stressed KO mice. Our results thus suggest that ROS might also contribute to the pathogenesis, but to a limited extent. The accumulation of malfunctioning mitochondria is likely the primary cause of the progression of myopathy in KO mice.

Regulation of ms^2 Modification by Oxidative Stress and Its Association with Human Disease

An important finding of this study is that the ms^2 modification levels were reduced in MELAS patients carrying the A3243G mutation in mt-$tRNA^{Leu}$. This result is surprising because mt-$tRNA^{Leu}$ does not contain an ms^2 modification. Previous studies have shown that the deficiency of taurine modification in mt-$tRNA^{Leu}$ carrying the A3243G mutation is the primary cause of MELAS (Kirino et al., 2005; Yasukawa et al., 2001). Given the significant association of the heteroplasmy level with the ms^2 modification level in MEALS patients, our results suggest that the myopathy in MELAS is caused not only by a decoding error at the Leu codon but also by decoding errors occurring at multiple codons, including Leu, Phe, Tyr, Trp, and Ser codons.

The reason A3243G in mt-$tRNA^{Leu}$ is associated with decreased modifications in other mt-tRNAs remains unclear. Although further studies are required to reveal the molecular mechanism, our results suggest that oxidative stress may be one of the reasons for this finding. Cdk5rap1 requires two [4Fe-4S] clusters for ms^2 group insertion (Forouhar et al., 2013); therefore, it is conceivable that ROS, such as H_2O_2 or $ONOO^-$, may oxidize these [4Fe-4S] clusters and inactivate Cdk5rap1. In support of our hypothesis, ROS-treated cells exhibited a rapid decrease in ms^2 modification that was effectively reversed by antioxidants. Thus, ROS generated by the mutation in mt-$tRNA^{Leu}$ might impair Cdk5RAP1-mediated ms^2 modification, which might further amplify mitochondrial dysfunction and ultimately accelerate myopathy in MELAS patients. In addition to mitochondrial disease, ms^2 modifications might be involved in a wide variety of human diseases in which ROS have been previously implicated, such as cardiac dysfunction and cancer (Schieber and Chandel, 2014).

In conclusion, this study reveals a unique quality control system in mitochondria by which the ms^2 modification of mt-tRNAs dynamically regulates mitochondrial protein synthesis and contributes to the development of myopathy in vivo. Our findings have important physiological implications for the basic mechanism of mitochondrial protein synthesis and provide insights into the pathological mechanism of mitochondrial disease.

EXPERIMENTAL PROCEDURES

Please see the Supplemental Experimental Procedures for additional details.

Animals

Cdk5rap1 KO mice were generated by crossing transgenic mice with exon 5 and 6 of *Cdk5rap1* floxed with LoxP sequence, with transgenic mice expressing Cre recombinase under the control of the CAG promoter. Mice were backcrossed to C57BL6/J mice for at least seven generations to eliminate Cre transgene and control genetic background. Littermates of WT and KO mice (8–12 weeks old) were used for experiments unless otherwise specified. Animals were housed at 25°C with 12 hr light and 12 hr dark cycles. A KD was purchased from Research Diets (D12369B). All animal procedures were approved by the Animal Ethics Committee of Kumamoto University (Approval ID, C25-163). Detailed information on genotyping can be found in the Supplemental Experimental Procedures.

Luciferase Assay

E. coli colonies were transformed with plasmids encoding dual *luciferase* for detecting decoding error, *GST-Cdk5rap1*, or dominant-negative *GST-Cdk5rap1*. Colonies were cultured at 37°C, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the cultures at a final concentration of 1 mM. After 1 hr of incubation, the cultures were harvested for the luciferase assay using the Dual-Luciferase Reporter Assay System (Promega). Detailed procedures for detecting decoding error can be found in the Supplemental Experimental Procedures.

Cell Culture and Transfection

Mammalian cells were grown in DMEM high-glucose medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, HyClone) at 37°C and 5% CO_2. Transfection of the plasmid DNA was performed with Lipofectamine 2000 (Invitrogen).

