Table 1
Patients' profile. The severity of mental retardation was assessed by the developmental quotient (DQ) test; profound: DQ < 20, severe; DQ < 35, moderate; DQ < 50, mild; DQ < 70.

	Gender	Age	Underlying disease	Mental retardation	Epilepsy classification	Seizure type	Seizure frequency	Number of AED used previously	The duration on the diet	Seizure frequency after 3 weeks	Seizure outcome at final visit
Pt-1	M	1y6 m		Profound	SLRE	CPS	3/week	8	7 days	–	
Pt-2	F	1y6 m	Trisomy 21	Severe	SGE (IS)	Tonic spasms	>50/day	4	6 months (ongoing)	Unchanged	Seizure-free
Pt-3	M	1y11 m		Profound	SGE (IS)	Tonic spasms	1–5/day	7	8 months (ongoing)	Seizure-free	Seizure-free
Pt-4	F	3y	TS	Profound	SGE (LGS)	Tonic	1–5/day	7	7 days	–	
						Atypical absence	1–5/day			–	
Pt-5	F	3y		Severe	SGE (Doose)	Myoclonic	1–5/day	6	1 months	Unchanged	Unchanged
						Myoclonic astatic	1–5/day				
Pt-6	F	5y		Profound	SLRE	Tonic	Many/day	8	2 years	75% decrease	Recurrence
						NCSE	Many/day			Seizure-free	Seizure-free
						Hypermotor	1–2/day			Seizure-free	Recurrence
Pt-7	F	5y	SBH	Mild	SLRE	GTC	1/week	3	5 months	Seizure-free	Recurrence
						NCSE	Many/day			Seizure-free	Recurrence
						Drop	Many/			Seizure-free	Recurrence
Pt-8	M	7y		Mild	SLRE	Tonic	3/day	10	3 weeks	Unchanged	
Pt-9	F	11y	TS	Moderate	SGE (LGS)	Tonic	1–5/week	12	14 days	–	
						Atypical absence	1–5/day			–	
Pt-10	F	17y	TS	Profound	SGE (LGS)	Tonic	1–5/day	11	3 weeks	Unchanged	
						Atypical absence	10/day			Unchanged	

M, male; F, female; TS, tuberous sclerosis; SBH, subcortical band heterotopia; 15q inv dup synd; 15q inversion duplication syndrome; SLRE, symptomatic localization-related epilepsy; SGE, symptomatic generalized epilepsy; IS, infantile spasms; LGS, Lennox-Gastaut syndrome; Doose, Doose syndrome; CPS, complex partial seizure; NCSE, nonconvulsive status epilepticus; GTC, generalized tonic clonic seizure.

Table 2
Daily diet composition based on the mean (standard deviation) amount of each of the dishes prepared at our hospital for patients. Pt-2 required step-down of the amount of carbohydrate in the MAD; 30P-20 to 10 g per day, and we calculated the data when the diet contained 10 g carbohydrate.

	Total calories (kcal) mean ± SD	Carbohydrates (g) mean ± SD	Proteins (g) mean ± SD	Fats (g) mean ± SD	Ketogenic ratio mean ± SD
Pt-1	796 (42)	8.9 (0.7)	27.3 (2.8)	70.9 (3.8)	1.97 (0.18)
Pt-2	885 (55)	10.6 (0.2)	36.2 (9.0)	75.1 (4.6)	1.66 (0.32)
Pt-3	1043 (73)	10.6 (0.2)	47.1 (10.2)	87.7 (7.6)	1.57 (0.34)
Pt-4	989 (33)	10.1 (0.4)	37.1 (3.3)	86.9 (3.3)	1.85 (0.15)
Pt-5	1059 (42)	10.3 (0.4)	51.2 (1.6)	87.5 (4.5)	1.42 (0.09)
Pt-6	1098 (50)	10.5 (0.3)	53.3 (6.9)	90.7 (4.9)	1.44 (0.22)
Pt-7	1302 (20)	10.3 (0.4)	54.5 (4.5)	112.4 (2.8)	1.75 (0.16)
Pt-8	1471 (54)	10.5 (0.4)	74.2 (9.6)	121.7 (5.8)	1.46 (0.23)
Pt-9	1514 (33)	9.4 (0.2)	59.7 (15.6)	132.9 (7.2)	2.04 (0.56)
Pt-10	1438 (21)	10.3 (0.5)	72.7 (6.5)	118.7 (3.0)	1.44 (0.14)

SD, standard deviation.

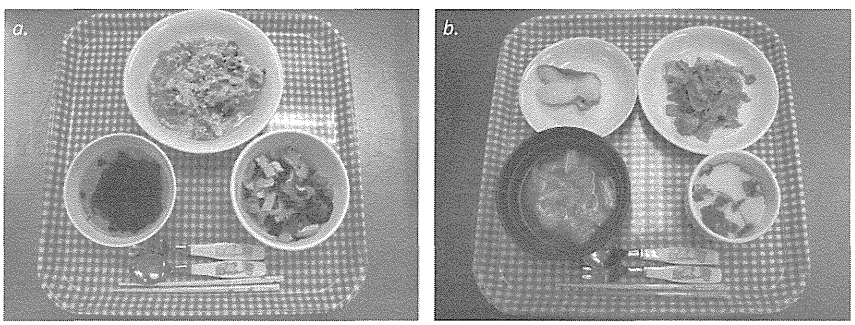

Fig. 1. Two examples of the Japanese-style lunch menu for pt-6. If rice had been added to the lunch menu, it would have been impossible to distinguish between a normal Japanese lunch and our MAD menu. (a) This lunch contained 16.5 g protein, 41.9 g fat, and 3.9 g carbohydrate, with a total calorie content of 471 kcal. The dishes illustrated in the figure: Upper: *Yanagawa*-style pod of beef: egg soup with beef and burdock. Left lower: Clear soup with seaweed. Right lower: Boiled *komatsuna* (mustard spinach) with soy sauce (*Yanagawa-style pod* originates from a local traditional food in Yanagawa city, Fukuoka). (b) This lunch contained 26.5 g protein, 35.8 g of fat, and 3.9 g carbohydrate, with a total carolie content of 460 kcal. Left upper: Japanese bluefish grilled with salt. Right upper: Sauteed pork with cabbage. Left lower: *Miso* (soybean paste) soup. Right lower: *Nanzenji temple-mushi*: savory steamed egg and tofu custard with assorted ingredients (The name, '*Nanzenji temple-mushi*' which contain steamed tofu, originates from Nanzenji temple in Kyoto, which is a historic old temple built 700 years ago, and now is very famous for both tourist attractions and tofu hot pot. Tofu hot spot is originally from veggy food for priests at Nanzenji temple).

unable to consume the MAD during the first week because of rotavirus enterocolitis. Pt-4 suddenly developed marked irritability and fatigue without hypoglycemia, and was reluctant to eat her meals any more. Therefore, her parents declined the MAD on the seventh day. Pt-9 was reluctant to eat her meals from the start and ate only half of her meals every day; therefore, her parents declined the MAD after 2 weeks. Pt-2 discontinued the MAD on the third day because she developed marked generalized fatigue with severe metabolic acidosis (pH 7.20, HCO3 9.1 mmol/L). Ten days later, however, she resumed the MAD with 30 g carbohydrate daily for 1 week and 20 g carbohydrate daily in the following week, and then, 10 g as in the original program. She did not develop fatigue or severe acidosis this time. Her carnitine profile was normal. In two patients (pt-8 and 10) who were able to continue adhering to the MAD for 3 weeks, the seizure frequency did not improve, therefore, they discontinued the diet at discharge. As a result, 3 patients (pt-3,6 and 7) who showed the tolerability and efficacy, and 2 patients (pt-2 and 3) who showed the tolerability but no efficacy decided to continue the diet after the discharge.

During the 3 weeks, there were no increase in the seizure frequency, constipation, kidney stones, or weight loss. The laboratory changes at the baseline and after 3 weeks on the diet are listed in Table 3. The laboratory data of pt-2 who started the MAD with 30 g carbohydrate daily were excluded from Table 3. Significant increase in the serum levels of total-cholesterol, LDL- cholesterol, triglycerides, blood urea nitrogen, and uric acid were observed in the third week. Of note, the mean values of total-cholesterol (272 mg/dL, normal <214 mg/dL), LDL-cholesterol (172 mg/dL, normal <139 mg/dL), and triglycerides (269 mg/dL, normal <146 mg/dL) after 3 weeks on the diet were elevated above the normal ranges. In pt-2, uric acid was elevated at 7.6 mg/dL, and she was treated with allopurinol.

