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Ⅳ．研究成果の刊行物・別刷

INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Taurine Ameliorates Impaired the Mitochondrial Function and Prevents Stroke-like Episodes in Patients with MELAS

Mitsue Rikimaru[1], Yutaka Ohsawa[1], Alexander M. Wolf[2], Kiyomi Nishimaki[2], Harumi Ichimiya[2], Naomi Kamimura[2], Shin-ichiro Nishimatsu[3], Shigeo Ohta[2] and Yoshihide Sunada[1]

Abstract

Objective Post-transcriptional taurine modification at the first anticodon ("wobble") nucleotide is deficient in A3243G-mutant mitochondrial (mt) $tRNA^{Leu(UUR)}$ of patients with myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). Wobble nucleotide modifications in tRNAs have recently been identified to be important in the accurate and efficient deciphering of codons. We herein examined whether taurine can alleviate mitochondrial dysfunction in patient-derived pathogenic cells and prevent clinical symptoms in MELAS patients.

Methods and Results The addition of taurine to the culture media ameliorated the reduced oxygen consumption, decreased the mitochondrial membrane potential, and increased the oxidative stress in MELAS patient-derived cells. Moreover, high dose oral administration of taurine (0.25 g/kg/day) completely prevented stroke-like episodes in two MELAS patients for more than nine years.

Conclusion Taurine supplementation may be a novel potential treatment option for preventing the stroke-like episodes associated with MELAS.

Key words: MELAS, post-transcriptional modification, taurine, stroke-like episodes

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Introduction

An A3243G or T3271C transition in the mitochondrial (mt) $tRNA^{Leu(UUR)}$ gene has been identified in approximately 80% and 10% respectively, of patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (1). Nearly 90% of patients with myoclonus epilepsy associated with ragged-red fibers (MERRF) possess an A8344G transition in the mt $tRNA^{Lys}$ gene (1). These mutations are located in the cloverleaf structure of each mt tRNA. However, it remains unknown how such point mutations in mt tRNAs induce mitochondrial dysfunction leading to the wide variety of MELAS or MERRF symptoms.

Post-transcriptional modifications in tRNAs play critical roles in modifying the genetic code. In 1966, Francis Crick proposed that the first anticodon ("wobble") nucleotide recognizes the third codon nucleotide through non-canonical Watson-Crick geometry; so-called "wobble" pairing (2). Growing evidence has shown that various post-transcriptional modifications at the wobble nucleotides in tRNAs are required to recognize their cognate codons accurately and efficiently (3). In normal human mt $tRNA^{Leu(UUR)}$ or mt $tRNA^{Lys}$, uridine at the wobble position is modified with taurine, a sulfur-containing β-amino acid (4-6). In contrast, the taurine modification is deficient in mutant mt $tRNA^{Leu(UUR)}$ or mutant mt $tRNA^{Lys}$ derived from clinical specimens of MELAS or MERRF patients (4-8). The taurine modification defect in the mutant mt tRNAs causes a deficiency in deciphering codons (1, 9). These findings have given rise to the intriguing possibility that MELAS and

[1]Department of Neurology, Kawasaki Medical School, Japan, [2]Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Nippon Medical School, Japan and [3]Department of Molecular and Developmental Biology, Kawasaki Medical School, Japan
Received for publication February 13, 2012; Accepted for publication August 6, 2012
Correspondence to Dr. Yoshihide Sunada, ysunada@med.kawasaki-m.ac.jp

MERRF are tRNA-modification disorders associated with the impairment of correct mitochondrial gene translation.

We hypothesized that high-dose taurine supplementation could restore the taurine modification of the mutant tRNAs in MELAS or MERRF patients. In the current study, we explored the potential therapeutic effect of taurine by examining the mitochondrial functions in patient-derived pathogenic cells and by observing the clinical symptoms in MELAS patients receiving taurine supplements.

Materials and Methods

The local ethics committee approved this study (No. 787) and all patients gave their informed consent for participation.

Construction of cybrid cells harboring mutant mtDNA

Immortalized cells possessing patient-derived mitochondrial (mt) DNA were constructed by the intercellular transfer of a patient's mtDNA to ρ^0 HeLa cells (EB8), which are mtDNA-less immortalized cells (10). EB8 cells were isolated by the long-term treatment of HeLa cells with ethidium bromide. Primary dermal fibroblasts were isolated from skin biopsy samples from an A3243G-MELAS, a T3271C-MELAS, and an A8344G-MERRF patient. The fibroblasts were enucleated by centrifugation in the presence of cytochalasin B. Then, the enucleated fibroblasts were fused with EB8 cells by treatment with polyethylene glycol. Control cytoplasmic hybrid (cybrid) strains (Ft2-11, A2) were constructed by fusing mtDNA-less HeLa cells with enucleated normal human fibroblasts.

The resulting cybrids were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μg uridine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen/Life Technologies Japan, Tokyo, Japan). Cybrids with more than 95% mutant mtDNA were used for the experiments. To decrease the endogenous taurine, the cells were also cultured in media with limited amounts of the taurine precursor, L-cysteine (1 mg/mL), and the taurine intermediate, L-methionine (high glucose, L-glutamine-minus, sodium pyruvate-minus Dulbecco's modified Eagle's medium; Gibco) supplemented with L-glutamine, sodium pyruvate, and uridine. The growth rate of mutant cybrids was unchanged after culture in limiting media for seven days.

