表1　ミトコンドリア脂肪酸代謝異常症の主な臨床症状・所見

1．全身のエネルギー産生障害に起因する症状・所見
　1）低ケトン性低血糖症（飢餓や感染に伴うことが多い）
　2）Reye 様症候群，乳幼児突然死症候群
2．各臓器の脂肪酸参加障害に起因する症状・所見
　1）筋緊張低下，筋力低下，労作時の筋痛，ミオグロビン尿症，横紋筋融解症
　　（筋型の場合はこれらの症状だけが出現する）
　2）肥大，心内膜弾性症
　3）肝腫大
　4）各臓器の脂肪変性
3．一般検査の異常所見
　1）代謝性アシドーシス
　2）高アンモニア血症
　3）CK，GOT，GPT の異常

（山本重則ほか，1995[14]）

利用率が高い時期なのでカルニチンは必須の栄養素と考えられている．また乳児期，小児期は筋肉量が少ないのでカルニチンの体内でのストックは少なく，カルニチン欠乏に陥りやすい．

カルニチンは以下の３つの理由からエネルギー産生代謝において重要な位置を占めるとされる．

① 長鎖脂肪酸のミトコンドリアへの輸送に必須である．

② ミトコンドリア内の CoA/acylCoA の比率を調整している．CoA とカルニチンの置換によりミトコンドリア内にフリーの CoA が生み出される．

③ 細胞毒であるアシル化合物をカルニチンエステルとして細胞内より除去し，尿中へ排泄する．

Ⅱ カルニチン欠乏症の症状と診断

1．症状

カルニチンはフリーカルニチンと脂肪酸と結合したアシルカルニチンとに大別される．さらにこの結合した脂肪酸の種類によりアシルカルニチンはいろいろな種類に分けられる．カルニチン欠乏症とはこのフリーカルニチンの血中濃度が低下した状態を示す．

カルニチン欠乏症においては，長鎖脂肪酸のミトコンドリアへの輸送の減少により，脂肪酸のβ酸化が障害される．これにより細胞のエネルギー産生は極端に低下して，ことにエネルギーを大量に消費する臓器，筋肉，心筋，脳に重大な症状が出現する．エネルギー枯渇が起きることからブドウ糖の過剰な利用が起き，糖新生系などで十分に補えなければ低血糖が生じる．症状・所見としては Reye 様症候群，心筋症，横紋筋融解症などの筋症状，低ケトン性低血糖，エネルギークライシスなどがキーワードである．

カルニチン欠乏症により引き起こされる脂肪酸のβ酸化障害による臨床症状・所見のまとめを表1に示す．

2．診断

カルニチン欠乏症が起きうる病態を表2にまとめて示す．さらにカルニチンは筋肉に含まれているので筋肉量の少ない痩せた患者はカルニチン欠乏症発症のリスクファクターである．

現在，血中カルニチン測定は酵素サイクリング法でのフリー・アシルカルニチン２分画測定とタンデムマス法によるフリー・アシルカルニチンのプロフィール一斉分析とが行われてい

表2　カルニチンが異常を示す病態

1．カルニチン輸送異常症（カルニチントランスポーター欠損症）
2．脂肪酸代謝異常症
　　カルニチンサイクル
　　脂肪酸β酸化異常症
3．先天性有機酸血症
4．薬物服用
5．腎尿細管障害（Fanconi 症候群など）
6．栄養障害（低栄養，特殊ミルク，経腸栄養剤投与時）
7．肝不全
8．腎不全，透析患者

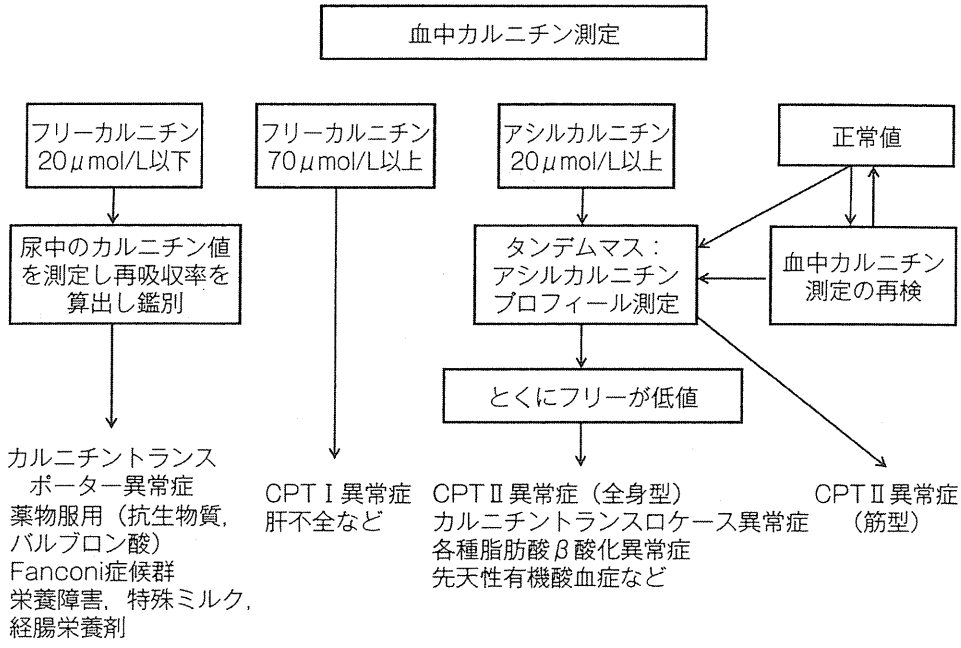

図1　血中カルニチン値における診断の流れ（高柳正樹，2009[15]）

る．血中カルニチンは年齢や採血時間などにより変動がみられるが，村上ら[1]は全年齢をまとめた平均値をフリー 47.8±12.9 μmol/L，アシル 5.8±4.9 μmol/L と報告している．したがって 20 μmol/L 以下は明らかに異常低値である．

図1にカルニチン異常を示す疾患の血中カルニチン値による診断フローチャートを示す．タンデムマススペクトロメーターを用いた血中アシルカルニチンプロフィール分析はカルニチンが関係している疾患，先天性有機酸代謝異常症，先天性脂肪酸代謝異常症の診断において非常に有効的な検査法である．これらの疾患が考えられたときには必須の検査である．

Ⅲ 医原性カルニチン欠乏症

1．バルプロン酸ナトリウムとカルニチン欠乏症

バルプロン酸ナトリウム投与によるカルニチン欠乏症に惹起される高アンモニア血症の問題も，1986 年に Matsuda ら[2]が報告して以来の古

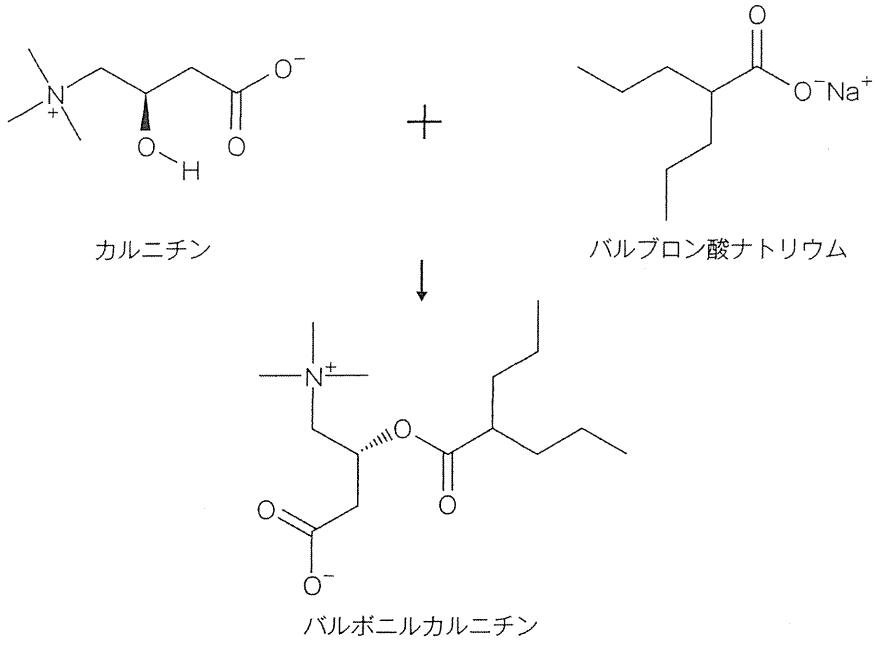

図2　バルプロン酸ナトリウムとカルニチン

くて新しい問題になっている．バルプロン酸ナトリウムはフリーカルニチンと結合してバルボニルカルニチンとなり尿中へ排泄される．このためカルニチンの枯渇による脂肪酸のβ酸化障害が生じ，高アンモニア血症が惹起される．**図2**にバルボニルカルニチン生成を図示する．しかしHiroseら[3]は健康な小児にバルプロン酸ナトリウムを投与してもカルニチン欠乏症は引き起こされないと報告している．おそらくバルプロン酸ナトリウムが投与される患者は，①抗けいれん薬多剤療法，②栄養不良（特殊ミルク，経腸栄養剤投与なども含む），③抗生物質療法併用，④Fanconi症候群などの併発症，⑤腎障害，などの他のカルニチン欠乏症の危険因子を有することが多く，バルプロン酸ナトリウムの投与により低フリーカルニチン状態が増悪するのではと考えられている．

2．ピボキシル基をもつ抗生物質とカルニチン欠乏症

2012年4月25日PMDAによりピボキシル基を含有する抗生物質によるカルニチン欠乏症による低血糖症状やけいれんを呈したなど38件の副作用が報告された[4]．これに基づき各製薬会社がこれらピボキシル基を含有する抗生物質の使用上の注意事項を変更し医療機関に連絡を行っている．