Oxygen Consumption

The oxygen consumption rate in MEF cells and intact mitochondria was measured using an XF24 Analyzer (Seahorse Bioscience). The oxygen consumption rate was normalized to the total protein concentration for measurement in cells. Detailed procedures for the respiratory assay can be found in the Supplemental Experimental Procedures.

Gene Expression Assay

RNA was extracted from tissues using Trizol (Invitrogen) following the manufacturer's instructions. Quantitative PCR (qPCR) was performed using SYBR Premix Ex Taq (TAKARA). For examination of the expression levels of oxidative response genes, the results were normalized to the geometric mean of multiple reference genes (Hprt1, RPL13A, B2M, GAPDH, ACT). Then, a Z-transformation was applied to the results to calculate the *Z* score and construct a heatmap (Cheadle et al., 2003). The sequences of primers used can be found in the Supplemental Experimental Procedures.

Analysis of tRNA Modification

Total RNAs were isolated from bacteria and tissues using Trizol reagent (Invitrogen). RNA was digested with Nuclease P1 (Sigma) and subjected to mass spectrometry (Agilent 6460). For detecting tRNA modification using the qPCR-based method, we adapted a protocol described previously (Xie et al., 2013). Detailed procedures for the mass spectrometry and qPCR method can be found in the Supplemental Experimental Procedures. To measure the tRNA modification level in blood samples, blood samples were collected from MELAS patients using standard procedures approved by Kurume University (IRB#9715).

ATP Measurement

Small pieces of skeletal muscle and heart tissue were immediately dissected after sacrificing mice and snap frozen in liquid nitrogen until measurement. ATP was measured using the ATP Bioluminescence Assay Kit following the manufacturer's protocol (TA100, WAKO). The luminescence was measured using a Centro XS^3 LB960 (Berthold) and normalized to total protein concentration.

Cardiac Function Examination

Echocardiographs were examined in M-mode while the mice were under anesthesia using the Vevo2100 system (Fujifilm VisualSonics, Inc.) according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using Prism 6 Software (GraphPad Software). An unpaired Student t test was used to test the differences between two groups. Analysis of variance (one-way ANOVA or two-way ANOVA) was used to test the difference among multiple groups followed by a post hoc examination of the p value between two groups. A two-tailed p value of 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2015.01.019.

AUTHOR CONTRIBUTIONS

F.-Y.W. and K.T. designed the experiments and wrote the manuscript. F.-Y.W. and B.Z. performed the experiments. Takeo Suzuki and Tsutomu Suzuki performed the mass spectrometry experiments. H.H., K.M., and Y.O. performed cardiac examinations. Y.U. and S.M. performed TAC surgery and cardiac examinations. H. Michiue, A.F., and H. Matsui performed electron microscopy. Y.K. provided the blood samples. N.T., P.X., and T.K. performed the qPCR-based examination of tRNA modifications.

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Case report

Leigh syndrome with Fukuyama congenital muscular dystrophy: A case report

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Abstract

We report the first case of Leigh syndrome (LS) with Fukuyama congenital muscular dystrophy (FCMD). A neonate suffered from lactic acidosis and subsequently presented with poor feeding, muscle weakness, hypotonia, cardiopulmonary dysfunction, and hydrocephalus. He died at 17 months. The findings of brain magnetic resonance imaging indicated some specific features of both LS and FCMD, and FCMD gene mutation was detected. Decreased mitochondrial respiratory complex I and II activity was noted. Mitochondrial DNA sequencing showed no pathogenic mutation. A case with complex I + II deficiency has rarely been reported, suggesting a nuclear gene mutation.

Keywords: Leigh syndrome; FCMD; Mitochondria; Complex I + II deficiency

1. Introduction

Fukuyama congenital muscular dystrophy (FCMD), one of the most common autosomal recessive disorders in the Japanese population, is characterized by congenital muscular dystrophy with cortical dysgenesis. The gene responsible for FCMD is located on 9q31. Most FCMD-bearing chromosomes (87%) have a 3-kb retrotransposal insertion in the 3′-untranslated region of the gene [1].

Leigh syndrome (LS) is a progressive neurodegenerative disorder with psychomotor retardation, signs and symptoms of brain stem and/or basal ganglia involvement, and raised lactate levels in blood and/or cerebrospinal fluid (CSF). In majority of the cases, dysfunction of the mitochondrial respiratory chain is responsible for the disease. LS is caused by either mitochondrial or nuclear gene mutations with large genetic heterogeneity [2]. Here, we report the first case of LS with FCMD.