The serum beta-hydroxybutyrate level increased to over 2000 μmol/L in all patients at 1 week on the diet (data not shown), and could be maintained at the third week in all but one patient whose level reduced to

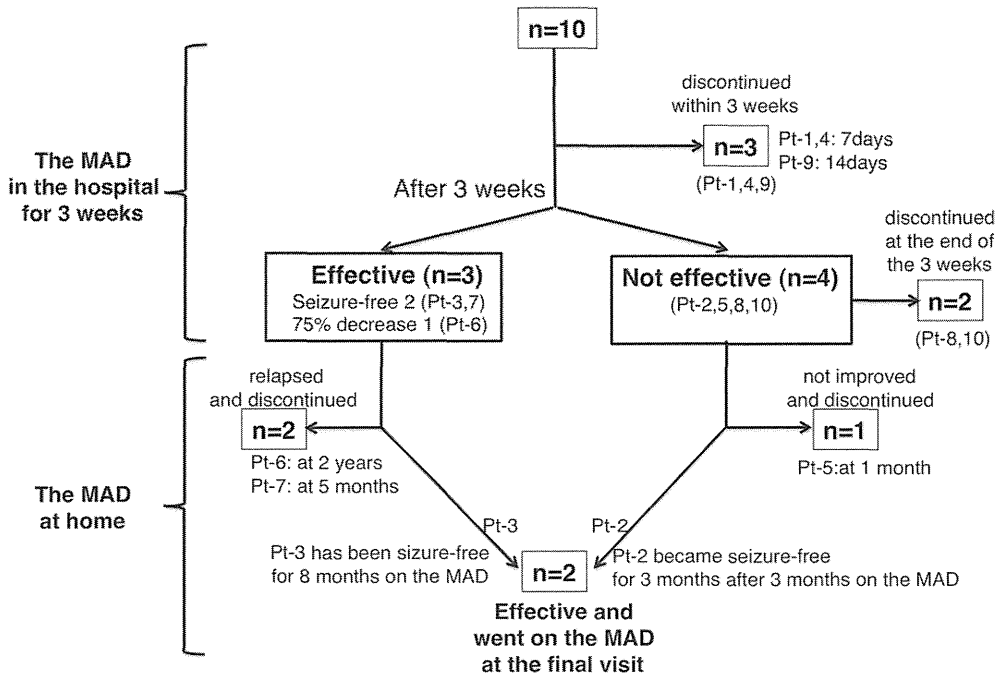

Fig. 2. The flow chart of the process of the MAD therapy.

479 μmol/L without achieving a reduction of seizure frequency (pt-8).

3.4. Treatment efficacy on seizures at the end of '3-week MAD program'

As was shown in Table 1 and Fig. 2, the diet could be maintained for all the 3 weeks in 7 of the patients. Three of these patients (pt-3, 6, and 7) showed marked decrease in seizure frequency after 3 weeks on the diet, and two of the three (pt-3 and 7) became seizure-free. Of note, nonconvulsive status epilepticus (NCSE) in both pt-6 and pt-7 could be successfully controlled by the MAD. The intervals from the start of the diet to the reduction of the seizures in pt-3, 6 and 7 were 4, 5 and 10 days, respectively.

3.5. Treatment efficacy on seizures in the children who continued the MAD after discharge

The MAD was continued after discharge from the hospital in 5 of the patients (pt-2,3,5,6 and 7) as shown in Fig. 2. Pt-2 became seizure-free 3 months after the start of the diet with carbohydrate restriction 10 g daily and remained seizure-free more than 3 months under the diet therapy. Pt-3 remained seizure-free for more than 8 months under the diet. Pt-5 discontinued the diet at 1 month after discharge because of the lack of any observable seizure reduction. Pt-6 discontinued the diet after 2 years, when the seizures reappeared. Several AEDs such as lamotrigine and topiramate were added to pt-6 after discontinuing the diet, however, she was still suffering from seizures at the final visit. Pt-7 developed recurrence

Table 3
The change in median (range) laboratory values at the baseline and after 3 weeks on the diet in 6 patients who could complete the 3-week MAD program (Pt-3,5,6,7,8 and10). The laboratory data of pt-2 who started the MAD with 30 g carbohydrate daily were excluded from Table 3. All the samples were obtained 2 h after breakfast and medication.

Laboratory value ($n = 6$: pt-3,5,6,7,8,10)	Base line	After 3 weeks	p Value
Aspartate aminotransferase	21 (19–39)	24.5 (22–32)	NS
Alanine aminotransferase	10 (8–20)	14 (11–16)	NS
Glucose 89 (70–98)	79 (68–94)	NS	
Total-cholesterol	159.5 (141–212)	272 (203–330)	<0.01
Low-density lipoprotein cholesterol	78 (72–118)	172 (123–221)	<0.01
High-density lipoprotein cholesterol	64 (53–86)	64.5 (47–93)	NS
Triglycerids	64 (43–84)	269 (76–449)	<0.01
Blood urea nitrogen	11.2 (8.2–15.5)	18.2 (14.8–21)	<0.01
Uric acids	4.1 (3.4–4.5)	5.5 (4.9–6.2)	<0.01
Beta hydroxybutyrate	122.5 (44–400)	3438.5 (479–6268)	<0.01

NS, no significant differences.

of the seizures by 5 months on the diet when her carbohydrate intake increased to 30 g daily, and she eventually discontinued the diet because her parents decided that she would undergo corpus callosotomy at another hospital. In total, 4 of the patients obtained remarkable seizure reduction with the MAD, although relapse occurred in 2 of the patients, at 5 months and 2 years after the seizure reduction, respectively.

4. Discussion

In Japan, the KD is not so commonly used in the treatment of epilepsy as in Western countries because the diet is very different from traditional Japanese food. Although the KD was introduced as early as in 1950's in Japan, only a limited number of institutions have employed the KD for the treatment of epilepsy, as the role of the KD became less prominent with the development of a variety of new AEDs [9]. The recent worldwide 'KD boom' originating from Johns Hopkins Hospital has drawn the attention of Japanese physicians and parents of Japanese children with epilepsy, however, the KD is still thought to be poorly tolerated by Japanese, whose customary diet contains less fat and more carbohydrate than Western foods.

The MAD was developed at Johns Hopkins Hospital as a more palatable and less restrictive diet for the treatment for medication-resistant epilepsy compared with the classical KD [4,5]. The MAD might be more acceptable than the classical KD among the Japanese people as it is easier to prepare and is closer in composition to the traditional Japanese food.

All 10 patients and their families in this study accepted the 3-week admission program for the initiation of the MAD, although not all of them accepted or tolerated the diet itself. The dietitians designed many Japanese menus; for example, the *Yanagawa*-style pod of beef (egg soup with beef and burdock) as shown in Fig. 1(a) and *Nanzenji temple-mushi* (savory steamed egg and tofu custard with assorted ingredients) shown in Fig. 1(b) were much like traditional Japanese foods. The dietary compositions of the MAD menus prepared by us were as ketogenic as those prepared in Western countries. The ketogenic ratio calculated retrospectively was in the range of 1.42–2.04 (median: 1.62), similar to that (about 1.0–2.0) reported in a previous review [10].

Only 2 children withdrew from our diet program before the end of 3 weeks because of dislike for the menus. When we explained about the MAD to the parents of the children with epilepsy, many expressed doubts whether their children would accept the MAD because their children would not tolerate a change from their usual carbohydrate-rich diet. However, our results showed that only 2 children would not accept the diet. Amari et al. [11] demonstrated that children with seizures exhibited significantly greater preference for fat-rich versus carbohydrate-rich foods as compared to control subjects, and that their parents underestimated the preferences. Therefore, it is very important to give children with epilepsy and their parents the opportunity at least to try the MAD in an attempt to decrease the seizure frequency.

Two patients (pt-2 and 4) in our study developed severe side effects. Pt-2 developed generalized fatigue with severe metabolic acidosis on the third day of the diet. After discontinuing the diet for 2 days, the MAD was resumed with 30 g carbohydrate for 1 week, 20 g carbohydrate in the following week, and then the patient could eventually continue the diet without developing acidosis with the carbohydrate restriction to 10 g daily. Her underlying disease was trisomy 21 and the laboratory data ruled out latent contraindications for the KD, such as inborn errors of fatty acid metabolism [12]. Our experience suggested that in some patients, MAD may need to be started with 20 or 30 g carbohydrates to ensure safety, although starting with 10 g carbohydrate may be the most suitable from the point of view of early seizure suppression [13]. Pt-4 suddenly developed marked irritability and fatigue, and was reluctant to eat her meals at the seventh day of the diet with unknown cause. She could not resume the MAD with 20 or 30 g carbohydrate because her parents declined.

Laboratory evaluation performed 3 weeks after the initiation of the diet revealed abnormal increases of the serum total cholesterol, LDL-cholesterol and triglycerides, however, these values subsequently decreased gradually even in the patients who continued adhering to the MAD for long periods of time, such as pt-3 and pt-6.

The types of seizure in our cases that could be controlled by MAD were tonic spasms, tonic seizures, generalized tonic-clonic seizures, drop attacks, hypermotor seizures, and nonconvulsive status epilepticus (NCSE). The fact that the NCSE in both pt-6 and pt-7 could be controlled by the MAD, which we described in detail in a previous report [14], was of great interest. NCSE, diagnosed as complex partial seizure epilepticus based on ictal EEGs, was characterized by intermittent loss or reduction in the level of consciousness throughout the day, while the patient was awake, for several months. It was completely abolished clinically and electrically by 5–10 days after the initiation of the MAD.