セフジトレンピボキシル，セフカペンピボキシル，セフテラムピボキシル，テビペネムピボキシル，ピブメシリナム塩酸塩などのピボキシル系抗生物質は，腸管吸収をよくするためのピボキシル基を有している．ピボキシル基は体内でピバリン酸になり，カルニチンと結合してピバロイルカルニチンとして尿中に排泄されるため，血清中のフリーカルニチン濃度が低下する．

このような抗生物質を長期投与され，低血糖などの症状を呈した患者が第25回日本小児救急学会などで多く報告されている．このカルニチン欠乏症を呈する病態はカルニチントランスポーター欠損症との区別が難しいことがあるので，慎重に診断を行うことが必要である．抗生物質をやめてカルニチン欠乏症が改善すれば抗生物質の投与によるものと判定しやすいが，Nakajimaら[5]はピボキシル基をもつ抗生物質投与によるカルニチン欠乏症は，正常値に復す

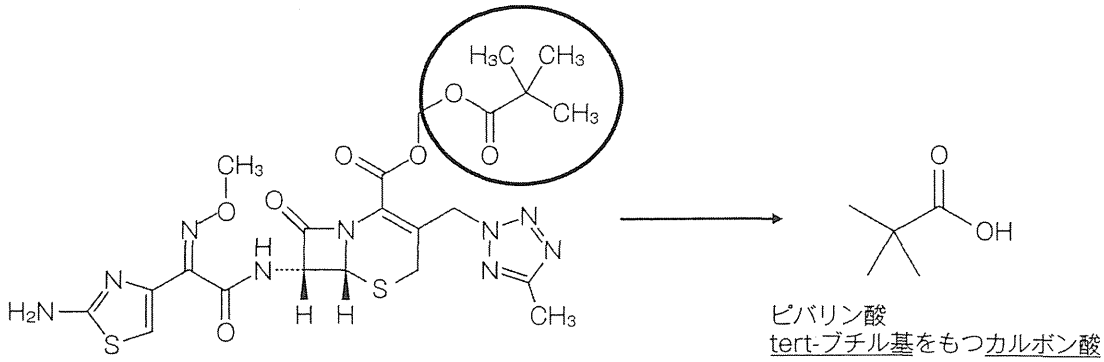

図3　ピボキシル基をもつ抗生物質とピバリン酸

るのに3カ月以上かかることを報告している．

図3にピボキシル基をもつ抗生物質の化学構造を例示する．

③．乳児用特殊医療用調製粉乳（ミルクアレルギー用乳，乳糖不耐用乳，先天代謝異常症用特殊ミルクなどを含む），経腸栄養剤，経静脈的栄養製剤使用時のカルニチン欠乏症

近年，乳児用特殊医療用調製粉乳（ミルクアレルギー用乳，乳糖不耐用乳，先天代謝異常症用特殊ミルクなどを含む）や経腸栄養剤において，カルニチンやビオチンなどのビタミン関連物質やセレン，亜鉛，ヨウ素などの微量元素の含有量の不足が問題になっている．児玉らはこれらの含有量をCODEXが推奨している含有量と比較して，一覧表として報告している[6]（**表3**）．また児玉らはこれら特別な栄養下におけるカルニチンを含めた必須栄養素の欠乏について，詳細な報告をしている[7]．

小澤の報告によれば[8]乳児用特殊医療用調製粉乳中にはその製造法の問題から，ビオチン，カルニチン，亜鉛，セレン，ヨウ素などの含有量が十分ではないとされている．測定したすべての特殊ミルク中には，一般乳に比べて1/4から1/3のカルニチンの含有量であったと報告している．ことにアミノ酸乳（明治エレメンタールフォーミュラ®など）はまったくカルニチンが含まれず，長期に使用すればカルニチン欠乏は必発である．

エンシュアリキッド®，エレンタール®，エレンタールP®をはじめ日本で使用されている大部分の経腸栄養剤・濃厚栄養剤中にはカルニチンはまったく含有されておらず，単独で長期に使用すればカルニチン欠乏は必発であり，カルニチン欠乏症の発症をみた症例の報告がある[9)10)]．経静脈的栄養（TPN）製剤にはカルニチンの添加がなされておらず，長期に使用したときにはカルニチン欠乏症となることが知られている．欧米ではTPN施行症例における肝障害および脂肪製剤使用時にはカルニチンの測定と補充が推奨されている．しかし本邦ではあまり注意が払われていない．田附らは12症例の中心静脈栄養施行症例を検討し，とくに経口，経腸栄養の導入に難渋している症例では，フリーカルニチンの低下が著明であると報告している[11)]．

大浦は[12)]先天代謝異常症用特殊ミルクは母乳などの自然タンパク摂取と併用して投与されることが基本であるので，これらビタミン関連物質や微量元素の欠乏は起きにくいのであろうと述べている．しかし先天代謝異常症用特殊ミルクの使用は，ビタミン関連物質，微量元素欠乏に陥る一つのリスクファクターであるということを十分認識しておくことは重要と考えられる．

医学中央雑誌にて報告されている乳児用特殊

表3　乳児特殊医療用調製粉乳および経腸栄養剤中の各種栄養素含有量

	ビオチン (μg)	カルニチン (mg)	セレン (μg)	ヨウ素 (μg)
CODEX 推奨量	1.5～10	1.2以上	1～9	10～60
通常の乳児用調製粉乳	0.5～1.0	1.6～4.0	1.0～1.5	5～12
主な特殊ミルク				
エレメンタルフォーミュラ	0.1以下	ND	ND	ND
ニューMA1	0.1以下	1.5	ND	ND
ペプディエット	0.1以下	NT	NT	NT
ラクトレス	0.1	ND	ND	ND
ノンラクト	0.1以下	0.68	ND	ND
ケトンフォーミュラ（817-B）	0.1	NT	NT	NT
必須脂肪酸強化MCTフォーミュラ（721）	0.6	NT	NT	NT
糖原病用フォーミュラ	ND～1.8	NT	NT	NT
高NH_4血症・シトルリン血症フォーミュラ（7925-A）	ND	NT	NT	9
蛋白除去粉乳（S-23）	未添加[1)]	未添加[1)]	未添加[1)]	6.6[2)]
イソロイシン・バリン・メチオニン・スレオニン・グリシン除去ミルク（S-22）	未添加[1)]	未添加[1)]	未添加[1)]	5.4[2)]
主な経腸栄養剤・濃厚流動食				
エンシュアリキッド	15.2	ND	ND	ND
エレンタール	13.1	ND	ND	6.5[3)]
エレンタールP	21.0	ND	ND	10.6[3)]
ラコール	3.86	ND	2.5	ND
MA-8	0.1	ND	1	2
MA-8プラス	5	ND	3	13
テルミールミニ	5.5	ND	5.0	29.0
CZ-Hi	5	ND	4	15

[1)]：分析値なし，配合上含有しないと考えられる．
[2)]：添加しているヨウ化カリウム量から計算
[3)]：KI：ヨウ化カリウムとしてND，検出感度以下：NT，測定未実施

（文献6）より引用）

医療用調製粉乳，経腸栄養剤，TPN製剤投与時のカルニチン欠乏症の国内の報告例を集めて表4に示した[7)]．

欧米ではカルニチンの補充が乳児用特殊医療用調製粉乳に認められている．今後欧米と同じ基準となるように各方面からの働きかけが必要であると考える．

Ⅳ 欠乏症の対応，予防

カルニチン欠乏症を示す時の治療指針を考える．現在カルニチンの補充療法については先天性有機酸異常症の一部以外ではカルニチンの補充療法についてはしっかりしたエビデンスに基づいた治療基準はない．血中のフリーカルニチンがどのくらいに低下すれば脂肪酸代謝に影響が出るのかについてもきちんとした報告はない．山本ら[13)]は特殊ミルクを飲んでいる乳児における検討で血中フリーカルニチンが26.9 μmol/Lでは脂肪酸酸化能に大きな問題のないことを報告している．表5に自験例カルニチントランスポーター欠損症2例と，ピボキシル基含有抗生物質によるカルニチン欠乏症3例のまとめを示した．表4と合わせてみると，大部分の症例がフリーカルニチンが20 μmol/L以下で発症している．筆者はフリーカルニチン20

表4 乳児特殊医療用調製粉乳，経腸栄養剤，TPNによるカルニチン欠乏症

症例	報告年齢	性別	症状	検査所見	栄養剤	試用期間	報告者名
1	3カ月	男	多呼吸	フリーカルニチン 0 μmol/L	明治 605Z	3カ月	高柳正樹
2	3歳	男	嘔吐，低血糖	フリーカルニチン 測定感度以下	エレンタールP	1年2カ月	中島浩司ほか
3	11カ月	女	臨床症状なし	フリーカルニチン 8.7 μmol/L	エレンタールP	11カ月	高柳正樹
4	2歳	男	拡張型心筋症	—	経管栄養剤	—	米田 哲
5	2カ月		拡張型心筋症，低血糖	フリーカルニチン 10.0 μmol/L	TPN	約20日	豊島勝昭
6	3カ月	男	肝機能障害，高CK血症	フリーカルニチン 13.8 μmol/L	MCTミルク	2カ月	後藤敦子
7	78歳	女	高脂血症	フリーカルニチン 22 μmol/L	エンシュアリキッド	2年6カ月	田中慎一郎
8	5カ月	女	低血糖，腸管拡張	—	TPN	5カ月半	大橋祐介
9	8カ月	男	肝機能障害	フリーカルニチン 19 μmol/L	TPN	8カ月	田附裕子
10	12症例			多数症例解析	TPN		田附裕子

