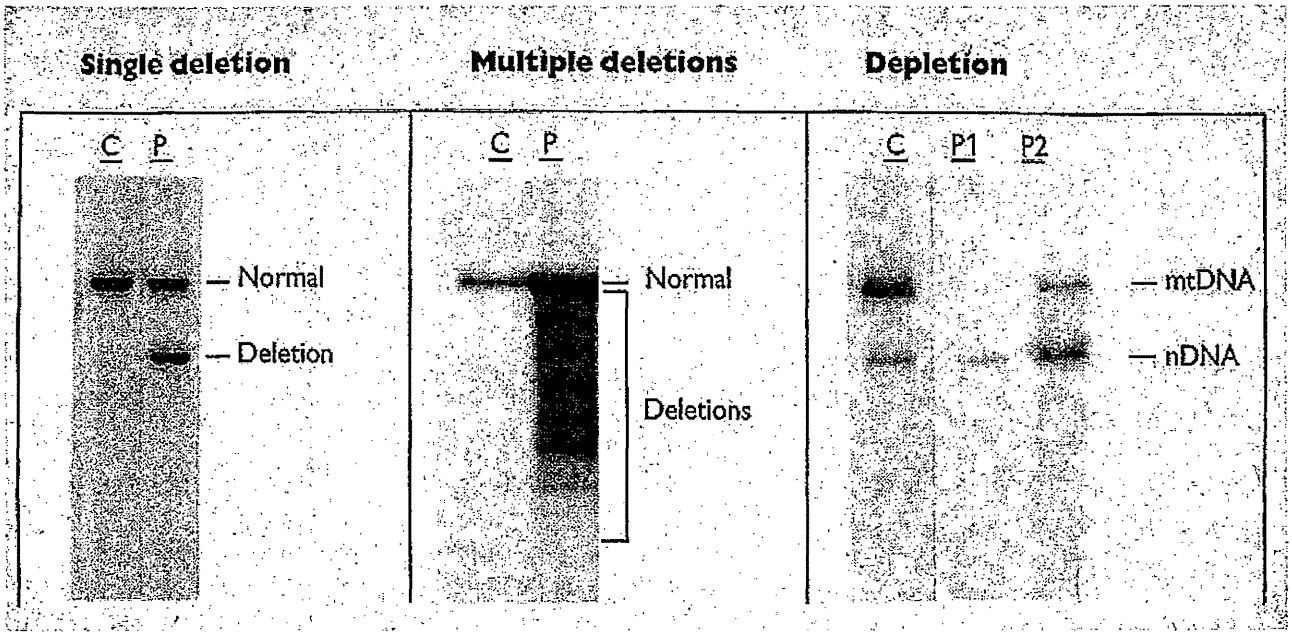

図2

ブとしては，精製ヒト全mtDNAもしくはmtDNA断片を用い遺伝子の欠失状態及び正常と欠失mtDNAの比率をデンシトメータで定量する．欠失が存在すれば，その部分をPCRで増幅し直接もしくはサブクローン後塩基配列を決定する．

治療法：これまで様々な治療法が試みられたが，有効な方法は知られていず，対症療法が主体となる．輸血，白血球輸注，血小板輸注等の補充療法は初期の骨髄抑制期に重要である．電子伝達系障害による代謝性アシドーシスには，重炭酸ナトリウムによるアルカリ療法がなされる．同時に，コエンザイムQ10，カルニチン（エルカルチン），カルジオクローム等の併用療法が試みられているがその有効性は不明である．

Ⅲ．乳児致死型ミトコンドリア病 Lethal Infantile Mitochondrial Disease：LIMD（McKusick No. 605711，No.614299，No.615330，No.613183など）

新生児期に重篤な代謝性アシドーシス，高乳酸血症で発症し，心不全，肝不全を来し，重篤な転機をとり，しばしば，敗血症，多臓器不全で死亡する重篤なミトコンドリア病の存在が知られている．その多くは，治療に抵抗性であり，生前に診断がつくことは稀である．敗血症など他の診断名で記載される事も多い．主に早期新生児期（生後2週間以内）に発症し乳児期（生後1年以内）に死亡するとされているが明確な基準は定まっていない．多くは，電子伝達系酵素の残存酵素活性が正常の5％以下と，酵素欠損が証明される．また，遺伝子異常では，電子伝達系酵素の構造遺伝子異常，アッセンブリー遺伝子異常，mtDNAの複製を司る遺伝子の異常（ミトコンドリアDNA枯渇症候群），*BOLA3*の異常など，多くの遺伝子異常が明らかにされてきた．ミトコンドリアDNA枯渇症候群では，核DNAに比較しmtDNAの含量が極端に減少している（図2）．疑ったら罹患臓器を用いて呼吸鎖complex酵素活性を測定するしかないが，治療法は無い．診断には，罹患臓器を用いた酵素活性が重要であり，酵素活性の組織特異性には注意を払う必要がある．我々も，生後1週間で重篤な代謝性アシドーシスを来し，拘束性肥大型心筋症で死亡したLIMD症例を経験した[3]．この症例は，症状経過から最重症型のミトコンドリア脳筋症を想定し，mtDNAの全周シーケンスを実施し，特異的変異を見出した．mtDNAの変異遺伝子の変異率はほぼ100％であり，これにより致死型の経過を取ったと考えられた．2011年に初めて報告された*BOLA3*異常症（McKusick No.613183）[4]は，常染色体劣性遺伝様式を示し，染色体の2p13.1に遺伝子座を有する．リポ酸を有する脱水素酵素の鉄硫黄クラスターのアッセンブリータンパクとしてミトコンドリア電子伝達系酵素複合体の1，2，3に共通する異常を来す．従って，電子伝達系酵素複合体の1，2，3の同時欠損を観た場合，本症を疑う事になる．また，電子伝達系酵素複合体の1，3，4，5の同時欠損を診た場合は，mtDNAの複製を司る遺伝子の異常（ミトコンドリア

DNA枯渇症候群) (McKusick No.609560, 188250, 251880, 601465, 203700, 613662, 174763, 612073, 603921, 256810, 137960, 271245, 606075, 612075, 612075, 604712, 245400, 611224, 221350, 610345, 615084, 615076, 615418, 103220 615471, 605654) やミトコンドリアtRNAの異常を疑う事になる. ミトコンドリアDNA枯渇症候群は, 遺伝的にも26種の遺伝子異常の報告があるほど多様であり, 単一の疾患群ではない. 生化学的酵素活性の検査パターンから, この疾患をまず疑う事が重要である.

Ⅳ. フロッピーインファント

生下時, あるいは乳児期早期より筋緊張が低下し, グニャグニャした感じのする小児は総称してフロッピーインファント (floppy infant) と呼ばれている. 筋緊張低下のために, 1) 奇妙なあるいは不自然な姿勢・肢位をとりやすく, 2) 受動運動に対する関節抵抗の減弱 (被動性の亢進), 3) 関節可動域の異常な拡大などの特徴を示す. 一般に新生児期の筋緊張の状態は, 在胎週数により異なる. 在胎28週の未熟児では, ほぼ全例が安静時に四肢を伸展しているが, 在胎32週までには四肢 (特に下肢) は屈曲する様になり, 満期産児では, すべての四肢は屈曲位をとっている. 従って, 筋緊張の低下した児をみた場合, 児の未熟性, 周産期の状態が筋緊張低下の原因と考えられない場合, フロッピーインファントとして, ミトコンドリア病の検索も必要となる. 特に, 周産期に原因が考えられない精神発達遅滞を合併したフロッピーインファントであれば, より強く小児期発症のミトコンドリア病を疑う.

フロッピーインファントで特徴的な主訴は, 筋力低下や易疲労性に起因する運動発達の遅れである. 病歴を聴取する場合, 前述の特徴的な症候に加えて, 妊娠中及び出生後の発育発達歴, 特に毒物などへの曝露歴も重要である. 先天的もしくは後天的に獲得された神経筋疾患の中枢神経系異常を来す周産期疾患は, 注意深く聴取する. 症状の発症時期, 程度, 仮死の状態, 生後の発達の状態は重要である. 発達では, 頸座り, 一人座り, 一人立ち, 一人歩きなどの時期は特に重要である. 筋に一次的原因のあるミオパチーでは, 近位筋優位に障害され, 末梢神経障害による筋萎縮症では, 遠位筋優位に障害される. しかし, 小児期においては生理的に筋の発達が十分でないため, 両者の明確な区別が出来にくい. 年長児では顔面筋罹患が特徴的な病気である先天性非進行性ミオパチー, 福山型先天性筋ジストロフィー, 筋強直性ジストロフィー, 顔面肩甲上腕型ジストロフィーも, 乳・幼児期には特徴的な顔貌とはならない. 重症筋無力症では, 眼瞼下垂とともに症状の日内変動がある. 家族歴としては, 家系図の中での罹患者の聴取は, 疾患の遺伝形式を推測する手がかりとなる. 少なくとも3世代の血縁者について全家族の性, 発症年齢, 流産, 死産, 血族結婚, 死因, 生存者の健康状態について詳細に調べる. 家系内男子のみの罹患であれば, X連鎖性劣性遺伝性疾患が, 両親が血族結婚であれば常染色体性劣性遺伝が, 両親のいずれかが同じ病気であれば常染色体性優性遺伝が考えられる.

明らかな周産期異常が無く, 精神発達遅滞およびけいれんを伴うようなフロッピーインファントであれば, まず小児期発症ミトコンドリア病を疑い, 乳酸, ピルビン酸などのバイオマーカーを提出する事が重要である.

Ⅴ. 低身長

ミトコンドリア病がエネルギー産生障害を伴うことから, エネルギー依存度の高い中枢神経系, 骨格筋, 心筋などの臓器障害を来す事が多いが, 間脳-下垂体系の調節障害の結果起こる成長ホルモン分泌不全性の低身長も, 本症の重要な臨床症状である. 我々は, 2001年から2006年にかけて, 日本国内で (MELAS mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) の実態を明確にする目的でコホート調査を実施した[5]. その際, 低身長の頻度・および程度について非常に頻度が高いことを報告した. また, 臨床・遺伝学的に確定診断した自験例39例のミトコンドリア病で, 成長障害 (低身長) についても検討した. なお, 低身長の定義は−2.0SD以下とした. 成長障害を認めたMELAS自験例8例について, 成長ホルモン分泌負荷を行い, 成長ホルモン分泌不全性低身長と診断した. 全例に成長ホルモン補充療法を開始し, その後の伸長率, 体脂肪率, 筋力, 重症度評価につき検討した.