Cell lines and in vitro analyses

Primary dermal fibroblasts obtained from skin biopsy samples from an A3243G-MELAS, a T3271C-MELAS, and an A8344G-MERRF patient were enucleated and subsequently fused with mt DNA-less HeLa cells (10). The resulting cybrid cells were treated with or without taurine and then were used in subsequent *in vitro* analyses of the mitochondrial oxygen consumption (11), membrane potential (12), and reduction and oxidation (redox) status (10). Taurine powder was purchased from Taisho Pharmaceutical Co., Ltd. (Tokyo, Japan).

Mitochondrial oxygen consumption

Cybrid cells cultured with or without taurine were trypsinized and resuspended in serum-free medium. The cell suspension was continuously stirred at 37℃ with an oxygen electrode (11). The cell concentration was determined using a hemocytometer. The oxygen consumption rates were measured using an Oxygen Meter Model 781 and a Mitocell MT200 closed respiratory chamber (Strathkelvin Instruments, North Lanarkshire, UK). The oxygen respiration rate was directly measured for the 40 mM taurine experiments. After treatment with the limiting media described above, the oxygen consumption was examined in the presence of 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial protonophore used to measure electron transport activity. The consumption value was subtracted from the 1 mM potassium cyanide-independent oxygen consumption value.

Mitochondrial membrane potential

To evaluate the mitochondrial membrane potential, cybrid cells were incubated for 30 minutes at 37℃ with 20 nM MitoTracker Red (Molecular Probes, Invitrogen, Carlsbad, CA, USA), a red-fluorescent dye that accumulates at the mitochondrial membrane (12) in response to the membrane potential. The MitoTracker Red signal increases in a membrane potential-dependent manner. The images were visualized with a confocal laser-scanning microscope (Fluoview FV300; Olympus, Tokyo, Japan) at an excitation wavelength of 594 nm. For the flow cytometric analysis, cells stained with MitoTracker Red were washed in phosphate-buffered saline, trypsinized, and analyzed using a Cell Lab Quanta™ instrument (Beckman Coulter, Inc., Brea, CA, USA). The fluorescent signal of more than 10,000 cells was examined for each experiment.

Mitochondrial redox status

The redox-sensitive green fluorescent protein, roGFP1, generates a unique fluorescence image after the formation (oxidation) of the disulfide bonds adjacent to the barreled β-sheets in the GFP protein (11). To allow real-time visualization of mitochondrial redox status, cybrid cells were stably transfected with the roGFP1 expression vector containing a mitochondrial-targeting sequence. Fluorescence images were recorded using a multi-dimensional imaging workstation (AS MDW; Leica Microsystems, Wetzlar, Germany) consisting of a tunable light source (Polychrome IV monochromator; Till Photonics, Gräfelfing, Germany), an inverted epifluorescence microscope (DM IRE2; Leica Microsystems) contained in a climate chamber maintained at 37℃, and a cooled charge-coupled device camera (CoolSnap HQ; Roper Scientific, Princeton, NJ, USA). The dual excitation ratio of roGFP1 from a single cell was recorded. The ratio of the reduced form of roGFP1 (roGFP1-SH) to the oxidized form