表5 カルニチントランスポーター欠損症およびピボキシル基含有抗生物質投与によるカルニチン欠乏症

症例	発症年齢	症状	抗生物質	投与期間	血中フリーカルニチン値	その他
1	1歳	意識障害，低血糖	セフジトレンピボキシル（メイアクト）	16日	9.8 μmol/L	
2	1歳11カ月	嘔吐，けいれん，低血糖	テビペネムピボキシル（オラペネム）	8カ月	低値	高アンモニア血症（186 μg/dL）
3	2歳	けいれん，低血糖	CDTR-PI，CFTR-PI（メイアクト，フロモックス）	8カ月	低値	高アンモニア血症（400 μg/dL以上）

カルニチントランスポーター欠損症

症例	報告年齢	性別	症状	検査所見
1	1歳6カ月	女	けいれん，低血糖	フリーカルニチン 7.1 μmol/L
2	3歳	男	腹痛，傾眠	フリーカルニチン 3.7 μmol/L

μmol/L以下がカルニチン補充の適応と考えている．

カルニチンの経口投与によるLD50は19.2 g/kgときわめて高い．副作用としては嘔気，嘔吐，体臭，胃炎，けいれんなどがあるが安全性は高い薬剤である．

筆者はバルプロン酸ナトリウム投与時で高アンモニア血症があれば当然だが，高アンモニア血症は認められない時でも20 μmol/L以下のカルニチン欠乏症のときは，基本的にはカルニチン投与を行うこととしている．ことに患者に低栄養，経管栄養，腎障害などの他にカルニチン低下のファクターがあるときには投与が勧められる．バルプロン酸ナトリウム投与症例で普通の食事を摂取しているならば10 mg/kg/日程度でもよいと考える．

ピボキシル基含有抗生物質投与時のカルニチン投与の適応については現在のところ明確な指針はない．ピボキシル基を含有しない抗生物質への変更はよい選択である．ピボキシル基含有抗生物質を長期に使用せざる得ないときには血中フリーカルニチンを測定して，低値ならばカルニチンの補充を行うべきである．カルニチン欠乏症を呈する投与期間としては1週間以内という短期のものも報告されているので，すべての投与患者の臨床症状に十分注意を払うべきである．とくにバルプロン酸ナトリウム投与症例，ミルクアレルギー用ミルク投与症例，低栄養症例，筋肉量の少ない症例へのこれら抗生物質の投与にはさらに十分な注意が必要である．

まったくカルニチンが含まれていない乳児用特殊医療用調製粉乳，経腸栄養剤を単独で長期に使用するときには，最初から同時にカルニチンを投与しておき，カルニチン欠乏症の発症を防ぐ事が望まれる．とくにバルプロン酸ナトリウムやピボキシル基含有抗生物質が投与される症例はカルニチン投与を積極的に考慮すべきである．この時には欠乏状態を補充するだけなので20～30 mg/kg/日で十分である．同時にビオチンの欠乏も必発なのでビオチンの補充も必須の対応である．エレンタール®，エレンタールP®は非常に多くの症例で単独で長期に使われているので十分な注意が必要である．長期にわたらないときでもカルニチン欠乏症発症の可能性があるので，各種ミトコンドリア脂肪酸酸化異常症の症状が認められた時には，血中カルニチンの検索と適切な治療が必要である．

中心静脈栄養施行患者では長期にわたるときはカルニチンの補充は必須事項である．短期であっても肝障害発症時および脂肪製剤使用時にはカルニチンの測定と補充を行うべきである．

カルニチントランスポーター欠損症では100～200 mg/kg/日以上が必要であり，有機酸血症などでは50～100（200）mg/kg/日が必要である．

おわりに

最近医原性のカルニチン欠乏症により障害を残す存在も知られるようになってきている．カルニチン欠乏に対する知識を小児科医のみならず，耳鼻科医，神経科医などにも広め，リスクのある患者に対しての十分な注意喚起の必要性を強調していくことが重要であると考える．

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お知らせ

第15回　国際乳幼児けいれん研究会

会　　期：2013年4月12日（金）～14日（日）
会　　場：順天堂大学医学部有山記念講堂　他
　　東京都文京区本郷2-1-1
会　　長：新島　新一（順天堂大学附属練馬病院小児科）
テ ー マ：新生児けいれん，新生児期発症てんかん，新生児脳障害の診断，治療，予後，発生予防（神経保護，再生）など
プログラム：国際抗てんかん連盟（ILAE）小児科委員会との共同企画による．すなわち国際的な一流の講師による特別講演，招待講演，教育講演，シンポジウム，一般演題（口演，ポスター），早朝セミナー，ランチョンセミナー，その他
参 加 者：日本全国-世界各国-特にアジア・オセアニア地域の小児神経科医，小児てんかん専門医，周産期医学・新生児学専門医，NICU担当医，小児神経救急医，発達神経科学基礎研究者，小児保健衛生関係
公 用 語：英語のみ
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Original Article

Molecular diagnosis of mitochondrial respiratory chain disorders in Japan: Focusing on mitochondrial DNA depletion syndrome

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Abstract ***Background***: Although mitochondrial respiratory chain disorders (MRCD) are one of the most common congenital metabolic diseases, there is no cumulative data on enzymatic diagnosis and clinical manifestation for MRCD in Japan and Asia.

Methods: We evaluated 675 Japanese patients having profound lactic acidemia, or patients having symptoms or signs of multiple-organ origin simultaneously without lactic acidemia on respiratory chain enzyme activity assay and blue native polyacrylamide gel electrophoresis. Quantitative polymerase chain reaction was used to diagnose mitochondrial DNA depletion syndrome (MTDPS). Mutation analysis of several genes responsible for MTDPS was also performed.

Results: A total of 232 patients were diagnosed with a probable or definite MRCD. MRCD are common, afflicting one in every several thousand people in Japan. More than one in 10 of the patients diagnosed lacked lactic acidemia. A subsequent analysis of the causative genes of MTDPS identified novel mutations in six of the patients. A 335 bp deletion in deoxyguanosine kinase (*DGUOK*; g.11692_12026del335 (p.A48fsX90)) was noted in two unrelated families, and may therefore be a common mutation in Japanese people. The proportion of all patients with MTDPS, and particularly those with recessive *DNA polymerase* γ (*POLG*) mutations, appears to be lower in Japan than in other studies. This is most likely due to the relatively high prevalence of ancient European *POLG* mutations in Caucasian populations. No other significant differences were identified in a comparison of the enzymatic diagnoses, disease classifications or prognoses in Japanese and Caucasian patients with MRCD.

Conclusion: MTDPS and other MRCD are common, but serious, diseases that occur across all races.

Key words *DGUOK* deletion mutation, enzymatic diagnosis, mitochondrial DNA depletion syndrome, mitochondrial respiratory chain disorder, racial difference.

Mitochondrial respiratory chain disorders (MRCD) are disorders of the oxidative phosphorylation system, which is responsible for ATP production. MRCD are the most common congenital metabolic diseases, afflicting at least 1 in 5000 persons.[1] Mitochondrial DNA depletion syndrome (MTDPS), in which mitochondrial DNA (mtDNA) level is lower than normal, is one of the major MRCD. A number of responsible genes of MTDPS have been identified, and the pathophysiology of this disease is partially characterized at the molecular level.[2–5] We have previously diagnosed and characterized MRCD cases in Japan using respiratory chain enzyme analysis.[6–9] Having recently analyzed the molecular diagnoses and clinical manifestations of MRCD in Japanese patients, and analyzing several genes responsible for hepatocerebral MTDPS, we herein discuss and compare the collected data to those reported for MRCD outside of Japan.

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Methods

Patients and samples

The subjects consisted of patients clinically suspected of having MRCD. We measured respiratory chain enzyme activity and quantity for patients with profound lactic acidemia, or patients with symptoms or signs of multiple-organ origin simultaneously without lactic acidemia. Other metabolic disorders were excluded on plasma tandem mass spectrometry and urine organic acid analysis. Approximately half of candidates were <1 year old,

and nearly 90% were <10 years old. In total, 1051 samples from 675 patients in 657 families were analyzed. Of the samples, 479 were cultured skin fibroblast cells, 239 were liver samples, 208 were muscle samples, 84 were myocardial samples, and 41 were other samples (including 25 kidney and seven brain samples).

Respiratory chain enzyme analysis

Both an *in vitro* respiratory chain enzyme activity assay[10] and blue native polyacrylamide gel electrophoresis (BN-PAGE)[11–13] were used to quantify the activity and amount of respiratory chain enzyme complexes. A diagnosis of MRCD was made when the results from the enzyme activity or BN-PAGE raised the diagnostic criteria assessment to definite or probable for MRCD according to the diagnostic criteria of Bernier *et al.*[14]

Entire mtDNA analysis

DNA was purified according to standard methods. The mitoSEQr™ system (Applied Biosystems, Foster City, CA, USA) was used for entire mtDNA analysis in each patient diagnosed with MRCD.

Quantitative polymerase chain reaction for diagnosis of MTDPS

Quantitative polymerase chain reaction (qPCR)[15] was used to determine whether mtDNA depletion was present in patients with decreased activity level of multiple respiratory chain enzymes (the mtDNA gene *MT-ND1* was compared against a nuclear gene, *CFTR* exon 24). A diagnosis of MTDPS was made when the relative copy number of mtDNA to nuclear DNA was <35% of that in healthy control tissue using four independent experiments.

