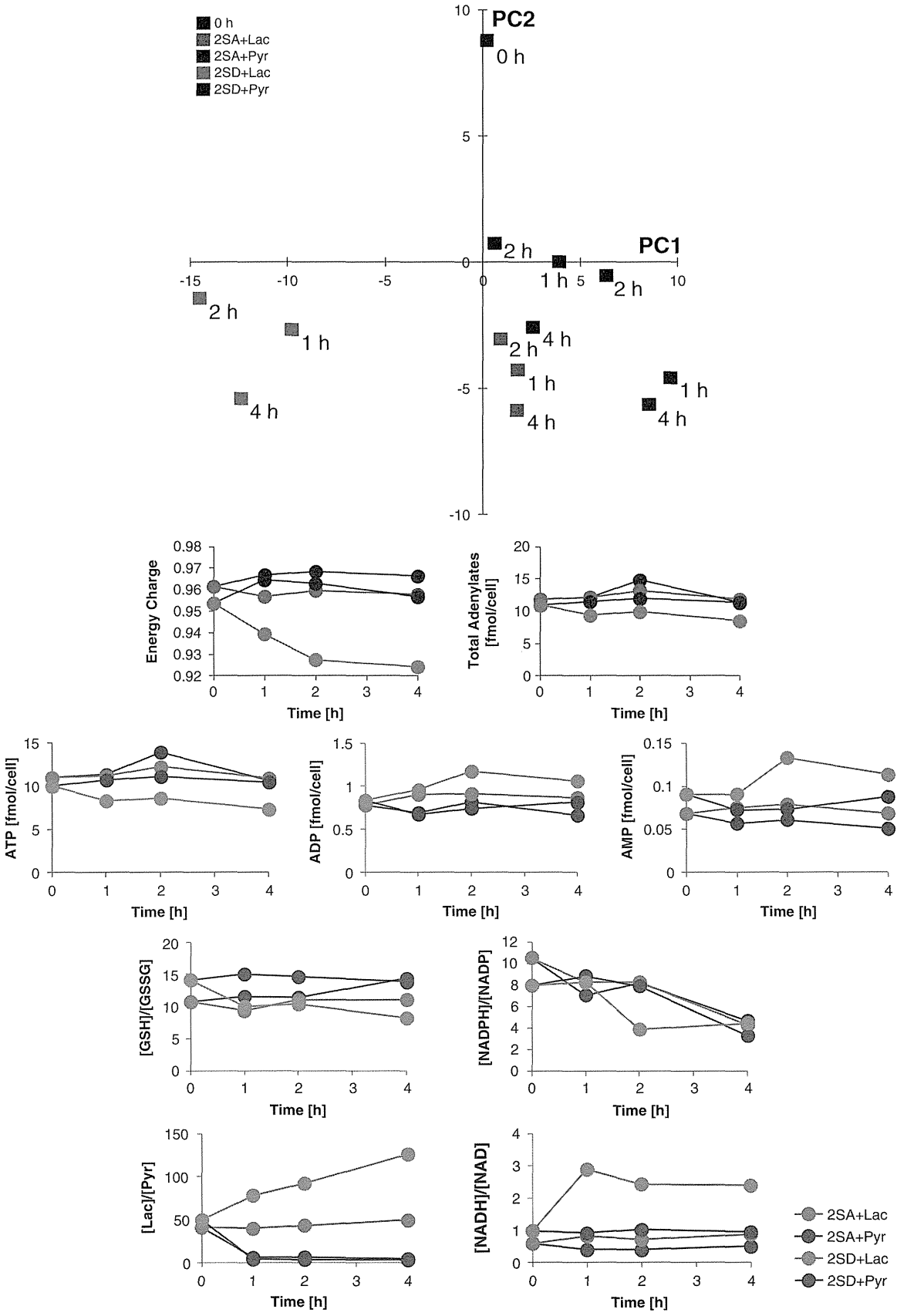

Fig. 2. A. Distributions of the principal component scores 1 (x-axis) and 2 (y-axis) of time-course metabolome data based on the intracellular and medium metabolites in 2SA and 2SD cells cultured with 10 mM lactate or 10 mM pyruvate. B. Time-course changes in metabolic parameters representing the energy status of 2SA and 2SD cells cultured with 10 mM lactate or 10 mM pyruvate. Energy charge was evaluated by ([ATP] + 0.5 × [ADP])/([ATP] + [ADP] + [AMP]); and total adenylates indicate the sum of ATP, ADP, and AMP levels.