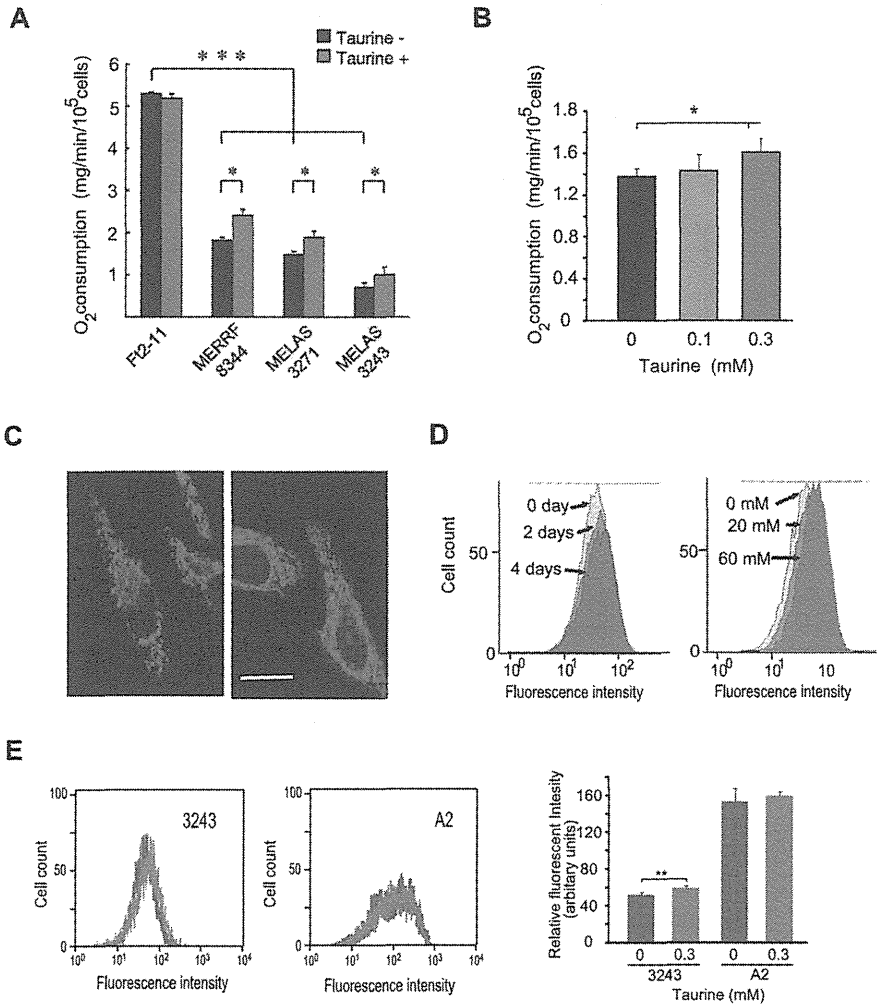

Figure 1. Taurine ameliorates the impaired mitochondrial function in patient-derived cybrid cells. (A) Patient-derived cybrid cells showed marked decreases in oxygen consumption (black bars). After four days in culture with taurine (40 mM), there was a significant increase in the oxygen consumption rates in patient-derived cybrids with mutant mtDNA, but not in wild-type control Ft2-11 cells (red bars) (*p < 0.05). (B) Cybrids were cultured in media with limited amounts of the taurine intermediate, L-methionine (1 mg/mL), and the taurine precursor, L-cysteine (5 mg/mL), for two days, followed by an additional four day culture with or without taurine (0, 0.1, or 0.3 mM). Taurine (0.3 mM) improved the oxygen consumption in the A3243G-MELAS cybrids cultured in the limiting media (*p < 0.05). (C) Cybrids were cultured in the presence (right) or absence (left) of 40 mM taurine for 4 days. Staining with the membrane potential-sensitive red-fluorescent dye MitoTracker Red (100 nM for 30 min) revealed an increased mitochondrial membrane potential with morphological improvement in the A3243G-MELAS cybrid cells. Scale bar: 100 μm. (D) The mitochondrial membrane potential was determined by a flow cytometric analysis after staining with 100 nM of MitoTracker Red for 30 min. The profiles in the left-hand panel show a time-dependent increase in membrane potential after incubation with 40 mM taurine. The right-hand profiles indicate that there was a dose-dependent shift in the membrane potential after four days of culture with the indicated amounts of taurine. (E) Cybrid cells were cultured in the limiting media described in (B). The reduced mitochondrial membrane potential in the A3243G-MELAS cybrid cells (3243) was significantly improved as judged by a flow cytometric analysis after a four-day incubation with 0.3 mM taurine (*p < 0.05). In contrast, the membrane potential in the control cybrid cells (A2) was unchanged after taurine treatment.

of roGFP1 (roGFP1-SS-) was obtained. The fluorescence ratio at 410:490 nm was used as the index of oxidation (11).

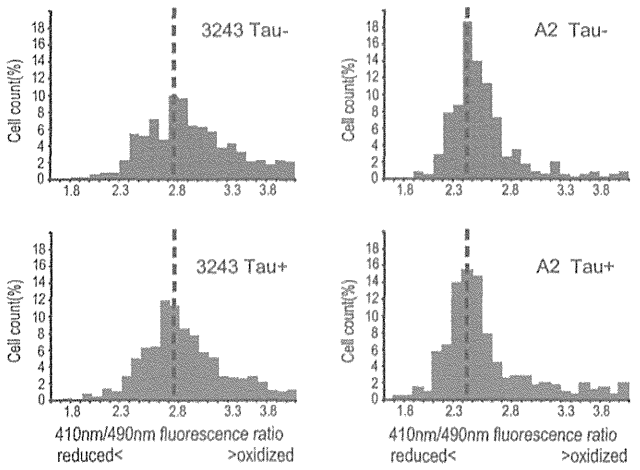

Figure 2. Taurine reduces the oxidative stress in patient-derived cybrid cells. The A3243G-MELAS cybrid cells (3243, left) and the control cybrid cells (A2, right) were stably transfected with a mitochondria-targeting redox-sensitive green fluorescent protein (roGFP). The histograms show the distribution of cells according to their 410:490 nm fluorescence ratio, an indicator of the oxidation status. Compared with the A2 cybrid cells, the ratio was increased in the 3243 cybrid cells, suggesting an increase in oxidative stress (green, upper) The addition of taurine (3 mM; red, lower) caused a shift towards a reduced status in the 3243 cybrid cells, but not in the A2 cybrid cells (red, lower). The data represent the mean values from eight independent experiments. *p < 0.05 between culture conditions with and without taurine.

Oral administration of taurine to patients with A3243G-MELAS

Taurine powder was orally administered three times a day, after a meal, to two patients with A3243G-MELAS at a dose of 0.25 g/kg/day. This corresponds to the maximal dose previously employed for Japanese patients with biliary obstructions (13).

Statistical analyses

Paired observations were statistically analyzed using a one-way analysis of variance followed by Bonferroni's test. p values <0.05 were considered to be statistically significant.