Mutation analysis of genes responsible for MTDPS

Mutation analysis was performed on the genomic DNA using primers designed to amplify the coding exons and the exon–intron boundaries of DNA polymerase γ (*POLG*; NM_002693.2), *deoxyguanosine kinase* (*DGUOK*; NM_080916.1 and NM_080918.1), and *MPV17* (NM_002437.4).[16] Fragments were analyzed by direct sequencing using ABI 3130XL (Applied Biosystems, Melbourne, Vic., Australia). Long-range PCR encompassing the 335 bp deletion was performed using primers shown in Figure 1(a).

DNA from healthy Japanese controls

A PSC Cell Line Purified DNA 100 set (Japan Health Sciences Foundation, Tokyo, Japan) was used as control DNA for healthy Japanese.

Statistical analysis

The log-rank test and Gehan-Breslow-Wilcoxon test were used to test for statistically significant differences.

Ethics

This study was approved by the Institutional Review Board in Saitama Medical University.

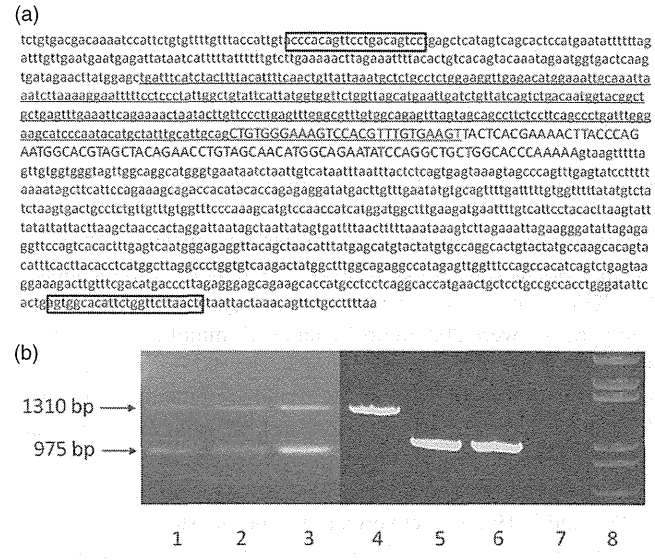

Fig. 1 Genomic sequence determination of 335 bp deoxyguanosine kinase (*DGUOK*) deletion in the family of patients 1 and 2. (a) Capitalization, sequence of exon 2; two rectangles, long-range polymerase chain reaction (PCR) primer sets; underline, 335 bp deletion. The large 335 bp deletion encompassing from the end of intron 1 to the beginning of exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90). (b) Lane 1, father; lane 2, mother; lane 3, middle healthy sister; lane 4, normal control; lane 5, patient 1; lane 6, patient 2; lane 7, no sample; lane 8, molecular weight marker. The 1310 bp band represents the normal sized PCR product. The 975 bp band represents the PCR product with 335 bp *DGUOK* deletion in this family.

Case reports: DGUOK deficiency in three Japanese patients

Patient 1

This Japanese girl was the first child to unrelated healthy parents and was born without any complications at 40 weeks of gestational age, weighing 2510 g. At 3 months of age, she was referred to hospital because of failure to thrive, nystagmus and incomplete head control. Laboratory tests showed mild liver dysfunction of unknown etiology. She was suspected to have hereditary tyrosinemia because her blood tyrosine level was 800 nmol/mL (cut-off, 500 nmol/mL), but urinary succinylacetone was not detected. At the age of 18 months, her liver dysfunction deteriorated to the level of liver failure with prolonged coagulation time (hepaplastin time 39%), and she underwent a liver transplantation, but died of cardiac tamponade at 19 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (0%, 9%, and 28% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (74% and 308%, respectively). On BN-PAGE analysis, the band corresponding to assembled complex I was invisible and those of complex III and IV were strikingly weak (data not shown). On qPCR, liver mtDNA was markedly decreased (3%), confirming a diagnosis of hepatocerebral MTDPS.

Patient 2

A healthy sister of patient 1 was born 2 years after her elder sister died. A third girl was born 4 years after her eldest sister died, without any complications at 40 weeks of gestation, with a weight of 2750 g. At 2 days of age, she was referred to hospital due to tachypnea, hypoglycemia, and metabolic acidosis. After that, mild liver dysfunction was found (total bilirubin, 4.2 mg/dL; direct bilirubin, 1.4 mg/dL; aspartate aminotransferase, 215 IU/L; alanine aminotransferase, 49 IU/L; γ-glutamyl transpeptidase, 842 IU/L) with hyperammonemia (180 μg/dL). Blood lactate and pyruvate were 20.9 mmol/L, and 0.27 mmol/L, respectively. Because of her eldest sister's course, she did not undergo liver transplantation and she died of liver failure at 9 months of age. The liver showed low activity of complexes I, III, and IV (0%, 6%, and 17% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (105% and 281%, respectively), as for the eldest sister. On qPCR, liver mtDNA was markedly decreased (6%) and she was diagnosed with hepatocerebral MTDPS.

Patient 3

A Japanese girl, unrelated to patients 1 and 2, was born as the third child to unrelated healthy parents at 37 weeks of gestational age weighing 1688 g. Symmetrical intrauterine growth retardation was noted from 30 weeks gestation. Her eldest brother died at 1 year 4 months with a hepatic disorder of unknown origin. Her elder sister was healthy. At 8 days of age, she was suffering from feeding difficulty with liver dysfunction and nystagmus. Developmental delay and failure to thrive gradually progressed. At the age of 8 months, her liver dysfunction deteriorated to the level of liver failure, and she underwent liver transplantation, but died at 18 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (12%, 12%, and 16% of normal control, respectively). In contrast, complex II and citrate synthase activity were normal (68% and 106%, respectively). On qPCR, liver mtDNA was markedly decreased (2%) and she was diagnosed with hepatocerebral MTDPS.

Results

Characteristics of Japanese children diagnosed with MRCD

In total, we diagnosed MRCD in 232 patients; these patients comprised 34% of the study group. The age distribution of these patients is as follows; nearly 40% before 1 month of age, three-fourths by age 1 year, and >90% by age 7 years. One hundred and twenty patients (52%) were male, and approximately half of the diagnosed patients were deceased. Diverse clinical diagnoses are shown in Figure 2. Eighty-seven patients (38%) had neurological disorders consisting of Leigh syndrome, neurodegenerative disorders, and so-called mitochondrial cytopathy. Fifty-nine (25%) had a lethal or non-lethal infantile mitochondrial disorder. Twenty-nine (13%) had mitochondrial hepatopathy, and 17 (7%) had mitochondrial cardiomyopathy. Among all MRCD, 28 patients (12%) lacked lactic acidemia, a feature that traditionally prompts suspicion of MRCD. The entire mitochondrial DNA sequence was determined for 139 patients, but a causative genetic abnormality was found in only 34 (24%) of these patients (data not shown); indicating that, in most cases, the causative gene or genes may be present in nuclear DNA.

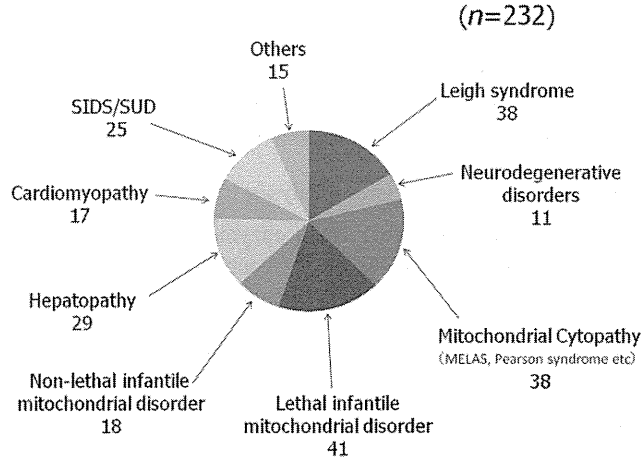

Fig. 2 Clinical diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan. Neurodegenerative disorders, neurodegenerative disorders unclassified to specific diseases. Patients with non-lethal infantile mitochondrial disorder started with symptoms such as lethal infantile mitochondrial disorder but survived beyond 1 year old. SIDS, sudden infant death syndrome; SUD, sudden unexplained death.

The enzymatic diagnoses were compared with Australian data (Fig. 3).[17] In Japanese patients, a respiratory chain complex I abnormality was most common (105 patients, 45%), followed, in decreasing order of prevalence, by respiratory chain abnormalities in multiple complexes (80 patients, 34%), a complex IV abnormality (33 patients, 13%), and a complex III abnormality (10 patients, 4%). No patient was given a probable or definitive

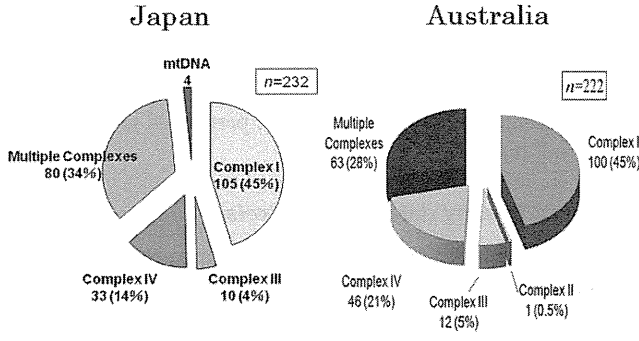

Fig. 3 Percentage distribution of enzymatic diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan and those reported previously in Australia. The enzymatic diagnosis of MRCD showed similar trends in prevalence between the Japanese and Australian patients,[17] with respiratory chain complex I being the most common type of MRCD, followed by abnormalities in multiple complexes, complex IV abnormalities, and complex III abnormalities. Complex II abnormalities were very rare among the two populations.

diagnosis of a complex II abnormality. Similarly, according to the Australian data, the most common abnormality was in complex I (45%), followed by abnormalities in multiple complexes (28%), complex IV (21%), and complex III (5%); only one patient had a complex II abnormality.