Seizure reduction was obtained within 10 days of starting the diet in three patients, while it took 3 months along with carbohydrate restriction to 10 g in one patient. A previous report [15] showed that the KDs work quickly when effective, typically within the first 1–2 weeks, in which 75% (74/99) of the patients showed improvement within the first 14 days, and 90% (89/99) within 23 days of the start of the KD. Thus, the 3-week duration of the MAD therapy program in our study was reasonable for determining the efficacy of MAD in controlling seizures, although the beneficial effect would be

missed in rare cases such as pt-2 of our study in whom KDs work more slowly.

In the case of pt-2, the parents decided to continue with the diet even after discharge despite the lack of change of the seizure frequency, because they were convinced that the diet was worth complying with. They persevered with the diet at home with the eager support from our dietitians, which could have led to the seizure cessation observed about 3 months after the initiation of the diet.

Thus, a good rapport between the patient's families and the professionals responsible for implementing the diet is probably a major factor in determining continued compliance with the diet for a long period of time.

In conclusion, the findings of this study revealed that the MAD appeared to be acceptable to a high proportion of Japanese children with epilepsy and their parents. Major reduction in the seizure frequency, and even complete freedom from seizures in some cases, was achieved in the children initiated on the MAD.

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Pyruvate therapy for mitochondrial DNA depletion syndrome ☆

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ABSTRACT

Background: Mitochondrial DNA depletion syndromes are a group of heterogeneous autosomal recessive disorders associated with a severe reduction in mitochondrial DNA in the affected tissues. Sodium pyruvate has been reported to have a therapeutic effect in mitochondrial diseases.
Methods: We analyzed the effects of 0.5 g/kg of sodium pyruvate administered through a nasogastric tube in a one-year-old patient with myopathic mitochondrial DNA depletion syndrome. To evaluate the improvement, we used the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) and manual muscle testing. As the improvement of motor functions in this severely disabled infant could not be comprehensively detected by NPMDS, we also observed the infant's ability to perform several tasks such as pouting, winking, and number of times she could tap a toy xylophone with a stick. Blood lactate and pyruvate levels were also monitored.
Results: After one month's treatment, the NPMDS score in section IV, the domain for the quality of life, improved from 17 to13. The infant became capable of raising her forearm, lower leg and wrist against gravity. The maximum number of times she could repeat each task increased and the movements became brisker and stronger. No significant change of the blood lactate level or lactate-to-pyruvate ratio, both of which were mildly increased at the initiation of the therapy, was observed despite the clinical improvement.
Conclusion: Sodium pyruvate administered at 0.5 g/kg improved the muscle strength and the NPMDS score of an infant with myopathic mitochondrial DNA depletion syndrome.
General significance: Sodium pyruvate may be effective for ameliorating the clinical manifestations of mitochondrial diseases. This article is part of a Special Issue entitled: Biochemistry of Mitochondria.
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1. Introduction

Mitochondrial DNA depletion syndromes (MDSs) are a heterogeneous group of autosomal recessive disorders manifesting mainly in infancy and childhood that are associated with a severe reduction of the mitochondrial DNA (mtDNA) copy number in the affected tissues [1]. Three different clinical forms of MDSs have been described: myopathic, encephalomyopathic, and hepatocerebral MDSs [2–6]. The clinical phenotypes can overlap and patients with myopathic MDS could develop encephalomyopathic MDS at a later date. Several causes of MDSs, which affect the mtDNA replication and maintenance, have been reported. These include defects of enzymes affecting the nucleotide pools (mitochondrial thymidine kinase, deoxyguanosine kinase, ribonucleotide reductase p53-R2 subunit and thymidine phosphorylase), defects of mtDNA replication proteins (mtDNA polymerase gamma and Twinkle), defects of succinyl-CoA ligase, which interacts with mitochondrial nucleotide diphosphate kinase, and defects of proteins of unknown function, including MPV 17 [5].

Like all of the other mitochondrial respiratory-chain disorders, there are no curative therapies for MDSs. For mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), however, which is associated with disturbance of the nucleotide pools, treatments that reduce the circulating levels of nucleotides can improve the symptoms, including peritoneal dialysis [7], enzyme replacement therapy [8] and allogenic stem cell transfusions [9]. Unfortunately however, such treatments cannot be applied to other types of MDSs. Treatments with vitamins, cofactors and respiratory substrates may improve some symptoms, however, the efficacy is limited.

Tanaka et al. recently reported the therapeutic promise of pyruvate for mitochondrial diseases [10]. According to their theory, pyruvate supplementation would improve the intracellular redox state by

Abbreviations: MDS, mitochondrial DNA depletion syndrome; L/P, lactate-to-pyruvate

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providing NAD^+ and reducing the lactate-to-pyruvate (L/P) molar ratio, which is high in cells with mitochondrial respiratory deficiency; as a result, ATP production by the glycolytic pathway would improve. In a preliminary study, they administered 5 g of sodium pyruvate to an adult patient with chronic progressive external ophthalmoplegia associated with mtDNA deletion. At 30 min after the administration of pyruvate, the blood lactate level decreased from 2.42 mM to 2.10 mM and the L/P ratio decreased from 25.65 to 16.29. No clinical improvement was, however, described in this report. So far, case reports on pyruvate therapy for mitochondrial diseases are very limited, and the efficacy of this treatment still remains inconclusive.

In the present report, we describe the clinical course of a one-year-old patient with myopathic MDS who was treated with sodium pyruvate, and discuss the efficacy of this newly proposed therapy for amelioration of the clinical manifestations of mitochondrial disorders.

2. Patients and methods

2.1. Patient

A one-year-old girl was born by Cesarean section (indication: breech presentation and placenta previa) to non-consanguineous parents at 37 weeks of gestation; the birth weight was 2970 g and the Apgar scores were 8 and 9. The family history was non-contributory. The infant began to have feeding difficulty on postnatal day 3 and developed respiratory failure and lactic acidosis (11.3 mM; normal range, 0.33–1.9 mM) on 10 days of age. She has been on a respirator ever since. The blood level of creatine phosphokinase was 3158 IU/L on postnatal day 3, but normalized later. There was no evidence of hepatomegaly and the blood levels of aspartate amino trasferase and alanine transaminase were mildly elevated (50 and 30 IU/L, respectively). Blood ammonia levels, acylcarnitine profile and urinary organic acids were normal. With improvement of the respiratory failure by mechanical ventilation, the blood lactate levels decreased, but remained between 3.0 mM and 6.5 mM, with high L/P ratios (between 36 and 97; normal <15), consistent with the diagnosis of a mitochondrial respiratory chain disorder. The lactate and pyruvate levels in the cerebrospinal fluid (CSF) were 4.2 mM and 0.18 mM, respectively with an L/P ratio of 23. Brain MRI at the age of 7 months showed mild dilatation of the lateral ventricles without any abnormal signals in the parenchyma. Treatment with coenzyme Q, thiamine, ascorbic acid and l-carnitine at the age of 3 months decreased the blood lactate levels (to between 1.4 mM and 3.1 mM), however, the L/P ratios remained high (between 16 and 45). The severe motor weakness and respirator dependence did not improve with this treatment.

Muscle biopsy performed at 10 months of age showed mild variations of the fiber size and predominance of the type 2A/2B fibers, comprising 71% of the fibers. A significant number of type 2C fibers were also found (22%). All fibers showed lipid droplets and glycogen accumulation. Ragged red fibers were found, however, strongly succinate dehydrogenase-positive vessels were not found. Cytochrome c oxidase staining was decreased, but not absent, in most fibers (Fig. 1).

Biochemical analysis of the respiratory chain enzymes in the muscle specimen revealed deficiencies of complex I (CoI), III (CoIII) and IV (CoIV), that were confirmed by the assay against citrate synthase (CS) or complex II (CoII) [11]: the activities of CoI, CoIII and CoIV relative to the activity of CS were 10.6%, 26.7% and 14.1%, respectively, and those relative to the activity of CoII were 6.5%, 16.4% and 8.8%, respectively (definite deficiency; <30% of CS or CoII).

Quantitative analysis of the mitochondrial DNA by real-time PCR [12] revealed that the ratio of the copy number of the mitochondrial NDI subunit relative to the nuclear CETR gene was 35.3% (normal; >40%), indicative of mitochondrial DNA depletion. Mutation analysis is underway.