Results

Taurine restores the reduced mitochondrial oxygen consumption in patient-derived cells

The cybrid cells harboring the disease-causing mutant mtDNAs showed lower oxygen consumption rates than the control cells (Fig. 1A). The addition of 40 mM taurine to the culture media increased the oxygen consumption rate in patient-derived cybrid cells, but not in control cells. Moreover, 0.3 mM taurine was also effective when the cybrid cells were cultured in limiting media lacking cysteine and methionine, which are a precursor and an intermediate, respectively, of taurine biogenesis (Fig. 1B).

Taurine improves the reduced mitochondrial membrane potential in A3243G-MELAS cells

MitoTracker Red-labeled mitochondria in the A3243G-MELAS cybrid cells displayed a weak signal with a granular appearance, suggesting that they had a decreased mitochondrial membrane potential compared to normal cells (Fig. 1C, left) (12). When the cybrid cells were cultivated in the presence of 40 mM taurine for four days, the mitochondria underwent changes in their morphology to a normal filamentous appearance, which was accompanied by an increase in the membrane potential (Fig. 1C, right) (12). The reduced mitochondrial membrane potential in the A3243G-MELAS cells was reversed by taurine in a time- and concentration-dependent manner (Fig. 1D). Moreover, 0.3 mM taurine increased the membrane potential in the A3243G-MELAS cybrids that were cultured in limiting media (Fig. 1E). In contrast, taurine did not alter the membrane potential in the control A2 cybrid cells.

Taurine improves the impaired redox status in patient-derived cells

We transfected the MELAS-cybrid cells with a gene encoding a redox-sensitive green fluorescent protein, roGFP, to monitor their redox status as judged by the ratio of fluorescence signals at 410 and 490 nm (11). The ratio in the A3243G-MELAS cybrid cells increased in comparison to that in the control cells, thus suggesting that they had an increased degree of oxidative stress (Fig. 2, upper). The addition of taurine to the culture media reduced the ratio in the A3243G-MELAS cybrid cells, but not in the control cells (Fig. 2, lower).

Taurine prevents stroke-like episodes in A3243G-MELAS patients

Case 1: A 29-year-old woman had an abrupt onset of generalized seizures and was admitted to our hospital in February 2001 (Fig. 3A). The lactate and pyruvate levels in her serum were elevated to 48.3 mg/dL (normal range, 3.0-17.0 mg/dL) and 1.7 mg/dL (normal range, 0.3-0.9 mg/dL), respectively. Brain magnetic resonance imaging (MRI) revealed a stroke-like lesion in the left occipital region (Fig. 3B). A biopsy from the left biceps brachii muscle showed a MELAS-like pattern, with cytochrome c oxidase-negative ragged-red fibers and succinate dehydrogenase-reactive blood vessels. A molecular genetic analysis of the mitochondrial DNA confirmed an A3243G transition. Treatment with coenzyme Q10 (180 mg daily) and phenytoin (250 mg daily) was commenced in February 2001. The anticonvulsant was switched from phenytoin to valproate (600 mg daily) in April 2001 because of repeated generalized seizures. A follow-up MRI in August 2001 revealed an additional right occipitotemporal lesion (Fig. 3C). The patient continued to experience epileptic seizures and had a stroke-