Manifestations, genetic diagnoses, and prognoses of MTDPS

A qPCR-based diagnosis of MTDPS was made for 16 of the 80 patients with an enzymatic diagnosis of a multiple complex abnormality, and for seven of the 105 patients with an enzymatic diagnosis of a respiratory chain complex I abnormality. Three of these 23 patients died due to sudden infant death syndrome and thus had no available records of clinical findings; the clinical findings from the remaining 20 patients were further analyzed.

The disease types among these 20 patients were compared with those reported by Sarzi *et al.*[4] (Fig. 4). Among the Japanese patients, 13 (65%) had acute hepatocerebral MTDPS, two (10%) had Alpers-like syndrome (delayed-onset hepatocerebral MTDPS), and five (25%) had encephalomyopathic MTDPS. This distribution is similar to that reported by Sarzi *et al.* We must note here that "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS. This is because no true case of Alpers syndrome has yet been identified in Japan. The results of analyses of the three main genes responsible for MTDPS are shown in Figure 5. Causative genetic anomalies were identified in six of the 20 Japanese patients (30%). No abnormality was identified in the three genes of the remaining 14 patients (70%). The responsible genes were *DGUOK* in three patients whose clinical reports are described in the previous section, *MPV17* in two patients,[7] and *POLG* in one patient whose clinical report will be published elsewhere. The individual genetic abnormalities are listed with the clinical findings in Table 1. Although three of the patients

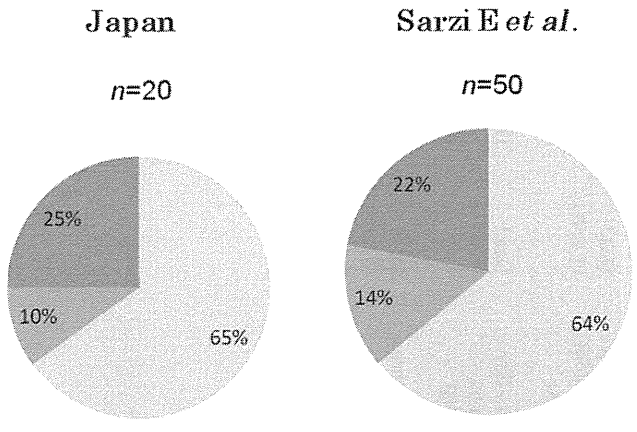

Fig. 4 Percentage distribution of disease types of mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS, because no true case of Alpers syndrome has yet been identified in Japan. The distribution of disease types in the present study did not differ from that reported by Sarzi *et al.*[4]. (▒) Hepatocerebral, (▒) Alpers–like syndrome, (▒) Encephalomyopathic.

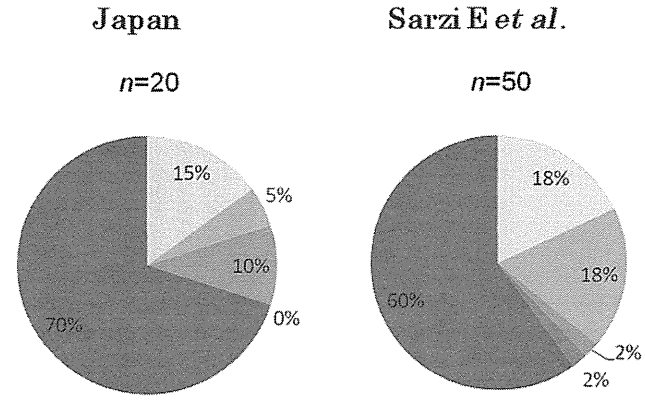

Fig. 5 Percentage distribution of responsible genes for mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* The causative gene was not identified in the majority of patients in each population. Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities identified by Sarzi *et al.*,[4] while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients. (▒) *DGUOK*, (▒) *POLG*, (▒) *MPV17*, (▒) *TK2*, (▒) *unknown*. DGUOK, deoxyguanosine kinase; POLG, DNA polymerase γ.

underwent liver transplantation during infancy, five of them died before 2 years of age. Patient 5 lived longer than the others because of dietary and pharmaceutical treatment targeting the mitochondrial respiratory chain complex II.[7]

The *DGUOK*-related patients were two sisters, with a homozygous 335 bp deletion (Fig. 1a; g.11692_12026del335; encompassing 308 bp of intron 1 and 27 bp at the start of exon 2), and a compound heterozygote patient, genetically unrelated to these sisters, with the same deletion and a c.743T>C (p.L248P) missense mutation. The large 335 bp deletion encompassing from intron 1 to exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90). Each parent and healthy sister is heterozygous for this mutation (Fig. 1b). The p.L248P variation is not listed as a polymorphism in the ensembl_mart_47 database (martdb.ensembl.org) and has not been reported as a disease-causing mutation. Moreover, the alignment shows that Leu248 is absolutely conserved in all species (Fig. 6).[18]

The *MPV17* patients were previously reported compound heterozygote siblings.[7] The *POLG* patient was a compound heterozygote. The genetic mutations noted in these six patients were confirmed to be absent in DNA of 100 healthy Japanese controls (data not shown).

Like Sarzi *et al.*, who did not find the responsible gene or genes in 60% of the patients, we were unable to identify the responsible gene or genes in a majority of the cases. We sequenced the whole exome of all the MTDPS patients to identify the underlying nuclear disease genes using next-generation sequencing system (data not shown). This did not identify pathogenic mutations in any of the known genes associated with MTDPS (*TK2, SUCLA2, RRM2B, SUCLG1, MGME1, C10orf2, TYMP, and AGK*) in the present MTDPS patients.

Table 1 Clinical and molecular characteristics for Japanese hepatocerebral MTDPS patients

Patient	Sex	Initial symptoms (age)	Outcome (age)	Clinical diagnosis	Complications	Liver transplantation	Blood lactate/ pyruvate (mmol/L)	%mtDNA in liver	Identified mutations	Ref
1	F	Failure to thrive (3 months)	Dead (1 year)	Hereditary tyrosinemia	Developmental delay	Done	Not available	3	*DGUOK* (g.11692_12026del335 (p.A48fsX90) homozygote)	
2	F	Tachypnea (2 days)	Dead (9 months)	Mitochondrial hepatopathy	Hypoglycemia	Not done	20.9/0.27	6	*DGUOK* (g.11692_12026del335 (p.A48fsX90) homozygote)	
3	F	Feeding difficulty (8 days)	Dead (1 year)	Mitochondrial hepatopathy	Developmental delay, failure to thrive	Done	2.9/0.14	2	*DGUOK* (g.11692_12026del335 (p.A48fsX90) / c.743T>C (p.L248P))	
4	M	Failure to thrive, acholic stool (3 months)	Dead (1 year)	Hepatic failure	Developmental delay	Done	Not available	8	*MPV17* (c.451insC (p.L151fsX189)/ c.509C>T (p.S170F))	7
5	M	Failure to thrive, vomiting (8 months)	Dead (6 years)	Hepatic failure	Developmental delay, gastroesophageal reflux, respiratory failure	Done (at 6 years)	Normal	7	*MPV17* (c.451insC (p.L151fsX189)/ c.509C>T (p.S170F))	7
6	F	Failure to thrive (4 months)	Dead (7 months)	Hepatic failure	Hypotonia	Not done	1.76/0.1	3	*POLG* (c.2869G>C (p.A957V) / c.3354T>C (p.I1185T))	

Shaded columns, two pairs of siblings. MTDPS, mitochondrial DNA depletion syndrome.

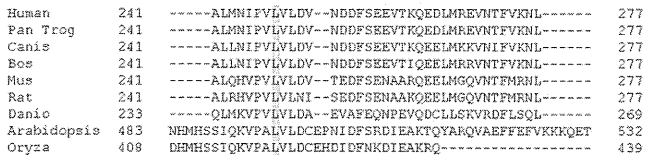

Fig. 6 ClustalW multiple sequence alignment of deoxyguanosine kinase (*DGUOK*) orthologs. The alignment shows that amino acid 248Leu mutated in the patient is absolutely conserved in all species. URLs: HomoloGene, http://www.ncbi.nlm.nih.gov/homologene (for the DGUOK ortholog amino acid sequences of human [accession no. NP_550438.1], Pan [accession no. XP_001153473.1], Canis [accession no. XP_533001.2], Bos [accession no. NP_001014888.2], Mus [accession no. XP_001107072.1], Rat [accession no. NP_001100072.1], Danio [accession no. XP_001093561.1], Arabidopsis [accession no. NP_565032.2], Oryza [accession no. NP_001044956.1]). ClustalW, http://www.ebi.ac.uk/Tools/clustalw/.[18]

Of the genetic mutations identified, *POLG* mutations were less prevalent than in Caucasian subjects. Only one of the present 15 cases of Alpers syndrome or hepatocerebral MTDPS were caused by recessive *POLG* mutations, compared with eight of 39 such cases diagnosed in France.

Sixteen of the 20 Japanese MTDPS patients were deceased. Sarzi *et al.* reported that 29 of the 50 MTDPS patients they analyzed were deceased. The data of the deceased patients were plotted to obtain curves of the ages of death (in months) in the two groups for comparison (Fig. 7). MTDPS patients had a short life in both study groups; many died during or before reaching early childhood. On log-rank test and Gehan-Breslow-Wilcoxon test no significant differences were seen between the survival data.

