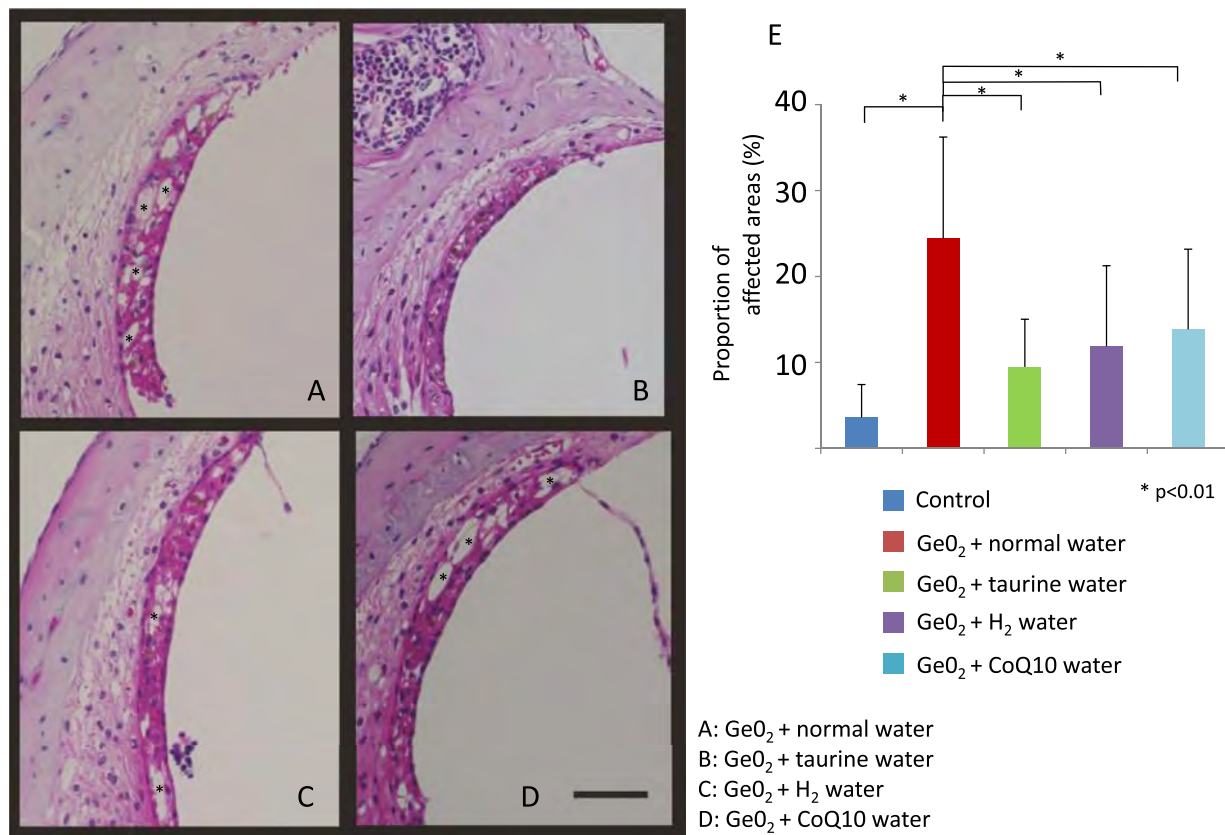
As mentioned above, we have shown that dietary intake of GeO<sub>2</sub> induces SNHL in CBA mice, which could be a good model to study SNHL associated with impairment of mitochondrial function. It has been reported that topical application of mitochondrial toxin, 3-nitropropionic acid (3-NP) on the round window of the cochlea can cause acute SNHL; both permanent and temporary threshold shifts were observed in this model depending on the amount of 3-NP used (Hoya et al., 2004; Okamoto et al., 2005). In the permanent threshold shift model, marked degeneration was observed in type 2 fibrocytes in the spiral prominence, type 4 fibrocytes in the spiral ligament, marginal cells, and intermediate cells in the stria vascularis 3 h after 3-NP administration, indicative that SNHL caused by topical application of 3-NP is primarily mediated by cellular degeneration in the lateral wall of the cochlea. Compared to this animal model, in our mouse model, the degeneration was observed not only in the stria vascularis but the SGCs. The difference of the affected sites may be due to the different methods of drug application. 3-NP was applied acutely and topically, whereas GeO<sub>2</sub> was applied chronically and systemically.