本邦でのミトコンドリア脳筋症で最も頻度の高い病型であるMELAS[6]では, その80%にミコンドリアDNAのtRNALeu (UUR) 遺伝子のA3243G変異を認めた[7]. この遺伝子異常は, 日本人糖尿病の約1.6%にみられ, 無症候性キャリアーを考えると, ヒトで最も多い遺伝子異常と考えられる[8]. 一次スクリーニングで判明した96名のMELAS患者では, 5年間の追跡調査を行った. その結果, MELASはその発症年齢で小

	全症例	小児型	成人型
コホート調査	96	58	38
低身長	53 (55.2%)	37 (64%)	16 (43.2%)
SDスコア	−2.9	−2.9	−2.8
文献調査	130	59	71
低身長	22 (16.9%)	12 (20.3%)	10 (14.1%)

18 cases (13.8%) published in 14 Pediatrics
73 cases (56.2%) published in 63 Internal Medicine
39 cases (30%) in 33 publications (Pathology)

図3

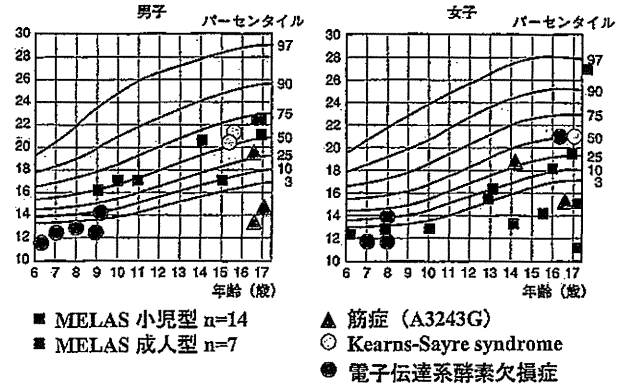

図4

児型（18歳未満）と成人型（18歳以上）に分類することができた．その中で全体の53人（55.2%）に低身長がみられ，その平均身長は−2.9SDであった（図3）．この調査に先立って，PubMedで1991年から2006年までで検索し，閲覧できるMELAS130症例を検討したところ，コホートデータと異なり低身長の頻度が16.9%と低いことがわかった．小児科医にとって，低身長は日常診療においても常に念頭におかなければならない重要な症状としてとらえることができるが，成人を中心とした神経内科医にとって，低身長を重要な症状ととらえることは難しいと思われ，実際の低身長の頻度は，さらに高いものと推測された．MELASに合併する低身長の成因として，1）下垂体性成長ホルモン分泌不全，2）血管内皮機能不全による視床下部-下垂体への脳虚血，3）ミトコンドリア異常によるホルモン産生細胞・分泌細胞の機能障害，4）GH分泌刺激を低下させる要因（低アルギニン血症，高血糖）などが考えられるが，明確な原因は不明である．当科フォロー中のMELAS患者で，8名に成長ホルモン分泌不全性低身長（GHD）を認め，成長ホルモン（GH）補充を行い，すべての症例で身長増加を認めた．しかし，耐糖能異常を来たした1例で，GH補充を中止した．また，伸長以外の効果として，筋力増強に有効に働くと考えられるが，耐糖能異常を来す可能性も併せて，MELAS患者へのGH補充に関しては今後も検討していく必要がある．

一方，小児型MELASでは，低身長の合併が64%に診られ，−2.9SDと著明な低身長を示した（図3）．小児における低身長の鑑別に，いままで指摘されていないミトコンドリア遺伝子異常のキャリアーも重要である．特に，家系に母系遺伝の低身長，DM＋難聴（MIDD: maternal inherited diabetes mellitus with deafness），片頭痛などがみられるときは，MELAS型の遺伝子異常を有する個体が含まれている可能性が高く，鑑別疾患の優先度は高くなると思われる．ミトコンドリア病における診断時のBMIを図4に示す．小児期発症のミトコンドリア病では，低身長のみでなく，やせも重要なサインであることが示される．本症に診られる低身長の治療介入には，耐糖能異常の問題もあり，成人成長ホルモン分泌不全症の治療も視野に入れた検討が必要である．

Ⅵ. 小児型MELAS

MELASは，40歳以前に，頭痛，嘔吐，痙攣，視野異常，四肢の運動麻痺，意識障害などで発症する脳卒中様発作を特徴とする．急性期の頭部画像では，脳卒中と類似した異常所見を呈するが，主な脳動脈の血管支配領域に一致せず，また，異常領域が時間的・空間的に発現・消失を繰り返す．病初期は，脳卒中様発作に伴う上記症状も可逆的であるが，発作を繰り返すうちに，明らかな後遺症として残り，最終的には梗塞様領域の脳は萎縮する．合併症に，片頭痛，易疲労性，筋力低下，るい痩，感音性難聴，外斜視，眼瞼下垂，神経症，肥大型心筋症，WPW症候群などの心伝導異常，DeToni Fanconi症候群，糖尿病，低身長，甲状腺機能低下症などの多内分泌疾患を伴う事も多い．最終的には脳血管性認知症類似の経過で寝たきりもしくは多臓器不全で死亡する．日本のMELASコホート研究では，発症年齢を確認した場合，小児期発症と成人期発症の2峰性分布を示していた（図5）．その結果，脳卒中様発作の発症時期を18歳以前と以後で重症度，症状・予後の違いがあるかを明確にする目的で，層別解析を行った．その結果，発症から死亡までの平均期間は，小児型で6年，成人型で10年，平均死亡年齢は，小児型で15歳2か月，成人型で40歳，平均変異率は小児型で75%，成人型で55%と，症状，重症度，進行度，変異率ともに小児期発症で有意に重症であった[5]．以

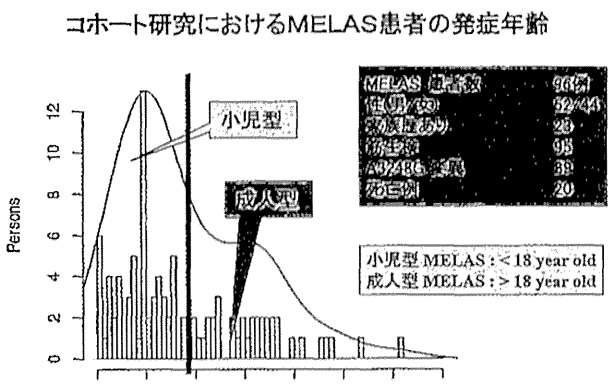

図5

上より，小児型MELASと成人型MELASでは，種々の特徴が異なっている事が明らかとなり，小児型MELASが有意に重症であった．生存曲線では，小児期発症MELASが成人型より3.2倍死亡率が高いことが示された[5]．

Ⅶ. Leigh脳症

幼少期（多くは2歳未満）から発症する精神運動発達遅滞，退行，食事摂取障害，痙攣，呼吸の異常，眼運動異常などを特徴とし，心，筋，腎，肝など多臓器の症状を示す重症型である．神経細胞の脱落，グリア増生を含む壊死・軟化病変があり，大脳基底核を中心に両側対称性に存在する．ミトコンドリアDNA異常では，T8993C/G変異や，すでに他の病型で報告された点変異でも，それが高度に蓄積した場合には本症を発症する．核DNAの異常では，電子伝達系酵素タンパクの核サブユニット，分子集合に影響を与えるassembly遺伝子の異常などの報告がある．Leigh脳症の多くは小児期に死亡する重症な疾患である．

Ⅷ. 終わりに

ミトコンドリア脳筋症の症状は，小児期と成人期では，発症様式，臨床症状，経過，予後などで大きく異なり，一見全く異なる病気のようにも映る．診断に際してもっとも重要な点は，まず，ミトコンドリア病を疑うことに尽きる．“any symptom in any organ at any age”を忘れない事である．

利益相反：(無)・有

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GDF15 is a novel biomarker to evaluate efficacy of pyruvate therapy for mitochondrial diseases

Yasunori Fujita [a], Masafumi Ito [a], Toshio Kojima [b], Shuichi Yatsuga [c], Yasutoshi Koga [c], Masashi Tanaka [d,*]

[a] Research Team for Mechanism of Aging, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi, Tokyo 173-0015, Japan
[b] Health Support Center, Toyohashi University of Technology, 1-1 Hibarigaoka Tenpaku-cho, Toyohashi, Aichi 441-8580, Japan
[c] Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan
[d] Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi, Tokyo 173-0015, Japan

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ABSTRACT

Pyruvate therapy is a promising approach for the treatment of mitochondrial diseases. To identify novel biomarkers for diagnosis and to evaluate therapeutic efficacy, we performed microarray analysis of 2SD cybrid cells harboring a MELAS-causing mutation and control cells treated with either lactate or pyruvate. We found that expression and secretion of growth differentiation factor 15 (GDF15) were increased in 2SD cells treated with lactate and that serum GDF15 levels were significantly higher in patients with mitochondrial diseases than in those with other diseases, suggesting that GDF15 could be a useful marker for diagnosis and evaluating the therapeutic efficacy of pyruvate.

1. Introduction

Mitochondrial diseases are caused by mitochondrial or nuclear genome mutations that affect the functions of mitochondria. The symptoms are caused by impaired energy metabolism due to mitochondrial dysfunction and manifest mostly in tissues with a high energy demand such as brain, heart, and muscle. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is one of the most common of the mitochondrial diseases (Pavlakis et al., 1984). The A-to-G transition at the 3243 position of the mitochondrial DNA (m.3243A > G) located in the mitochondrial $tRNA^{Leu\ (UUR)}$ gene is a MELAS-causing mutation, and it is detected in approximately 80% of patients with MELAS (Goto et al., 1990, 1992; Kirino et al., 2004; Yasukawa et al., 2000).

These pathogenic mutations typically result in defective ATP synthesis in mitochondria, and therefore ATP production depends on the glycolytic pathway. Since lactate production is aberrantly increased by the acceleration of glycolysis when energy demand is elevated, the lactate to pyruvate (L/P) ratio in serum is often increased in patients with mitochondrial diseases and has been clinically used for estimating the dysfunction of mitochondrial respiration. It is well known that the L/P ratio reflects the intracellular NADH/NAD^+ ratio. Since NAD^+ is indispensable for oxidation of glyceraldehyde 3-phosphate (GAP) to 1,3-bisphosphoglycerate (BPG) by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the glycolytic pathway, a shortage of NAD^+ interrupts this reaction, resulting in decreased ATP biosynthesis. Tanaka et al. (2007) proposed that the addition of pyruvate would facilitate oxidation of NADH to NAD^+ via the lactate dehydrogenase reaction, which would restore ATP production by the glycolytic pathway even under defective respiratory conditions. Indeed, positive effects of sodium pyruvate on clinical manifestations of mitochondrial diseases have been reported (Koga et al., 2012; Saito et al., 2012). However, useful biomarkers for evaluating the therapeutic efficacy of pyruvate remain to be developed.

Cybrid cell lines established by the fusion of enucleated myoblast cells from a patient with a cultured cell line depleted of mtDNA have been used to elucidate the pathogenesis and underlying molecular mechanisms of mitochondrial diseases. We previously reported increased expression of amino acid starvation-responsive genes in cybrid cells with MELAS and NARP (neuropathy, ataxia, and retinitis pigmentosa) mutations (Fujita et al., 2007). In our earlier study (Kami et al., 2012), we found that exposure to excessive sodium lactate significantly increases the intracellular L/P and NADH/NAD^+ ratios in cybrid cells harboring the MELAS mutation (m.3243A > G), which implies worsening of lactic acidosis and NAD^+ shortage. On the other hand, we found that treatment with sodium pyruvate facilitates the ATP production and improves the energy status, as indicated by a decrease in the L/P ratio and retention of the NADH/NAD^+ ratio. Taken together, we considered that these experimental conditions would be ideal for identifying biomarker candidate genes, whose expression levels reflect

* Corresponding author. Tel.: +81 3 3964 3241; fax: +81 3 3579 4776.
E-mail address: mtanaka@tmig.or.jp (M. Tanaka).

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the intracellular energy deficiency and the effect of pyruvate on energy metabolism.

In the present study, we performed a global gene expression analysis of cybrid cells with the MELAS mutation (m.3243A > G: 2SD cells) and control cybrid cells (2SA cells) treated or not with lactate or pyruvate. We identified several biomarker candidate genes, among which we focused on growth differentiation factor 15 (GDF15). The level of GDF15 in the conditioned medium was significantly higher in 2SD cells than in 2SA cells, which level was further increased by lactate but was not affected by pyruvate in 2SD cells. We also demonstrated that the concentration of GDF15 in the serum was markedly elevated in patients with mitochondrial diseases compared with that in those with other pediatric diseases. Thus, we identified GDF15 as a novel serum marker for the diagnosis of mitochondrial diseases and possibly for monitoring the disease status and progression and for evaluating the therapeutic efficacy of pyruvate.

2. Materials and methods

2.1. Cell culture

The 2SA and 2SD cybrid cell lines were previously established by Chomyn et al. (1992). Briefly, 14 cybrid clones were isolated after the fusion of enucleated myoblasts derived from a MELAS patient with mtDNA-deficient $\rho^0$206 cells generated from a human 143B osteosarcoma cell line. Among those clones, 10 clones had homoplasmic wild-type mtDNA, and 4 clones harbored strongly predominant mutant mtDNA. For our experiments, we chose two clones, 2SA and 2SD cybrid cell lines carrying 100% wild-type mtDNA and 94% m.3243A > G mutant mtDNA, respectively. The 2SD but not 2SA cybrid cells were shown to be defective in mitochondrial protein synthesis and respiratory capacity (Chomyn et al., 1992). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.4 mM uridine at 37 °C under a humidified atmosphere of 5% CO_2.