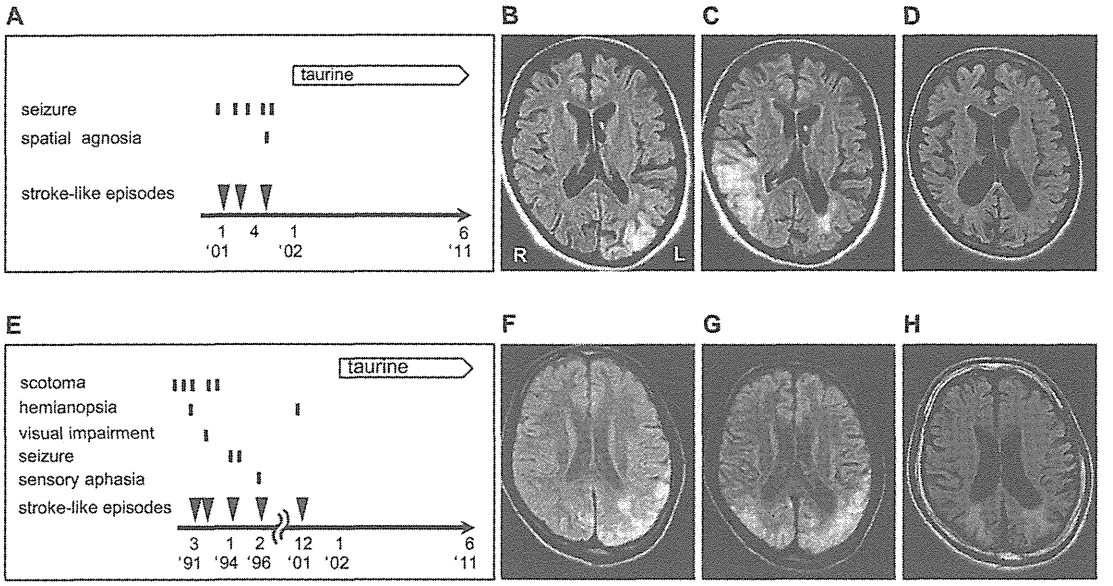

Figure 3. Oral administration of taurine reduces the stroke-like episodes in MELAS patients. The clinical courses of two MELAS patients (Cases 1 and 2) harboring the A3243G mutation in the mt $tRNA^{Leu(UUR)}$ (A, E) are shown. Taurine administration completely prevented stroke-like episodes in both patients for more than nine years. Fluid Attenuated Inversion Recovery (FLAIR) images of brain MRI revealed that multiple stroke-like lesions had developed in the occipitotemporal region before oral taurine administration (B, C, F, G). The most recent MRI showed no additional stroke-like lesions after taurine treatment in both patients (D, H).

like episode presenting hemispatial agnosia over the next seven months. Oral taurine supplementation was started in January 2002. From the beginning of the taurine treatment, her epileptic and stroke-like episodes ceased completely. In July 2007, her blood concentration of taurine was 481.3 μM, more than 5-fold higher than the normal range (39.5-93.2 μM). In December 2010, the elevated levels of serum lactate and pyruvate had declined to near normal levels, at 24.3 mg/dL and 0.9 mg/dL, respectively. The most recent brain MRI exhibited no new lesions, but mild cerebral atrophy was present (Fig. 3D). The patient has been doing well for the last nine years with the taurine treatment still ongoing.

Case 2: A 21-year-old man was admitted to another hospital in March 1991 because of repeated scintillating scotoma and right homonymous hemianopsia (Fig. 3E). He was diagnosed with A3243G-MELAS based on typical muscle biopsy findings and a mtDNA analysis. He was treated with coenzyme Q10 (120 mg/dL) and phenytoin (150 mg daily); however, he soon developed vision loss on the right side. He was admitted to our hospital in July 1991. The serum levels of lactate and pyruvate were elevated to 38.7 mg/dL and 1.2 mg/dL, respectively. The anticonvulsant was switched from phenytoin to valproate (600 mg daily) in January 1994 because of repeated generalized seizures. Over the next eight years he suffered from several stroke-like episodes, including sensory aphasia and visual impairment. Brain MRI scans in October 1991 and January 1994 revealed an accumulation of stroke lesions in the bilateral occipital regions (Fig. 3F, G). In December 2001 he had a stroke-like episode presenting with left hemianopsia. Taurine supplementation was started in January 2002, and since then, no stroke-like episodes have occurred. In September 2007, his blood taurine concentration was 996.0 μM, approximately 10-fold higher than the normal range. In February 2010, the serum values of lactate and pyruvate had declined to 29.1 mg/dL and 0.38 mg/dL, respectively. The most recent brain MRI exhibited no additional stroke-like lesions (Fig. 3H).

Discussion

Post-transcriptional modifications at the wobble nucleotide are crucial for the maturation mechanisms of tRNAs and they are required for the correct decoding of codons. In A3243G-MELAS patients, the taurine modification is defective at the wobble nucleotide in the mutant mt $tRNA^{Leu(UUR)}$ (5). In the present study, we showed that taurine ameliorates the mitochondrial dysfunction in patient-derived pathogenic cells carrying mutant $tRNA^{Leu(UUR)}$, but did not reinforce the normal mitochondrial function in control cells. Oral taurine administration also achieved long-term prevention of stroke-like episodes in two patients with MELAS.

We previously showed that when taurine (τ) is added to the culture media of HeLa cells, it is transported to the mitochondria and used as a substrate to synthesize taurine-modified uridine, 5-taunomethyluridine (τm^5U), in mt $tRNA^{Leu(UUR)}$ (Fig. 4A) (1, 4-7). Considering that τm^5U formation proceeds through an enzymatic reaction, the present results suggest that an increased concentration of taurine ac-