The current study revealed previously unrecognized pathways associated with GeO<sub>2</sub>-induced SNHL, such as the down-regulation of genes involved in the mitochondrial respiratory chain. The DNA microarray analysis revealed that chronic application of GeO<sub>2</sub> down-regulated 27 genes in the respiratory chain complexes I, II, III, IV and V. Someya et al. (S Someya et al., 2007) reported changes of gene expression in the cochlea of DBA/2 J mice, which show severe progressive age-related hearing loss. In their study, gene analysis revealed that the aged DBA/2 J mice showed significant down-regulation of genes encoding components of the mitochondrial respiratory chain complexes I, II, III, IV, and V. Deficiency of complex IV is reported to be associated with SNHL (Horváth et al., 2005; Lamperti et al., 2012) in other reports. Gutiérrez Cortés et al. (Gutiérrez Cortés et al., 2012) also suggested that mutations of genes in complex I, III, and IV could be the cause of maternally

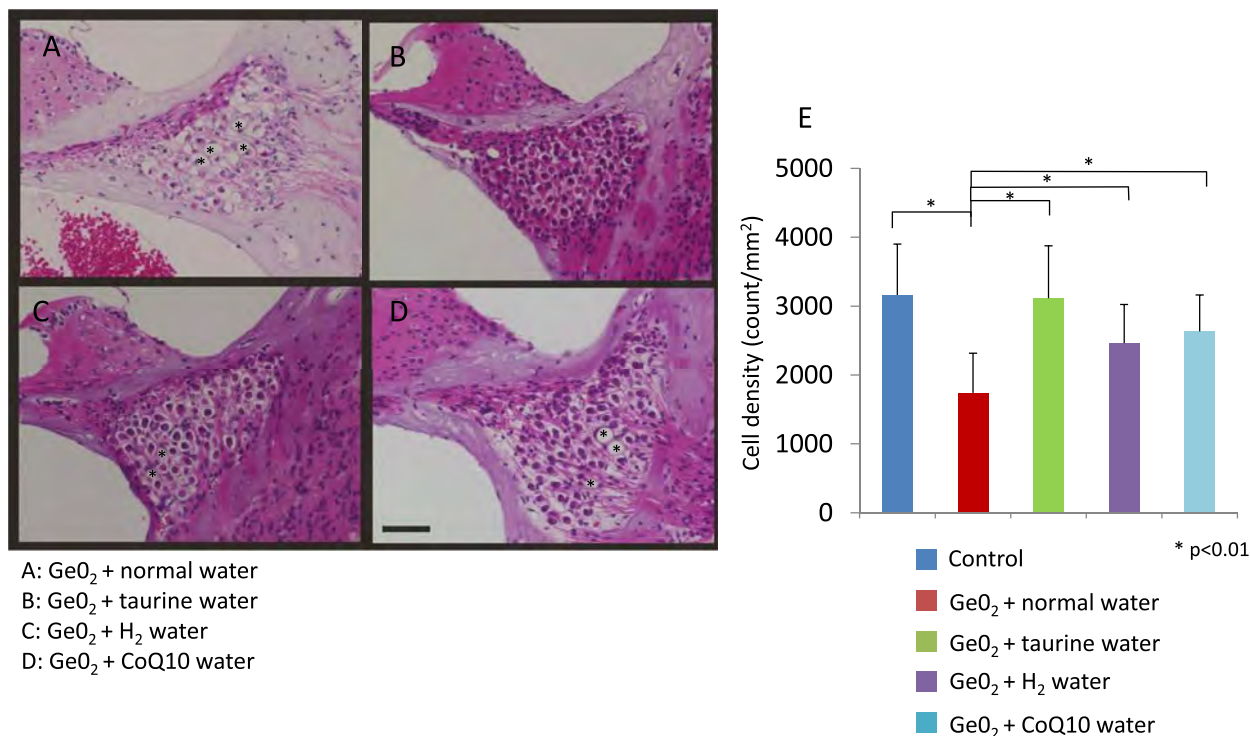
**Table 2**  
List of down-regulated genes encoding components of the mitochondrial respiratory chain in the cochlea.