2.2. Microarray analysis

Total RNA was isolated from cells by using a miRNeasy mini kit (Qiagen, Venlo, Netherlands). One hundred nanograms of total RNA was labeled and amplified with a low input quick amp labeling kit (Agilent Technologies, Santa Clara, CA, USA) used according to the manufacturer's instructions. The labeled cRNA was hybridized to the Agilent SurePrint G3 Human GE 8x60K Microarray in a rotating hybridization oven at 10 rpm for 20 h at 65 °C. After hybridization, the microarrays were washed according to the manufacturer's instructions and scanned on an Agilent DNA Microarray Scanner with Scan Control software. The resulting images were processed, and raw data were collected by using Agilent Feature Extraction software. Expression data were analyzed by using GeneSpring GX 11 (Agilent Technologies). The signal intensity of each probe was normalized by a percentile shift, in which each value was divided by the 75th percentile of all values in its array. For pairwise comparison analysis, only the probes that had expression flags present under at least one condition were considered. The list was analyzed with Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood, CA, USA)

2.3. Quantitative RT-PCR

Total RNA was reverse transcribed to cDNA with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) used according to the manufacturer's protocols. Real-time PCR was performed on the StepOnePlus Real-Time PCR System (Life Technologies) using Power SYBR Green PCR Master Mix. 18S rRNA gene was used as an internal control for normalization. The sequences of primers are listed in Supplementary Table 1.

2.4. Patients

A written informed consent was obtained from all patients or their legal guardians. Enrolled patients were diagnosed with mitochondrial diseases by medical doctors in Kurume University Hospital over the period of 2005–2013. Seventeen patients diagnosed at this hospital as having mitochondrial diseases were recruited for this study. As a control group, 13 patients diagnosed as having other pediatric diseases such as dwarfism were also recruited. The clinical information of the patients is listed in Supplementary Table 2. This study was approved by the Institutional Review Board (Kurume University #13099).

2.5. ELISA and multiplex suspension array

Cells were placed on 60-mm dishes 1 day before replacing the medium with fresh medium. Conditioned medium cultured for 24 h was collected, and the particulates were removed by centrifugation (at 500 ×*g* for 10 min, at 10,000 ×*g* for 30 min). The GDF15 and INHBE concentrations in the supernatants and in the sera of patients were determined in duplicate by using a Human GDF-15 Immunoassay (R&D Systems, Minneapolis, MN, USA) and enzyme-linked immunosorbent assay kit for Inhibin Beta E (Uscn Life Science, Wuhan, Hubei, PRC) according to the manufacturer's instructions. For measuring other cytokine concentrations, the sera were subjected to a multiplex suspension array, Bio-Plex Pro Human Cytokine Grp II Panel 21-Plex (Bio-Rad, Hercules, CA, USA). The cytokines measured by use of this array were the following: IL-1α, IL-2Rα, IL-3, IL-12 (p40), IL-16, IL-18, CTACK, GRO-α, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, MIG, β-NGF, SCF, SCGF-β, SDF-1α, TNF-β, and TRAIL. We measured the FGF21 (BioVendor, Czech Republic) concentration in duplicate samples by ELISA. Unmeasurable high-concentration samples of FGF21 and GDF15 were diluted 10-fold prior to measurement. The value from each assay was determined by reference to the linear portion of the standard curves for FGF21 and GDF15. All assays were performed by a trained scientist or technical staff.

2.6. Statistical analysis

Statistical analyses were performed by using IBM SPSS statistics (IBM, Armonk, NY, USA). We used the nonparametric Mann–Whitney *U* test to validate differences in cytokine levels in serum between mitochondrial disease patients and controls. The correlation between GDF15 and FGF21 concentrations in serum was assessed by Spearman correlation analysis. We plotted the receiver operating characteristics (ROC) curve for GDF15, HGF, SCF, SCGF-β, and FGF21 and calculated the area under the curve (AUC). The data for the sensitivity and 100 minus the specificity were plotted on a continuous scale.

3. Results

3.1. Gene expression changes in response to intracellular energy deficiency in 2SD cells

We performed microarray analysis of 2SD cybrid cells harboring the MELAS mutation (m.3243A > G) and 2SA control cybrid cells treated with 10 mM lactate or 10 mM pyruvate for 0, 4 or 8 h (Fig. 1A). The numbers of gene probes whose signal intensities were altered by 2-fold for each comparison are given in Supplementary Tables 3–6. We found remarkable changes in gene expression in 2SD cells, but not in 2SA cells, treated with lactate for 8 h. As shown in Supplementary Fig. 1A, we then selected gene probes that were increased by lactate treatment for 8 h compared with those without treatment and concurrently up-regulated by lactate but not by pyruvate at 8 h after treatment and thereby identified 313 probes that were specifically up-regulated by lactate in 2SD cells at 8 h

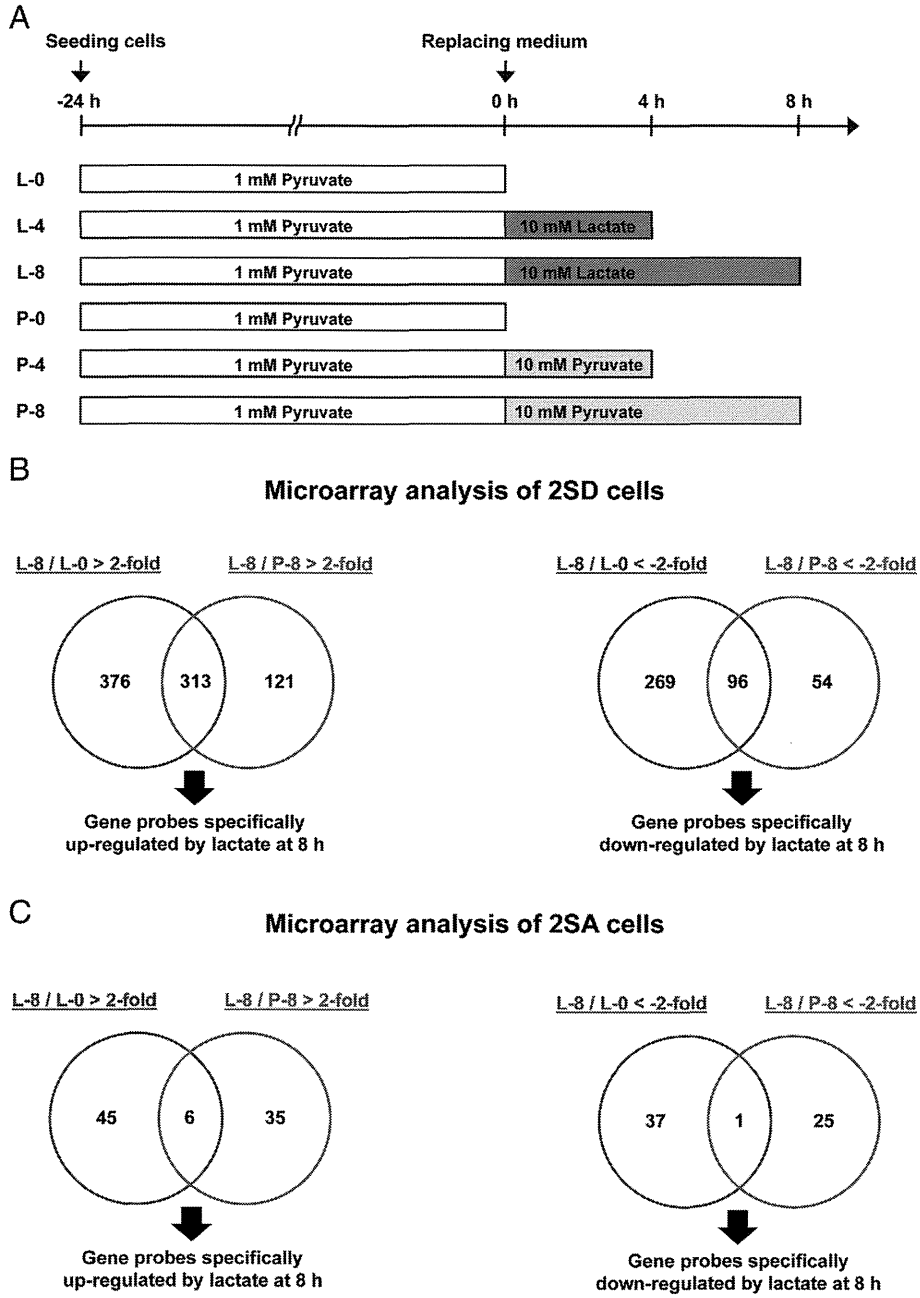

Fig. 1. Microarray analysis of 2SD and 2SA cells (A) Diagram of treatment protocols. Total RNA isolated from 2SD and 2SA cells treated with 10 mM lactate or 10 mM pyruvate for 0, 4, or 8 h were subjected to microarray analysis ($n = 2$). (B, C) Venn diagrams show the number of probes for genes in 2SD cells (B) or 2SA cells (C) that were increased (left panels) or decreased (right panels) in expression by lactate treatment for 8 h compared with their expression at 0 h and concurrently up-regulated by lactate but not by pyruvate after 8-h treatment. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

(Fig. 1B). Using similar criteria (Supplementary Fig. 1B), we also identified 96 probes that were specifically down-regulated in 2SD cells by lactate treatment for 8 h (Fig. 1B). In 2SA cells, having normal mitochondrial function, the numbers of gene probes that responded to lactate treatment were limited (Fig. 1C). The clustering analysis of the 313 up-regulated (corresponding to 231 genes) and 96 down-regulated (corresponding to 75 genes) gene probes highlighted significant differences in gene expression patterns between 2SD and 2SA cells and also between lactate and pyruvate treatments (Fig. 2). These results suggest that a defective energy metabolism caused by exposure to a high dose of lactate resulted in significant changes in gene expression in 2SD cells.

3.2. Gene networks associated with intracellular energy deficiency in 2SD cells

In order to identify gene networks associated with a defective energy metabolism in the lactate-treated 2SD cells, a gene network analysis was performed on 231 up-regulated genes and 75 down-regulated ones. This analysis identified 11 and 5 gene networks for up- and down-regulated genes, respectively (Fig. 3 and Supplementary Figs. 2 and 3). The top-ranked gene network identified for the up-regulated genes contained those related to the amino-acid starvation response, such as ASNS, ATF3, NUPR1, DDIT3, CTH, TRIB3, STC2, and PCK2 (Fig. 3A). It is worth noting that GDF15, on which we focused in the