2. Case report

2.1. Index case

A Japanese boy was born at term as the third child to non-consanguineous healthy parents. His serum creatine

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kinase concentration was extremely high (45149 IU/L) on the day of birth without any anomaly. Serum lactate level, plasma amino acid profiles, and carnitine profiles were normal. Urinary organic acid profiles showed no specific abnormalities. The patient suddenly suffered from severe lactic acidosis, hyperglycemia, and acute heart failure at day 17. Levels of lactate and pyruvate in the CSF were 4.9 mM and 0.21 mM. A mitochondrial disorder was suspected and treatment was started with carnitine, ubiquinone, and other vitamins in addition to cardiotonics and insulin. The infant's condition improved, but he subsequently presented with poor feeding, muscle weakness, and hypotonia at 1 month. Hypertrophic cardiomyopathy occurred at 3 months and cardiopulmonary function worsened after repeated lactic acidosis, and he required mechanical ventilation from the age of 6 months. He presented with an enlarged head circumference and a tense anterior fontanelle at 12 months, and died of pneumonia at 17 months.

Magnetic resonance imaging (MRI)at 2 months revealed cerebellar cysts, pachygyria, and T2-hyperintense lesions in white matter and the brainstem, but basal ganglia were normal (Fig. 1A). A follow-up investigation at 4 months indicated extended T2-hyperintense lesions (Fig. 1B). A brain computed tomography (CT) scan at 14 months showed severe hydrocephalus and extensive cerebral atrophy (Fig. 1C).

Cerebellar cysts and pachygyria are characteristic of FCMD, genetic testing for *FCMD* was performed. We examined retrotransposal insertion into the 3′-untranslated region (UTR) of the *FCMD* gene using a polymerase chain reaction (PCR)-based diagnostic method involving peripheral blood leukocytes of this case and his parents [1]. A homozygous mutation of this case and heterozygous mutation of his parents were detected. Repeated lactic acidosis and brain stem lesions led us to suspect LS. A skin biopsy was performed for mitochondrial analysis at 1 month. Activities of mitochondrial respiratory chain complex (Co) I, II, III, and IV were assayed from skin fibroblasts, as described previously [3]. The activities were also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker and to Co II activity, and evaluated according to the diagnostic criteria [4]. Respiratory chain complex I and II activities were very low, but CS, Co III, and Co IV activities were normal (Table 1). Expression of the mitochondrial respiratory chain CoI, II, III, and IV proteins was concurrently examined by Western blotting