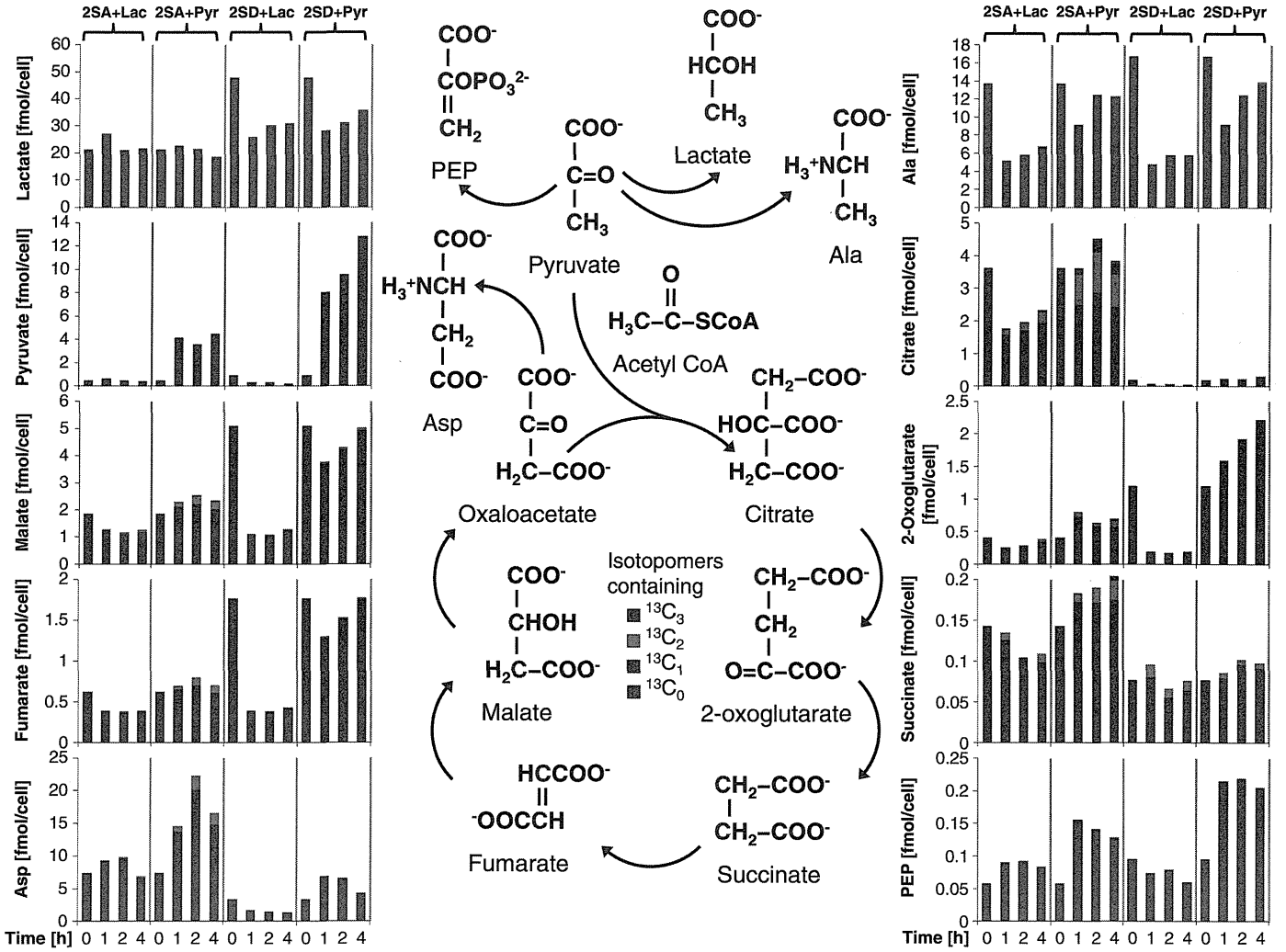

Fig. 3. Time-dependent changes in isotopomer proportions of intracellular pyruvate, lactate, PEP, Ala, Asp, and TCA cycle intermediates in 2SA and 2SD cells cultured with 10 mM lactate or 10 mM pyruvate. The colors of the bars represent the number of ^{13}C replaced with ^{12}C in the metabolites and isotopomers examined. The carbon atoms shown in black and red in each chemical structure represent the expected positions of ^{12}C and ^{13}C, respectively, in the first rotation of the TCA cycle. There are 2 possibilities considered for the ^{13}C position in succinate, fumarate, malate, oxaloacetate, and Asp.

pyruvate increased the metabolic flux not mainly from the pyruvate but from other sources to 2-oxoglutarate, fumarate, and malate. In this perspective, the balanced [NADH]/[NAD] ratio upon pyruvate treatment possibly enhanced the reactions involving NAD^+ as a cofactor. For example, the improved [NADH]/[NAD] ratio would have activated the glyceraldehyde 3-phosphate → 1,3-bisphosphoglycerate reaction by glyceraldehyde 3-phosphate dehydrogenase, which requires NAD^+ as a cofactor, and this would be the key for an optimal glycolytic ATP production in the pyruvate-supplied 2SD cells. In addition, the transaminase reaction involving the oxaloacetate + Glu → Asp + 2-oxoglutarate conversion might have been enhanced and contributed to the increased Asp and 2-oxoglutarate levels in the pyruvate-supplied 2SD cells. Since the malate dehydrogenase reaction also involves NAD^+, the significant increase in Asp, 2-oxoglutarate, fumarate, and malate levels in the pyruvate-supplied 2SD cells might have been due to the enhanced transamination cycle coupled with the urea cycle. Moreover, the glutamate → 2-oxoglutarate reaction by glutamate dehydrogenase and 2-oxoglutarate → succinyl-CoA reaction by 2-oxoglutarate dehydrogenase also require NAD^+ as a cofactor; thus, the activation of these reactions may have facilitated the succeeding succinyl CoA → succinate conversion for GTP production. This replenishment of TCA cycle intermediates and the balanced [NADH]/[NAD] ratio are considered essential for a limited but steady production of ATP via oxidative phosphorylation in 2SD cells, given that the expression of respiratory complexes I, III, and IV in 2SD cells is known to be decreased but not lost and that the expression of complex V is as high as that in their parental cells (Fujita et al., 2007). Although the pyruvate → acetyl CoA reaction by pyruvate dehydrogenase also involves NAD^+ as a cofactor, the pyruvate treatment did not significantly increase the intracellular citrate level. This result might have been due to a shortage of oxaloacetate, which combines with acetyl CoA for citrate production, or alternatively to a defect in pyruvate dehydrogenase and/or citrate synthase. In fact, pyruvate dehydrogenase deficiency and resulting altered oxidative phosphorylation function have been reported in a MELAS patient (Wilichowski et al., 1998), whereas citrate synthase activity in such a patient is reportedly nearly normal (Yoneda et al., 1989).

In the lactate-supplied 2SD cells, the levels of intracellular essential amino acids such as Ile, Leu, Met, His, Val, and Phe (cluster 1 in Fig. 1) and those of essential amino acids in the medium, such as Lys and Phe (cluster 2 in Fig. 1), accumulated significantly. The alteration of intracellular free amino acid pools in MELAS mutant cells was proposed based on our previous finding that ASNS gene expression is up-regulated in these cells (Fujita et al., 2007). MELAS mutant cybrids are known to exhibit autophagic cell death triggered by a combination of nitrosative and metabolic stress (Sandhu et al., 2005); thus, 2SD cells may have a constitutively high level of autophagic activity, and this might also have contributed to the generation of free amino acids by autophagic degradation of proteins and the resulting accumulation of essential

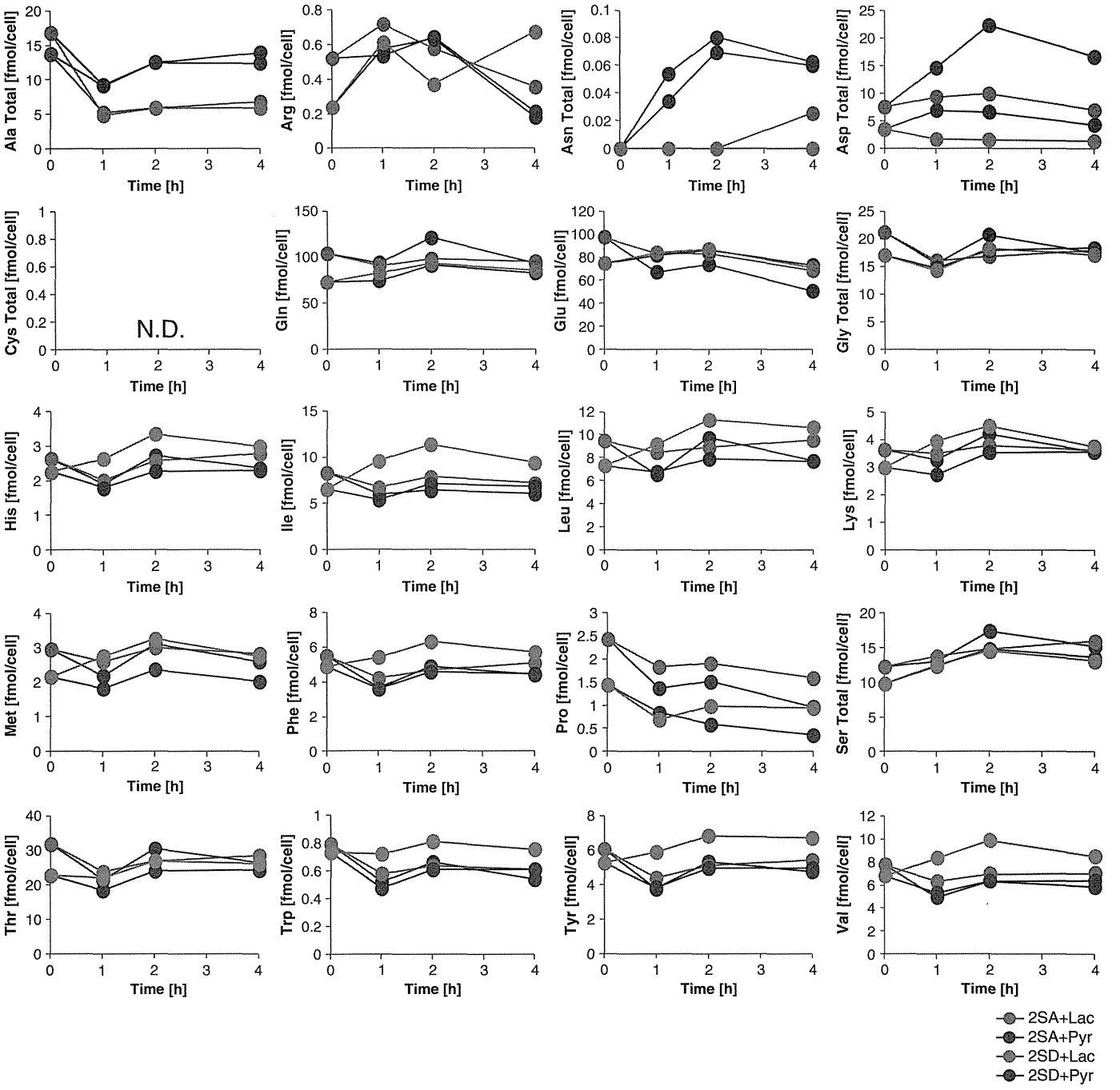

Fig. 4. Time-dependent changes in intracellular amino acid concentrations in 2SA and 2SD cells cultured with 10 mM lactate or 10 mM pyruvate. "N.D." indicates that the metabolite level was under the detection limit of CE-TOFMS analysis.