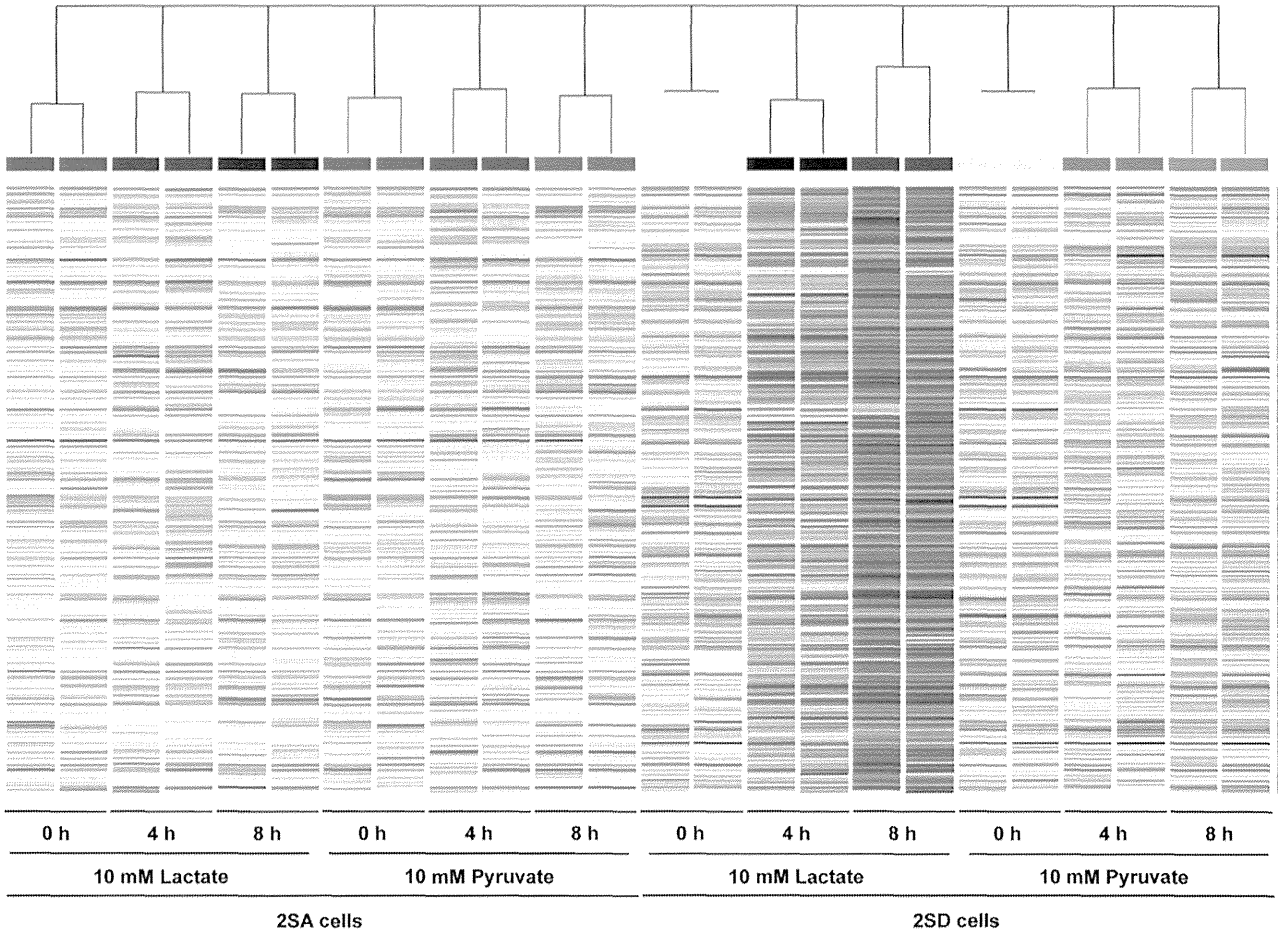

Fig. 2. Clustering analysis of the microarray data The gene probes up-regulated ($n = 313$) and down-regulated ($n = 96$) at 8 h after lactate treatment were subjected to clustering analysis. Part of the data are shown. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

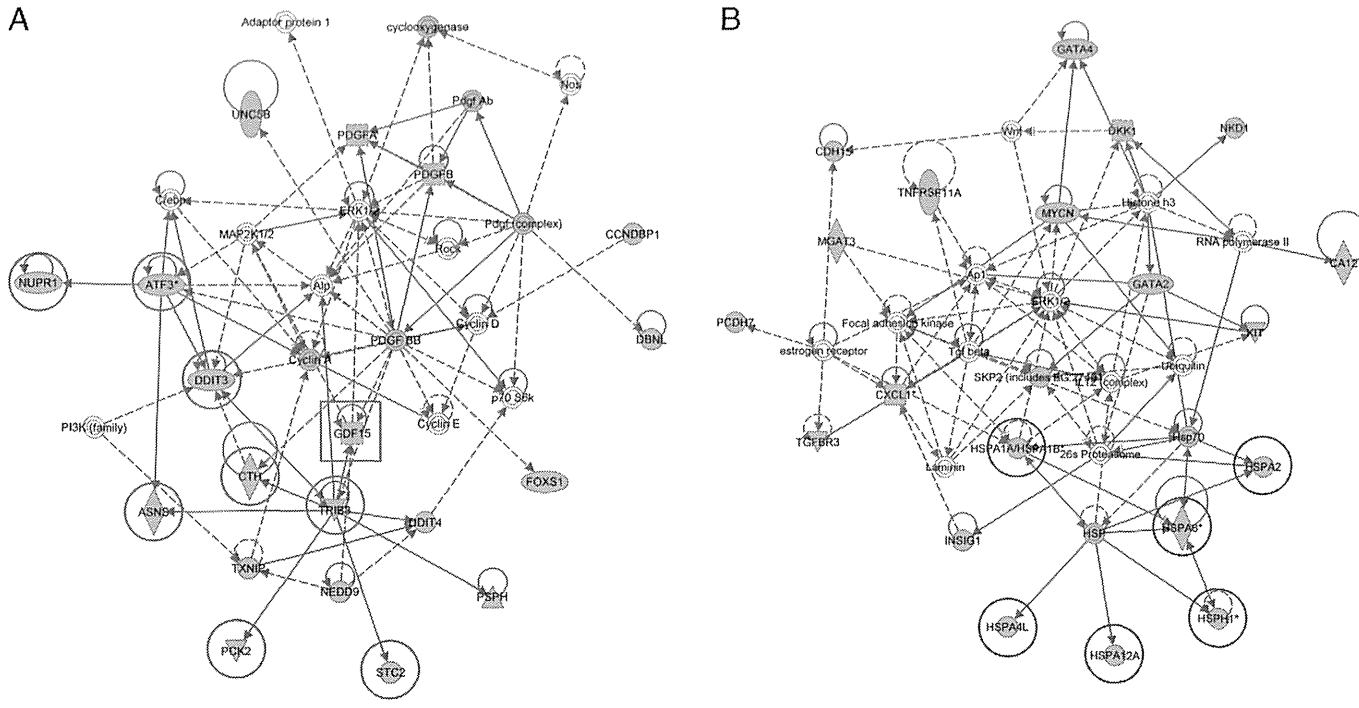

Fig. 3. Gene network analysis of the microarray data The genes specifically up-regulated ($n = 231$) and down-regulated ($n = 75$) at 8 h after lactate treatment were subjected to gene network analysis. The top-ranked gene networks in terms of the number of genes included are shown for up-regulated (A) and down-regulated (B) genes. Genes involved in the amino-acid starvation response (red circles) and heat-shock response (blue circles) as well as GDF15 (red square) are denoted. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

present study, was included in this network. On the other hand, the gene network for down-regulated genes included those linked to the heat-shock protein response, such as HSPA1A, HSPA2, HSPA4L, HSPA8, HSPA12A, and HSPH1 (Fig. 3B).

3.3. GDF15 as a potential biomarker for diagnosis and evaluating the therapeutic efficacy of pyruvate

Proteins encoded by genes related to intracellular energy deficiency in 2SD cells and secreted into the medium could be potential biomarkers for mitochondrial diseases. Gene annotation analysis revealed the location of gene products that were specifically up- and down-regulated by lactate at 8 h (231 and 75 genes, respectively) (Table 1). Twenty-three up-regulated genes and 4 down-regulated genes were annotated to the extracellular space, each of which is listed in Tables 2 and 3. Among them, we focused on the top 2 ranked up-regulated genes, growth differentiation factor 15 (GDF15) and inhibin beta E (INHBE).

To validate the intracellular expression levels of these genes, we performed quantitative RT-PCR for GDF15 and INHBE. The expression levels of GDF15 (Fig. 4A) and INHBE (Fig. 4B) in the 2SD cells were increased by treatment with 10 mM lactate, but not with 10 mM pyruvate, for 4 or 8 h. Furthermore, GDF15 expression at 0 h was higher in 2SD cells than in 2SA cells. These results confirmed the reproducibility of our microarray data and identified GDF15 and INHBE as candidate biomarkers. To determine whether the secretion of GDF15 and INHBE proteins was increased in 2SD cells in response to lactate treatment, we measured their concentrations in medium from 2SA and 2SD cells cultured for 24 h in the presence of 1 mM pyruvate, 10 mM lactate, or 10 mM pyruvate. ELISA showed that the GDF15 levels were higher in the conditioned medium of 2SD cells than in that of 2SA cells under all of the culture conditions (Fig. 4C). Moreover, treatment with 10 mM lactate, but not with 10 mM pyruvate, promoted secretion of GDF15 in 2SD cells in comparison with treatment with 1 mM pyruvate, whereas 2SA cells did not respond to the high dose of lactate and pyruvate treatment. In contrast, INHBE protein was not detectable by ELISA in the conditioned medium of either 2SD or 2SA cells under any culture conditions (data not shown). These results indicate that GDF15 could be a potential biomarker for diagnosis and monitoring the disease status and progression as well as for assessing the therapeutic efficacy of pyruvate for the treatment of mitochondrial diseases.

3.4. GDF15 as a biomarker for diagnosis of mitochondrial diseases

In order to validate the feasibility of GDF15 as a serum biomarker, we measured its concentration in the serum of 17 patients with mitochondrial diseases as well as in that of 13 patients with other pediatric diseases as a control (Supplementary Table 2). ELISA showed that the average concentration of GDF15 in the serum of mitochondrial disease patients was 2632.9 pg/mL, whereas that for other pediatric disease patients was 285.2 pg/mL, suggesting that GDF15 levels were significantly increased in the serum of mitochondrial disease patients and could clearly distinguish mitochondrial disease patients from control patients (Fig. 5A).

Table 1
The location of probes (genes) up- and down-regulated in 2SD cells with lactate treatment for 8 h.

Location	Up-regulated		Down-regulated	
	Probe number	Gene number	Probe number	Gene number
Nucleus	39	35	14	14
Cytoplasm	51	47	25	19
Plasma membrane	37	33	16	16
Extracellular space	26	23	5	4
Unknown	160	93	36	22

Since fibroblast growth factor 21 (FGF21) was recently proposed as a diagnostic marker for mitochondrial diseases (Davis et al., 2013; Suomalainen et al., 2011), we also measured the FGF21 levels in the serum of the same mitochondrial disease patients and control patients (Fig. 5B). The serum FGF21 levels were higher in patients with mitochondrial diseases than in those with other diseases. Furthermore, there was a good correlation between the serum GDF15 and FGF21 levels (Fig. 5C).

In an attempt to find additional biomarkers, we determined the serum levels of 21 cytokines in the same patients by using the multiplex suspension array. As shown in Supplementary Fig. 4A, the serum concentrations of HGF and SCF were higher in patients with mitochondrial diseases than in control patients, whereas the serum levels of SCGF-β were lower in the former than in the latter.

Finally, we performed ROC curve analysis of GDF15, HGF, SCF, SCGF-β, and FGF21. As shown in Fig. 5D, the area under the curves (AUC) for GDF15 (0.986) was higher than that for FGF21 (0.787). The AUC for FGF21 was similar to those for HGF (0.747), SCF (0.729), and SCGF-β (0.837) (Supplementary Fig. 4B), indicating that GDF15 had the maximum sensitivity and specificity for diagnosis of mitochondrial diseases. These results suggest that GDF15 has the greatest potential as a novel diagnostic marker for MELAS and other mitochondrial diseases.

4. Discussion

Based on the global gene expression analysis of cybrid cells with mitochondrial dysfunction, we identified GDF15 as a potential biomarker whose expression and secretion reflected the intracellular energy deficiency and the effect of pyruvate therapy on the energy metabolism. We then determined the serum levels of GDF15 in patients with mitochondrial diseases and other diseases and identified GDF15 as a novel diagnostic marker for mitochondrial diseases. Although additional clinical studies are needed, the serum GDF15 concentration may be a useful biomarker not only for diagnosis of mitochondrial diseases but also for monitoring the disease status and progression as well as for determining the efficacy of pyruvate therapy.