The patient showed slowly progressive motor regression despite the treatment; by the age of 12 months, she lost the ability to smile, hold her arms above her chest against gravity or raise the lower legs, all of which she had been able to do until 8 months of age. At the age of 12 months, the patient was referred to our hospital for further treatment. Physical examination on admission showed severe

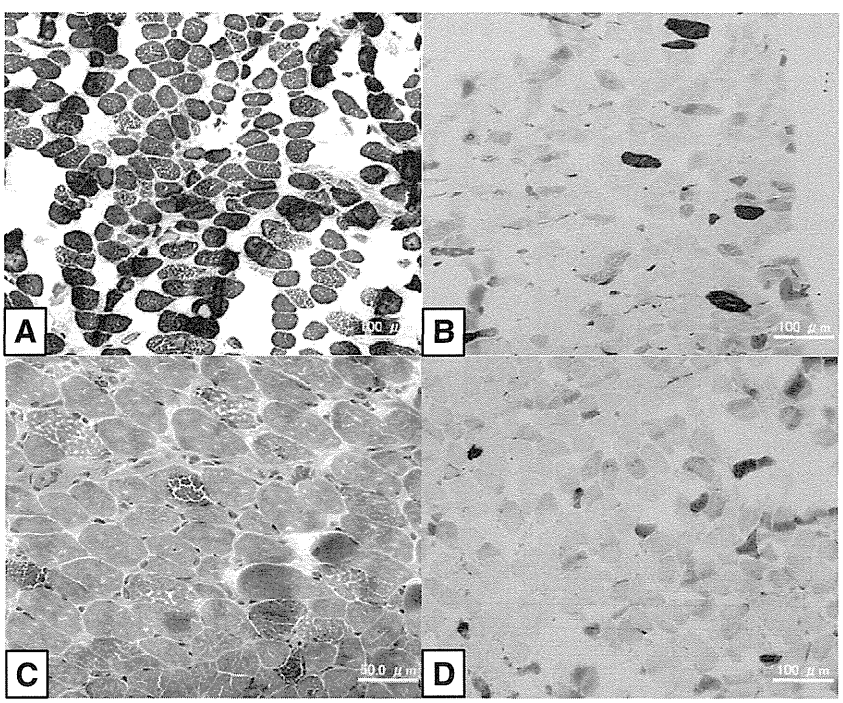

Fig. 1. Histochemistry of the biopsied muscle. ATPase staining shows type 2 fiber predominance at pH 10.6 (A) and an increased number of type 2C fibers at pH4.2 (B). The percentages of type 1, 2A/B and 2C fibers were 7%, 71% and 22%, respectively. Modified Gomori trichrome staining (C) shows scattered ragged-red fibers. Cytochrome *c* oxidase staining (D) shows decreased, but not absent, staining in most fibers.

generalized hypotonia and muscle weakness. Echocardiography was normal. She had dysphagia and was fed via a nasogastric tube. Her cognition ability seemed normal despite the mild ventricular dilatation on MRI. Her hepatic dysfunction was limited to mild elevation of the serum transaminases. Therefore, the infant was diagnosed as having myopathic-type MDS.

2.2. Pyruvate therapy

The pyruvate treatment was approved by the ethics committee of Shiga Medical Center for Children and written informed consent was obtained from the parents. Sodium pyruvate (Musashino Chemical Laboratory, Tokyo), dissolved at 0.5 g/kg in water at the concentration of 0.06 g/ml was given through a nasogastric tube in three divided doses (although the recommended concentration of sodium pyruvate to avoid osmotic diarrhea is about 0.02 g/ml, we chose the higher concentration to avoid water overload). During the pyruvate therapy, other treatments, including vitamins and coenzyme Q, remained unchanged. Pyruvate was administered throughout the study period and the effects of the therapy were examined one month and two months after the initiation of the therapy.

2.3. Evaluation of the treatment effect

To evaluate the treatment effect, we used the Newcastle Paediatric Mitochondrial Disease Scale (NPMDS) for 0–24 months [13]. The measurements were performed on the day of the start of treatment before taking the first dose of pyruvate and one month and two months after the initiation of therapy with pyruvate. Considering that the motor disabilities of this patient were probably too severe for any changes to be detected by this scale, we also tried to evaluate the changes in the motor activities or muscle power by performing manual muscle testing (MMT) on the extremities as well as observing the patient's ability to perform tasks including pouting, pulling the corner of the mouth laterally, winking repeatedly, and tapping a toy xylophone with a stick by rotating the wrist while resting the arm on the floor. These tasks were the ones which her mother had let her do almost daily either as play or as a communication tool for more than two months before the initiation of the pyruvate therapy. We coaxed her to repeat the movements as many times as possible and counted the number of times she could repeat them. The measurement of each task was conducted only once because of development of fatigue. The examination was done on the day of initiation of the pyruvate therapy and one month and two months after the treatment initiation. During the treatment, the frequency of performance of the tasks which the infant's mother let her do almost daily was the same as that before the treatment, and the patient was not particularly trained to show better performance of the tasks.

2.4. Results

The pyruvate therapy did not cause any side effects, including diarrhea. The overall NPMDS score before the treatment initiation was 35, which decreased (improved) to 31 after one month of pyruvate therapy (Table 1). However, the improvement was only observed in the domain of the quality of life (section IV of the scale), which reflects the parent's subjective opinion. The scale measured two months after the initiation of therapy was the same as the one measured after one month of therapy. We also found that the patient became able to raise her forearms briefly by about 30° after one month of treatment, and by almost 90° after two months. She regained the ability to raise and hold the lower legs briefly by 2 months after the start of the therapy. She could move the wrist only horizontally before the treatment, but became able to also move it vertically after 1 month of the treatment. These observations indicated that the power of the biceps brachii, quadriceps femoris and brachioradialis muscles increased from grade 2 to grade 3 on MMT (Table 2). One month after the start of the pyruvate therapy, the number of times of pouting increased from 6 times to 15, winking from 6 times to 10, and tapping a xylophone from 5 times to 7. She could barely move the mouth corner before and until one month after the start of the therapy; however, she could move it 8 times by the second month (Table 2). Some other improvements which we observed, but could not measure quantitatively, included extended duration of each movement such as pouting and stretching of the mouth corner, increase in the speed and strength of the tapping, as well as more vivid facial expressions.

The blood lactate levels and L/P ratios did not change with the therapy. The lactate levels measured twice on separate days before the start of the treatment were 2.1 mM and 2.5 mM, with L/P ratios of 18 and 18, respectively. The lactate levels after one month and two months of pyruvate treatment were 2.7 mM and 2.3 mM, with L/P ratios of 18 and 18, respectively.

Table 1
Changes of the NPMDS scores with pyruvate therapy.

Section	Before Tx	1 month after Tx	2 months after Tx
I	7	7	7
II	6	6	6
III	5	5	5
IV	17	13	13
Overall	35	31	31

NPMDS, Newcastle Paediatric Mitochondrial Disease Scale; Tx, treatment.

3. Discussion

Tanaka et al. proposed several possible mechanisms by which pyruvate may improve the energy metabolism in respiratory chain-deficient mitochondria (Fig. 2) [10]: (a) Pyruvate reacts non-enzymatically with hydrogen peroxide to yield acetate, carbon dioxide and water, thereby eliminating hydrogen peroxide which is increased due to leakage of reactive oxygen species from the respiratory-chain deficient mitochondria. (b) In the presence of lactate dehydrogenase, pyruvate provides NAD^+ from NADH. NAD^+ is essential for oxidation of glyceraldehyde 3-phosphate by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to form 1,3-bisphosphoglycerate, which donates a phosphate group to ADP to produce ATP. Mitochondria with respiratory-chain disturbance are deficient in NAD^+, causing inhibition of the glycolytic pathway via GAPDH and an increase in the NADH-to-NAD^+ ratio, which is equivalent to the L/P ratio. Pyruvate supply reactivates the glycolysis which is impaired secondarily due to disturbance of the respiratory chain, and lowers the NADH/NAD^+ and L/P ratio. (c) Pyruvate dehydrogenase kinase (PDK) inhibits pyruvate dehydrogenase (PDH) activity, and pyruvate inhibits PDK activity. As a result, pyruvate activates PDH.

Table 2
Changes in motor function and lactate levels with pyruvate therapy.

	Before Tx	1 month	2 months
Lip pouting	6	15	ND
Winking	6	10	11
Pulling the mouth corner	None	None	8
Tapping a xylophone with a stick	5	7	ND
Raising the forearms from the bed floor	None	30°	90°
Raising the lower legs against gravity	Barely	Possible	Can hold
Flexing the wrists against gravity	Impossible	Possible	Possible
Blood lactate level	2.5 mM	2.7 mM	2.3 mM
Lactate-to-pyruvate ratio	18	18	18

The patient was asked to repeat the tasks as many times as possible. The number of times she could repeat the tasks was observed before, one month and two months after the start of the treatment. For raising the forearms, angles from the floor at which the arms could be raised were measured. Tx, treatment; ND, not done because the patient was not willing to perform.

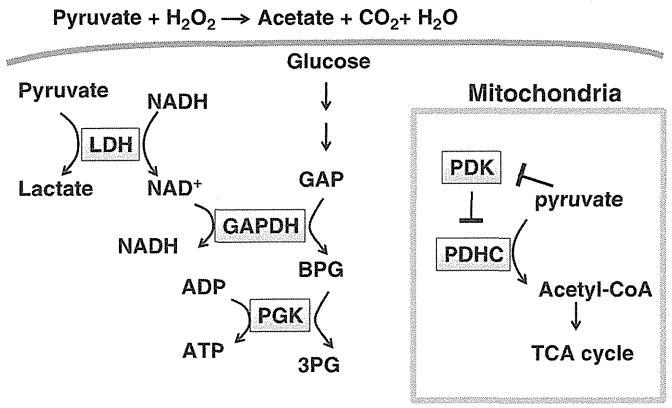

Fig. 2. Effects of pyruvate on energy metabolism and cell injury. Pyruvate eliminates hydrogen peroxide by a non-enzymatic reaction. Pyruvate provides NAD^+ from NADH with lactate dehydrogenase (LDH). In the presence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NAD^+ oxidizes glyceraldehyde 3-phosphate (GAP) to form 1,3-bisphosphoglycerate (BPG). BPG then provides its phosphate group to ADP to form ATP by phosphoglycerate kinase (PGK), and becomes 3-phosphoglycerate (3PG). In the mitochondrial matrix, pyruvate inhibits pyruvate dehydrogenase kinase (PDK) which inactivates pyruvate dehydrogenase complex (PDHC). As a result, PDH activates PDHC and provides acetyl-CoA, which enters TCA cycle.