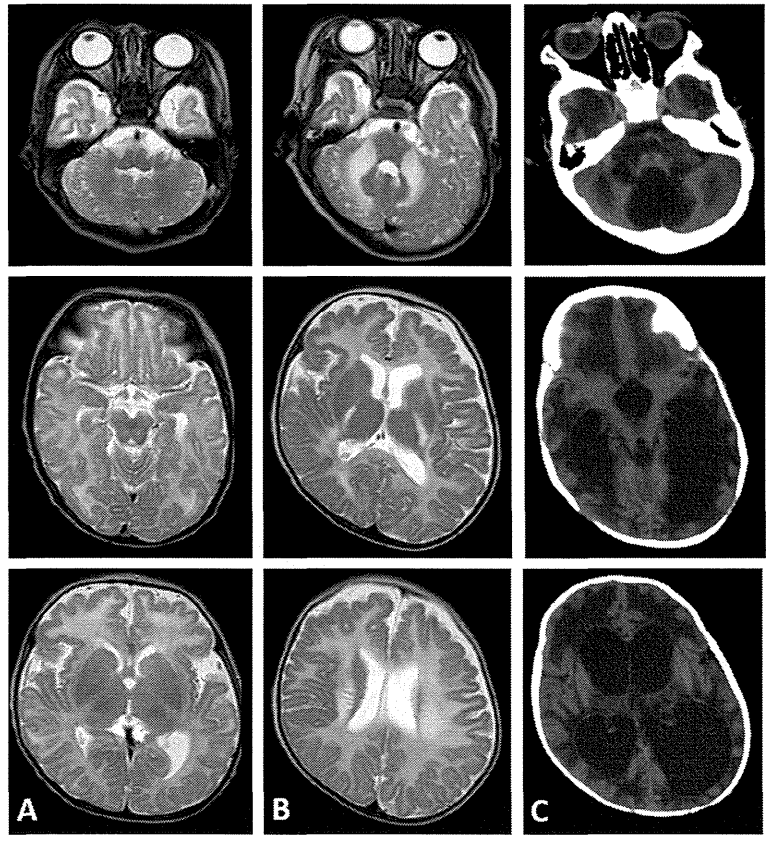

Fig. 1. Magnetic resonance image (MRI) at the age of 2 months (A) shows cerebellar cysts (A, top), bilateral symmetrical lesions in the brainstem (A, middle), pachygyria, and T2-hyperintensity in white matter, predominantly in the frontal lobes (A, bottom). An MRI at 4 months of age indicated T2-hyperintensity extending into the middle cerebellar peduncles, posterior limb of the internal capsule, and the corona radiata (B). A brain computed tomography scan at 14 months of age showed severe hydrocephalus, widespread hypodensity of white matter, and extensive cerebral atrophy (C).

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Table 1
Activities of mitochondrial respiratory chain complex (Co) I, II, III, and IV; citrate synthase (CS) from skin fibroblasts. Enzyme activities are expressed as a percentage of mean relative activity of 35 normal controls and relative to CS and Co II.

	Co I	Co II	Co II + III	Co III	Co IV	CS
Crude activity (%)	32	18	21	56	45	80
CS ratio (%)	38	21	24	65	55	
Co II ratio (%)	177		112	312	259	

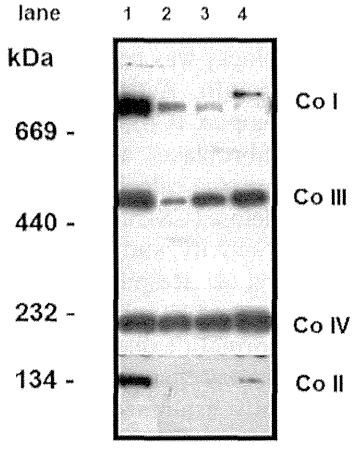

Fig. 2. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of skin fibroblasts 1: control; 2: this case; 3: a double of lane 2; 4: a triple of lane 2. The bands corresponding to Co II were almost invisible and those corresponding to Co I were markedly weak, whereas the intensities of the Co III and IV bands gradually became strong.

using blue native polyacrylamide gel electrophoresis (BN-PAGE), as described previously [5]. The BN-PAGE analysis showed that the bands corresponding to Co II were almost invisible and those corresponding to Co I were markedly weak (Fig. 2). This finding was in agreement with the enzyme activity assay results. The mitoSEQr™ system (Applied Biosystems, Foster City, CA, USA) was used for the entire mitochondrial DNA analysis. Genomic DNA was extracted from skin fibroblasts. Data were analyzed with SeqScape Software v2.5 and compared with mitochondrial DNA sequences (Mitomap: www.mitomap.org). Several base substitutions were detected, but no pathogenic mutation was detected in the entire mitochondrial DNA sequence. High resolution chromosome analysis was normal.

2.2. Family history

The index case's older brother had repeated afebrile convulsions since the age of 2 months, but brain MRI findings were normal. Laboratory tests showed elevated level of lactate (2.92 mM) in CSF and a normal level of serum creatine kinase (75 IU/L). He died of sudden cardiac dysfunction at 4 months. The second child was a healthy girl with normal development.

3. Discussion

A case of FCMD and mitochondrial respiratory chain disorder (MRCD) has never been reported. The pathophysiology of FCMD and MRCD is quite different, therefore, low activities of the respiratory chain complexes in this case were probably not due to FCMD. LS is clinically characterized by a wide variety of manifestations involving multiple organs in infancy or early childhood. Thus, the early onset of his symptoms suggested that LS was the main cause.