amino acids both intracellularly and in the medium. A high autophagic activity in 2SD cells can also be inferred by the increased production of reactive oxygen species (ROS) in MELAS syndrome (Rusanen et al., 2000), since ROS have been identified as signaling molecules to induce autophagy (Scherz-Shouval et al., 2007). In fact, the intracellular ROS level in 2SD cells is higher than that in 2SA cells (Fujita et al., 2007) and oxygen exposure-induced apoptosis is higher in MELAS mutant cells than in normal controls (Zhang et al., 1998). These findings thus imply that excessive ROS generated by the impaired respiratory chain facilitated autophagy in lactate-supplied 2SD cells, which eventually generated free amino acids and contributed to the increase in the levels of essential amino acids. In this perspective, the diminished accumulation of these essential amino acids in pyruvate-supplied 2SD cells may be explained by the fact that pyruvate is in fact a strong antioxidant and reacts with and reduces H_2O_2 (Desagher et al., 1997; Nath et al., 1995); thus, the pyruvate treatment might have alleviated oxidative stress and the accompanying autophagic activity in the 2SD cells.

Lactate is a well-known sensitive metabolic marker of MELAS (Castillo et al., 1995), which was also supported by its constantly high intracellular level and high increasing rate in the medium, as observed in this study. We also identified a few other metabolites that might function as potential MELAS markers. For example, the average increasing rate of medium Lys (Supplementary Fig. 3) in lactate-supplied 2SD cells (~171 fmol/cell/h) was significantly higher than that in the pyruvate-supplied 2SD cells (~53.7 fmol/cell/h) and 2SA cells (~33.8 fmol/cell/h under both lactate- and

pyruvate-supplied conditions). A possible reason for this high Lys level in the medium of the lactate-supplied 2SD cells might be the fact that the catabolism of Lys to acetyl CoA is known to involve 4 reactions that require NAD^+ as a cofactor; and thus this catabolic pathway may have been slowed down because of the constitutively low [NADH]/[NAD] ratio in the 2SD cells. Though to a lesser extent, a similar trend was observed in the increasing rate of Val in the medium (Supplementary Fig. 3; ~270 fmol/cell/h in lactate-supplied 2SD cells and ~202 fmol/cell/h under the other conditions). This may be again due to the shortage of NAD^+ in the lactate-supplied 2SD cells, as the catabolic pathway of Val to succinyl CoA is known to involve 3 reactions that require NAD^+ as a cofactor. The balanced [NADH]/[NAD] ratio seems critical also for the catabolism of these essential amino acids, since the increasing rate in these amino acids was lowered to the levels observed in 2SA cells by the pyruvate treatment. Although the overall trend of medium levels of Lys and Val appeared not to be considerably different among the conditions (Supplementary Fig. 3), this significant difference in the increasing rate of medium Lys and Val between the lactate-supplied 2SD cells and cells under the other conditions may be amplified in the long term. Accordingly, Lys and Val might be manifested in the blood or urine of MELAS patients and detectable as a diagnostic marker for MELAS and most likely other mitochondrial diseases showing imbalanced [NADH]/[NAD] as a pathologic condition. Moreover, gamma-aminobutyric acid (GABA) was one of the few metabolites that showed a clear cell line-specific trend independent of pyruvate- or lactate-administration (Supplementary Fig. 4). The intracellular GABA level in 2SA cells was nearly twice as high as that in 2SD cells throughout the experiment, and this trend was unchanged by either pyruvate or lactate treatment. Thus, GABA administration might be effective to somehow alleviate the symptoms of MELAS. Indeed, it has been speculated that treatment with inhibitory neurotransmitters such as GABA is theoretically effective to lower hyperexcitability in MELAS patients (Iizuka and Sakai, 2005).

Pyruvate administration does not always exhibit the expected efficacies in MELAS patients and not necessarily allow an optimistic outlook. This is perhaps by reason of the polygenetic nature of the cause of MELAS, which is known to be associated with at least 29 specific point mutations. There are at least 7 identified point mutations in the mitochondrial tRNA(Leu) gene, as well as mutations affecting many other mitochondrial tRNA genes (His, Lys, Gln, and Glu) and protein-coding genes (MT-ND1, MT-CO3, MT-ND4, MT-ND5, MT-ND6, and MT-CYB; (Sproule and Kaufmann, 2008)). Nevertheless, it is likely that the symptoms associated with lactic acidosis, *i.e.*, a high [NADH]/[NAD] ratio and possibly oxidative stress, would be alleviated by pyruvate administration. Current treatment regimens for MELAS patients involve indiscriminative administration of vitamins, cofactors, and oxygen-radical scavengers, which aims at the mitigation, postponement, or circumvention of the postulated damage to the respiratory chain (DiMauro and Schon, 2003). But pyruvate treatment could be a more effective, affordable, side effect-free, and most importantly, metabolically rational treatment regimen to improve symptoms associated with MELAS and even those of many other mitochondrial diseases. Such treatment would do so by facilitating efficient anaerobic glycolysis and probably supporting a limited but steady activity of oxidative phosphorylation for enhancing ATP production. Metabolome analysis of 2SA cells and MELAS mutant 2SD cells not only highlighted the basal metabolic differences between these cell lines but also their metabolic alterations and flux profiles in response to a high dose of lactate or pyruvate administration. The results showed a dramatic and sustainable effect of pyruvate administration on the energy metabolism of 2SD cells, supporting the idea that balancing the [NADH]/[NAD] ratio is crucial for facilitating anaerobic glycolysis for sufficient energy production in MELAS mutant cells. In this perspective, the efficacy of pyruvate treatment may not be limited to only alleviation of the symptoms associated with MELAS but rather also to that of those associated with a wider range of mitochondrial diseases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mito.2012.07.113.

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Case report

Late-onset Leigh syndrome with myoclonic epilepsy with ragged-red fibers

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Abstract

We report the case of a boy with myoclonic epilepsy with ragged-red fibers (MERRF) who had astatic seizures since 2 years of age and later developed ataxia, absence seizures, and myoclonus. Almost homoplasmic A8344G mutation of mitochondrial DNA (m.8344A>G mutation) was detected in lymphocytes. He developed late-onset Leigh syndrome (LS) when he contracted pneumonia at 6 years. He developed bulbar palsy and deep coma. MRI demonstrated lesions in the brainstem, basal ganglia, and cerebral cortex. Three similar cases have been reported; two carried the almost-homoplasmic m.8344A>G mutation in muscle tissue. These suggested that almost homoplastic m.8344A>G mutation developed clinical phenotype of MERRF in the early stage and late-onset Leigh syndrome in the late course of the disease.

Keywords: Leigh syndrome; MERRF; m.8344A>G

1. Introduction

Slowly progressive myoclonic epilepsy, ataxia, and myopathy are the main clinical features of myoclonic epilepsy with ragged-red fibers (MERRF) (OMIM #545000) [1]. MERRF onset varies from childhood to adulthood, after normal early development. The A → G mutation at nucleotide 8344 of mitochondrial DNA (m.8344A>G mutation) accounts for 80–90% of MERRF cases [2]. Biochemically, enzyme complexes of the respiratory chain, mainly NADH-CoQ reductase (complex I) and cytochrome c oxidase (complex IV), are deficient [3].