Discussion

We started an enzyme diagnosis referral service for children suspected of MRCD in 2007 and have diagnosed MRCD in 30–40 patients from around Japan annually since then. In the last year we have made >100 new MRCD diagnoses. Approximately half of the diagnoses are for neonates. There are approximately one million births in Japan annually. Under the assumption that the patients referred for enzyme diagnosis represent approximately half of all Japanese MRCD patients, the prevalence of neonatal-onset MRCD becomes $50 \times 2/1\,000\,000 = 1/10\,000$. When patients with juvenile-onset and adult-onset mitochondrial disease are factored in, the prevalence of these diseases in Japan becomes one in several thousand, which is comparable to the prevalence in Western countries.[1]

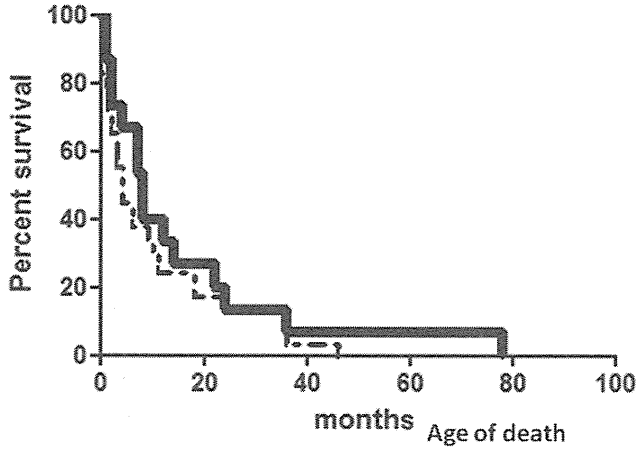

Fig. 7 Comparison of the ages of death (in months) in the two studies. A commonality between the Japanese patients and the Sarzi *et al.* patients[4] was observed. No significant difference in disease severity was identified (log-rank test, $P = 0.3637$; Gehan-Breslow-Wilcoxon test, $P = 0.2667$). (▬) Japanese, $n = 16/20$; (▪▪▪) Sarzi *et al.*, $n = 29/50$.

It is noteworthy that >10% of the patients lacked lactic acidemia, which many physicians still regard as synonymous with mitochondrial disease. Hence, mitochondrial disease must also be considered in lactic acidemia-free patients with unexplained signs and symptoms in multiple organs.

The enzymatic diagnosis of MRCD showed similar trends in prevalence between Japanese and Australian patients, with respiratory chain complex I being the most common type of MRCD, followed by abnormalities in multiple complexes, complex IV abnormalities, and complex III abnormalities. Complex II abnormalities were very rare in both populations.

Twenty percent of the patients with multiple respiratory chain disorders in the present study and 50% of the patients in the Sarzi *et al.* study[4] had MTDPS. Although MTDPS was the leading cause of MRCD in both groups, MTDPS represented a smaller proportion of the MRCD in Japan. According to the Online Mendelian Inheritance in Man database, MTDPS can be classified as encephalomyopathic, hepatocerebral, or specific (a classification that includes mitochondrial neurogastrointestinal encephalopathy [MNGIE] and Sengers syndrome). Encephalomyopathic MTDPS features respiratory failure and myopathy. Hepatocerebral MTDPS is characterized by liver disorders, growth disorders, and hypoglycemia. The distribution of the disease type classifications of the Japanese patients did not differ from the distribution reported by Sarzi *et al.*

Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities in the Sarzi *et al.* study, while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients. The causative gene, however, was not identified in the majority of patients in each study. The six Japanese hepatocerebral MTDPS patients in whom the responsible gene was identified are listed in Table 1. The serious nature of this disease is evident, given that all six experienced onset as neonates or infants and died during or before reaching early childhood.

Deoxyguanosine kinase deficiency was originally described as the cause of infantile-onset hepatocerebral mitochondrial disease, typically featuring hepatic failure, nystagmus and hypotonia.[19] Recently it has been shown that patients with *DGUOK* mutation may present with neonatal hemochromatosis[20] or adult-onset myopathy and mitochondrial DNA multiple deletions, with or without liver involvement.[21,22] We found two novel *DGUOK* mutations in two apparently unrelated Japanese families. Three patients in two families had typical signs and symptoms of hepatocerebral MTDPS, and both parents in each family were

heterozygous for these mutations. A 335 bp deletion in *DGUOK* was found in both families, and may therefore be a common mutation in the Japanese population.

The present analysis of MTDPS patients concludes with a comparison of the ages of death (in months) in the two groups (Fig. 7). A commonality between the Japanese patients and the Sarzi *et al.* patients was the early age of death: most patients died during or before reaching early childhood. *DGUOK* deficiency was most serious in both studies. Likewise, many patients in each study experienced onset as neonates or infants. No significant difference in disease severity was identified between the two studies.

The present results indicate a lower prevalence of *POLG* mutations in the Japanese population, which is likely attributable to several common mutations found in Caucasian people that appear to be ancient European founder mutations (p.A467T, p.G848S, and p.W748S).[23] In children with recessive *POLG* mutations, these three mutations represented seven of 16 mutant alleles reported by Sarzi *et al.*[4] A recent study collated the prevalence of these three mutations in 10 studies reporting a total of 249 POLG patients and found that they represented 49% of mutant alleles in predominantly Caucasian patients.[24] Most Caucasian POLG patients will thus have at least one allele carrying one of these three founder mutations, and Hakonen *et al.* suggested that they may have been spread during Viking times.[23] The carrier frequency of these mutations is as high as 2% in some European countries. Their expected absence in Asian patients likely explains a lower prevalence of recessive POLG disease in Asian populations.

Conclusion

Mitochondrial DNA depletion syndrome and other mitochondrial respiratory chain disorders are common, but serious, diseases that occur across all races.

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Diagnosis and molecular basis of mitochondrial respiratory chain disorders: Exome sequencing for disease gene identification☆,☆☆

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ABSTRACT

Mitochondrial disorders have the highest incidence among congenital metabolic diseases, and are thought to occur at a rate of 1 in 5000 births. About 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities, while the rest occur due to defects in genes encoded in the nucleus. The most important function of the mitochondria is biosynthesis of ATP. Mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorder, as respiratory chain complexes serve a central role in ATP biosynthesis. By next-generation sequencing of the exome, we analyzed 104 patients with mitochondrial respiratory chain disorders. The results of analysis to date were 18 patients with novel variants in genes previously reported to be disease-causing, and 27 patients with mutations in genes suggested to be associated in some way with mitochondria, and it is likely that they are new disease-causing genes in mitochondrial disorders. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

1. Introduction

1.1. Mitochondrial disorders

Mitochondrial disorders have the highest incidence among congenital metabolic disorders, and are thought to occur at a rate of 1 in 5000 births [1]. The common view of mitochondrial disorders is that they include mitochondrial encephalopathy and myopathy, with onset due to mitochondrial DNA defects inherited through the maternal line. In fact, however, only about 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities [2,3], while the rest occur due to defects in genes encoded in the nucleus. Most cases are sporadic (do not have a clear genetic association), and a majority of cases resulting from nuclear gene abnormalities are autosomal recessive. Mitochondrial DNA has a circular structure with a length of 16.6 kbp, and encodes only 13 proteins [4]. These 13 proteins are part of the structural composition of complex I (7 proteins), complex III (1 protein), complex IV (3 proteins) and complex V (2 proteins) in the respiratory chain. They do not include any complex II structural proteins. The remaining genes encoded in mitochondrial DNA are 22 tRNAs and two ribosomal RNAs, and mitochondrial disorders due to defects in these RNAs have also been reported. Meanwhile, a certain amount of the gene products encoded in the nucleus exists in the mitochondria, and roughly 1500 are thought to serve important roles in mitochondrial function [5]. In this analysis, we focused on mitochondrial disorders thought to occur due to defects in genes encoded in the nucleus. Mitochondria have many functions, one of the most important being biosynthesis of energy (ATP), and we assume for the following discussion that mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorders (MRCD), as respiratory chain complexes [6] serve a central role in ATP biosynthesis.

1.2. Mitochondrial disorders of nuclear origin

As stated above, of the approximately 1500 genes encoded in the nucleus that are thought to be involved in biosynthesis and mitochondrial function, more than 100 have been reported to be causes of mitochondrial disorders [7–9] (Table 1). Among these, about 90% of genes have an autosomal recessive inheritance pattern, and only a small portion

Abbreviations: MRCD, mitochondrial respiratory chain disorder; BN-PAGE, blue native polyacrylamide gel electrophoresis; iPS, induced pluripotent stem cells; LIMD, lethal infantile mitochondrial disease; LCSH, Long Contiguous Stretch of Homozygosity

☆☆ This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

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Table 1
The genetic basis of MRCD.