GDF15 is a member of the transforming growth factor-β (TGF-β) superfamily and is widely expressed in mammalian tissues (Unsicker et al., 2013). GDF15 plays important roles in multiple pathologies including cardiovascular diseases, cancer, and inflammation. It has been shown that GDF15 is up-regulated by tumor suppressor p53 in response to high glucose or treatment with anti-cancer compounds (Baek et al., 2002; Li et al., 2013; Yang et al., 2003). The p53 protein is a transcription factor that responds to a variety of stresses such as DNA damage, oxidative stress, hypoxia, and metabolic stress, and it activates the expression of genes to induce cell cycle arrest, DNA repair, senescence, and cell death (Sermeus and Michiels, 2011; Sperka et al., 2012; Zhang et al., 2010). CDKN1A (p21), a potent cyclin-dependent kinase inhibitor, is a major downstream effector of p53, which induces cell-cycle arrest (Sperka et al., 2012). In our microarray data, the CDKN1A expression level was 3.5-fold increased by lactate treatment of 2SD cells (data not shown). Previous reports demonstrated increased expression of CDKN1A in the skeletal muscle of patients with mitochondrial diseases and a cell line depleted of mitochondrial DNA (Behan et al., 2005; Crimi et al., 2005). Besides CDKN1A, we found other p53 effector genes in the list of genes up-regulated in the lactate-treated 2SD cells, including GADD45A, EGR2, DDIT3, CHMP4C, SESN2, ULBP1, DDIT4, and NUPR1 (data not shown). These results suggest that p53 activation may have played an important role in the induction of GDF15 expression in 2SD cells treated with lactate. It has been also demonstrated that p53 activation caused by metabolic stress is mediated by AMP-activated protein kinase (AMPK; Zhang et al., 2010). Our previous metabolomic profiling revealed that the ATP level drops but that the ADP and AMP levels are increased in lactate-treated 2SD cells (Kami et al., 2012), implying that elevation of the AMP/ATP ratio may activate p53 through AMPK activation. Taken together, it is possible that p53 induced GDF15 expression in

Table 2
Genes annotated to the extracellular space among those specifically up-regulated by lactate treatment for 8 h.

Gene symbol	Accession number	Entrez gene name	Fold change	
			L-8/L-0[a]	L-8/P-8[b]
GDF15	NM_004864	Growth differentiation factor 15	27.4	14.8
INHBE	NM_031479	Inhibin, beta E	15.0	9.4
AREG	NM_001657	Amphiregulin	14.0	2.2
ECM2	NM_001393	Extracellular matrix protein 2, female organ and adipocyte specific	11.8	9.0
ADM2	NM_024866	Adrenomedullin 2	10.3	3.0
MMP3	NM_002422	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	9.8	4.2
IL1A	NM_000575	Interleukin 1, alpha	7.6	6.0
C12orf39	ENST00000256969	Chromosome 12 open reading frame 39	6.3	6.7
APOL6	NM_030641	Apolipoprotein L, 6	6.2	3.8
SCG5	NM_003020	Secretogranin V (7B2 protein)	5.2	3.0
SPOCK2	NM_014767	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	5.1	6.6
AMTN	NM_212557	Amelotin	5.0	3.9
IL23A	NM_016584	Interleukin 23, alpha subunit p19	4.4	2.8
ADAMTS17	NM_139057	ADAM metallopeptidase with thrombospondin type 1 motif, 17	3.5	2.2
VEGFA	NM_001025370	Vascular endothelial growth factor A	3.4	2.5
STC2	NM_003714	Stanniocalcin 2	3.4	2.6
PDGFB	NM_002608	Platelet-derived growth factor beta polypeptide	2.8	3.8
C1QTNF1	NM_198594	C1q and tumor necrosis factor related protein 1	2.6	2.9
HECW2	NM_020760	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	2.4	2.1
IGFALS	NM_004970	Insulin-like growth factor binding protein, acid labile subunit	2.3	2.5
IGFBP1	NM_000596	Insulin-like growth factor binding protein 1	2.3	2.1
PDGFA	NM_002607	Platelet-derived growth factor alpha polypeptide	2.2	2.2
CLEC3B	NM_003278	C-type lectin domain family 3, member B	2.1	2.2

[a]Fold change between 8 h and 0 h after lactate treatment
[b]Fold change between lactate treatment and pyruvate treatment at 8 h

response to AMPK activation caused by the intracellular energy deficiency. However, it remains to be determined whether other stresses such as oxidative stress may also have participated in p53 activation and GDF15 induction in the lactate-treated 2SD cells.

Gene network analysis demonstrated that the top-ranked network contained not only genes associated with the amino-acid starvation response but also the GDF15 gene (Fig. 3A). In a mouse model of late-onset mitochondrial myopathy, the expression of amino-acid starvation-responsive genes was shown to be elevated (Tyynismaa et al., 2010). The asparagine synthetase (ASNS), which is a representative gene involved in the amino-acid starvation response, has been reported to be up-regulated in the skeletal muscle of patients with mitochondrial diseases and in cybrid cells established from a mitochondrial disease patient (Crimi et al., 2005; Fujita et al., 2007). Activating transcription factor 4 (ATF4) is a master regulator of integrated stress responses (ISR), in which a variety of stresses, including amino-acid starvation as well as glucose starvation, ER stress, hypoxia, and oxidative stress, induce phosphorylation of eIF2α followed by up-regulation of ATF4 to activate expression of stress-responsive genes (Harding et al., 2003; Jiang et al., 2004; Rouschop et al., 2010; Rzymski et al., 2010; Teske et al., 2011). It is noteworthy to point out that GDF15 has been shown to be up-regulated by ATF4 in mouse embryonic fibroblasts (Jousse et al., 2007). Taken together, such findings suggest that the ISR pathway may also contribute to the induction of GDF15 in response to defective energy metabolism and play a role in the pathogenesis of mitochondrial diseases.

In the present study, we validated the clinical usefulness of GDF15 as a diagnostic marker by determining the serum GDF15 levels in patients with mitochondrial diseases and in those with other pediatric diseases. The results showed that serum GDF15 levels were significantly elevated in patients with mitochondrial diseases, which finding is consistent with a recent report (Kalko et al., 2014). We also demonstrated that GDF15 had higher sensitivity and specificity than FGF21, which was recently identified as a sensitive and specific blood biomarker for muscle pathology in a wide range of mitochondrial diseases in adults and children (Suomalainen et al., 2011). Our small-scale study, however, may have underestimated the clinical usefulness of FGF21, because the AUC for FGF21 reported by 2 independent groups (0.95 and 0.91) was higher than that in the present study (0.787).

Using the multiplex suspension array, we also identified HGF, SCF, and SCGF-β as potential diagnostic markers for mitochondrial diseases. The ROC curve analysis, however, revealed that GDF15 had the maximum sensitivity and specificity for diagnosis of mitochondrial diseases compared with HGF, SCF, SCGF-β, or FGF21. Based on the microarray analysis, we also selected INHBE as the next best candidate gene (Table 2). INHBE is a member of the activin beta family, which has been reported to be primarily expressed in the liver and up-regulated by drug-induced ER stress, cysteine deprivation, and insulin treatment (Bruning et al., 2012; Dombroski et al., 2010; Hashimoto et al., 2009; Lee et al., 2008). Although secreted INHBE protein was not detectable in the conditioned medium from the cell cultures, we are currently investigating the clinical usefulness of INHBE as a biomarker for diagnosis and monitoring of the disease status and progression.

Table 3
Genes annotated to the extracellular space among those specifically down-regulated by lactate treatment for 8 h.

Gene symbol	Accession number	Entrez gene name	Fold change	
			L-8/L-0[a]	L-8/P-8[b]
CXCL1	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	−3.4	−2.6
PDZRN3	NM_015009	PDZ domain containing ring finger 3	−2.4	−2.0
SLC39A10	NM_020342	Solute carrier family 39 (zinc transporter), member 10	−2.3	−2.9
DKK1	NM_012242	Dickkopf 1 homolog (Xenopus laevis)	−2.1	−2.3

[a]Fold change between 8 h and 0 h after lactate treatment
[b]Fold change between lactate treatment and pyruvate treatment at 8 h

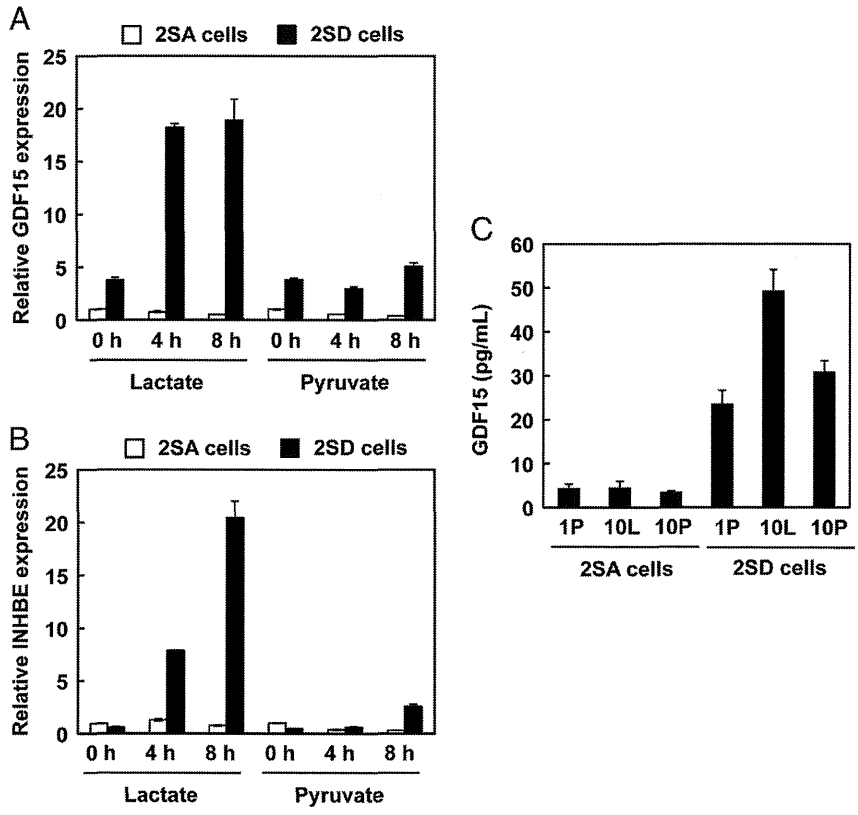

Fig. 4. Quantitative RT-PCR and ELISA for GDF15 and INHBE Total RNA isolated from 2SA and 2SD cells treated with 10 mM lactate or 10 mM pyruvate for 0, 4 or 8 h (n = 3) were subjected to quantitative RT-PCR for GDF15 (A) and INHBE (B). (C) The conditioned medium collected from 2SA and 2SD cell cultures treated with 10 mM lactate (10 L), 10 mM pyruvate (10P) or 1 mM pyruvate (1P) for 24 h was subjected to ELISA for GDF15 protein ($n = 3$).

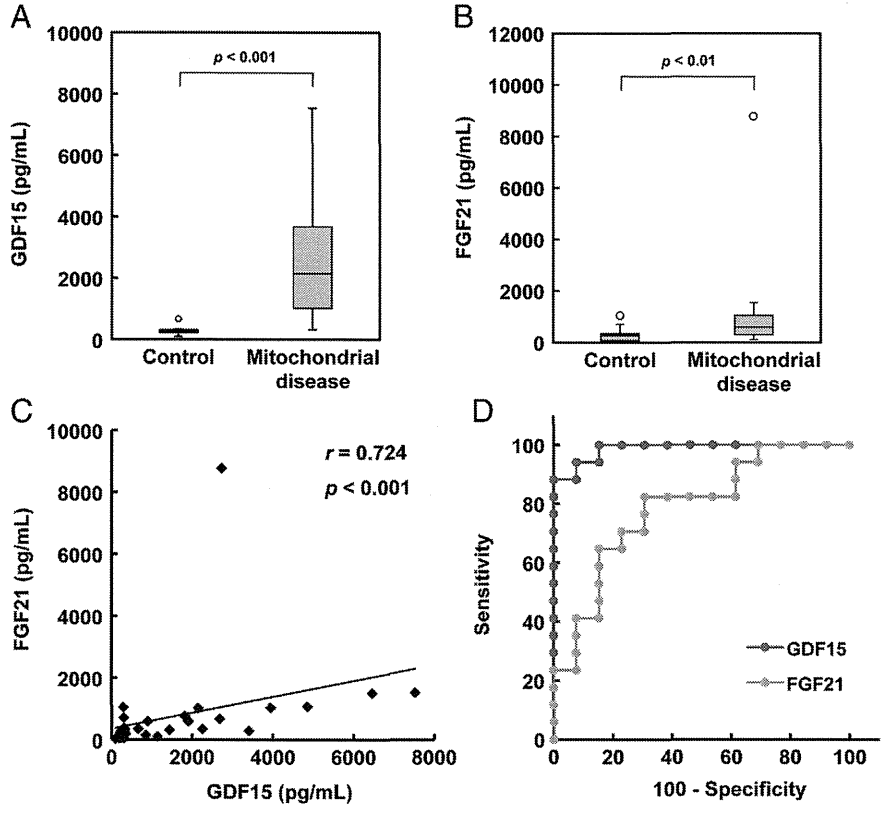

Fig. 5. Measurement of the GDF15 and FGF21 concentrations in the serum of patients. The serum GDF15 (A) and FGF21 (B) concentrations in 17 patients with mitochondrial diseases as well as those in 13 patients with other pediatric diseases were determined by ELISA. The outlier is shown with an open symbol. (C) A correlation analysis between the serum GDF15 and FGF21 levels was performed for the patients described above by use of IBM SPSS statistics. (D) The ROC curve analysis for GDF15 and FGF21 was performed. Areas under the curves (AUC) for GDF15 and FGF21 were 0.986 (95% CI 0.957-1.000) and 0.787 (95% CI 0.621-0.953), respectively.