White matter abnormalities in patients with FCMD are often detected by MRI as transient T2-hyperintensity. Kato et al. reported that the pathological origin of white matter lesions is dysmyelination and that the lesions are masked by brain development [6]. In this case, the extended signal abnormalities had different features compared with those of FCMD. Some cases of complex II deficiency with extensive T2-hyperintensities in white matter have been reported [7,8]. The white matter abnormalities in our case may have been associated with the complex II deficiency. The patient presented with progressive hydrocephalus, but he had no prior clinical signs of intraventricular hemorrhage or infection in CSF. Patients with FCMD, who are homozygotes for the insertion mutation with hydrocephalus have never been reported [9]. A few patients with LS develop cerebellar atrophy or ventricular enlargement [10].

Many cases of combined complex deficiencies have been reported, but a case with a complex I + II deficiency has rarely been reported. The entire mitochondrial DNA sequencing in this case showed no pathogenic mutation. These findings suggest that LS in this case was the result of a nuclear gene mutation.

The genes responsible for mitochondrial disease located contiguous to the FCMD gene have not been identified. The infant's older brother was suspected to have MRCD without obvious clinical signs of FCMD. Therefore, we speculated that the present case was unlikely to be a contiguous gene syndrome. We are investigating this patient's fibroblasts using next-generation sequencing to identify the causative nuclear gene mutation and the relation between the two diseases.

Potential conflict of interest report

The authors indicated no potential conflict of interest.

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Original Article

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

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

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

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

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

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

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

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Methods

Patients and samples

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

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

Respiratory chain enzyme analysis

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

Entire mtDNA analysis

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

Quantitative polymerase chain reaction for diagnosis of MTDPS

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

Mutation analysis of genes responsible for MTDPS

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

DNA from healthy Japanese controls

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

Statistical analysis

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

Ethics

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

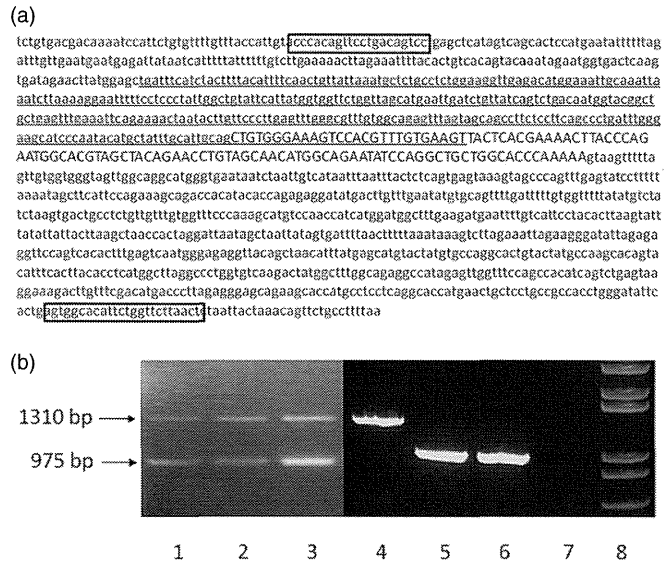

Fig. 1 Genomic sequence determination of 335 bp deoxyguanosine kinase (*DGUOK*) deletion in the family of patients 1 and 2. (a) Capitalization, sequence of exon 2; two rectangles, long-range polymerase chain reaction (PCR) primer sets; underline, 335 bp deletion. The large 335 bp deletion encompassing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

Case reports: DGUOK deficiency in three Japanese patients

Patient 1

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

Patient 2

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

Patient 3

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

Results

Characteristics of Japanese children diagnosed with MRCD

In total, we diagnosed MRCD in 232 patients; these patients comprised 34% of the study group. The age distribution of these patients is as follows; nearly 40% before 1 month of age, three-fourths by age 1 year, and >90% by age 7 years. One hundred and twenty patients (52%) were male, and approximately half of the diagnosed patients were deceased. Diverse clinical diagnoses are shown in Figure 2. Eighty-seven patients (38%) had neurological disorders consisting of Leigh syndrome, neurodegenerative disorders, and so-called mitochondrial cytopathy. Fifty-nine (25%) had a lethal or non-lethal infantile mitochondrial disorder. Twenty-nine (13%) had mitochondrial hepatopathy, and 17 (7%) had mitochondrial cardiomyopathy. Among all MRCD, 28 patients (12%) lacked lactic acidemia, a feature that traditionally prompts suspicion of MRCD. The entire mitochondrial DNA