The efficacy of pyruvate in improving the energy metabolism was observed in ρ^0 cells, which lack mtDNA. By adding pyruvate to the culture media, the ρ^0 cells survived, probably because of improved ATP production by pyruvate [14]. As the cells of the affected tissues in MDS are similar to the ρ^0 cells, it is reasonable to assume that pyruvate may be effective for ameliorating the clinical manifestations of MDS.

The weakness of the present study lies in the incomplete quantitative analysis of the treatment effect. Because of the patient's age and the severe weakness, it was not possible to measure the muscle strength accurately. As we anticipated, the NPMDS did not show any changes in scores in the domains that can show improvement in the muscle power, because the disability was too severe to allow detection of any improvement using this scale; in the domain for the current clinical assessment (section III), for example, the severity of myopathy is rated as severe when a patient is wheelchair dependent and the grade is defined as moderate when a patient has proximal weakness limiting functional movement. The improvement of the motor weakness in our patient was not sufficient to cause the rating to change from severe to moderate. However, even under this situation, the score for the quality of life showed improvement. One can argue that the improvement in the NPMDS score was due to the normal developmental process with age. However, the patient showed motor regression during the 11 months prior to the start of the treatment, and the parents noticed improvement by one month after the start of the treatment.

The tasks we chose to evaluate the muscle function can be influenced by skill rather than muscle strength. Therefore, the improvement in the performance of tasks could be simply due to a training effect, as the patient had been doing the same tasks daily. However, the patient had started to perform the tasks at least two months before the start of treatment, and no improvement was noticed during this pre-treatment period. On the other hand, improvement began to be noticed within a few weeks after the start of pyruvate therapy. Besides the improvement noted in the performance of these tasks which need skill, and may, therefore, be influenced by training, a significant increase in the muscle power in the biceps brachii, quadriceps femoris and brachioradialis muscles was observed; the patient became able to raise her forearms, lower legs and wrists against gravity, all of which she had become unable to do during the course of illness since 8 months of age. Our findings therefore suggest that the pyruvate therapy significantly improved the muscle strength and quality of life of the patient by a month after the start of treatment.

Contrary to the observed clinical improvement and the theory proposed by Tanaka et al., no significant changes of the blood lactate levels and L/P ratio were observed in this patient. One explanation for this discrepancy is that the blood lactate levels at the time of the therapy were too low (although higher than normal) to allow detection of any changes; the lactate level and the L/P ratio shortly before the start of pyruvate therapy were 2.5 mM and 18, respectively while those at the age of 3 months, by which time the patient was more active, were between 3.0 mM and 6.5 mM and 36 and 97, respectively. This apparent improvement in the blood lactate levels even before the start of pyruvate therapy might be due to the decrease in the muscle bulk as well as the severely weak muscle activity, which decreased the lactate production. Another factor which may have contributed to this discrepancy is the normal mitochondrial function in the liver. In myopathic MDS, mtDNA in the liver is not depleted; therefore, lactate released from the muscle might be metabolized in the liver, causing the blood lactate levels and L/P ratios to become near normal. On the other hand, when the lactate levels were very high at the age of 3 months, this factor did not contribute significantly. To prove that pyruvate does decrease the lactate levels and L/P ratios and increases the ATP production within the muscles, changes in these parameters in the muscles must be shown *in vivo*, possibly by magnetic resonance spectroscopy. We conducted no such evaluation in this study.

Thus, more clinical studies are necessary to precisely evaluate the efficacy of pyruvate therapy in patients with MDSs. However, there is only one published report, and several unpublished case reports on pyruvate therapy for mitochondrial diseases so far. Komaki et al. reported that an 11-year-old patient with Leigh syndrome associated with cytochrome c oxidase deficiency, who had easy fatigability and ataxic gait, became capable of participating in athletic games after treatment with oral sodium pyruvate at 0.5 g/kg [15]. They reported decrease of the blood lactate level from 2.3 mM to 1.1 mM and decrease of the L/P ratio from 18.1 to 11.7 in this patient. They also found an improvement in the cardiac dysfunction in the patient after one year's treatment. Other unpublished case reports include improvements in the MRI findings and cardiac dysfunction in a patient with Leigh syndrome (Wakamoto et al.) [15], cardiac improvement in another patient with Leigh syndrome (Koga et al.) [15], and activation of PDH activity which was estimated by measuring the $^{13}CO_2$ in exhaled air per unit time after administration of [1–^{13}C] pyruvate in two patients with PDH deficiency (Hamada et al. presented at the 52nd annual meeting of Japanese Society for Inherited Metabolic Diseases). We also treated a one-year old patient with Leigh syndrome associated with T9176C mutation in the mtDNA. The patient was severely disabled with tetraplegia at the time of the therapy, and showed no clinical improvement with pyruvate therapy. The severity of the symptoms at the time of pyruvate therapy may have differed between the patient treated by Komaki et al. and our own patient with Leigh syndrome. This finding highlights the limitation of this therapy and the possibly superior effects of the therapy in patients with an earlier stage of the disorder. No case reports on MDS are available.

Unlike non-physiological chemical drugs, such as dicholoroacetate, which can have some serious adverse effects, pyruvate is a physiological metabolite. The only possible side effects are sodium overload and osmotic diarrhea. Our patient did not develop diarrhea even though we did not dilute the sodium pyruvate as recommended, to avoid water overload. No serious adverse effects have been reported so far.

4. Conclusions

Oral (through a nasogastric tube) administration of 0.5 g/kg of sodium pyruvate improved the muscle power and quality of life of our patient with myopathic MDS. There are some case reports describing

the efficacy of pyruvate in patients with different mitochondrial diseases. Considering that pyruvate can activate glycolysis even in cells without any mitochondria, as shown in ρ^0 cells, pyruvate therapy is a promising treatment for mitochondrial diseases. More clinical and biochemical studies are necessary to clearly prove the efficacy of this treatment.

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Case report

Liver-specific mitochondrial respiratory chain complex I deficiency in fatal influenza encephalopathy

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Abstract

We report on a 4-year-old boy who died from influenza encephalopathy. The clinical course and microscopic findings of the autopsied liver were compatible with Reye's syndrome. We examined the mitochondrial respiratory chain function by blue native polyacrylamide gel electrophoresis (BN-PAGE), western blotting, and respiratory chain enzyme activity assays. The activity of liver respiratory chain complex (CO) I was markedly decreased (7.2% of the respective control activity); whereas, the other respiratory chain complex activities were substantially normal (CO II, 57.9%; CO III, 122.3%; CO IV, 161.0%). The activities of CO I–IV in fibroblasts were normal (CO I, 82.0%; CO II, 83.1%; CO III, 72.9%; CO IV, 97.3%). The patient was diagnosed with liver-specific complex I deficiency. This inborn disorder may have contributed to the fatal outcome. We propose that relying only on fibroblast respiratory chain complex activities may lead to the misdiagnosis of liver-specific complex I deficiency.
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Keywords: Influenza encephalopathy; Reye's syndrome; Mitochondria; Complex I deficiency; Liver-specific

1. Introduction

Influenza encephalopathy is a critical complication of influenza infection. Although the pathological mechanism is poorly understood, mitochondrial malfunction is suggested to play a role in the pathogenesis [1]. We describe a boy with liver-specific mitochondrial respiratory chain complex I deficiency who developed fatal encephalopathy associated with influenza A infection. The possible contribution of the mitochondrial respiratory chain disorder to the clinical course is discussed.

2. Case report

A 4-year-old Japanese boy developed pyrexia. He was treated with acetaminophen once and visited the family doctor. Influenza A infection was diagnosed by nasal antigen test in a clinic and he was treated with oseltamivir. He was admitted to a nearby hospital due to a generalized seizure in the evening; then, he was transferred to our institute because of highly elevated serum transaminase. He was the first child born to healthy parents with no consanguinity. No other child had died in early

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infancy within three degrees of relationship. He had normal psychomotor development and had not been vaccinated against influenza.

On arrival, he was comatose and had a temperature of 38.9 °C, heart rate of 136 beats per minute, and blood pressure of 106/62 mm Hg. Neither arrhythmia nor cardiac hypertrophy was seen in the electrocardiogram or echocardiography. Blood examination showed marked liver dysfunction and ammonemia (aspartate aminotransferase, 4282 IU/l; alanine aminotransferase, 1750 IU/l; ammonia, 156 μg/dl). Blood gas analysis showed marked acidosis (pH 6.964, pCO_2 59.6 mm Hg, HCO_3 11.2 mol/l, BE −23.7 mmol, and lactate 9.0 mmol/l). Blood glucose was 128 mg/dl under intravenous infusion. Influenza encephalopathy was diagnosed and intensive therapy, including mechanical ventilation, steroid, and heart stimulants, was started. A few hours later, he developed cardio-pulmonary arrest and died 36 h after developing pyrexia. This clinical course led us to suspect Reye's syndrome and mitochondrial disorders. The parents consented to resection of the patient's liver and skin fibroblasts. Urine organic acid analysis, blood amino acid profile, and carnitine profile did not show any findings suggestive of congenital metabolic disorders. Microscopical finding showed microvesicular fatty droplets in hepatic cytoplasm in hematoxylin-eosin and oil red O staining (Fig. 1), that was compatible with Reye's syndrome. The grade of histological hepatic changes was milder than the fulminant clinical course.