It is well known that mitochondrial dysfunction is associated with the pathology of various diseases such as Parkinson's disease, Alzheimer's disease, diabetes, and aging (Exner et al., 2012; Lopez-Otin et al., 2013; Martin and McGee, 2014). GDF15, which may reflect mitochondria dysfunction, could be a useful marker for those diseases and the aging process. In support of this idea, the serum GDF15 level was reported to be elevated under various pathological conditions such as cancers, cardiovascular diseases, diabetes, and obesity (Dostalova et al., 2009; Kempf et al., 2007; Welsh et al., 2003); however, in most cases, it was not as high as that observed in mitochondrial diseases. Recent cohort studies also demonstrated that the serum GDF15 level is a novel predictor of all-cause mortality and is associated with cognitive performance and cognitive decline (Fuchs et al., 2013; Wiklund et al., 2010). We thus anticipate that GDF15 will attract more interest with respect to a variety of diseases and aging associated with mitochondrial dysfunction.

In conclusion, we identified GDF15 as a novel serum marker for the diagnosis of mitochondrial diseases and possibly both for monitoring the disease status and progression and for evaluating the therapeutic efficacy of pyruvate. Large-scale clinical trials including combined use of other markers such as FGF21 should confirm the clinical usefulness of GDF15.

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Appendix A. Supplementary data

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Efficacy of pyruvate therapy in patients with mitochondrial disease: A semi-quantitative clinical evaluation study

Tatsuya Fujii [a,*], Fumihito Nozaki [a], Keiko Saito [a,1], Anri Hayashi [a], Yutaka Nishigaki [b,2], Kei Murayama [c], Masashi Tanaka [b], Yasutoshi Koga [d], Ikuko Hiejima [a], Tomohiro Kumada [a]

[a] Department of Pediatrics, Shiga Medical Center for Children, 5-7-30 Moriyama, Shiga 524-0022, Japan
[b] Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakane-cho, Itabashi, Tokyo 173-0015, Japan
[c] Department of Metabolism, Chiba Children's Hospital, 579-1 Heta-cho, Midori, Chiba 266-0007, Japan
[d] Department of Pediatrics and Child Health, Kurume University Graduate School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan

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ABSTRACT

Background: Disorders of oxidative phosphorylation (OXPHOS) cause an increase in the NADH/NAD^+ ratio, which impairs the glycolysis pathway. Treatment with pyruvate is expected to decrease the ratio and thereby restore glycolysis. There are some case reports on the efficacy of pyruvate treatment for mitochondrial diseases. However, few of these reports assessed their results using a standardized scale.
Methods: We monitored 4 bedridden patients with OXPHOS disorders who continued therapies of 0.5–1.0 g/kg/day of sodium pyruvate for more than 12 months. The efficacies of these treatments were evaluated with the Newcastle Pediatric Mitochondrial Disease Scale and the Gross Motor Function Measure with 88 items.
Results: The ages of the patients at the treatment initiation ranged from 8–100 months. Of the 4 patients, 3 exhibited improvements within 1–3 months from the initiation of treatment. Among these 3 patients, one maintained the improvement for over 2 years. The remaining 2 regressed 3–6 months after the initiation of treatment. The blood lactate/pyruvate ratios did not correlate with the efficacy of treatment.
Conclusion: Pyruvate was effective even in bedridden patients with OXPHOS disorders, at least in the short term. Clinical trials with more patients and less severe disabilities are necessary to evaluate the long-term efficacy of this treatment. Biomarkers other than lactate and pyruvate need to be identified to biochemically monitor the efficacy of this treatment.

1. Introduction

Tanaka et al. [1] proposed that pyruvate has therapeutic potential for patients with oxidative phosphorylation (OXPHOS) disorders in which the intracellular NADH/NAD^+ ratio is increased. Such an increased ratio impairs the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the glycolysis pathway. Theoretically, with lactate dehydrogenase, pyruvate provides NAD^+ and decreases this ratio and thereby restores the activity of GAPDH, which produces ATP. Additionally, pyruvate activates pyruvate dehydrogenase and non-enzymatically eliminates hydrogen peroxide.

There are several case reports on the efficacy of pyruvate in patients with OXPHOS disorders [2–4]. However, few of these reports have evaluated the clinical outcomes using a standardized clinical assessment scale. We semi-quantitatively evaluated the efficacy of pyruvate therapy in 4 patients with OXPHOS disorders using standardized scales. This study was approved by the Ethical Committee of our institution. Written informed consent was obtained from the parents of every patient.

2. Patients and methods

2.1. Patients

Four patients who had been on pyruvate for more than 12 months were studied (Table 1). Two patients had Leigh syndrome associated with m.8993 T>G or m.9176 T>C mutations. One patient had non-specific encephalomyopathy associated with complex I and IV combined deficiency. Another patient had myopathic mitochondrial DNA depletion syndrome. All patients were bedridden, and all but one

Abbreviations: NPMDS, Newcastle Pediatric Mitochondrial Disease Scale; GMFM-88, Gross Motor Function Measure with 88 items; JMDRS, Japanese Mitochondrial Disease Rating Scale; OXPHOS, Oxidative phosphorylation; MELAS, Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; FGF-21, Fibroblast growth factor 21.

* Corresponding author at: Department of Pediatrics, Shiga Medical Center for Children, 5-7-30 Moriyama, Moriyama-City, Shiga 524-0022, Japan.
E-mail address: tatsufu@gmail.com (T. Fujii).
[1] Present address: Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Shogoinkawahara-cho, Sakyo, Kyoto, Kyoto 606-8507, Japan.
[2] Present address: Nishigaki Clinic & Research Laboratory, 1-177 Uchinaka, Nakagawa, Nagoya 454-0927, Japan.

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Table 1
Profiles of the patients.

Patients	Clinical Dx	Molecular or biochemical Dx	Age at the start of the Tx	ADL at the start of the Tx	Dose of sodium pyruvate (g/kg/day)	Duration of the Tx
Patient 1	Leigh syndrome	m.8993 T>G	8 y 4 m	Bedridden Unable to roll over Tube fed	0.5	27 m
Patient 2	Leigh syndrome	m.9176 T>C	8 m	Bedridden Unable to roll over Tube fed	0.5	66 m
Patient 3	Non-specific encephalomyopathy	Complex I + IV deficiency	1 y 8 m	Able to roll over to one direction Unable to creep Orally fed	0.5 then 1.0	17 m
Patient 4	Myopathic mitochondrial depletion syndrome	mtDNA depletion	1 y 7 m	Bedridden Unable to roll over On a respirator Tube fed	0.5	41 m

Dx, diagnosis; Tx, treatment; mt, mitochondrial; ADL, activities of daily living.

(namely, the patient with combined deficiencies of complex I and IV) were tube fed. The ages at the initiation of pyruvate therapy were 8–100 months (median 20 months). The durations of therapy were 17–66 months (median 34 months). During the pyruvate therapy monitoring period, all other concomitant mitochondrial disease medications were maintained unchanged.

2.2. Pyruvate

Sodium pyruvate was obtained from Musashino Chemical Laboratory (Tokyo). Sodium pyruvate was administered at 0.5 g/kg/day orally or through a feeding tube in 2 divided doses. This dose was increased to 1.0 g/kg/day in one patient. To avoid osmotic diarrhea, the pyruvate was dissolved in water at concentrations of approximately 2%–10%. Higher concentrations were utilized if the dilution caused overhydration or the volume was too large to drink.

2.3. Clinical evaluation

The efficacy of the pyruvate therapy was clinically evaluated with 3 standard scales: the Newcastle Pediatric Mitochondrial Disease Scale (NPMDS) [5], the Gross Motor Function Measure with 88 items (GMFM-88) [6], and the Japanese Mitochondrial Disease Rating Scale (JMDRS) [7]. The NPMDS is composed of 4 domains: Section I, current function; Section II, systemic specific involvement; Section III, current clinical assessment; and Section IV, quality of life. Sections I–III are scored based on objective observations, and Section IV takes the subjective views of the parents into account. Higher scores indicate more severe clinical situations. There are 3 sets of age-specific NPMDSs. Depending on the patient's age at the time of the evaluation, the NPMDS for 0–24 months or that for 2–11 years was used. The GMFM-88 is composed of 5 dimensions: A, lying and rolling; B, sitting; C, crawling and kneeling; D, standing; and E, walking, running and jumping. The scores are expressed in percentages relative to the maximum score in each dimension. The total score is expressed as the mean of percentages across all 5 dimensions. As the patients were bedridden, only dimensions A and B could be assessed, and the scores for the dimensions C to E were considered to be zero %. Higher scores indicate better motor abilities. The JMDRS is the modified Japanese version of the European Neuromuscular Conference (ENMC) Mitochondrial Disease Rating Scale [8]. Higher scores in this scale indicate more severe symptoms. With the exception of Patient 4, who was only assessed with the NPMDS, all other patients were evaluated with the NPMDS and the GMFM at the same time. Patient 2 was initially monitored with the JMDRS. Then, after a 4-week-washout period, the patient was reassessed with the NPMDS and GMFM. Changes in motor functions that were too subtle to be detected with these scales were descriptively recorded. Serum lactate and pyruvate levels as well as plasma amino acids were monitored.

2.4. Statistical analysis

Statistical analysis of the biochemical data was performed using Mann–Whitney *U*-test. A value of $p < 0.05$ was considered as statistically significant.

3. Results

The changes in motor function and assessment scores are summarized in Table 2.

3.1. Patient 1 (m.8993 T>G Leigh syndrome)

The therapy was initiated at the age of 8 years and 4 months, and at this time, this female patient was unable to roll over. In the supine position, she could not raise her legs more than 45 degrees from the floor (as measured at the hip joint). One month after the initiation of therapy, the patient gained the abilities to roll over and raise her legs vertically from the floor. The movement of her arms became more active and rapid. The overall NPMDS score changed from 42.3 to 38.6. The sum of the scores for sections I–III changed from 31 to 29, which indicates that the objective findings improved by 2 points over one month. Dimension A of the GMFM-88 also changed from 31.4% to 47.1%, which resulted in a change from 6.3% to 9.4% in the total score. Thus, this patient's improvement was confirmed semi-quantitatively with 2 scales. Next, pyruvate was withdrawn to confirm the effect of the pyruvate treatment. Within 1 to 2 weeks, the patient became lethargic and less active. After 19 days of washout, she developed status epilepticus. Resumption of pyruvate therapy restored her clinical status to the pre-washout state. Upon re-evaluation at the age of 10 years and 7 months (after 26 months of treatment excluding the washout period), the patient exhibited maintained improved motor ability as confirmed by the unchanged GMFM-88 score. The NPMDS was not administered at this point.

Blood lactate levels and lactate/pyruvate ratios measured twice during the pre-treatment period and once after the 19-day-washout were from 1.2 mM to 1.5 mM (median 1.2 mM), and from 14.2 to 25.6 (median 19.7), respectively. Those measured at 1, 4, 18 and 20 months after the treatment resumption following the washout period ranged from 0.81 mM to 1.2 mM (median 0.85 mM), and from 15.7 to 27.3 (median 20.0), respectively (Table 3). Thus, lactate levels decreased with pyruvate therapy, but the difference was not significant. Lactate/pyruvate ratio was not reduced. Plasma alanine, valine and lysine levels were measured after the washout and 1 month after the treatment resumption. None of these decreased with the therapy (Table 3).