Gene	Gene ID	Affy ID	P Value	FC
<b>Oxidative Phosphorylation</b>				
Atp5a1	C78762	1,420,037_at	0.011	-2.028
Atp5d	BC008273	1,423,716_s_at	0.033	-1.571
Atp5e	NM_025983	1,416,567_s_at	0.000	-1.835
Atp5g1	NM_007506	1,416,020_a_at	0.000	-1.567
Atp5g2	NM_026468	1,415,980_at	0.002	-1.727
Atp5j	NM_016755	1,416,143_at	0.008	-1.191
Atp5k	AV216686	1,434,053_x_at	0.004	-1.727
Atp5l	NM_013795	1,448,203_at	0.009	-1.593
Atp5o	NM_138,597	1,416,278_a_at	0.000	-1.563
Atp5o /// LOC432676	AV066932	1,437,164_x_at	0.005	-1.558
Atp6ap1	AI316502	1,449,622_s_at	0.000	-1.795
Cox5a	NM_007747	1,448,153_at	0.025	-1.345
Cyc1	NM_025567	1,416,604_at	0.005	-1.266
Cycs	NM_007808	1,422,483_a_at	0.007	-1.235
Ndufc2	NM_024220	1,416,366_at	0.000	-2.120
Ndufs7	BC013503	1,451,312_at	0.001	-1.458
Ndufv2	AV046532	1,438,159_x_at	0.003	-1.259
<b>Tricarboxylic acid cycle</b>				
Aco1	BB504570	1,456,728_x_at	0.003	-1.360
Aco2	AU019938	1,436,934_s_at	0.047	-1.345
Cs	AB056479	1,450,667_a_at	0.022	-1.248
Dlst	BC006702	1,423,710_at	0.005	-1.616
Idh3b	NM_130,884	1,418,886_s_at	0.001	-1.316
Idh3g	NM_008323	1,416,789_at	0.000	-1.736
Mdh2	NM_008617	1,416,478_a_at	0.000	-1.342
Polr3h	AK019868	1,424,227_at	0.000	-1.181
Sdhb	BC013509	1,418,005_at	0.000	-1.387
Sdhc	NM_025321	1,448,630_a_at	0.012	-1.537

Column titles: Gene, gene symbol; Gene ID, representative public gene ID. Affy ID, Affymetrix probe set ID; FC, fold change.



**Fig. 6.** Representative light micrographs of the stria vascularis in animals given germanium and water (A), taurine (B), hydrogen water (C), and CoQ10 (D). The extent of the degeneration is significantly attenuated in animals given one of the antioxidants compared to those given water without antioxidant. Taurine provides the strongest effect, with the extent of degeneration not being different compared to the controls without germanium treatment (E). Representative vasculoar degeneration area are marked with “\*”. Bar = 50 μm.



**Fig. 7.** Representative light micrographs of the spiral ganglion cells in animals given germanium and water (A), taurine (B), hydrogen water (C), and CoQ10 (D). The extent of the degeneration is significantly attenuated in animals given one of the antioxidants compared to those given water without an antioxidant. Taurine provides the strongest effect, with the extent of degeneration not being different compared to the controls without germanium treatment (E). Representative degenerated areas are marked with “\*”. Bar = 50  $\mu$ m.

inherited non-syndromic hearing loss. These results are in line with our findings that the dysfunction of mitochondrial respiratory chain complexes was closely related to SNHL. Moreover, the deficiencies of mitochondrial respiratory chain complexes have been reported to be closely related to neural degeneration. For example, Atp5a1 deficiency has been reported to cause severe neonatal encephalopathy, and Atp5e causes early onset lactic acidosis, 3-methylglutaconic aciduria, mild mental retardation, and severe peripheral neuropathy development. Both genes were confirmed to be down-regulated in the current study. Complex V deficiency mediated by other genes also has been observed in neurodegenerative diseases (Kantrow et al., 1997). Complex II deficiency also has been reported to cause neurodegenerative disorders (EA. Shoubridge, 2001; EA. Shoubridge, 2001). Although there has been no report examining the degeneration of SGCs, one of the peripheral neurons, in these deficiencies, it is speculated that mitochondrial dysfunction caused by the down-regulation of genes in complex I, III, and IV may be related to the degeneration of the SGCs.