Leigh syndrome (LS; OMIM #256000) is a rapidly progressive neurodegenerative disorder characterized by necrotizing changes in the basal ganglia and brainstem. Psychomotor retardation, seizures, nystagmus, ophthalmoplegia, optic atrophy, ataxia, dystonia, and respiratory failure are the main clinical features [4]. Most patients developed LS until 2 years of life and diseased in several days to months after the onset. LS has a heterogeneous genetic background, and mitochondrial and nuclear genes coding respiratory chain complexes or the pyruvate dehydrogenase complex are responsible for this disease [5].

The m.8344A>G mutation may rarely be a cause for LS [6]. The development of LS in a patient with the MERRF phenotype is very rare. To our knowledge, only three cases have been reported [1,2,7]. We report the case of a boy diagnosed with LS at 6 years who showed the MERRF phenotype from 2 years of age.

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2. Case report (Fig. 1)

The patient was born at term by normal delivery from nonconsanguineous parents. There was no family history of neurological disorders. Developmental milestones were normal until 1 year of age, when he could walk on his own. At 2 years, he developed astatic seizures well-controlled by valproic acid (VPA). At 3 years, he developed cerebellar ataxia with dysarthria and wide-based gait. At 4 years, he presented with atypical absence and myoclonic seizures, well-controlled with ethosuximide and clonazepam in addition to VPA.

Laboratory data at 5 years were as follows: Blood gas analysis (in vein revealed the following: pH, 7.405; pCO_2, 41.0 mmHg; pO_2, 87.7 mmHg; HCO_3^- 19.1 mmol/l; and base excess, −4.7 mmol/l. Lactate and pyruvate levels in serum were elevated to 33.2 mg/dl and 1.89 mg/dl, respectively. In the cerebrospinal fluid (CSF), lactate and pyruvate levels were 22.3 mg/dl and 1.33 mg/dl, respectively. Ictal EEG during astatic seizures at 2 years showed bilateral occipital-dominant, 3–4 Hz diffuse spike and wave complexes (Fig. 2). Visual evoked potentials (VEPs) demonstrated high amplitude. Somatosensory evoked potentials were normal. MRI revealed cerebral and cerebellar cortex atrophy. Molecular genetic analysis examined the A → G transition at position 8344 in the $tRNA^{Lys}$ gene of mtDNA. The mtDNA mutation in the investigated lymphocytes were demonstrated by the method of Yoneda et al. [8] (Fig. 4). PCR products were digested by Nae 1. The RELP analysis of PCR products generated from wild type and mutant mtDNAs. The mutation band was evaluated by measuring scanned photographs in NIH image J software (available at http://rsb.info.nih.gov/nihimage/Default.html) to determine the relative intensity. The wild type mutation was not detected in the bands, which was considered to be almost homoplasmic in this case.

After MERRF diagnosis at 4 years, VPA was discontinued and CoQ_{10} and Vit B1 were administered. At

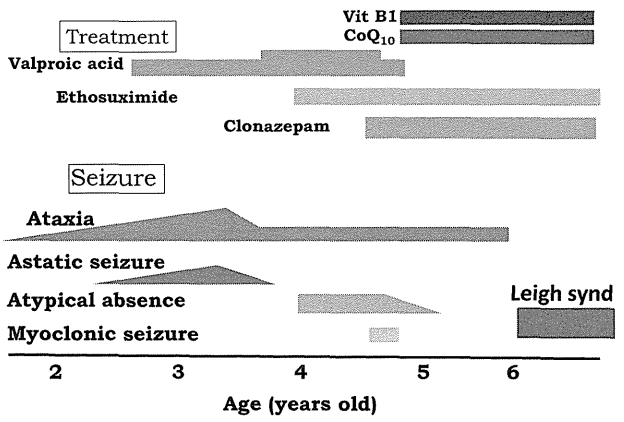

Fig. 1. Clinical course.

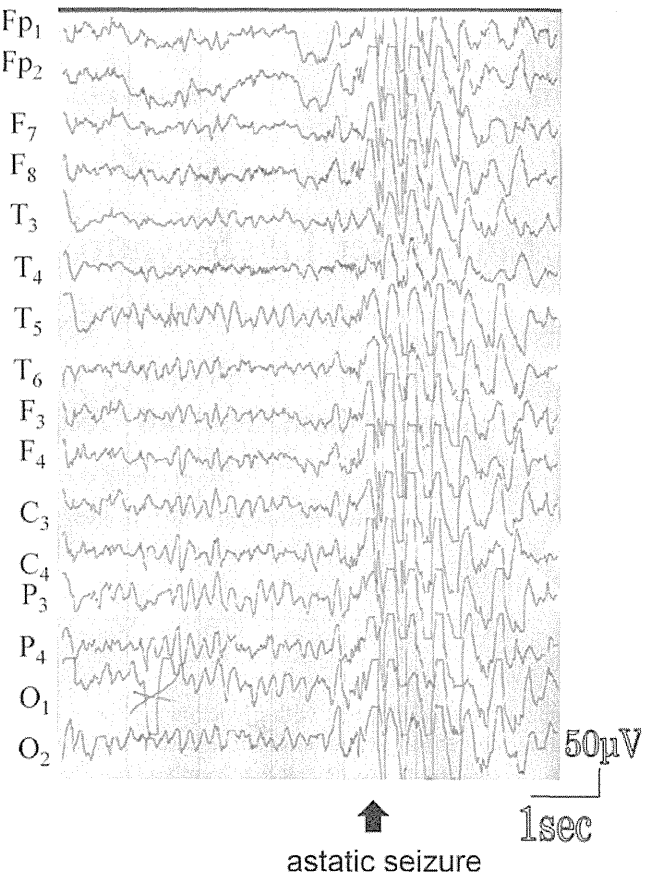

Fig. 2. Ictal EEG in astatic seizure.

6 years, he developed bacterial pneumonia with slight fever (from 98 to 100 °F) for 10 days. He did not have any neurological disturbance at that time. However, he suddenly presented with progressive bulbar palsy and marked generalized hypotonia two days after the body temperature was elevated above 102 °F. Brain T2-weighted MRI demonstrated high-signal bilateral lesions from the pons to the medulla. His consciousness slowly deteriorated, and lesions extending to the thalamus and bilateral cerebral hemispheres were noted on MRI (Fig. 3 (A)–(C)) 4–6 weeks after the initial episode. Within 6 weeks, he went into deep coma without spontaneous movements and was placed on permanent mechanical ventilation.

3. Discussion

Age of onset was 2 years; the male patient had astatic seizures with 3–4 Hz diffuse spike and wave complexes on EEG. The initial differential diagnosis was myoclonic astatic epilepsy, Dravet syndrome and Lennox–Gastaut syndrome. At 4 years, he had absences and myoclonic seizures with elevated lactate and pyruvate levels in serum and CSF and high-amplitude VEPs, and the patient was diagnosed with MERRF with almost