Table 2
Clinical effects of pyruvate therapy.

Patient 1, Leigh syndrome with m.8993 T>G				
		At the Tx initiation (Age 8 y 4 M)	1 month Tx (Age 8 y 5 m)	26 months Tx (Age 10 y 7 m)
ADL		Unable to roll over Unable to raise the legs > 45° in supine position	Able to roll over Able to raise the legs 90° Moves arms more rapidly	The same as the ADL at 8 y 5 m
NPMDS	I	18	18	ND
	II	2	1	ND
	III	11	10	ND
	IV	11.3	9.6	ND
	Overall	42.3	38.6	ND
GMFM	A	31.4%	47.1%	47.1%
	Total	6.3%	9.4%	9.4%
Patient 2, Leigh syndrome with m.9176 T>C. First treatment				
		At the Tx initiation (Age 8 m)	1-month Tx (Age 9 m)	12-month Tx (Age 20 m)
ADL		Unable to roll over Partially tube-fed	Unable to roll over Partially tube-fed	Able to roll over Orally fed
JMDRS		52	52	53
Patient 2. Second treatment after washout.				
		After 4-week washout (Age 5 y 3 m)	2 months after the Tx resumption (Age 5 y 5 m)	11 months after the resumption (Age 6 y 5 m)
ADL		Unable to roll over Tube-fed	Unable to roll over Tube-fed	Unable to roll over Tube-fed
NPMDS	I	13	13	15
	II	3	3	5
	III	14	14	17
	IV	4.2	4.2	16.7
	Overall	34.2	34.2	53.7
GMFM	A	5.9%	5.9%	3.9%
	Total	1.2%	1.2%	0.8%
Patient 3, complex I + IV deficiency				
		At the Tx initiation (Age 1 y 8 m)	2-month Tx (1 y 10 m)	12-month Tx (2 y 8 m)
ADL		Roll over one direction Head control fair Mild dysphagia	Roll over bilaterally Head control fair No dysphagia	Roll over bilaterally Head control poor
NPMDS	I	7	6	6
	II	6	6	2
	III	15	13	13
	IV	16.7	7.3	7.3
	Overall	44.7	32.3	28.3
GMFM	A	54.9%	66.7%	60.8%
	B	13.3%	13.3%	3.3%
	Total	13.6%	16.0%	12.8%
Patient 4, mitochondrial DNA depletion syndrome				
		At the Tx initiation (Age 1 y 7 m)	2-month Tx (Age 1 y 9 m)	41-month Tx (Age 5 y 0 m)
ADL		On respirator Unable to raise the forearm above the floor Myopathy only	On respirator Able to raise the forearm 90° at the elbow. Myopathy only	On respirator Unable to raise the forearm Encephalomyopathy
NPMDS	I	7	7	15
	II	6	6	15
	III	5	5	24
	IV	17	13	10.8
	Overall	35	31	64.8

Tx, treatment; ADL, Activities of daily living; NPMDS, Newcastle Pediatric Mitochondrial Disease Scale; GMFM, Gross Motor Function Measure; JMDRS, Japanese Mitochondrial Disease Rating Scale; I–IV, Sections I–IV of NPMDS; A and B, Dimensions A and B of GMFM; ND, not done.

3.2. Patient 2 (m.9176 T>C Leigh syndrome)

Pyruvate therapy was initiated at the age of 8 months for this male patient who was unable to roll over and had poor head control. Oral feeding was partially possible. After one-month of treatment, motor function was not altered and neither was the JMDRS score, which was 52. After 12 months of treatment, at the age of 1 year and 8 months, the patient was able to roll over and full oral feeding became possible. However, these subtle changes were not detected by JMDRS. The JMDRS score actually increased by 1 point due to seizures. At 3 years

Table 3
Changes in blood lactate and amino acids levels with pyruvate therapy.

	Lactate (mM) Median (range) (n) (normal: 0.3–1.9)		Lactate/Pyruvate ratio Median (range) (n) (normal: 10–15)		Alanine (μM) Median (range) (n) (normal: 209–523)		Valine (μM) Median (range) (n) (normal: 148–307)		Lysine (μM) Median (range) (n) (normal: 109–242)	
	Before	After	Before	After	Before	After	Before	After	Before	After
Patient 1	1.2 (1.2–1.5) (3)	0.85 (0.81–1.2) (4)	19.7 (14.2–25.6) (3)	20.0 (15.7–27.3) (4)	256 (1)	439 (1)	165 (1)	263 (1)	104 (1)	200 (1)
Patient 2	2.8 (1.2–4.4) (2)	2.4 (0.9–3.1) (5)	23.2 (19.2–27.2) (2)	23.1 (14.7–30.5) (5)	402 (360–443) (2)	340 (320–428) (5)	173 (172–174) (2)	168 (135–171) (5)	139 (96.6–180) (2)	112 (96.2–172) (5)
Patient 3	3.9 (2.5–8.0) (4)	5.6 (3.7–9.3) (7)	25.0 (14.7–35.3) (4)	30.5 (17.7–45.9) (7)	543 (427–659) (2)	729 (549–840) (7)	171 (154–188) (2)	219 (149–280) (7)	117 (87.8–146) (2)	122 (88.7–172) (7)
Patient 4	2.3 (2.1–2.7) (4)	2.5 (2.3–2.7) (5)	16.9 (14.9–18.7) (4)	17.3 (14.1–21.2) (5)	350 (1)	384 (381–386) (2)	140 (1)	187 (182–191) (2)	108 (1)	158 (157–158) (2)

Mann–Whitney U-test did not show any significant differences.

of age, the patient developed acute encephalopathy associated with a viral infection and lost the abilities of oral feeding and rolling over. To re-evaluate the efficacy of pyruvate, the patient was reassessed with the NPMDS and GMFM-88 at the age of 5 years and 3 months after a 4-week pyruvate washout period. The washout did not cause any deterioration. Two months after the resumption of the pyruvate therapy, neither the NPMDS (overall score, 34.2) nor the GMFM-88 (total score 1.2%) scores changed. After 11 months of therapy after the washout, the scores for all sections of the NPMDS increased, and the overall score increased by 19.5 points. The total GMFM-88 score decreased from 1.2% to 0.8%. Thus, pyruvate was not effective for this patient.

Blood lactate levels and lactate/pyruvate ratios measured twice during 2 months before the first pyruvate therapy at the age of 8 months were 1.2 mM and 4.4 mM (median, 2.8 mM), and 19.2 and 27.2 (median, 23.2), respectively. Those at 1, 2, 3, 4 and 12 months after the therapy ranged from 0.9 mM to 3.1 mM (median, 2.4 mM) and from 14.7 to 30.5 (median, 23.1), respectively. Lactate levels and lactate/pyruvate ratios did not change significantly with the therapy (Table 3). Plasma alanine, valine and lysine levels measured twice before and at 1, 2, 3, 4 and 12 months after the therapy showed a mild but non-significant decrease with the therapy (Table 3).

3.3. *Patient 3 (combined deficiencies of complex I and IV)*

This male patient presented with developmental delay, nystagmus, hypertrophic cardiomyopathy and mild hearing disturbance (38 dB). At the age of 11 months, he developed status epileptics followed by regression. Increased lactate levels and lactate/pyruvate ratio in the cerebrospinal fluid (CSF) (lactate:5.2 mM, lactate/pyruvate ratio: 20.0) and blood (lactate: 12.3 mM, lactate/pyruvate ratio: 41.6) led to a skin biopsy, which revealed deficiencies in complexes I and IV: the activities of complex I and IV relative to the activity of citrate synthase were 24.7% and 22.9% of normal controls ($n = 12$), respectively, and those relative to the activity of complex II were 33.5% and 31.4% of normal, respectively. Muscle biopsy could not be obtained. The clinical signs and symptoms fulfilled the mitochondrial disease criteria for definite mitochondrial disorder proposed by Morava et al. [9]. No mutation was revealed in the mitochondrial DNA. Molecular analysis of the nuclear genes is under way. Treatment with coenzyme Q_{10} at the age of 1 year and 6 months did not produce any improvement. Pyruvate therapy was initiated at the age of 1 year and 8 months, and at this time the patient had mild dysphagia and incomplete head-control. He could roll over only in one direction. After 2 months of pyruvate therapy with a maintenance dose of 1.0 g/kg/day, he gained the ability to roll over bilaterally and the dysphagia disappeared. The total scores for sections I–III decreased from 28 to 25, and the score for IV also decreased from 16.7 to 7.3. The GMFM-88 score increased from 13.6% to 16.0%. Thus, the efficacy of the 2-month pyruvate therapy was confirmed by both scales. However, over the next 10 months, a slow regression in motor function was observed, and at 2 years and 8 months of age (after 12 months of treatment), this patient's GMFM-88 score decreased from 16.0% to 12.8%. However, the scores for section II of the NPMDS (the version for 2–11 year-olds was used) decreased by 4 points due to improvements in seizures and gastrointestinal and hepatic function. The regression of motor function that was evident in the GMFM-88 was not detected by the NPMDS (the scores for sections I and III were unchanged).

Blood lactate levels and lactate/pyruvate ratios measured 4 times during the 9-month pre-treatment period ranged from 2.5 mM to 8.0 mM (median, 3.9 mM), and from 14.7 to 35.3 (median, 25.0), respectively. Those measured 1, 2, 3, 4, 6, 9 and 12 months after the therapy ranged from 3.7 mM to 9.3 mM (median, 5.6 mM), and from 17.7 to 45.9 (median 30.5), respectively (Table 3). Thus, neither the blood lactate levels nor the lactate/pyruvate ratios decreased with the pyruvate therapy. Among the measurements, those measured twice during the first 2-month treatment, which was clinically effective, did not show any decrease either. Plasma alanine, valine and lysine levels were

measured twice before the treatment and 7 times after the therapy. None of these decreased significantly with the therapy (Table 3).

Throughout the therapy, the patient exhibited chronic diarrhea that seemed to be a side effect of the treatment.

3.4. Patient 4 (myopathic form of the mtDNA depletion syndrome)

The short-term efficacy of pyruvate therapy for this female patient and her clinical and biochemical profile have been reported in detail elsewhere [3]. Briefly, the patient developed severe generalized weakness including facial muscles and respiratory failure during the neonatal period. The patient had a tracheostomy and was on a respirator. She had lactic acidosis (3.0 mM to 6.5 mM) with high lactate/pyruvate ratio (36 to 97). Muscle biopsy revealed ragged red fibers and decreased cytochrome c oxidase staining. The activities of complex I, III and IV relative to the activity of citrate synthase in the muscle were 10.6%, 26.7% and 14.1% of the control, respectively. Those relative to the activity of complex II were 6.5%, 16.4% and 8.8%, respectively. Quantitative analysis of the mtDNA revealed that the copy number of the mitochondrial ND1 subunit relative to the nuclear CFTR gene was 35.3% (normal: >40%). Exome sequencing is under way to detect a mutation in causative genes. The clinical signs and symptoms were compatible with Morava et al.'s criteria for definite mitochondrial disease [9]. As reported elsewhere, after 2 months of pyruvate therapy, the patient exhibited a mild improvement in the movement of her extremities at the age of 1 year and 9 months [3]. The overall NPMDS scores decreased from 35 to 31, but this decrease was limited to section IV. As the patient was not assessed with the GMFM, we were unable to semi-quantitatively demonstrate the improvement in motor function. One month later (after 3 months of treatment), the patient developed status epilepticus. An MRI revealed lesions in the occipital areas, which indicated a progression from the myopathic form to the encephalomyopathic form. At 5 years of age, after 41 months of treatment, scores in all sections of the NPMDS increased, and the increase in overall NPMDS score was 33.8 points compared to the score at the 2-month treatment.

Blood lactate and lactate/pyruvate ratios measured 4 times during the 2-month pre-treatment period ranged from 2.1 mM to 2.7 mM (median, 2.3 mM), and from 14.9 to 18.7 (median, 16.9), respectively. Those measured 1, 4, 6, 8 and 13 weeks after the therapy ranged from 2.3 mM to 2.7 mM (median, 2.5 mM), and from 14.1 to 21.2 (median, 17.3), respectively (Table 3). Plasma alanine, valine and lysine levels were measured once before the therapy and 4 and 8 weeks after the therapy. None of these decreased with the pyruvate therapy (Table 3).