In the current study, the up-regulation of genes involved in apoptosis was also observed. It has been reported that the defect of the respiratory chain system is associated with the induction of apoptosis (Kantrow et al., 1997). An in vivo study using Neuro-2A cells showed that treatment of GeO<sub>2</sub> to the neuron A2 cell induced the release of cytochrome c from mitochondria, loss of mitochondrial membrane potential, and translocation of the Bax, resulting in apoptosis by the mitochondrial-dependent pathway (Lin et al., 2006). Interestingly, such phenomena have also been reported by applying similar semiconductor elements, such as arsenic, indium, and gallium (Bustamante et al., 1997; Chang et al., 2003; Hu et al., 2003; Milton et al., 2004). Studies investigating the mechanism for arsenic-induced apoptosis have revealed that the apoptosis is triggered by inhibition of mitochondrial respiratory function, resulting in the induction of ROS. ROS inactivate enzymes and damage

DNA molecules by the direct chemical attack on their structure (Pelicano et al., 2003; Shen et al., 2003). Considering these, it can be assumed that the accumulation of germanium in the mitochondria would affect mitochondrial respiratory function, thereby resulting in ROS generation and induced mitochondria-mediated apoptosis of the stria vascularis and SGCs.

Taken together, these reports suggest that GeO<sub>2</sub> accumulation causes mitochondrial dysfunction, leading to the degeneration of the cochlea and subsequent progressive hearing loss via apoptotic pathway. However, the current study does not directly demonstrate a causal relationship between mitochondrial dysfunction and cochlear degeneration, which should be evaluated by future studies.

In the current study, we observed that antioxidants, such as taurine, CoQ10, and hydrogen water, attenuated GeO<sub>2</sub>-induced SNHL and degeneration of the stria vascularis and SGCs, which implies that ROS play a key role in GeO<sub>2</sub>-mediated damage in the cochlea. Those antioxidants have been proven to have powerful antioxidant effects in various fields. For example, taurine has been exhibited to protect various organs from oxidative stress caused by aluminium (Qiao et al., 2015), diabetes (Koh et al., 2014), and various drugs (Das et al., 2009; Manna et al., 2009; Alam and Hafiz, 2011; Roy and Sil, 2012). It has also been reported that administration of taurine induces a significant reduction of intracellular ROS level and recovery of mitochondria membrane potential caused by arsenic in mouse neuroblastoma N2a cells. (Chou et al., Apr). CoQ10 has been shown to protect neuronal cells from UVB- and ROS-induced damage (M Sikorska et al., 2014), brain ischemia/reperfusion, gentamicin-induced cochlear damage and hearing loss, and hepatic oxidative stress and inflammation (M Sikorska et al., 2014). In addition, supplementation of CoQ10 has been reported to show a therapeutic effect in patients with mitochondrial respiratory chain disorders (Hargreaves, 2014).

Hydrogen gas has shown protective effects from ischemia/reperfusion injuries in cerebral (Sato et al., 2008) and myocardial infarction (Hayashida et al., 2008; Yoshida et al., 2012), hepatic injury (Fukuda et al., 2007), cisplatin-induced nephrotoxicity (Nakashima-Kamimura et al., 2009), and noise-induced hearing loss (Lin et al., 2011; Fransson et al., 2021). Although these antioxidants showed protective effect in the current study, hydrogen showed weakest effect compared to other supplements. This may be explained by the limited concentration of hydrogen when given in water. The solubility of the hydrogen is limited and easily leaked from water. Although the glass bottle used minimized the leakage of hydrogen from water and the water was changed every other day to keep the hydrogen concentration above 0.4 mM, the dose may be not sufficient to achieve satisfactory effects. Another reason may be the unique mechanism of scavenging system of hydrogen, which selectively scavenges free hydroxyl radicals ( $\bullet\text{OH}$ ) (Sato et al., 2008; Ohsawa et al., 2007). Other types of free radicals may be more relevant to  $\text{GeO}_2$ -induced damage, and as a result, the effects of hydrogen may be limited.