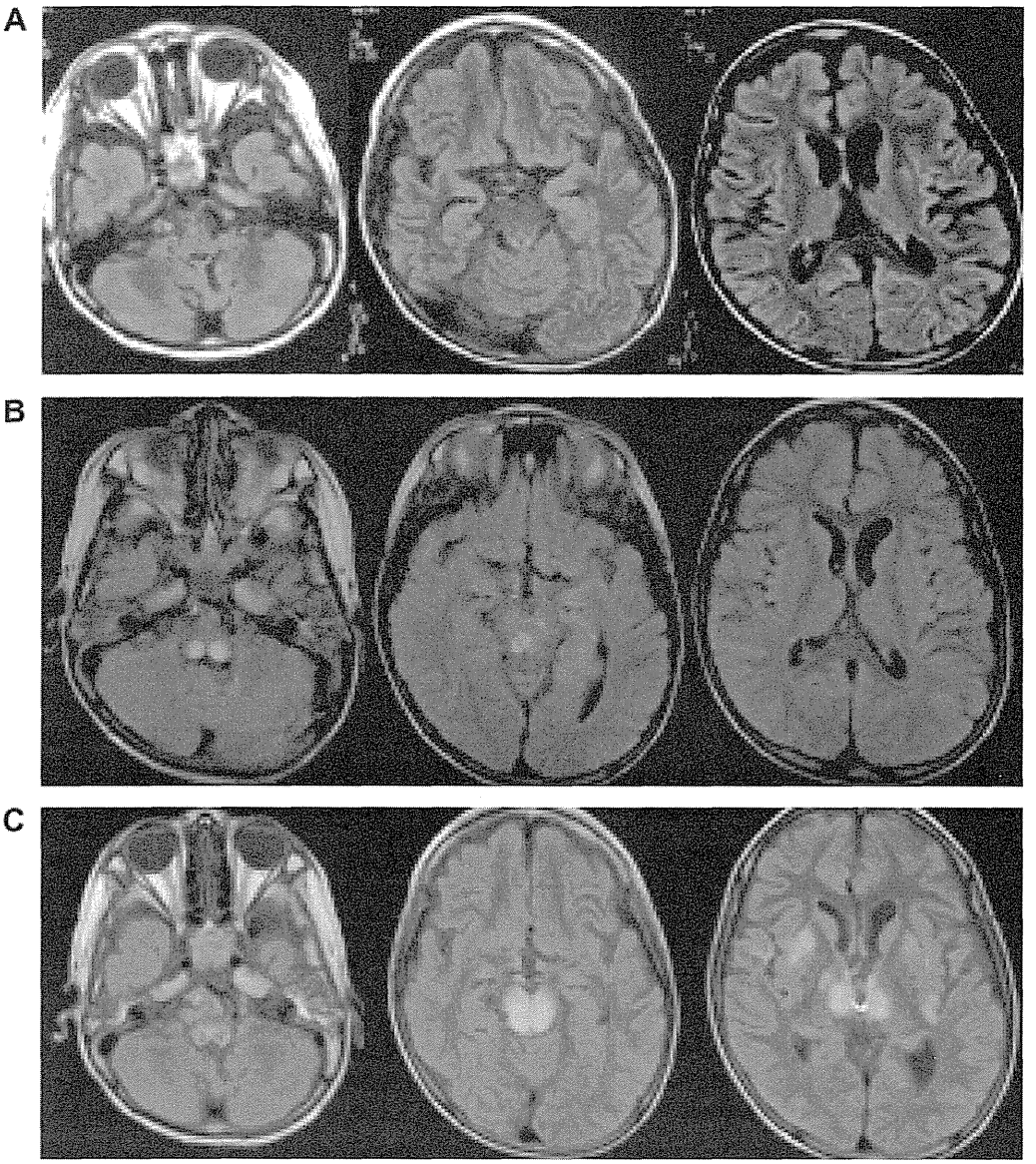

Fig. 3. Brain MRI (FLAIR images) (A) At 4 years of age, when MERRF was diagnosed: diffuse cerebral cortex atrophy. (B) At 6 years of age, when LS was diagnosed: Bilateral, symmetric, high-intensity lesions are seen in the brainstem from the pons to medulla. (C) Six weeks after diagnosis: the lesions extend to the thalamus and bilateral cerebral hemispheres.

homoplastic m.8344A>G mutation in lymphocytes. At 6 years, he rapidly developed LS after pneumonia was developed with high grade fever.

MERRF with LS and m.8344A>G mutation is very rare; only three cases have been reported [1,2,7]. Sweeney et al. [1] reported MERRF with ataxia and myoclonic epilepsy in a 7-year-old boy who developed LS at 18 years when he contracted bacterial pneumonia. The m.8344A>G mutation rates were detected in the lymphocytes (81%), skeletal muscle (99%), cerebellum (97%), cerebral cortex (97%), cardiac muscle (97%), liver (99%), and kidney (98%). Silvestri et al. [2] reported that only two of 150 cases with m.8344A>G mutation developed LS. High mutation rates were detected in muscle (100%) and lymphocytes (93%), but detailed clinical information was not written. Berkovic et al. [7] reported the case of a 4-year-old boy diagnosed with MERRF who died at 9 years, after contracting measles and pneumonia. Brain autopsy demonstrated LS but the mtDNA mutation rate was not investigated.

Including our patient, three of four reported cases developed MERRF before 7 years of age and LS 5–11 years later. Therefore, early-onset MERRF patients should be closely monitored for LS symptoms.

Factors affecting the LS progression have not been clearly described, but the mechanism is considered to be the compromised oxidative phosphorylation (OXPHOS) function due to mutations in nuclear or

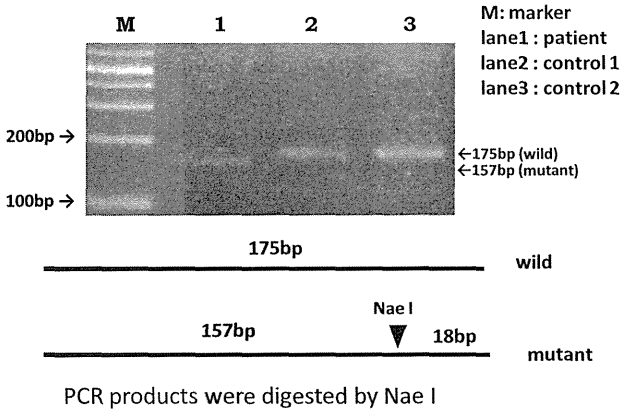

Fig. 4. Restriction fragment length polymorphism (RFLP) analysis for the A → G transition at position 8344. Mutated PCR products were digested by Nae 1. A 175-bp band corresponds to the wild-type sequence; 175- and 18-bp bands correspond to the Nae 1 digested fragments derived from the mutated mtDNA sequence. The patient demonstrated homoplasmic mutation.

mitochondrial genes encoding respiratory chain components or their assembly factors. In addition, sudden clinical and metabolic deterioration can happen with additional stress, such as fever, resulting in the pathological picture of LS [7]. Our case also suggested the affecting factor was high fever with pneumonia. Two of the three cases also described the development of LS after pneumonia was diagnosed, though these cases did not describe the febrile status or special events in infection.

The relationship between the percentage of mtDNA mutation rate and the severity of phenotype has been controversial. Piccolo et al. [9] reported that both severe MERRF phenotype and unaffected cases showed a high percentage of m.8344A>G. On the other hand, Tatuch et al. [10] reported that the high rate of LS m.8933T>G was related with the risk of LS. The mutation present in the ATP 6 gene was considered to cause failure of ATP synthesis. Including our case, the three cases reported that mtDNA mutation homoplasmy was detected in lymphocytes or muscle. The result suggests that the m.8344A>G mutation was potentially indicative of a broad expressive spectrum, and mtDNA homoplasmy or high mutation of heteroplasmy in lymphocytes or muscle may be a risk factor for LS. However, the matter of small sample size needs further investigation.