The activities of respiratory chain complexes (Co) I, II, III, and IV were assayed in the crude post-600 g supernatant of the liver and in isolated mitochondria from skin fibroblasts as described previously [2]. The activity of each complex was presented as a percent ratio relative to the mean value obtained from 12 healthy controls. The activities of Co I, II, III, and IV were also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker, or Co II activity [2]. Liver respiratory chain complex I activities were very low, but CS, Co II, III, and IV activities were normal. In contrast to the liver, the fibroblast complex I activity was normal (Table 1).

The expression of the mitochondrial respiratory chain Co I, II, III, and IV proteins in the liver and fibroblasts were examined by Western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to methods described previously [3]. The results of BN-PAGE are shown in Fig. 2. The band corresponding to Co I was not visible; while, the intensities of the Co II, III, and IV bands remained normal. Several base substitutions were detected by polymerase chain reaction, but there was no pathogenic mutation in the genomic DNA extracted from the autopsied liver tissue.

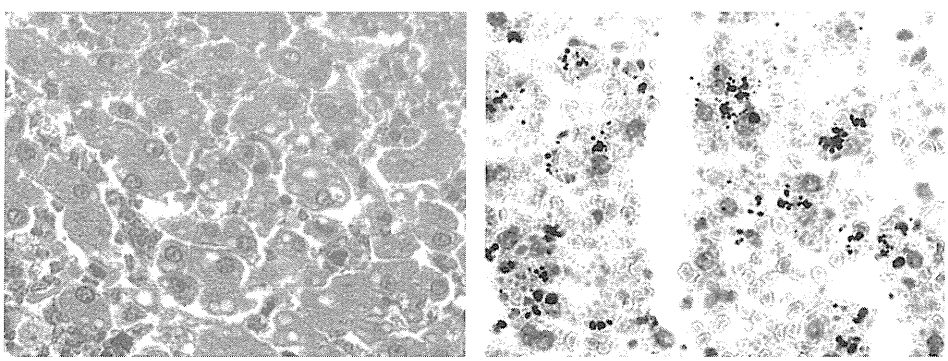
3. Discussion

Mitochondrial malfunction has been described in influenza encephalopathy. There are no reports of mitochondrial respiratory chain diseases, although disorders of fatty acid oxidation have been discussed [1]. Complex I deficiency was first recognized in 1979 by Morgan-

Table 1
Enzyme assay of respiratory chain complexes.

%	Co I	Co II	Co III	Co IV	CS
Liver					
% of normal	7.2	57.9	122.3	161.0	78.1
CS ratio	9.2	74.1	155.0	203.8	–
Co II ratio	12.3	–	212.2	272.2	–
Fibroblast					
% of normal	82.0	83.1	72.9	97.3	120.4
CS ratio	66.2	66.8	56.5	76.3	–
Co II ratio	98.2	–	83.7	112.5	–

Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthase.
Enzyme activities are expressed as a % of the mean relative activity of the normal control and relative to CS and Co II.



A. Hematoxylin-Eosin staining (× 400) B. Oil Red O staining (× 400)

Fig. 1. Autopsy liver samples show preserved hepatic architecture with scattered distribution of micro-vesicular fatty droplets in the hepatic cytoplasm (A). Marked congestion, focal necrosis, and mild inflammatory cellular infiltration without fibrosis were noted. Fat deposition was also suggested with oil red O staining (B). The grade of histological hepatic changes was milder than the fulminant clinical course.

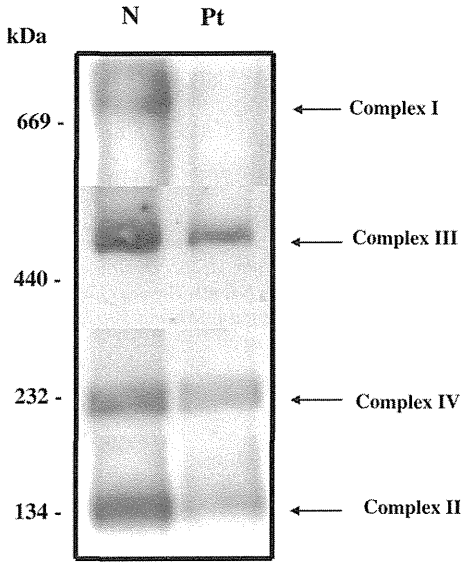


Fig. 2. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of liver respiratory chain enzymes showed markedly decreased protein expression of complex I, while the protein bands of complex II, III, and IV were comparable to the control (N) samples.

Hughes; yet, studies have not progressed because of technical difficulties. More recently, complex I deficiency was regarded as the most common energy generation disorder. The manifestations range from typical mitochondrial diseases, such as Leigh syndrome, to obscure conditions such as slow regression or intractable secretory diarrhea [4].

Complex II activity has been shown to be more labile than complex I when measuring respiratory chain enzymes in patients with a wide range of metabolic disorders, liver failure, or liver disease [5]. In the present case, only complex I activity was very low; this indicates primary complex I deficiency rather than a secondary effect of influenza A infection. Complex I includes seven mitochondrial DNA-encoded subunits and at least 39 nuclear-encoded subunits. In our case, no mutation was detected in the mitochondrial DNA (mtDNA). The detection rate for mutations in mitochondrial or nuclear DNA in complex I deficiency is as small as 20% [6,7].

In the present case, complex I was deficient only in the liver, not in fibroblasts. Mitochondrial respiratory complex disorders can show clinical and biochemical tissue specificity [2,4,6,8,10]. For this reason, it is difficult to diagnose by suspension cells or serum enzyme assays. The possible mechanisms of tissue specificity are tissue-specific subunits of complex I [9], the ratio between normal and mutant mtDNA in a specific tissue [7], and tissue differences in RNA processing [10]. To our knowledge, very few cases with liver-specific complex I deficiency have been reported [2,8]. These reported cases had chronic neurological symptoms such as epilepsy, hypotonia, or developmental regression, with the exception of one case that had severe cardiomyopathy in early infancy [2]. There was one case without evidence of liver dysfunction [8]. Clinically there was no definite difference from usual Co I deficiency. One reason for the small number of cases is that the liver is not the prime diagnostic tissue. Respiratory chain complex deficiency is usually confirmed by tissue biopsy. Muscle is usually the prime diagnostic tissue, and cultured skin fibroblasts are also often analyzed [10]. False-negative diagnostic results may occur because the liver is not examined.

This case was determined to be complex I deficiency by BN-PAGE Western blotting and determination of enzyme activities. This is the first report of respiratory chain complex I deficiency in influenza encephalopathy. We suggest there may be many undiagnosed cases of this metabolic disorder. Here, we described a healthy child, who had never been suspected of having any disease, diagnosed with a metabolic disorder after acute encephalopathy with subsequent death. Future studies are needed to focus on the development of a method to detect this inborn metabolic disorder before onset.

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CLINICAL STUDY

Analysis of plasma ghrelin in patients with medium-chain acyl-CoA dehydrogenase deficiency and glutaric aciduria type II

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Abstract

Objective: Ghrelin requires a fatty acid modification for binding to the GH secretagogue receptor. Acylation of the Ser3 residue of ghrelin is essential for its biological activities. We hypothesized that acyl-CoA is the fatty acid substrate for ghrelin acylation. Because serum octanoyl-CoA levels are altered by fatty acid oxidation disorders, we examined circulating ghrelin levels in affected patients.
Materials and methods: Blood levels of acyl (A) and des-acyl (D) forms of ghrelin and acylcarnitine of patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and glutaric aciduria type II (GA2) were measured.
Results: Plasma acyl ghrelin levels and A/D ratios increased in patients with MCAD deficiency or GA2 when compared with normal subjects. Reverse-phase HPLC confirmed that *n*-octanoylated ghrelin levels were elevated in these patients.
Conclusion: Changing serum medium-chain acylcarnitine levels may affect circulating acyl ghrelin levels, suggesting that acyl-CoA is the substrate for ghrelin acylation.

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Introduction

Ghrelin, an endogenous ligand for the GH secretagogue receptor, is an acylated peptide produced by gastrointestinal endocrine cells (1). Ghrelin is the only peptide known to require a fatty acid modification. Octanoylation of the Ser3 residue is essential for ghrelin-mediated stimulation of GH secretion and regulation of energy homeostasis via increased food intake and adiposity (2, 3). Other than octanoylation (C8:0), the hormone is subject to other types of acyl modification, decanoylation (C10:0), and possibly decenoylation (C10:1) (4, 5). Recently, ghrelin *O*-acyltransferase (GOAT), which octanoylates ghrelin, was identified (6, 7). The fatty acid substrate that contributes to ghrelin acylation, however, has not been clarified, although the presumed donor is acyl-CoA.