## 5. Conclusion

Chronic dietary intake of  $\text{GeO}_2$  in CBA mice could induce SNHL due to the degeneration of stria vascularis and the SGCs, which was associated with down-regulation of mitochondrial respiratory chain associated genes and up-regulation of apoptosis-associated genes. Antioxidant supplements, such as taurine, CoQ10, and hydrogen water, could attenuate cochlear damage and SNHL induced by  $\text{GeO}_2$  intake. SNHL induced by oral intake of  $\text{GeO}_2$  can be a promising animal model to investigate SNHL associated with mitochondrial dysfunction. Daily supplements of antioxidants may be one of the solutions to prevent or slow SNHL associated with mitochondrial dysfunction.

## Author statement

Akinori Kashio: investigation and writing of the original draft preparation; Chikako Yamada: investigation; Kazuo Yasuhara: investigation; Teru Kamogashira: investigation; Shinichi Someya: investigation and analysis; Tatsuya Yamasoba: conceptualization, methodology, writing of the review and editing, supervision, and funding acquisition.

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## Data availability

Data will be made available on request.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.heares.2022.108678.

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## Sec.3

# ミトコンドリア病

### 1 ミトコンドリア病とは

私たちの体は何10兆個もの小さな細胞が集まって形作られています。その細胞の一つ一つの中にミトコンドリアが存在しています。ミトコンドリアは二重膜に囲まれた細胞内小器官であり、一つの細胞に数百から数千存在していると言われています。主な役割は、細胞で利用される主要なエネルギーであるアデノシン三リン酸（ATP）の産生です。取り込まれた栄養を利用して、細胞の、ひいてはその集合体である私たち生物の活動を支えるエネルギーを作り出す、いわば発電所のような役割を果たしています。そ

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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低下して、さまざまな症状が出現します。それがミトコンドリア病です。

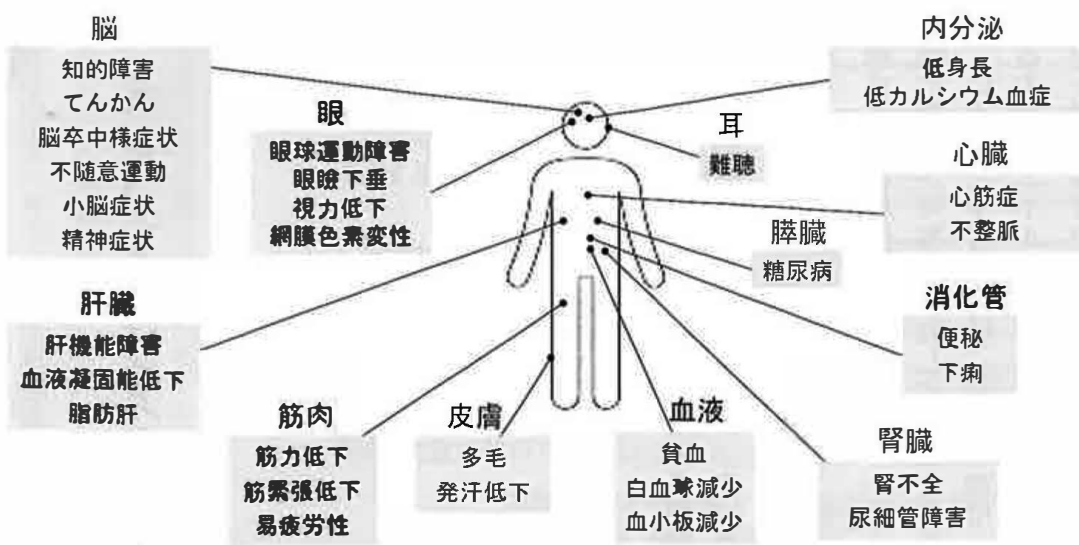
エネルギーを多く使う脳や筋肉などに症状が出やすいため、ミトコンドリア病は「ミトコンドリア脳筋症」と呼ばれることもあります。エネルギーを特にたくさん必要とする神経細胞の機能異常は、知的発達症（知的障害）、てんかん、不随意運動、小脳失調などの症状をもたらします。筋肉の症状としては、筋力低下、筋緊張低下、眼球運動障害、眼瞼下垂などが見られます。しかし、ミトコンドリアは体中のほぼ全ての細胞に存在してい