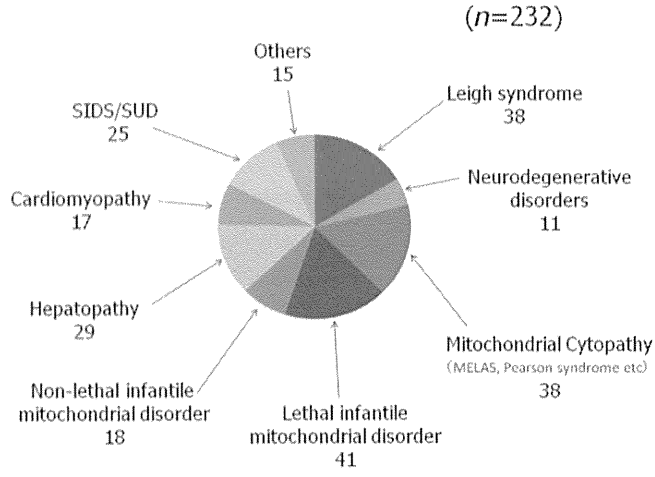

Fig. 2 Clinical diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan. Neurodegenerative disorders, neurodegenerative disorders unclassified to specific diseases. Patients with non-lethal infantile mitochondrial disorder started with symptoms such as lethal infantile mitochondrial disorder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

sequence was determined for 139 patients, but a causative genetic abnormality was found in only 34 (24%) of these patients (data not shown); indicating that, in most cases, the causative gene or genes may be present in nuclear DNA.

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

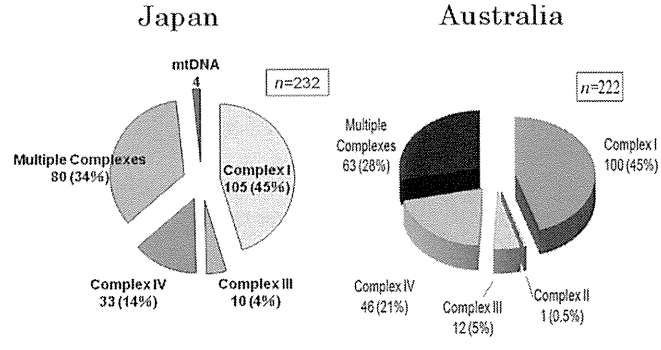

Fig. 3 Percentage distribution of enzymatic diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan and those reported previously in Australia. The enzymatic diagnosis of MRCD showed similar trends in prevalence between the Japanese and Australian patients,[17] with respiratory chain complex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

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

Manifestations, genetic diagnoses, and prognoses of MTDPS

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

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

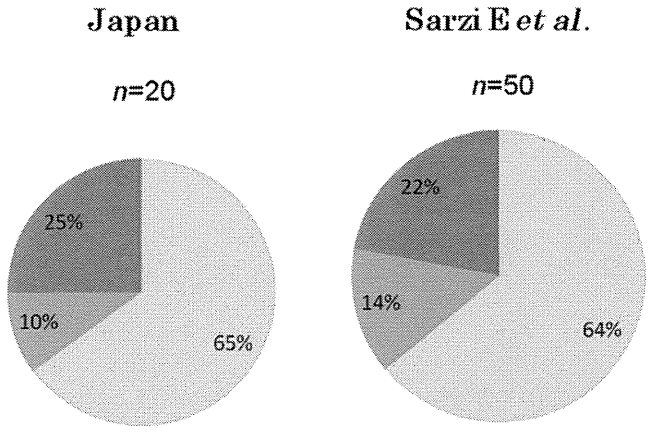

Fig. 4 Percentage distribution of disease types of mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS, because no true case of Alpers syndrome has yet been identified in Japan. The distribution of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