mtDNA mutations: 35/37 genes	
tRNAs, subunits, rRNAs, and deletions & duplications	
Nuclear mutations: 117 genes	
Nuclear-encoded subunits: 27/~80 genes	**mtDNA replication: 5 genes**
Complex I: *NDUFV1, 2, NDUFB3, 9 NDUFA1, 2, 9, 10, 11, 12, NDUFS1, 2, 3, 4, 6, 7, 8*	*POLG, POLG2, C10orf2, MPV17, AGK*
Complex II: *SDHA, SDHB, SDHC, SDHD*	**mtDNA expression: 24 genes**
Complex III: *UQCRB, UQCRQ*	*LRPPRC, TACO1, MTPAP, MRPS16, MRPS22,MRPL3, GFM1,TSFM,TUFM,TRMU,C12orf65,MTFMT, DARS2, RARS2,YARS2,SARS2,AARS2,HARS2,MARS2,EARS2, RMND1, MTO1, FARS2, GFM2*
Complex IV: *COX6B1, COX4I2, COX7B*	
Complex V: *ATP5E*	
Import, processing, assembly: 38 genes	**Nucleotide transport, synthesis: 9 genes**
Complex I: *C8orf38, C20orf7, NDUFAF1, F2, F3, F4, FOXRED1, NUBPL, ACAD9, AIFM1*	*SLC25A4, SLC25A3, TYMP, DGUOK, TK2, PUS1, SUCLA2, SUCLG1, RRM2B*
Complex II: *SDHAF1, SDHAF2*	**Membrane composition: 14 genes**
Complex III: *BCS1L, HCCS, TTC19*	*COQ2, COQ6, COQ9, PDSS1, PDSS2, CABC1, SERAC1, MPC1, NMT, TAZ, CYCS, OPA1, MFN2, DNM1L*
Complex IV: *SURF1, SCO2, SCO1, COX10, COX15, ETHE1, FASTKD2, C2orf64, C12orf62*	
Complex V: *ATPAF2, TMEM70*	
Multiple: *TIMM8A, SPG7, HSPD1, AFG3L2, DNAJC19, GFER*	
Iron/FeS: *FXN, ISCU, GLRX5, ABCB7, NFU1, BOLA3*	
117 nuclear gene defects	Categories are based on D.R Thorburn's paper[7]

95: autosomal recessive·
10: autosomal dominant·
5: recessive or dominant·
7: X-linked·

have a dominant inheritance pattern [10]. There have also been seven reported cases of mitochondrial disorders from defects in genes encoded by the X chromosome. By function, these include genes involved in the structural composition of the complexes and mitochondrial biosynthesis, genes involved in membrane composition, genes involved in the synthesis and transport of nucleic acids, genes involved in regulating the expression of mitochondrial DNA, and genes involved in mitochondrial DNA replication.

We have actively analyzed the exomes of patients with MRCD in order to identify the cause. Here, we briefly describe our project and discuss the results of exome analyses performed to date, touching on some of the problems that have been encountered.

2. Outline of exome analysis project for MRCD patients

Fig. 1 outlines our current project. It is supported by the Ministry of Education, Culture, Sports, Science and Technology's Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) (http://www.cell-innovation.org/english/html/program/theme_010_okazaki.html). First, analyses of enzyme activity [11], quantity and size were performed using fibroblasts from patient skin or biopsy specimens from diseased organs of patients suspected of having MRCD in clinical practice [12]. Quantity and size were analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE) [13]. Next, among patients in whom decreased enzyme activity or complex formation abnormalities were seen biochemically, whole exome analysis was performed in those with no known mitochondrial DNA abnormalities, and the obtained candidate causal genes were confirmed at the cellular level by rescue experiment or other methods, such as siRNA experiment. Many patients with mitochondrial disorders have primary symptoms in the central nervous system, but brain biopsy in these patients is untenable. Therefore, induced pluripotent stem (iPS) cells were created using fibroblasts from the skin of patients from whom informed consent was obtained. These iPS cells were then differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction that occurs specifically in the nervous system, based on the notion that this may lead to treatment at the cellular level and ultimately to treatment in humans.

3. Clinical diagnosis of MRCD

Mitochondria exist in all tissues, and symptoms are presented in various organs and/or pathological entities. In pediatric MRCD, symptoms are broadly divided into: (1) encephalomyopathy symptoms; (2) gastrointestinal/hepatic symptoms; and (3) myocardial symptoms [14]. So-called "mitochondrial encephalomyopathy," which has traditionally been considered the main form of mitochondrial disease, belongs among the relatively mild mitochondrial diseases and occurs mostly in older people. Fig. 2 shows a breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013 [15]. Patients with the traditionally described nerve and muscle symptoms numbered 111 in total, including 50 with Leigh syndrome, 11 with neurodegenerative disorders for which no clear cause could be identified, and 50 with so-called "mitochondrial encephalomyopathy." These 111 patients accounted for 40% of the total of 275 patients. Conversely, other forms accounted for two-thirds of cases, among which were 49 cases of lethal infantile mitochondrial disease (LIMD). Together with non-lethal infantile mitochondrial disease (NLIMD), which follows the same course but in which patients survive beyond 1 year of age, the number reached 71, and was by far the most common clinical diagnosis. LIMD encompasses hyperlactacidemia occurring in the neonatal period together with multiple organ failure. Most cases have poor outcomes, and it is thought that most of these patients died with the cause remaining unknown and no diagnosis established. Next were mitochondrial disorders showing single organ dysfunction only, such as mitochondrial hepatopathy (12%) and cardiomyopathy (7%).

4. Exome analysis of MRCD patients

As most mitochondrial diseases occur sporadically with only a few cases discovered in one family line, linkage analysis using a large pedigree cannot be applied, thus suggesting that we cannot use information on chromosomal localization for causal gene identification. When identifying disease-causing genes using bioinformatics analysis for exome data, knowledge of the inheritance patterns is very important [16]. As approximately 90% of MRCD-causing genes show a recessive mode of

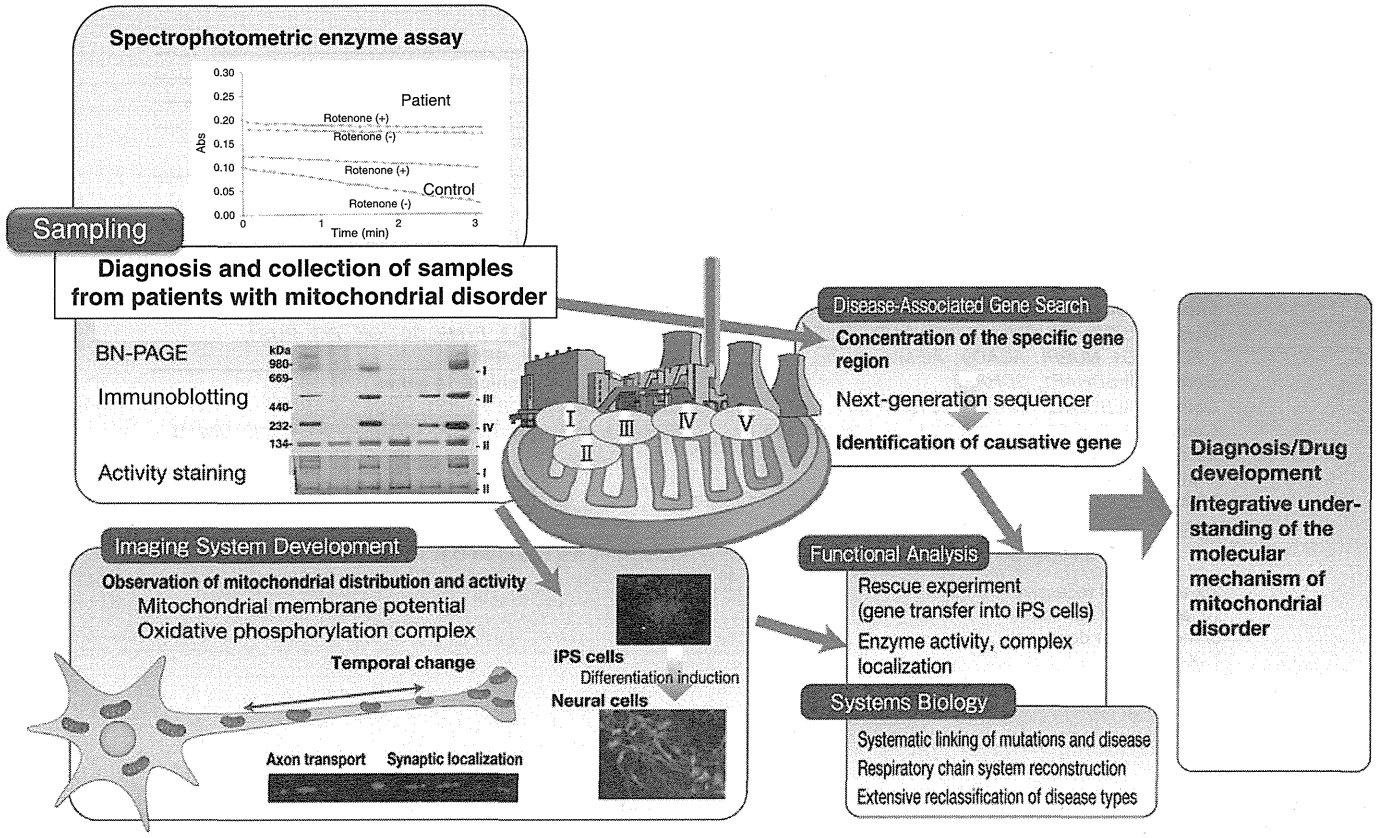

Fig. 1. Outline of exome analysis project for MRCD patients. The first step is 'Sampling', which refers to diagnosis and collection of samples from patients with mitochondrial disorders using both spectrophotometric enzyme assay [11] and BN-PAGE [13]. The next step is 'Disease-Associated Gene Search' using exome analysis. In 'Functional Analysis' and 'System Biology', candidate causal genes are confirmed at the cellular level by rescue experiment or other means. In 'Imaging System Development', induced pluripotent stem cells are created using fibroblasts and differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction. The final purpose of our project is integrative understanding of the molecular mechanisms of mitochondrial disorders.

inheritance (as shown in Table 1), we prioritized such genes as harboring rare variants in a homozygous or compound heterozygous fashion. Low priority is given to the analysis of genes showing mutation in only one allele because patients and healthy control individuals harbored a comparable number of rare heterozygous alleles; we were unable to prioritize dominant-acting genes.