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Metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy: Diagnosis of mitochondrial respiratory chain disorders

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ABSTRACT

Mitochondrial respiratory chain disorders are the most common disorders among inherited metabolic disorders. However, there are few published reports regarding the relationship between mitochondrial respiratory chain disorders and sudden unexpected death in infancy. In the present study, we performed metabolic autopsy in 13 Japanese cases of sudden unexpected death in infancy. We performed fat staining of liver and postmortem acylcarnitine analysis. In addition, we analyzed mitochondrial respiratory chain enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. In heart, 11 cases of complex I activity met the major criteria and one case of complex I activity met the minor criteria. In liver, three cases of complex I activity met the major criteria and four cases of complex I activity met the minor criteria. However, these specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. In cultured fibroblasts, only one case of complex I activity met the major criteria and one case of complex I activity met the minor criteria. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. These cases might not have been identified without postmortem cultured fibroblasts. In conclusion, we detected one probable case and one possible case of mitochondrial respiratory chain disorders among 13 Japanese cases of sudden unexpected death in infancy. Mitochondrial respiratory chain disorders are one of the important inherited metabolic disorders causing sudden unexpected death in infancy. We advocate metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy cases.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as sudden unexpected death occurring before 12 months of age. If SUDI remains unexplained after thorough investigations, it is classified as sudden infant death syndrome (SIDS). The more common causes of SUDI are infection, cardiovascular anomaly, child abuse, and metabolic disorders. However, the many potential inherited metabolic disorders are more difficult to diagnose at autopsy as compared to cardiovascular defects and serious infection. Inherited metabolic disorders may, therefore, be underdiagnosed as a cause of SUDI or misdiagnosed as SIDS. Fatty acid oxidation disorders (FAODs) are one type of the inherited metabolic disorders and may cause as much as 5% of SUDI cases after thorough investigations including metabolic autopsy [1–5]. In a review of SUDI cases with respect to potential FAODs, we found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we performed fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis, advocating the importance of metabolic autopsy in SUDI cases.

Mitochondrial respiratory chain (MRC) disorders were first identified in 1962 [7]. MRC disorders have a frequency of about at least 1:5000 newborns and are the most common disorders among inherited metabolic disorders [8]. However, there are few published reports regarding the relationship between MRC disorders and SUDI. Studies of MRC disorders have not progressed because of technical difficulties or variability in clinical manifestations [9]. In sudden death cases especially, clinical features are unclear and postmortem changes complicate molecular analysis.

In the present study, we performed metabolic autopsy in 13 Japanese cases of SUDI in order to determine whether MRC disorders could be detected or not. We performed fat staining of liver and postmortem

Abbreviations: CS, citrate synthetase; FAODs, fatty acid oxidation disorders; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; SIDS, sudden infant death syndrome; SUDI, sudden unexpected death in infancy.

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acylcarnitine analysis according to the previous methods. In addition, we analyzed MRC enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. With such metabolic autopsy, we were able to detect one probable case and one possible case of MRC disorders. These cases might not have been identified without metabolic autopsy. MRC disorders are important diseases causing SUDI and metabolic autopsy might be helpful for forensic scientists and pediatricians to diagnose MRC disorders that might not otherwise be identified.

2. Materials and methods

2.1. Subjects

Between October 2009 and September 2011, forensic autopsy was performed on 588 cases at our institute, 22 of whom were under 12 months of age. Following macroscopic examination, nine cases could be diagnosed but 13 cases (Table 1) did not have any characteristic appearance and remained undiagnosed. In this study, we reviewed these 13 undiagnosed cases (8 males, 5 females) with age ranging from 1 to 10 months.

2.2. Autopsy

Autopsies were performed within 24 h following death. Blood was obtained from the femoral vein. Heart and liver specimens were immediately cut and frozen at −80 °C. Dermis, which was cut and sterilized, was cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 1% penicillin streptomycin glutamine, and 2.5% amphotericin B (Life Technologies, Indianapolis, IN). Once cultures were established, fibroblasts were frozen at −80 °C.

2.3. Sudan III staining

Liver samples preserved in 4% phosphate-buffered formaldehyde solution were frozen, cut into 10-μm sections, and stained by the Sudan III method for fat staining.

2.4. Postmortem blood acylcarnitine analysis by tandem mass spectrometry

Whole blood samples obtained at autopsy were blotted onto one spot on Guthrie cards. They were subjected to acylcarnitine analysis by tandem mass spectrometry and compared with the previously determined normal range [6].

Table 1
SUDI cases.

Case no.	Age/sex	Height/weight (cm/kg)	Circumstances	Fever	Remarks
1	4 mo/M	68/7.5	Sleeping	−	
2	10 mo/F	70/8.8	Sleeping	−	Sister: undiagnosed encephalitis
3	10 mo/F	71/7.7	Sleeping	+	Cesarean section
4	9 mo/M	67/7.5	Sleeping	−	
5	4 mo/M	60/5.7	Sleeping	−	Hydrocephalia
6	6 mo/M	68/8.0	Sleeping	−	
7	1 mo/F	51/3.6	Sleeping	−	Twins, preterm birth
8	10 mo/M	72/9.9	Sleeping	−	Developmental disease (right side of the body paralysis)
9	6 mo/F	64/8.9	Sleeping	−	Bronchitis
10	4 mo/M	65/7.4	Sleeping	−	Cesarean section
11	1 mo/M	58/4.8	Sleeping	−	
12	5 mo/M	59/4.2	Sleeping	−	Preterm birth
13	2 mo/F	53/3.9	Sleeping	−	Low-birth-weight infant

Abbreviations: F, female; M, male; mo, month; SUDI, sudden unexpected death in infancy.

2.5. Enzyme analysis

The activity of mitochondrial respiratory chain complexes I, II, III, and IV was assayed in the crude post-600-g supernatant of heart and liver, and in isolated mitochondria from skin fibroblasts as described previously [10]. The activity of each complex was presented as a percent ratio relative to the mean value [9]. The activity of complexes I, II, III, and IV was also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker or complex II activity [10].

2.6. Ethics

This study was approved by the Ethics Committee of the Osaka University Graduate School of Medicine.

3. Results

3.1. Microscopic examination

One of the common features in diagnosing MRC disorders is hepatic steatosis. We therefore performed Sudan III staining to examine whether vacuoles caused by fatty degeneration were present in hepatocytes. Diffuse microvesicular steatosis was detected in case 5 (Fig. 1A). No Sudan III-positive vacuole was detected in case 13 (Fig. 1B) and the other cases, for example, case 2 (Fig. 1C).

3.2. Postmortem blood acylcarnitine analysis

We performed acylcarnitine analysis by tandem mass spectrometry using whole blood samples. In all samples, data were within the normal range. These data suggested that no case was affected by FAODs (data not shown).

3.3. Enzyme analysis of MRC complexes in heart, liver, and cultured fibroblasts

The enzyme activity of each complex was compared with the CS ratio and complex II ratio. Lower than 20% activity of any complex in a tissue or lower than 30% activity of any complex in a cell line meets the major criteria. Lower than 30% activity of any complex in a tissue or lower than 40% activity of any complex in a cell line meets the minor criteria according to Bernier et al. [11].

In heart, 11 cases of complex I activity met the major criteria of MRC disorders and one case of complex I activity met the minor criteria (Fig. 2A). In liver, three cases of complex I activity met the major criteria of MRC disorders and four cases of complex I activity met the minor criteria (Fig. 2B). In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria of MRC disorders and one case (case 13) of complex I activity met the minor criteria (Fig. 2C, Table 2). The activity of complexes II, III, and IV was maintained in almost all cases.

3.4. Diagnosis

A definite diagnosis is defined as the identification of either two major criteria or one major plus two minor criteria. A probable diagnosis is defined as either one major plus one minor criterion or at least three minor criteria. A possible diagnosis is defined as either a single major criterion or two minor criteria, one of which must be clinical [11].

All the cases had a clinical symptom of sudden death, meeting one minor criterion. In the enzyme activity, eleven cases (cases 2, 4–13) met the major criteria and we could make a probable diagnosis in these 11 cases. The other two cases (cases 1 and 3) met the minor criteria and we could make a possible diagnosis.