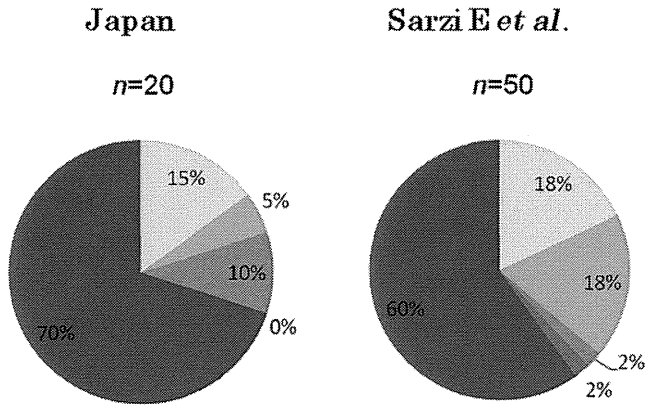

Fig. 5 Percentage distribution of responsible genes for mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* The causative gene was not identified in the majority of patients in each population. Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the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

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

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

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

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

Table 1 Clinical and molecular characteristics for Japanese hepatocerebral MTDPS patients

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

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

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Human          241   -----ALMNIPVLVLDV--NDDFSEEVTKQEDLMREVNTFVKNL------   277
Pan Trog       241   -----ALMNIPVLVLDV--NDDFSEEVTKQEDLMREVNTFVKNL------   277
Canis          241   -----ALLNIPVLVLDV--NDDFSEEVTKQEELMKKVNIFVKNL------   277
Bos            241   -----ALLNIPVLVLDV--NDDFSEEVTIQEELMRRVNTFVKNL------   277
Mus            241   -----ALQHVPVLVLDV--TEDFSENAARQEELMGQVNTFMRNL------   277
Rat            241   -----ALRHVPVLVLNI--SEDFSENAAKQEELMGQVNTFMRNL------   277
Danio          233   -----QLMKVPVLVLDA--EVAFEQNPEVQDCLLSKVRDFLSQL------   269
Arabidopsis    483   NHMHSSIQKVPALVLDCEPNIDFSRDIEAKTQYARQVAEFFEFVKKKQET   532
Oryza          408   DHMHSSIQKVPALVLDCEHDIDFNKDIEAKRQ-------------------   439
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Fig. 6 ClustalW multiple sequence alignment of deoxyguanosine kinase (*DGUOK*) orthologs. The alignment shows that amino acid 248Leu mutated in the patient is absolutely conserved in all species. URLs: HomoloGene, http://www.ncbi.nlm.nih.gov/homologene (for the DGUOK ortholog amino acid sequences of human [accession no. NP_550438.1], Pan [accession no. XP_001153473.1], Canis [accession no. XP_533001.2], Bos [accession no. NP_001014888.2], Mus [accession no. XP_001107072.1], Rat [accession no. NP_001100072.1], Danio [accession no. XP_001093561.1], Arabidopsis [accession no. NP_565032.2], Oryza [accession no. NP_001044956.1]). ClustalW, http://www.ebi.ac.uk/Tools/clustalw/.[18]

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

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

Discussion

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

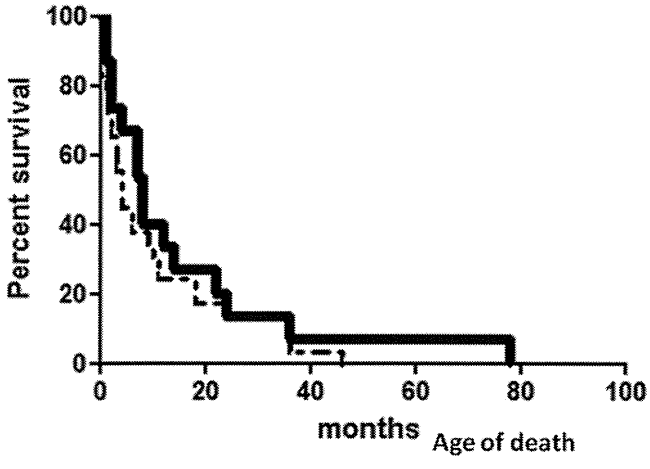

Fig. 7 Comparison of the ages of death (in months) in the two studies. A commonality between the Japanese patients and the Sarzi *et al.* patients[4] was observed. No significant difference in disease severity was identified (log-rank test, $P = 0.3637$; Gehan-Breslow-Wilcoxon test, $P = 0.2667$). (▬)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