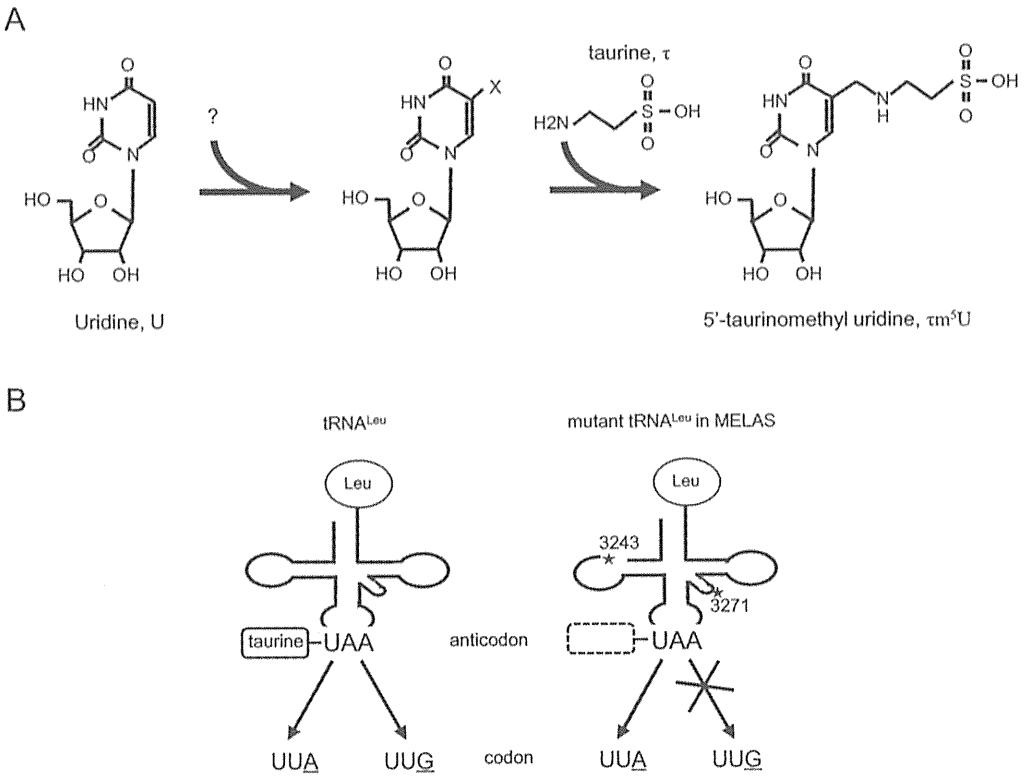

Figure 4. A proposed pathomechanism of MELAS, an RNA-modification disorder. (A) A mechanism of post-transcriptional taurine modification at the first wobble anticodon [uridine (U)] in normal mt $tRNA^{Leu(UUR)}$. Taurine (τ) is incorporated into the C5 position of the uracil ring to generate the final modification product, 5-taurinomethyluridine (τm^5U) (4). (B) Taurine modification functions to stabilize the wobble anticodon-codon pairing. Normal mt $tRNA^{Leu(UUR)}$, with a taurine-modification at the wobble uridine (U), efficiently pairs with codons UUA and UUG (right). In contrast, the MELAS-causing mutant mt $tRNA^{Leu(UUR)}$ lacks the wobble taurine modification, resulting in a specific reduction of UUG codon-specific translation but not UUA codon-specific translation. Defective taurine modification in the mutant mt $tRNA^{Leu(UUR)}$ results in a deficiency in mitochondrial protein synthesis caused by an inability to decipher codons (left) (7).

celerates the enzymatic formation of τm^5U, thereby reversing impaired codon recognition by the mutant mt $tRNA^{Leu(UUR)}$ (Fig. 4B). The pathogenic mutations in MELAS and MERRF might hinder the specific recognition by an RNA-modifying enzyme (4-7). Further studies will be required to clarify the precise molecular mechanisms underlying the wobble taurine modification in mt $tRNA^{Leu(UUR)}$, and how much supplemented taurine incorporates into the wobble uridine in mutant mt $tRNA^{Leu(UUR)}$ in clinical samples from patients.

Low plasma concentrations of taurine induce cardiomyopathy in cats. This particular species has no biosynthetic pathway for endogenous taurine (14). In agreement with our results, high-dose oral administration of taurine to cats increased the plasma and cardiac concentrations, and ameliorated the cardiac dysfunction. Because the cardiac muscles are composed of slow myofibers that are rich in mitochondria (14), taurine supplementation could alleviate the cardiomyopathy via increased τm^5U formation in mt tRNAs.

The present results provide new insight into our understanding of MELAS, and possibly MERRF, as putative RNA-modification disorders that lack the wobble taurine modification. Our results also suggest that the oral administration of taurine may be an effective therapy for these disorders.

The authors state that they have no Conflict of Interest (COI).

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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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Lactate-to-pyruvate ratio

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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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