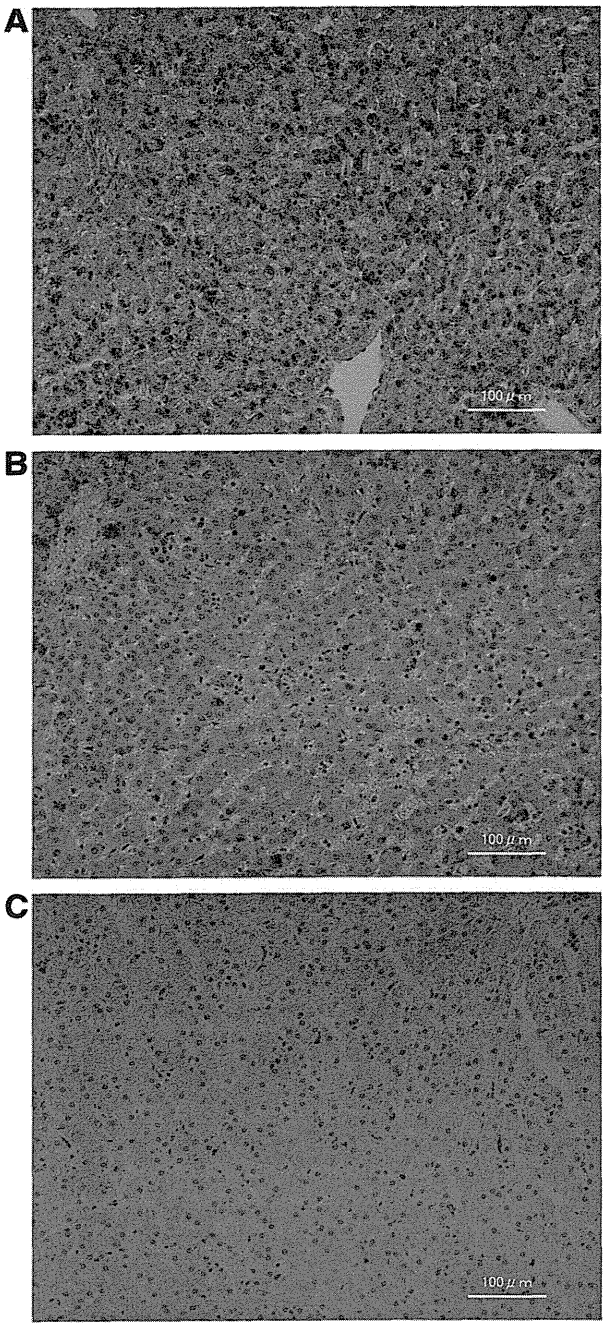

Fig. 1. Microscopic examination of liver (Sudan III staining): (A) case 5, (B) case 13, and (C) case 2. Diffuse microvesicular steatosis was detected in case 5 (A). No Sudan III-positive vacuole was detected in case 13 (B) and the other cases, for example, case 2 (C).

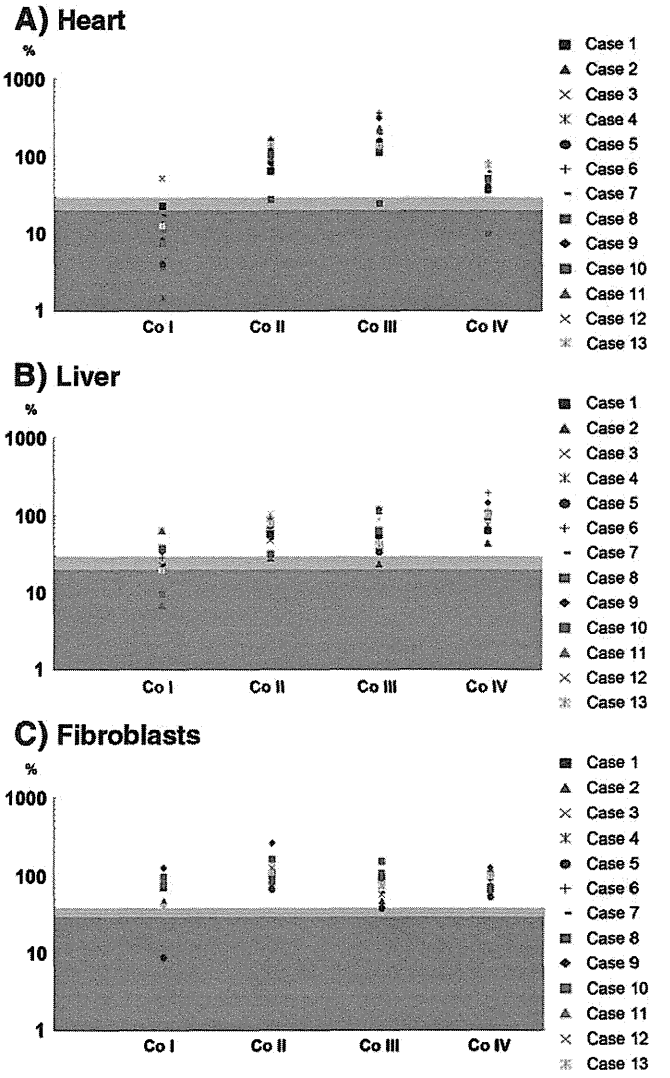

Fig. 2. Enzyme activity of MRC complexes in heart (A), liver (B), and cultured fibroblasts (C). In heart, 11 cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and one case of complex I activity was under 30% of the CS ratio, meeting the minor criteria (A). In liver, three cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and four cases of complex I activity were under 30% of the CS ratio, meeting the minor criteria (B). In cultured fibroblasts, one case (case 5) of complex I activity was under 30% of the CS ratio, meeting the major criteria and one case (case 13) of complex I activity was under 40% of the CS ratio, meeting the minor criteria (C). The activity of complexes II, III, and IV was maintained in almost all cases. The enzyme activity of each complex was compared with the CS ratio. Lower than 20% activity in a tissue or lower than 30% activity in a cell line (dark blue) meets the major criteria. Lower than 30% activity in a tissue or lower than 40% activity in a cell line (light blue) meets the minor criteria.

4. Discussion

Mitochondria are essential organelles that exist in all nucleated mammalian cells. They provide the energy required for normal cell function through oxidative phosphorylation (OXPHOS). OXPHOS includes MRC complexes (complexes I, II, III, and IV) and ATP synthase (complex V) [12], which use reduced coenzymes from the tricarboxylic acid cycle and molecular oxygen, generating cellular energy in the form of ATP [13].

The infantile or early neonatal period demands high energy. Patients with MRC disorders are unable to produce adequate energy, which may thus compromise them in the first days of life or during infancy. MRC disorders affect most organ systems and present variable clinical manifestations from prenatal complications through acute neonatal decompensation and death to adult-onset disorders. Therefore, it is not surprising that MRC disorders are also one of the causes of SUDI. However, there are few reports on a relationship between MRC disorders and SUDI [12,14].

We have previously reviewed SUDI cases with respect to FAODs and found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we advocated the importance of metabolic autopsy [15], including fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis. Using this protocol, most FAODs, some amino acid oxidation disorders, and some organic acid oxidation disorders could be diagnosed.

However, MRC disorders are difficult to diagnose. First, they present variable clinical manifestations and non-specific features such as failure to thrive or hepatic, cardiac, renal, gastrointestinal, endocrine, hematological, or other symptoms [10,16]. Second, although blood

Table 2
Enzyme assay of mitochondrial respiratory chain complexes in cultured fibroblasts.