るので、ミトコンドリア病の症状は全身のあらゆる臓器・器官に現れる可能性があります。体のどの部分のミトコンドリアが障害されるかによって、個々の患者の症状は異なってきます。それぞれの臓器・器官に出やすい症状や病気を図表1に示します。

このようにさまざまな症状を呈するミトコンドリア病は、国の指定難病医療費助成制度の対象となっており、小児慢性特定疾病の一つにも定められています。指定難病として「ミトコンドリア病」と診断されるには、決められた診断基準を満たす必要があります。具体的には、筋肉、脳、心臓、肺、腎臓、脾臓、内分泌、血液、眼、耳のいずれかに、ミトコンドリア病でよく見られる症状があることが要件になります。

従来、ミトコンドリア病の患者数は非常に少ないと思われていました。しかし、近年の調査研究から、一般人口においてミトコンドリア病と診断されている患者は、以前考えられていたほど少なくないことがわかってきました。成人人口10万人あたりで、イギリスでは23人、スペインでは5・7人と報告されています。一

方、日本の正確な患者数は不明ですが、全国の入院患者の医療保険のデータベースから、平成30年度の患者数は3629人、一般人口10万人あたり2.9人と推



図表1 ミトコンドリア病の主な症状

定されています。この調査は入院患者のみを対象としており、実際の患者数はこれより多い可能性があると思われる。

## 2 ミトコンドリア病の原因

ミトコンドリア病は、体の設計図である遺伝子(DNA)の変化によって引き起こされます。近年の遺伝子検査技術の進歩により、ミトコンドリア病の原因となる遺伝子の種類は極めて多いことがわかってきました。ミトコンドリアでは1500にものぼる因子(タンパクなど)が機能していると言われてます。それらのうち、実に約400種類の因子において、その設計図である遺伝子の変化がミトコンドリア病患者で報告されています。

これらの遺伝子は、ほとんどが細胞の核の中に存在しますが(核遺伝子)、核の外(細胞質)にあるミトコンドリアもその内部に独自の環状DNAであるミトコンドリアDNA(ミトコンドリア遺伝子)をもっており、その変化もミトコンドリア病を引き起こします。小児期発症

のミトコンドリア病では、70%〜85%の患者が核遺伝子の変化、残りの患者がミトコンドリア遺伝子の変化が原因であることが知られています。一方、成人になってから発症するミトコンドリア病患者では、逆に70%〜80%程度の患者がミトコンドリア遺伝子の変化、残りの患者が核遺伝子の変化が原因であると言われています。

## 3 ミトコンドリア病の診断

指定難病の「ミトコンドリア病」と診断されるには、先に紹介した種々の症状に加え、検査でミトコンドリアの異常を示す所見があることが基準になります(図表2)。まず、医療機関で行われる血液や髄液検査、あるいは脳画像検査や眼底検査の異常が診断基準で示されています。体内の乳酸が高くなることも多いです。血液や脳脊髄液の乳酸値の上昇は診断の一助となります。神経症状のある患者では、脳CTやMRI検査等の画像検査を行い、大脳基底核や脳幹等のエネルギー需要度の高い部位の病変の有無を調べます。眼底に特徴的所見を認めること

があるので、眼科検査も行われます。さらに、症状に応じて各臓器の評価を行い、ミトコンドリア病に特徴的な障害をきた

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|---|
| <p>1. 臨床検査</p> <p>①血液または髄液の乳酸値が繰り返して高い、または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。</p> <p>②脳CTやMRIにて、大脳基底核、脳幹に両側対称性の病変等を認める。</p> <p>③眼底検査に異常を認める（急性期の視神経乳頭の発赤・腫脹、毛細血管の蛇行、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、慢性期の視神経萎縮を両眼に認める）。</p> <p>2. 特殊検査</p> <p>①遺伝学的検査所見：ミトコンドリアDNAに質的、量的異常、また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。</p> <p>②生化学検査所見：ミトコンドリア関連酵素の活性低下、またはコエンザイム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。</p> <p>③病理検査所見：骨格筋生検や培養細胞また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。</p> |
|---|