4. Discussion

All 4 of the treated patients were severely disabled and bedridden. Therefore, objective and semi-quantitative assessments of the outcomes were difficult because the expected improvements were subtle. The NPMDS is a scale that was designed to specifically monitor mitochondrial disease, which results in a variety of multi-organ symptoms. Therefore, the scale encompasses all aspects of mitochondrial disease. Consequently, this scale cannot detect small changes in motor function. The logic applies to the JMDRS. In contrast, the GMFM-88 evaluates motor function with as many as 88 items; therefore, this assessment may detect small changes in motor abilities. However, the GMFM was designed to assess cerebral palsy, and its reliability in monitoring mitochondrial disease has not been validated. In contrast to the GMFM-66, which can only be used for cerebral palsy, the GMFM-88 has been validated for the monitoring of motor functions in disorders other than cerebral palsy, such as spinal muscular atrophy, Down syndrome and traumatic brain injuries. [10–12] Therefore, we assumed that the GMFM-88 could also be used to monitor motor functions in mitochondrial disease. Nevertheless, given that the GMFM-88 has not been validated for using in mitochondrial disease, we assessed the outcomes via a combination of the GMFM-88 and NPMDS scores with the exception of patient 4, who was assessed only with the NPMDS. We also tried using other scales including Pediatric Evaluation of Disability Inventory (PEDI) [13] and Functional Independence Measure for Children (Wee-FIM) [14]. Our preliminary study, however, showed that these could not detect clinical changes in our patients.

Patients 3 and 4 were assessed with 2 different sets of age-specific NPMDSs as they matured into ages suitable for the application of the older age-specific NPMDSs during the monitoring period. The number of items scored in each section of the NPMDS for 2–11-year-olds is greater than that of the NPMDS for 0–24-month-olds. Therefore, it is possible that total NPMDS scores may increase when the version for older patients is used even if clinical severity remains unchanged. In Patient 3, the score for section II as assessed 2 years and 8 months decreased compared to the score assessed at 1 year and 10 months, whereas the scores for the other sections remained unchanged. Thus, a "pseudo-increase" in the score due to the use of a different set of NPMDS scales did not occur in this patient. In Patient 4, the scores for sections I, II and III increased by 8, 9 and 19 points, respectively, at 5 years of age compared to the scores observed at 21 months of age. Given that the maximum scores for sections I, II and III are higher by 6, 3 and 6 points, respectively, in the NPMDS for 2-11 year-olds than in the NPMDS for 0–24 month-olds, the increases in the scores that were higher than the maximum possible increases due to the differences in the versions of the NPMDS indicated that the increases were real.

The most noteworthy result of this study was that 3 of the 4 severely disabled patients (Patients 1, 3 and 4) exhibited improvement within 1 to 2 months of the initiation of pyruvate therapy. These improvements were confirmed by both the NPMDS and GMFM-88 (Patients 1 and 3) or the NPMDS only (Patient 4). The semi-quantitative improvement observed in Patient 4 was limited to section IV of the NPMDS, which accounts for the parents' subjective assessments. However, a descriptive observation record also revealed improvement in muscle power. [3] Given that no improvements were observed prior to pyruvate therapy in these patients and that the improvements were observed with 1–2 months of the initiation of pyruvate therapy, it is unlikely that the observed ameliorations were simply due to natural motor development rather than the effects of the therapy. The efficacy was particularly evident in Patient 1 who had m.8993 T>G and exhibited improvements in motor function that were maintained for over 2 years. The worsening of symptoms during pyruvate withdrawal also supported the efficacy of pyruvate treatment in this patient. In contrast, 2 of the 3 responsive patients did not maintain the improvements for longer than several months. Notably however, the overall NPMDS score for Patient 3 decreased (i.e., symptoms improved) after 12 months of therapy compared to this patient's score after 2 months of the therapy despite the worsening of the GMFM score. These findings indicated that the patient's overall health improved during long-term therapy, although this patient's motor abilities regressed. In Patient 4, the disease progression overwhelmed the effect of the pyruvate therapy shortly after the responsiveness was confirmed after 2 months of therapy; this finding indicated a limitation of this therapy. We could not explain why Patient 2, who had m.9176 T>C, did not respond to pyruvate therapy. Given the age of this patient, the mild improvements in motor function after 12 months of pyruvate therapy, which could not be detected with the JMDRS, seemed to be due to natural motor development rather than resulting from the treatment.

The only adverse effect of pyruvate therapy was the mild but chronic diarrhea that was observed in one patient who was on 1.0 g/kg/day of sodium pyruvate.

An *in vitro* study that utilized cybrid cells harboring MELAS m.3243A>G mutant mitochondria found that pyruvate treatment facilitates the pyruvate-to-lactate conversion, decreases the lactate/pyruvate ratio, normalizes the NADH/NAD^+ ratio, and enhances ATP production and energy charge without significantly altering the intracellular lactate level. [15] These data support the theory that the effects of pyruvate

therapy are mediated via the normalization of the NADH/NAD^+ ratio, which provides the NAD^+ that is deficient in OXPHOS disturbances. In contrast to the theory and the result of this *in vitro* study, none of our responsive patients exhibited decreases in blood lactate/pyruvate ratios, which are equivalent to the NADH/NAD^+ ratios, during the effective short-term therapy. Blood lactate levels decreased in 2 patients, especially in Patient 1, but the differences were non-significant. Thus, the blood lactate/pyruvate ratios and blood lactate levels of our patients could not be used as biochemical markers to monitor the effects of the therapy. The discrepancy between the clinical data from our patients and the *in vitro* data may be partly explained by the fact that blood lactate levels vary depending on the physical activity of the patient at the time of blood sampling, the interval between meal and sampling, as well as on the time required for the blood sampling procedure. However, all of our patients were bedridden and the data were from multiple samplings in different days. The blood samplings were done either after overnight-fast or several hours after a meal. Therefore, it is unlikely that the discrepancy was artifactual. Still, monitoring the lactate levels and lactate/pyruvate ratios in the CSF rather than in the blood would further reduce the possible artifact. Komaki et al. treated an ambulatory patient with Leigh syndrome associated with cytochrome c oxidase deficiency [2]. With pyruvate therapy, blood lactate level and lactate/pyruvate ratio decreased from 2.3 mM to 1.1 mM, and from 17.7 to 11.4, respectively. However, the measurements were done only once before and after the therapy, so the statistical significance could not be evaluated. Koga et al. found statistically significant decreases in blood lactate, pyruvate and alanine levels with pyruvate therapy in a non-ambulatory patient with pyruvate dehydrogenase (PDH) deficiency [4]. Blood lactate/pyruvate ratio in this patient also decreased, but the difference was non-significant (the ratios in PDH deficiency are generally normal). Differences between Komaki et al. and Koga et al.'s patients from ours were that 1) Komaki et al.'s patient was ambulatory, and 2) the pre-treatment blood levels of lactate and alanine in Koga et al.'s patient were much higher than those in our patients: the blood lactate and alanine levels in this patient were 9.6 ± 0.54 mM ($n = 8$) and 1700 ± 280 μM ($n = 8$), respectively, while the median values of pre-treatment lactate levels in our 4 patients ranged from 1.2 to 3.9 mM and those of alanine were from 256 to 543 μM. This may indicate that the blood lactate and alanine levels and lactate/pyruvate ratio are not sensitive biochemical markers to monitor the pyruvate therapy unless the patients are ambulatory or their pre-treatment blood levels of lactate and alanine are very high.

If the blood lactate/pyruvate ratio does not necessarily reflect the intracellular NADH/NAD^+ ratio, the identification of a marker other than blood lactate and pyruvate is crucial. Kami et al. found that the lysine and valine levels in media in which MELAS-mutant cybrid cells were incubated with 10 mM lactate were higher than those of controls. These increases may be because catabolisms of lysine to acetyl CoA and valine to succinyl CoA require NAD^+, which is deficient due to the imbalance in the NADH/NAD^+ ratio [15]. Plasma levels of lysine and valine in our patients, however, did not decrease with the therapy. We do not know if the levels of these amino acids may decrease with pyruvate therapy in patients with very high blood lactate levels: Koga et al. did not measure valine and lysine levels in their responsive patient [4]. Fibroblast growth factor 21 (FGF-21), a circulating hormone-like cytokine, is reported to be one of the best biomarker with high sensitivity and specificity for detecting muscle-manifesting mitochondrial respiratory chain deficiencies [16]. Although FGF-21 has higher sensitivity than lactate or lactate/pyruvate ratio to diagnose mitochondrial disease, its utility in monitoring the disease is unknown. Further study is necessary to find biomarkers to monitor the effect of pyruvate therapy biochemically.

In conclusion, as confirmed by the GMFM-88 and/or NPMDS, pyruvate therapy was safe and effective even in severely disabled patients with OXPHOS disorders, at least in the short-term. Further studies utilizing greater numbers of patients with less severe disabilities are necessary to evaluate the long-term efficacy of this treatment. The blood lactate and pyruvate levels did not correlate with the efficacy of the pyruvate therapy in our patients as has been reported in *in vitro* studies. The identification of more sensitive biomarkers that reflect the intracellular NADH/NAD^+ ratio or improvements in ATP production is crucial for monitoring the clinical and biochemical efficacy of this therapy.

Conflict of interest

The authors have no conflicts of interest to disclose.

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Original article

New TRPM6 mutation and management of hypomagnesaemia with secondary hypocalcaemia

Koujyu Katayama [a], Nataliya Povalko [a], Shuichi Yatsuga [a], Junko Nishioka [a], Tatsuyuki Kakuma [b], Toyojiro Matsuishi [a], Yasutoshi Koga [a,*]

[a] *Department of Pediatrics and Child Health, Kurume University Graduate School of Medicine, Kurume, Japan*
[b] *Department of Biostatistics, Kurume University Graduate School of Medicine, Kurume, Japan*

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Abstract

Background: TRPM6 gene mutation has been reported to cause hypomagnesemia with secondary hypocalcemia (HSH). However, the genotype–phenotype correlation for TRPM6 gene mutations has not been clarified.

Objective: To elucidate the factors underlying the severe neurological complications in HSH and evaluate the potential association between the location of TRPM6 gene mutations and clinical data of HSH.

Methods: A Japanese patient diagnosed with HSH at 10 weeks of age exhibited neurological damage and failed to thrive. Magnesium supplements were therefore started at 12 weeks of age. Mutational analysis of the TRPM6 gene was performed using a direct sequencing method to determine the position and type of mutation. Using the data of 29 HSH patients reported in the literature, linear regression analysis was also performed to examine the association between TRPM6 gene mutation location and HSH onset age, initial serum magnesium and calcium concentrations, and dose of oral magnesium.

Results: A novel stop-codon homozygous mutation [c.4190 G > A] W1397X was identified in exon 26 of the patient's TRPM6 gene. No statistical correlation was found between the location of mutations in the TRPM6 gene and the clinical data for 4 clinical indicators of HSH.

Conclusions: We identified the first Japanese HSH patient with a novel nonsense mutation in the TRPM6 gene. Regression analysis of mutation locations in the protein-coding region of TRPM6 and the reported clinical data for 4 clinical indicators of HSH in 30 HSH patients did not detect a genotype–phenotype correlation.

Keywords: TRPM6; HSH; Genotype–phenotype correlation; Magnesium; Mental retardation; Failure to thrive

1. Introduction

Hypomagnesemia with secondary hypocalcemia (HSH, OMIM #602014) is a rare autosomal recessive disorder that is characterized by the development of neurological symptoms, including tetany, muscle spasms, and seizures, in early infancy due to low serum magnesium [1]. The low serum magnesium levels

Abbreviations: HSH, hypomagnesemia with secondary hypocalcemia; TRPM6, transient receptor potential channel melastatin 6

* Corresponding author. Address: Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-Machi, Kurume City, Fukuoka 830-0011, Japan. Tel.: +81 942 31 7565; fax: +81 942 38 1792.

E-mail address: yasukoga@med.kurume-u.ac.jp (Y. Koga).

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