Mitochondrial fatty acid oxidation (FAO) disorders result from genetic defects in transport proteins or enzymes involved in fatty acid β-oxidation (8, 9). The clinical phenotypes have recently been associated with a growing number of disorders, such as Reye syndrome, sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications during pregnancy (10). Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, the most common inherited defect in FAO, causes elevated serum octanoylcarnitine levels (11), reflecting elevated octanoyl-CoA levels. Glutaric aciduria type II (GA2), which is caused by defects in electron transfer flavoprotein (ETF), ETF-ubiquinone oxidoreductase, or other unknown abnormalities in flavin metabolism or transport, is characterized by elevated serum acylcarnitine levels, including octanoylcarnitine (8, 9). In carnitine palmitoyltransferase II (CPT II) deficiency and very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, serum octanoyl-CoA levels do not increase, but at times actually decrease (8, 9).

We hypothesized that octanoyl-CoA is the fatty acid substrate for ghrelin acylation. To examine this hypothesis, we measured circulating ghrelin levels in patients with MCAD deficiency (MCADD) and GA2.

Materials and methods

Subjects

Five female patients with FAO deficiency (two with MCADD one with GA2, one with CPT II deficiency (12),

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and one with VLCAD deficiency) were recruited for this study. The study protocol was approved by the ethics committee on human research at the Kyoto University Graduate School of Medicine. Written informed consent was obtained prior to enrollment.

Measurement of plasma ghrelin concentrations

Because FAO patients tend to develop hypoglycemia by fasting, it was difficult to do overnight fasting. Therefore, blood samples for ghrelin analyses were drawn from a forearm vein in the morning after fasting as long as possible. Plasma samples were prepared as described previously (13). Blood samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Kyoto, Japan: 1000 kallikrein inactivator units/ml = 23.6 nmol/ml (23.6 pM)) and centrifuged at 4 °C. One-tenth volume of 1 M HCl was immediately added to the separated plasma. The acylated and desacylated forms of ghrelin were measured using a fluorescence enzyme immunoassay (FEIA; Tosoh Corp. Tokyo, Japan). The minimal detection limits for acyl and des-acyl ghrelin in this assay system were 2.5 and 10 fmol/ml respectively. The interassay coefficients of variation were 2.9 and 3.1% for acyl and des-acyl ghrelins respectively.

Reverse-phase HPLC

Reverse-phase HPLC (RP-HPLC) was performed as described previously (4, 5, 14). Briefly, plasma diluted 50% with 0.9% saline was applied to a Sep-Pak C18 cartridge pre-equilibrated with 0.9% saline. The cartridge was washed with saline and 10% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). Adsorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA. The eluate was evaporated and separated by RP-HPLC. All HPLC fractions were quantified using RIAs for ghrelin (4, 14, 15, 16). RIAs for a ghrelin C-terminal region (C-RIA) and a ghrelin N-terminal region (N-RIA) measure des-acyl ghrelin and octanoyl-ghrelin respectively (15). A RIA for N-terminal ghrelin showed ~20–25% cross-reactivity values for the *n*-decanoylated and *n*-decenoylated forms (16). Authentic human ghrelin-(1–28) was chromatographed with the same HPLC system.

Tandem mass spectrometry

Acylcarnitines in sera and dried blood spots were measured according to previously reported methods (17, 18), without derivatization. Briefly, 3 μl serum and 110 μl methanol solutions (99%) with deuterium-labeled acylcarnitines as internal standards were mixed and centrifuged, and 5 μl of the supernatant was introduced into liquid chromatography flow of methanol/acetonitrile/water (4:4:2) with 0.05% formic acid using a SIL-20AC autoinjector (Shimadzu, Kyoto, Japan). Flow injection and electrospray ionization tandem mass spectrometric (MS/MS) analyses were performed using an API 4000 LC/MS/MS system (AB Sciex, Tokyo, Japan). Positive ion MS/MS analysis was performed in precursor ion scan mode with an m/z value of 85 for the product ion. Data were recorded for 0.7 min after every sample injection and the recorded intensities of the designated ions were averaged using Chemoview Software (Foster City, CA, USA). All samples were measured serially within 1 day.

Results

We measured plasma ghrelin concentrations in patients with MCADD and GA2 (Table 1) and also in patients with CPT II and VLCAD deficiency. Elevated C8-acylcarnitine serum levels were observed in MCADD and GA II, whereas they were unchanged or lower in CPT II or VLCAD deficiency (Table 1). Levels of acyl ghrelin but not des-acyl ghrelin appeared to be elevated in patients with MCADD or GA2 in comparison with those in patients with CPT II or VLCAD deficiency, or those in female normal subjects from a previous study.

We then performed RP-HPLC analysis of ghrelin using plasma from patient 1 with MCADD. It demonstrated an eluted peak that corresponded to *n*-octanoylated human ghrelin-(1–28) in an N-RIA and a C-RIA, indicating that the detected acyl ghrelin was octanoylated (Fig. 1A). When plasma from patient 3 with GA2 was examined using the same method, the N-RIA revealed that the major peak corresponded to *n*-octanoylated human ghrelin-(1–28) (Fig. 1B). In addition, a small peak, which corresponded to decanoylated ghrelin, was observed in fraction 16 (arrow c), reflecting that serum C10-acylcarnitine levels were also elevated in patient 3 (Table 1).

Discussion

Ghrelin is the sole peptide hormone known to have a fatty acid modification. When we started this study in 2007, the catalytic enzyme and fatty acid substrate that mediate ghrelin acylation had not been identified. During this study, the GOAT enzyme was shown to be essential for ghrelin acylation (6, 7). Octanoic acid and octanoyl-CoA were candidates for the fatty acid substrate. We hypothesized that octanoyl-CoA was the substrate, because acylation of ghrelin should be an intracellular process. In fact, Ohgusu *et al.* (19) showed that acyl-CoA can be the substrate for ghrelin acyl-modification using the *in vitro* assay system. We tested this hypothesis in patients with MCADD and GA2,

Table 1 Clinical features, serum acylcarnitine levels, and plasma ghrelin concentrations in female patients with FAO disorders.

Subjects	Disease	Age (years)	BMI	Height (cm)	Acylcarnitine (nmol/ml)								AG (fmol/ml)	DAG (fmol/ml)	A/D ratio
					C4	C6	C8	C10:1	C10	C12	C14	C16	C18			
Patients ($n=5$)																
1	MCAD	6	15.1	119.5	0.30	0.55	4.61	0.95	0.29	0.04	0.01	0.05	0.01	45.09	57.23	0.79
2	MCAD	11	16.0	125.3	0.07	0.36	2.26	0.40	0.20	0.02	0.02	0.07	0.01	30.11	40.83	0.74
3	GA2	6	15.8	116.1	0.39	0.31	1.24	0.32	1.86	0.35	0.12	0.16	0.05	56.55	50.80	1.11
4	CPT II def.	10	17.8	141.5	0.10	0.06	0.21	0.20	0.40	0.15	0.03	0.08	0.02	19.76	34.49	0.57
5	VLCAD def.	5	14.8	109.5	0.07	0.09	0.07	0.08	0.28	0.42	2.17	2.00	0.87	27.02	113.07	0.24
Normal subjects ($n=20$; mean±S.D.)[a]		32.6±10.3	20.3±1.9											19.66±11.26	47.71±43.71	0.48±0.17
Reference range ($n=34$; mean±S.D.)					0.25±0.09	0.04±0.02	0.07±0.06	0.08±0.05	0.13±0.12	0.06±0.05	0.03±0.02	0.09±0.04	0.04±0.02			

C8, octanoyl acylcarnitine; C10, decanoyl acylcarnitine; C10:1, decenoyl acylcarnitine; AG, acyl ghrelin; DAG, des-acyl ghrelin; def., deficiency.
[a]See reference 13. All samples were reassayed using the FEIA.

which are characterized by higher intracellular octanoyl-CoA levels. Indeed, plasma A/D ratios tended to be elevated in these FAO deficiencies. A relationship between age and ghrelin levels may exist (20, 21). Concerning children, Ikezaki reported that the circulating ghrelin levels tended to correlate negatively with age in children and adolescents, but the correlation was not significant (22). Thus, the relationship has not been confirmed yet. Although we did not compare them directly with those in age- and body mass index (BMI)-matched normal children, they appeared to be higher than those in children with CPT II and VLCAD deficiencies with similar BMIs. BMIs of these patients were comparable to those of normal Japanese female children (23). These findings support the hypothesis that octanoyl-CoA is a primary substrate for ghrelin, although medium-chain triglyceride dietary lipids are a direct source for ghrelin acylation (7, 16, 24). Moreover, GOAT is a membrane-bound molecule in the endoplasmic reticulum (ER). Although how octanoyl-CoA gets into the ER lumen is unclear, Yang *et al.* (6) speculated that GOAT might mediate the transfer of octanoyl-CoA from the cytosol to the ER lumen. Although serum acylcarnitine levels tended to correlate with acyl ghrelin levels, further studies using more patients with FAO disorders are needed to confirm this relationship.