図表2 ミトコンドリア病の検査所見（指定難病医療費制度 診断基準を参考に作成）

していないかチェックします。心臓を例にとれば、心電図や心エコーを行い、ミトコンドリア病で起こりうる不整脈や心筋症の有無を確認します。難聴や糖尿病も診断の手がかりになります。いかなる臓器・器官にも障害が起こりうることを念頭に、特徴的な所見がないか、丁寧な診察と検査によって評価することが大切です。

臨床的にミトコンドリア病を疑った場合には、遺伝子検査や生化学的検査などの特殊検査によって、ミトコンドリアの異常を証明します。ミトコンドリア内に存在する、エネルギーを産生するために必要な酵素の働きを評価するためには、酵素活性測定などの生化学的検査が行われます。また、症状のある臓器（筋肉や肝臓、心臓等）の組織を用いた病理学的検査によってミトコンドリア病に特徴的な所見が見られれば、診断の一助となります。そして、前述のように遺伝子の変化がミトコンドリア病の原因となるので、確定診断のためには遺伝学的検査（遺伝子検査）が大切です。原因となりうる遺伝子の数が非常に多いため、検査には時間と労力がかかりますが、近年の検査

技術の長足の進歩によって、多くの患者の遺伝子診断が可能となりました。遺伝子の変化は全ての患者で検出されるわけではありませんが、ミトコンドリア病疑いの患者の60%以上で、遺伝子検査による確定診断が可能になったと言われています。

#### 4 ミトコンドリア病の病型

ミトコンドリア病の患者の症状は多彩です。症状によってさまざまな病気に分類されます。主な病型を紹介します。

##### ①新生児ミトコンドリア病

ミトコンドリア病はあらゆる年齢で発症する可能性があります。最も早期の新生児期、すなわち出生から1カ月以内に発症するミトコンドリア病を指します。出生直後から全身状態が悪化する重症例も多く見られます。原因としては、ミトコンドリア遺伝子よりも、核遺伝子の変化が多いことが知られています。

##### ② Leigh脳症（リー脳症）

乳幼児期、多くは2歳までに発症する進行性の疾患で、脳幹及び（あるいは）大脳基底核の左右対称性の病変が特徴で

す。精神運動発達遅滞やてんかん、不随意運動、眼球運動障害や呼吸障害などの脳幹の症状が見られます。筋緊張や筋力低下といった筋肉の症状も見られます。感染症を契機にして、急激に状態が悪化することのある疾患です。患者の20%程度にミトコンドリア遺伝子異常、残りの約80%は核遺伝子異常が原因とされ、現在まで約100種類に及ぶ原因遺伝子が報告されています。

### ③脳卒中様症状を伴うミトコンドリア病 (MELAS/メラス)

あらゆる年齢で発症しうる、ミトコンドリア病のなかで最も頻度の高い疾患です。けいれんや意識障害、麻痺などを呈する脳卒中のような発作を特徴としますが、低身長、難聴、心筋症、糖尿病、腎症等多彩な症状を呈しうる症候群です。ミトコンドリア遺伝子の配列の3243番目の変化 (m.3243A>G) を有する患者が80%を占めます。

### ④慢性進行性外眼筋麻痺症候群 (CPEO/シーピーイーオー)

眼球運動が麻痺する、脛が下がるなどの眼の症状が次第に進行する病気です。MELAS同様発症年齢は小児期から成

人期まで幅広く、筋力低下や糖尿病、難聴など多臓器の障害を伴うことがあります。特に心臓の伝導障害(不整脈)と眼の網膜変性を合併する場合は Kearns Sayre 症候群(カーンズ・セイヤー症候群)と呼ばれます。遺伝子検査では、多くの場合ミトコンドリア遺伝子の欠失(配列の一部が失われる状態)が見られます。欠失の原因が核遺伝子の変化にあることもあり、原因は多彩です。

### ⑤ミオクローヌスを伴うミトコンドリア病 (MERRF/マーフ)

自分の意志とは無関係に筋肉が素早く収縮するミオクローヌスが最初に見られ、続いててんかん発作や体がふらつく小脳症状が出現します。筋力低下、知能低下、低身長も合併しやすい疾患です。約80%の患者に、ミトコンドリア遺伝子の配列の8344番目の変化 (m.8344A>G) が見られます。