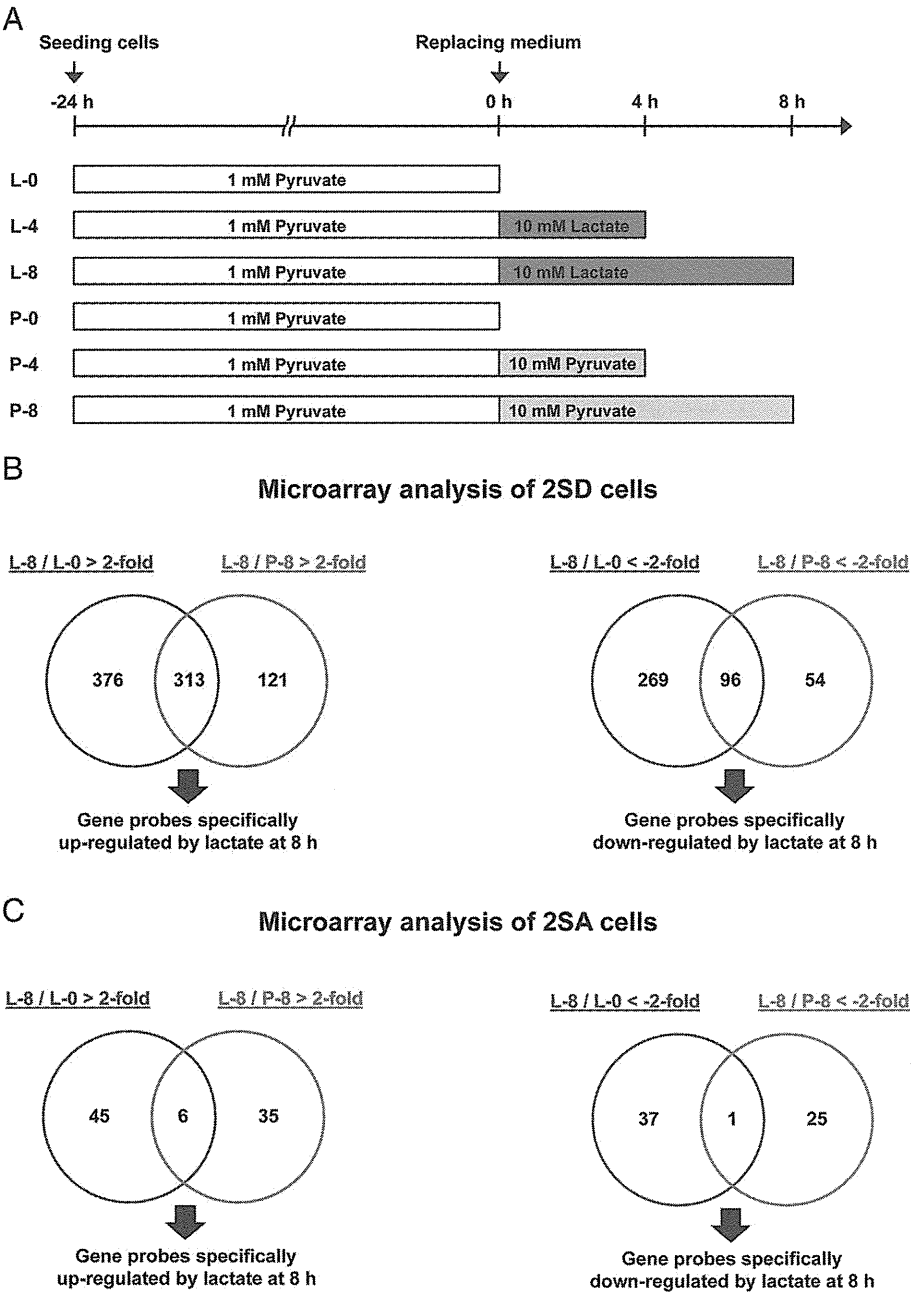

Fig. 1. Microarray analysis of 2SD and 2SA cells (A) Diagram of treatment protocols. Total RNA isolated from 2SD and 2SA cells treated with 10 mM lactate or 10 mM pyruvate for 0, 4, or 8 h were subjected to microarray analysis ($n = 2$). (B, C) Venn diagrams show the number of probes for genes in 2SD 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