In addition to *n*-octanoylated ghrelin, other molecular forms of the ghrelin peptide exist, including des-acyl ghrelin lacking an acyl modification and such minor acylated ghrelin species as *n*-decanoylated ghrelin (Ser3 is modified by *n*-decanoic acid) (4, 5). Serum from a patient with GA2 showed the presence of acylated ghrelin that was not octanoylated and was possibly decanoylated (16). In a patient with GA2, intracellular levels of a variety of acyl-CoAs, including octanoyl- and decanoyl-CoAs, were increased, whereas MCADD was associated with specific elevation of octanoyl-CoA levels. In fact, the patient with GA2 had elevated octanoylcarnitine and decanoylcarnitine levels: 1.24 and 1.86 nmol/ml respectively. Nonetheless, the HPLC peak representing *n*-decanoylated ghrelin was much smaller than that representing *n*-octanoylated ghrelin. Although this is possibly because GOAT acylates ghrelin more efficiently with octanoyl-CoA than decanoyl-CoA, it is more likely because the cross-reactivity between *n*-octanoylated and *n*-decanoylated ghrelins is 20–25% in the N-RIA. In fact, the HPLC peaks of fraction 15–17 in the C-RIA, which detects similarly both *n*-octanoylated and *n*-decanoylated ghrelins, were large, strongly suggesting that a substantial amount of *n*-decanoylated ghrelin comparable to the elevated decanoylcarnitine level was present. Our observation that acyl ghrelin levels were not elevated in VLCAD and CPT II deficiencies, in which medium-chain acyl-CoAs levels are not higher, supported the idea that GOAT specifically acts on medium-chain acyl-CoAs. Although C16 and C18 levels were not increased in the patient with CPT II deficiency (Table 1), they may be normalized during

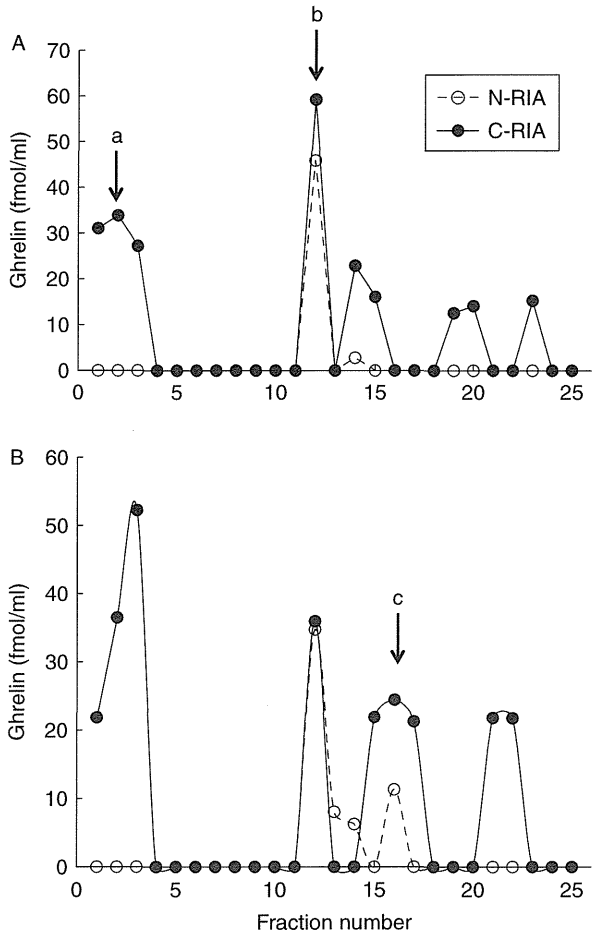

Figure 1 Representative RP-HPLC profiles of ghrelin immunoreactivity in patients with MCADD (A) and GA2 (B). Closed circles, data obtained using a RIA for a ghrelin C-terminal region (C-RIA); open circles, data obtained using a RIA for a ghrelin N-terminal region (N-RIA). Patient plasma extracts from a Sep-Pak C18 cartridge were fractionated using a Symmetry300 C18 column (5 mm packing, 3.9×150 mm, Waters). A linear gradient of 10–60% CH_3CN containing 0.1% TFA was passed over the column for 40 min at 1.0 ml/min. The fraction volume was 1.0 ml. Arrows indicate the elution positions of des-acyl human ghrelin-(1–28) (a), *n*-octanoylated human ghrelin-(1–28) (b), and *n*-decanoylated ghrelin (c).

a stable period in a mild form of CPT II deficiency (25). In fact, this patient did not manifest any marked signs or symptoms at the measurement.

Ghrelin modification with the fatty acid is essential for its biological action. Octanoylation of ghrelin may also be linked to energy homeostasis and fat metabolism. For instance, when serum *n*-octanoic acid levels increase following fat degradation, ghrelin octanoylation is enhanced, resulting in stimulation of fat synthesis. Thus, ghrelin may play an important role in energy homeostasis through its own fatty acid metabolism. Related to this concept, Kirchner *et al.* (24) speculated that signaling via GOAT and ghrelin might act as a fat sensor for exogenous nutrients and support fat storage as nutrients are ingested.

FAO deficiency contributes to such clinical problems as sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications (8, 9). Early diagnosis and appropriate management are required to reduce mortality and morbidity associated with this class of disorders. Recently, newborn screening has been expanded in this area. Measuring plasma ghrelin levels may support a diagnosis of MCADD or GA2, for example. Moreover, our results have pathophysiological implications for these disorders. Plasma ghrelin levels are changed by energy demands and food intake (e.g. glucose and fat), and ghrelin affects appetite and adiposity (2, 3). Alterations of plasma ghrelin levels in FAO disorders may reflect and/or influence the patient's metabolic status. In addition, higher acyl ghrelin levels may affect the GH/insulin-like growth factor 1 (IGF1) system. There are reports that higher AG levels would increase GH and IGF1 levels (26, 27, 28, 29) and thereby linear growth could be affected. Although none of our patients manifested markedly abnormal growth velocity, we did not measure their serum GH/IGF1 levels. Thus, further studies are warranted to detail a variety of metabolic parameters in this setting.

There are several limitations in this study. At first, the number of FAO patients tested is small. Unfortunately, the incidence of FAO patients in the Japanese population is much smaller than that in Caucasians. Although we asked pediatricians on a nationwide scale, we could successfully collect only five female patients. No adult case has yet been reported in Japan. Secondly, as mentioned above, the normal female subjects were not matched in age or BMI, although patients with MCADD and GA2 exhibited higher plasma A/D ratios than those in child CPT II and VLCAD deficiencies with similar BMIs. To supplement the correlation study, we performed RP-HPLC analysis to prove the increased octanoylation of ghrelin in MCADD and GA2 directly. Further, the presence of *n*-decanoylated ghrelin is also demonstrated in GA2. Thirdly, the disturbance in the hepatic carbohydrate regulation and the altered peripheral glucose uptake may occur in FAO patients. Hence, abnormal carbohydrate regulation could influence acyl ghrelin levels. Since none of our patients manifested abnormal fasting glucose and HbA1c levels, we speculated that no significant effects occurred.

In summary, we have demonstrated increased levels of acyl ghrelin in patients with MCADD or GA2, which are also characterized by increased intracellular octanoyl-CoA levels. These findings provide mechanistic insights into the biosynthesis of ghrelin. Furthermore, analyzing plasma ghrelin levels may help elucidate pathophysiological processes in FAO deficiencies and aid in the diagnosis of these disorders. Detailed studies using more patients are certainly needed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Case report

Two Japanese patients with Leigh syndrome caused by novel *SURF1* mutations

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Abstract

We report two patients with Leigh syndrome that showed a combination of facial dysmorphism and MRI imaging indicating an *SURF1* deficiency, which was confirmed by sequence analysis. Case 1 is a 3-year-old girl with failure to thrive and developmental delay. She presented with tachypnea at rest and displayed facial dysmorphism including frontal bossing, lateral displacement of inner canthi, esotropia, maxillary hypoplasia, slightly upturned nostril, and hypertrichosis dominant on the forehead and extremities. Case 2 is an 8-year-old boy with respiratory failure. He had been diagnosed as selective complex IV deficiency. Case 2 displayed facial dysmorphism and hypertrichosis. Since both patients displayed characteristic facial dysmorphism and MRI findings, we sequenced the *SURF1* gene and identified two heterozygous mutations; c.49+1 G>T and c.752_753del in Case 1, and homozygous c.743 C>A in Case 2. For patients with Leigh syndrome showing these facial dysmorphism and hypertrichosis, sequence analysis of the *SURF1* gene may be useful.
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Keywords: Leigh syndrome; *SURF1* deficiency; Facial dysmorphism; Hypertrichosis

1. Introduction

Leigh syndrome (OMIM 256000) is a progressive neurodegenerative disorder with the usual onset in infancy or early childhood. It is a genetically heterogeneous disease and the most common cause is a molecular defect in mitochondrial energy production system, including the respiratory chain complexes and pyruvate dehydrogenase complex. An isolated generalized defect of complex IV, (Cytochrome C oxidase) is the most common biochemical abnormalities found in Leigh syndrome [1]. Leigh syndrome with *SURF1* mutations, which encode the putative assembly protein of complex IV, have been reported [2] with specific clinical features of facial dysmorphism [3], hypertrichosis [4], and MRI findings [5]. Here, we report two patients with these clinical features and novel *SURF1* mutations.

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