## 5 ミトコンドリア病の治療

これら多様な病型のあるミトコンドリア病に対する治療法は、大きく分けて二つあります。

一つは対症療法です。個々の患者で病型や臓器障害の種類や程度が異なりますので、それぞれの症状に応じた治療を行います。例えば、てんかんに対しては薬物によるコントロールを目指します。最近では新しいてんかん治療薬が増えているので、選択肢が広がっています。ミトコンドリア機能に悪影響を与えにくい薬剤を、発作症状にあわせて使用します。糖尿病に対するインスリン療法、難聴に対する補聴器や人工内耳、不整脈に対するペースメーカーなど、障害された臓器に対して適切な治療法が用いられます。薬物や医療機器の進歩によって、さまざまな治療法が可能となっているので、各臓器の症状、病気に応じて、それぞれの分野の専門医の診療を受けることが望ましいと考えます。そのため、多くの診療科をもつ病院を中心に医療が受けられる体制づくりが求められています。

もう一つの治療法は、ミトコンドリア病の原因であるミトコンドリア機能の低下を回復させる原因治療法です。ミトコンドリアに存在する酵素の働きを補助するビタミン類などが使用されていますが、ミトコンドリア病全般に対する十分

な効果は明らかにされていません。一方で、ミトコンドリア病が疑われる患者の一部には、ビタミン等が特異的に効果を発揮する疾患があるので、遺伝子診断などを用いた正確な診断が重要になります。

前述したMELASについては、アミノ酸の一種であるタウリンの内服治療が、脳卒中様発作の抑制効果をもつことが認められ、保険適応をもつ薬剤として承認されています。現在、脳卒中様発作以外の糖尿病などの症状に対する効果についても検討されており、今後の適応拡大が期待されます。

## 6 今後の課題と展望

ミトコンドリア病に対する根本的治療法はまだ十分に確立されていませんが、細胞のエネルギー産生能を高める新しい物質、抗酸化作用（異常なミトコンドリアが発する活性酸素による細胞障害を抑制する効果）をもつ薬物、ミトコンドリア遺伝子異常を減らす化合物等、さまざまな治療法開発研究が進行中です。一部の薬剤は国内外で臨床試験が始まってい

ます。特定の病型や症状をターゲットとした治療法開発も進んでくると思われ、個々の患者に合わせた診療を実現するためには、正確な診断が一層重要になると思われます。

診断においては、遺伝子診断が重要だと述べましたが、病気の原因となる遺伝子が極めて多いことが課題となっています。多数の遺伝子を一度に調べることは技術的には可能となりましたが、それに必要な費用と労力の問題があります。また、検査で遺伝子の変化が検出されなくても、ミトコンドリア病は否定できないことにも注意が必要です。遺伝子解析技術は非常に進歩していますが、遺伝子の変化の検出に至らず「ミトコンドリア病疑い」のまま診断が定まらない患者が多いことも問題です。最近、ミトコンドリア病の遺伝子検査の保険適応が認められたので、今後、検査体制の整備が進むことで、病気の原因がわかって正確な診断に至る患者が増えることが期待されます。

まだ課題の多い状況ではありますが、診断技術が進歩して以前より多くの患者の確定診断がえられるようになったこ

と、新規の治療法開発の研究が進んでいくことから、ミトコンドリア病診療は新しい時代に入っていると言えます。新しい治療法の効果を検証するためには、多くの患者に対する臨床試験が必要になります。また、多種多様なミトコンドリア病について、それぞれの疾患の臨床経過、患者の状況を知り、比較検討して治療効果を判断する必要があります。ミトコンドリア病は、患者数の多くない、いわゆる稀少疾患であるので、複数の医療機関・施設が協力して取り組まなければなりません。そこで、治療法開発の促進と、円滑な臨床試験実施を目的に、全国の患者を対象とした患者登録制度が運用されています。現在、小児期発症のミトコンドリア病を対象とした「MO Bank (<http://mo-bank.com/index.html>)」、MELAS、CPEO、MERRF等を対象としたRemudy (<http://www.remudy.jp/mitd/index.html>) が利用できます。登録を希望される方は、主治医に相談していただければと思います。登録いただいた情報から、症状や経過、治療の効果を分析することで、最適な医療につながることを期待されます。