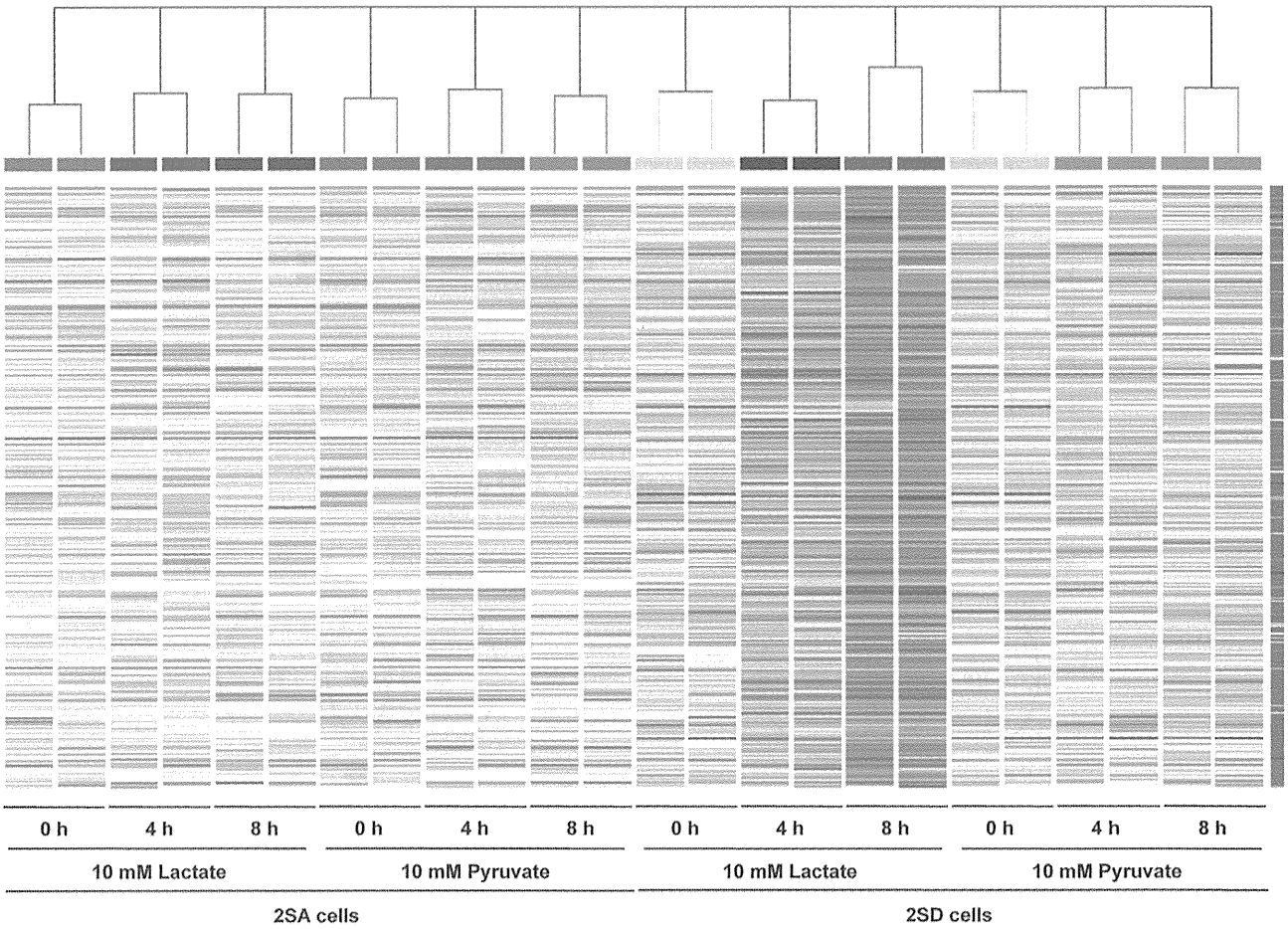

Fig. 2. Clustering analysis of the microarray data The gene probes up-regulated ($n = 313$) and down-regulated ($n = 96$) at 8 h after lactate treatment were subjected to clustering analysis. Part of the data are shown. (For interpretation of the references to colour in this figure, the reader is referred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

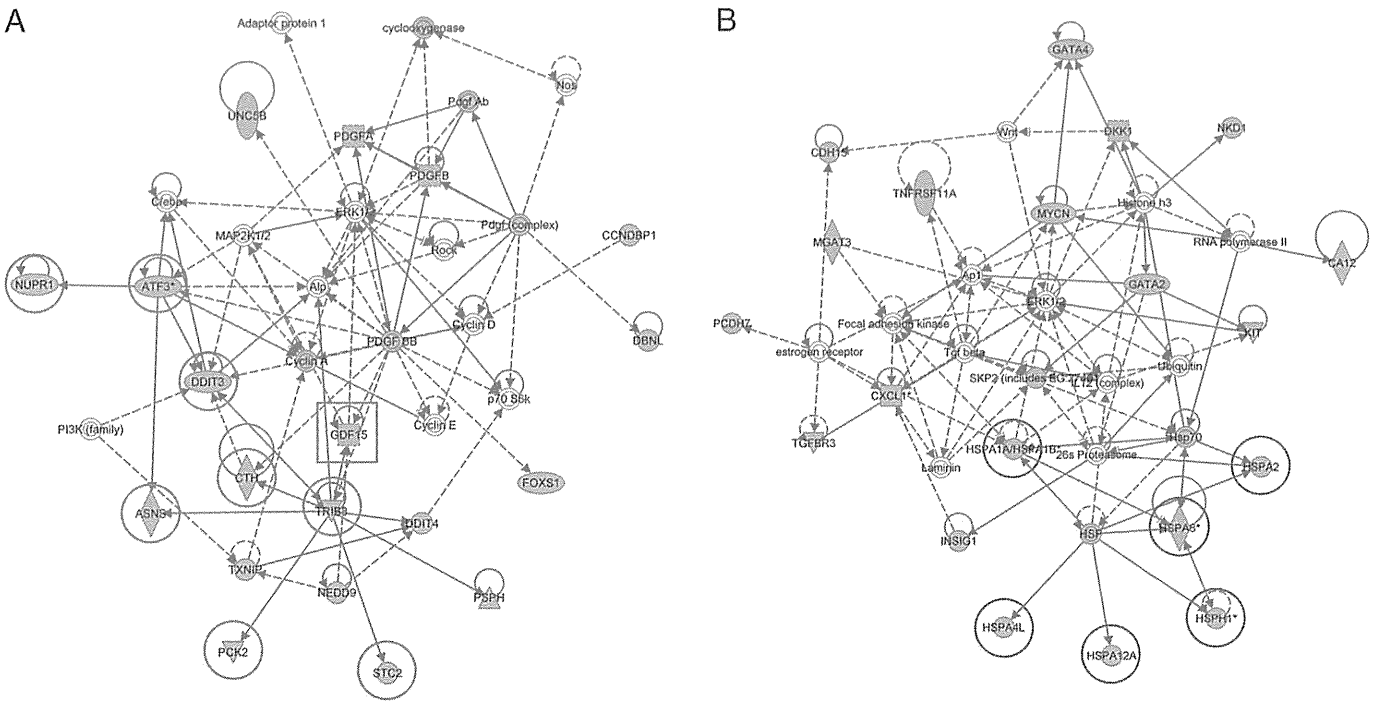

Fig. 3. Gene network analysis of the microarray data The genes specifically up-regulated ($n = 231$) and down-regulated ($n = 75$) at 8 h after lactate treatment were subjected to gene network analysis. The top-ranked gene networks in terms of the number of genes included are shown for up-regulated (A) 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.)