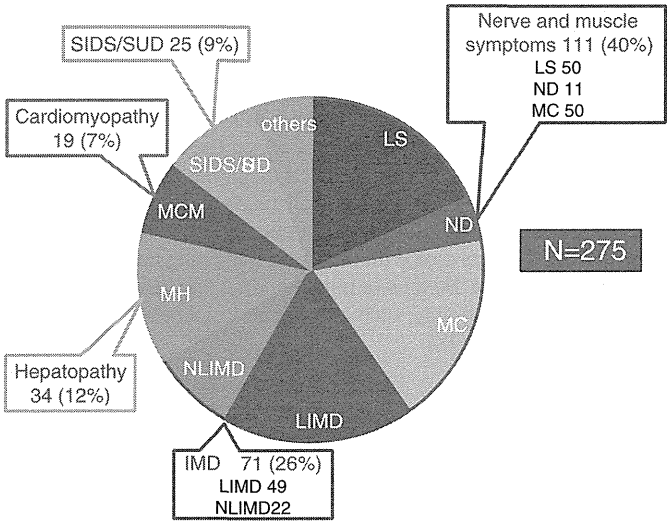

Fig. 2. Breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013. LS, Leigh syndrome; ND, neurodegenerative disorder; MC, mitochondrial cytopathy; IMD, infantile mitochondrial disease (lethal and non-lethal); MH, mitochondrial hepatopathy; MCM, mitochondrial cardiomyopathy; SIDS, sudden infant death syndrome; SUD, sudden unexpected death.

Our current bioinformatics analysis pipeline is as follows: read alignment was performed with a Burrows–Wheeler Aligner (BWA, version 0.7.0) [17] using the 1000 Genomes project phase II reference genome (hs37d5.fa). PCR duplicate reads were removed using Picard (version 1.89) (http://picard.sourceforge.net) and non-mappable reads were removed using SAMtools (version 0.1.19) [18]. After filtering out these reads, the Genome Analysis Toolkit (GATK) version 2.4-9-nightly-2013-04-12-g3fc5478 [19] was used to realign insertions and deletions, and for quality recalibration and variant calling (UnifiedGenotyper). Detected variants were annotated using ANNOVAR (version 2013Feb21) [20] and custom ruby scripts. The effect of the mutations on protein function was assessed by SIFT and GERP using dbNSFP [21]. The positions of mutations were based on RefSeq transcript sequences. Variants were assessed by comparing allele frequencies in the dbSNP135, Exome Sequencing Project (ESP5400) data set, and 1000 Genomes Projects (based on phase 1 release v3 called from 20101123 alignments). As mitochondrial disorders are rare, we excluded variants present in dbSNP with a frequency > 0.1%. After filtering out these variants, the VAAST program [22] was used to create a candidate gene list in each patient showing recessive characteristics.

As stated above, because mitochondrial disease patients have very high heterogeneity, the number of patients sharing the same gene mutation is quite low. Hence, attention should be directed towards removing these mutations from the disease candidates when the same amino acid substitutions are shared among multiple patients in our study, because these variants are highly likely to be SNPs unique to the Japanese population. Using these criteria, we are able to narrow down the number of variants to a mean of several genes for each patient. After listing

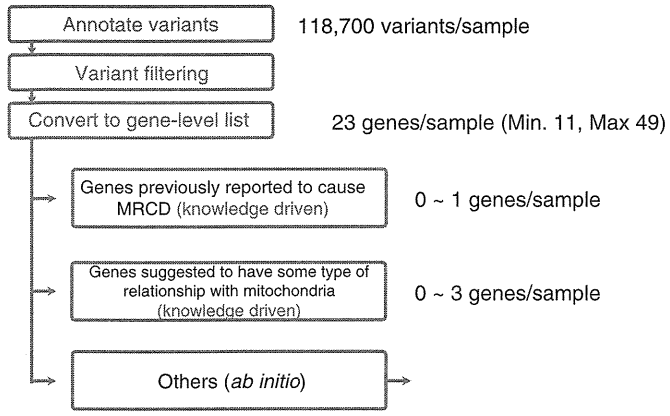

Fig. 3. Narrowing down of gene mutations discovered by exome analysis. After filtering out variants with the methods described in the 'Exome analysis of MRCD patients' section, genes were divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (*ab initio*).

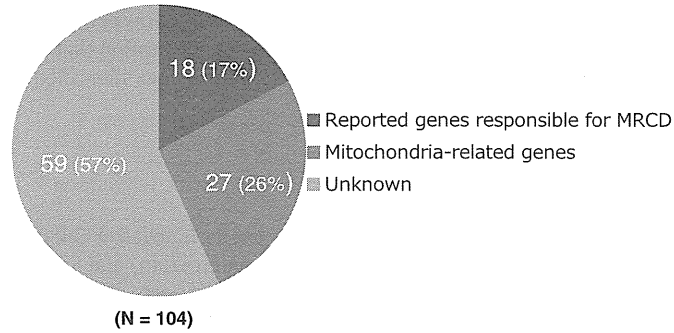

Fig. 4. Candidate genes with exome analysis for MRCD patients. Results of analysis for 104 patients to date (as of January 2013) are shown. Eighteen patients (17%) had variants previously reported to be disease-causing. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria. The remaining 59 patients (57%) are highly likely to contain completely novel disease-causing mutations for which no data have been obtained to date.

these candidate variants, we further investigated whether these variants are located within genes related to mitochondrial function. When genes overlapped with those reported to be related to mitochondrial function, we found that they were likely to be causative genes and were further subjected to experimental analysis such as haplotype phasing or functional assay including rescue experiments. To prepare a list of genes reported to be related to mitochondria, we included genes annotated as somehow related to mitochondria in the UniProt (http://www.uniprot.org/) [23] database, as well as the MitoCarta database (http://www.broadinstitute.org/pubs/MitoCarta/index.html) [24], which includes approximately 1000 gene products listed with the use of shotgun proteomics and mitochondrial localization analysis.

We also investigated whether there is Long Contiguous Stretch of Homozygosity (LCSH) using Affymetrix SNP arrays in a majority of patients. Although no cases of consanguineous marriage were reported in the interviews with the primary physician, about 5% of cases harbor LCSH proven by SNP arrays. When homozygous mutations are localized in these LCSH regions, the mutations are highly likely to be causative of disease.

5. Results of exome analysis for MRCD patients

The variants (mutations) found in the process of narrowing down the gene mutations discovered to date are shown in Fig. 3. These genes were narrowed down to the final candidate genes and divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (*ab initio*). The results of analysis of 104 patients to date (as of January 2013) are shown in Fig. 4. Eighteen patients (17%) had variants previously reported to be disease-causing. Among these 18 patients, one had a homozygote of a previously reported mutation and two had a compound heterozygote of a reported and a novel mutation (data not shown). All other mutations found in this study were new. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria, and it is likely that they are novel disease-causing genes in mitochondrial disorders. Table 2 lists the functions of the genes in these 27 cases. For the remaining 59 cases, each patient has about 20 gene variants that are unique to each patient, and it is necessary to confirm whether any of these mutations can actually cause the disease. These 59 patients are highly likely to contain completely novel disease-causing mutations for which no clues have been obtained to date. The biggest issue we currently face is how to confirm the disease-causing gene from these 20 gene variants for each patient.

6. Conclusion and future prospects

The above describes the progress we have made in exome analysis of neonatal or infantile MRCD patients. While we have identified many candidate genes, the causes of MRCD are extremely diverse and heterogeneous. Thus, in many cases, it is difficult to demonstrate conclusively that a mutation in a candidate gene is the true cause. We have performed analyses focusing on cases in which a biochemical diagnosis was established at the cellular level in addition to clinical symptoms such as enzyme activity and complex formation abnormalities. Nonetheless, confirmation of the causal genes with rescue experiments or other means is difficult. In the future, it will be necessary to increase the case number or search for patients with similar symptoms and similar gene mutations in collaboration with researchers throughout the world. We are currently conducting analyses of pediatric patients with a focus on MRCD, and gene mutations (amino acid substitutions) harbored by patients of the childhood onset type are probably variants conferring major damage on enzyme activity or protein function. Onset is also thought to occur in adulthood rather than in childhood in some cases of milder (hypomorphic: partial loss of function) variants with the same gene defect. As these are thought to include nerve diseases,

Table 2
Functions of new disease-causing candidate genes for MRCD.

MtoX#1	Non-receptor tyrosine kinase
MtoX#2	Acyl-CoA thioesterase
MtoX#3	Fatty acid β oxidation
MtoX#4	tRNA synthetase
MtoX#5	ABC transporter superfamily
MtoX#6	ATR-dependent AMP-binding enzyme family
MtoX#7	Heme biosynthesis
MtoX#8	AAA ATPase family
MtoX#9	Pre-mRNA splicing factor
MtoX#10	Creatine kinase
MtoX#11	Synaptic transmission
MtoX#12	Synthesis of Coenzyme Q
MtoX#13	Heme biosynthetic process
MtoX#14	Citrate synthase family.
MtoX#15	Cholesterol metabolism
MtoX#16	Mitochondrial fission
MtoX#17	Muscle organ development
MtoX#18	Cholesterol biosynthetic process
MtoX#19	Ribosomal protein
MtoX#20	Tumor suppressor
MtoX#21	A component of complex I
MtoX#22	A protease, located in inner membrane
MtoX#23	Regulation of PDH
MtoX#24	Mitochondrial translation
MtoX#25	Queuosine biosynthetic process
MtoX#26	Mitochondrial carrier family
MtoX#27	Methyltransferase superfamilya