	Enzyme activity (%)[a]			
	Co I	Co II	Co III	Co IV
Case 5				
CS ratio	9	66	38	53
Co II ratio	13	—	71	58
Case 13				
CS ratio	39	106	76	98
Co II ratio	37	—	73	92

Abbreviations: Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthetase.
[a] Relative to mean CS and Co II of the normal controls.

lactate levels and muscle morphology can be used as a screening test, some confirmed patients were normal [10]. Third, genomic mutational analysis is difficult because MRC complexes are composed of 13 subunits encoded by mitochondrial DNA and over 70 subunits encoded by nuclear genes. In addition, nuclear genes are related to many assembly factors, membrane dynamics, nucleotide transport synthesis, and mitochondrial DNA replication and expression. Therefore, enzyme analysis still remains the most significant diagnostic tool. A definite diagnosis thus requires enzyme analysis [8].

In the present study, we performed enzyme analysis in frozen heart, frozen liver, and cultured fibroblasts. Eleven cases were supposed to be a probable diagnosis and two cases were supposed to be a possible diagnosis. However, it seemed unlikely that such a high proportion would have real MRC disorders. Did we have to take the effect of postmortem changes into consideration?

For forensic autopsy, organ specimens are often preserved in formaldehyde solution and sometimes frozen. These specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. Based on the previous report that artifactual loss of complex II activity in autopsy samples preceded that of complex I and the data that complex II activity in the present study was maintained, this low complex I activity might be decreased before death. However, postmortem changes cannot be completely ruled out and this low complex I activity may not therefore be consistent with premortem activity.

We therefore analyzed activity in cultured fibroblasts. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria and one case (case 13) of complex I activity met the minor criteria. In case 5, complex I activity was distinctively decreased. Sudan III staining of the case revealed hepatic steatosis, consistent with Reye-like syndrome. Reye-like syndrome is one of the characteristic features of MRC disorders [9]. We could therefore make a probable diagnosis (case 5) and a possible diagnosis (case 13) from metabolic autopsy with postmortem cultured fibroblasts.

Case 5 had hydrocephalia and case 13 was a low-birth-weight infant. However, neither was severe. Macroscopic examination did not reveal any abnormal appearance and microscopic examination showed no pathological findings except for steatosis. These cases might not have been identified without postmortem cultured fibroblasts. As with such cases, some MRC disorders reveal no clinical manifestation and no pathological characteristic. We believe it is important to perform metabolic autopsy with postmortem cultured fibroblasts when encountering SUDI cases.

We emphasized the advantage of metabolic autopsy with cultured fibroblasts. First, despite lacking obvious preceding symptoms, MRC disorders could be diagnosed. Second, cultured cells are the only method to retrieve premortem information from the deceased. Third, even frozen samples are affected by postmortem changes and may lead to a false positive diagnosis. However, we have to discuss the disadvantage. MRC disorders showed tissue specificity and the activity of cultured fibroblasts represent normal in some cases. Some of the low complex I activity in heart or liver could represent premortem MRC disorders despite normal activity in cultured fibroblasts. Thus, other molecular investigations may well be added to enzyme analysis. Recently, systematic gene analysis using next-generation sequencing has been reported for the diagnosis of patients with MRC disorders [17]. Further investigations are thus needed.

In conclusion, we detected one probable case and one possible case of MRC disorders among 13 Japanese cases of SUDI. MRC disorders are one of the important inherited metabolic disorders causing SUDI. We advocate metabolic autopsy with postmortem cultured fibroblasts in SUDI cases.

Acknowledgments

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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.

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

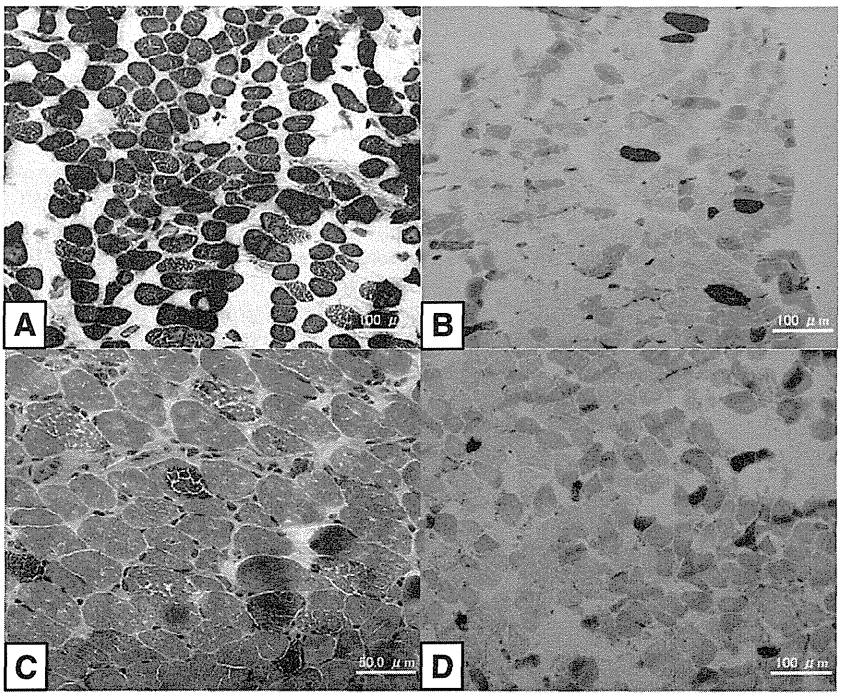

Fig. 1. Histochemistry of the biopsied muscle. ATPase staining shows type 2 fiber predominance at pH 10.6 (A) and an increased number of type 2C fibers at pH4.2 (B). The percentages of type 1, 2A/B and 2C fibers were 7%, 71% and 22%, respectively. Modified Gomori trichrome staining (C) shows scattered 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.

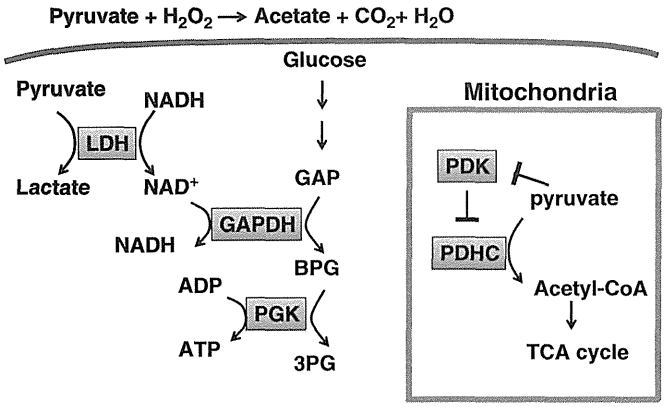

Fig. 2. Effects of pyruvate on energy metabolism and cell injury. Pyruvate eliminates hydrogen peroxide by a non-enzymatic reaction. Pyruvate provides NAD^+ from NADH with lactate dehydrogenase (LDH). In the presence of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NAD^+ oxidizes glyceraldehyde 3-phosphate (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.

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.

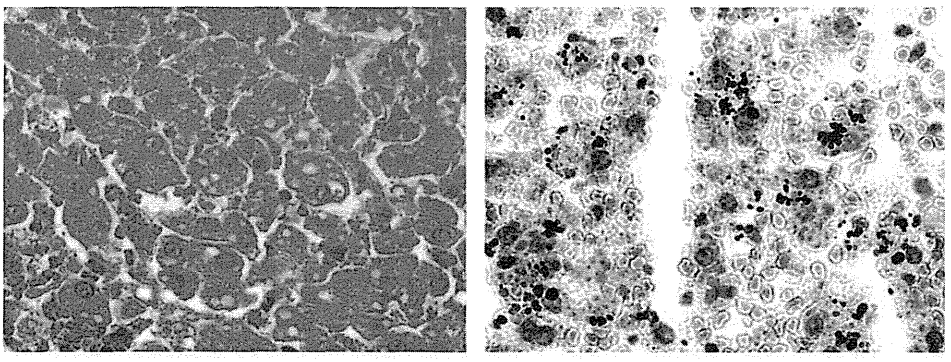
3. Discussion

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.