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

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

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

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

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

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

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

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

Conclusion

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

Acknowledgments

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SHORT COMMUNICATION

A girl with West syndrome and autistic features harboring a *de novo TBL1XR1* mutation

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Recently, *de novo* mutations in *TBL1XR1* were found in two patients with autism spectrum disorders. Here, we report on a Japanese girl presenting with West syndrome, Rett syndrome-like and autistic features. Her initial development was normal until she developed a series of spasms at 5 months of age. Electroencephalogram at 7 months showed a pattern of hypsarrhythmia, which led to a diagnosis of West syndrome. Stereotypic hand movements appeared at 8 months of age, and autistic features such as deficits in communication, hyperactivity and excitability were observed later, at 4 years and 9 months. Whole exome sequencing of the patient and her parents revealed a *de novo TBL1XR1* mutation [c.209 G>A (p.Gly70Asp)] occurring at an evolutionarily conserved amino acid in an F-box-like domain. Our report expands the clinical spectrum of *TBL1XR1* mutations to West syndrome with Rett-like features, together with autistic features.
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TBL1XR1 at 3q26.32 encodes transducin β-like 1 X-linked receptor 1, a co-repressor of nuclear hormone transcription factors that is required for β-catenin–Tcf-mediated Wnt signaling.[1–3] Recently, two *de novo TBL1XR1* mutations were found in 2 of 2446 patients with autism spectrum disorders, suggesting an association between *TBL1XR1* mutations and autism.[4,5] Here, we present a third case with a *de novo TBL1XR1* mutation, showing West syndrome, Rett-like and autistic features.

CASE REPORT

This report concerns a 5-year-old girl who is the offspring of unrelated healthy Japanese parents. She was born at 39 weeks of gestation without asphyxia after an uneventful pregnancy. Her birth weight, birth length and head circumference were 3088 g (+0.3 standard deviation (s.d.)), 51.1 cm (+1.0 s.d.) and 34.0 cm (+0.5 s.d.), respectively. She showed social smiling and head control at 3 and 4 months of age, respectively. Then at 5 months she developed a series of spasms occurring 5–6 times a day, when her head control became unstable. Electroencephalography at 7 months of age showed hypsarrhythmia patterns (Figure 1a), which led to a diagnosis of West syndrome. Brain magnetic resonance imaging showed no structural brain anomalies (Figures 1a and c). Administration of adrenocorticotropic hormone therapy only temporarily reduced the frequency of spasms.

On examination at 7 months of age, the patient's weight, height and head circumference were 8720 g (+1.2 s.d.), 69.5 cm (+0.9 s.d.) and 42 cm (−0.6 s.d.), respectively. Mild dysmorphic features were observed, including a long palpebral fissure, thick eyebrows and downturned corners of the mouth. The patient showed no eye fixation and pursuit, as well as no social smile. Her muscle tone was mildly hypotonic. Despite being given extensive treatments including adrenocorticotropic hormone therapy in combination with valproic acid, nitrazepam, vitamin B6, topiramate, clonazepam and clobazam, she continued to exhibit frequent subtle tonic seizures with eyelid opening. At 8 months of age, stereotypical hand movements appeared that resembled hand-washing.

Laboratory examination revealed elevated serum levels of several components: lactic acid (39.9 mg dl^{-1}, compared with a normal range of 5.0–20.0 mg dl^{-1}), pyruvate (2.79 mg dl^{-1}, normal range 0.3–0.9 mg dl^{-1}) and alanine (1447 nmol ml^{-1}, normal range 180–470 nmol ml^{-1}). However, following vitamin B1 treatment, both pyruvate and alanine serum levels returned to normal. The levels of lactic acid and pyruvate in cerebrospinal fluid appeared normal (14.6 and 0.65 mg dl^{-1}, respectively). Respiratory-chain enzymes, using muscle homogenates and fibroblasts, and mitochondrial DNA sequence analysis were all normal, and pathological examination of muscle specimens revealed no specific findings. Repeated examination of both serum lactic acid and pyruvate levels also showed no abnormalities.

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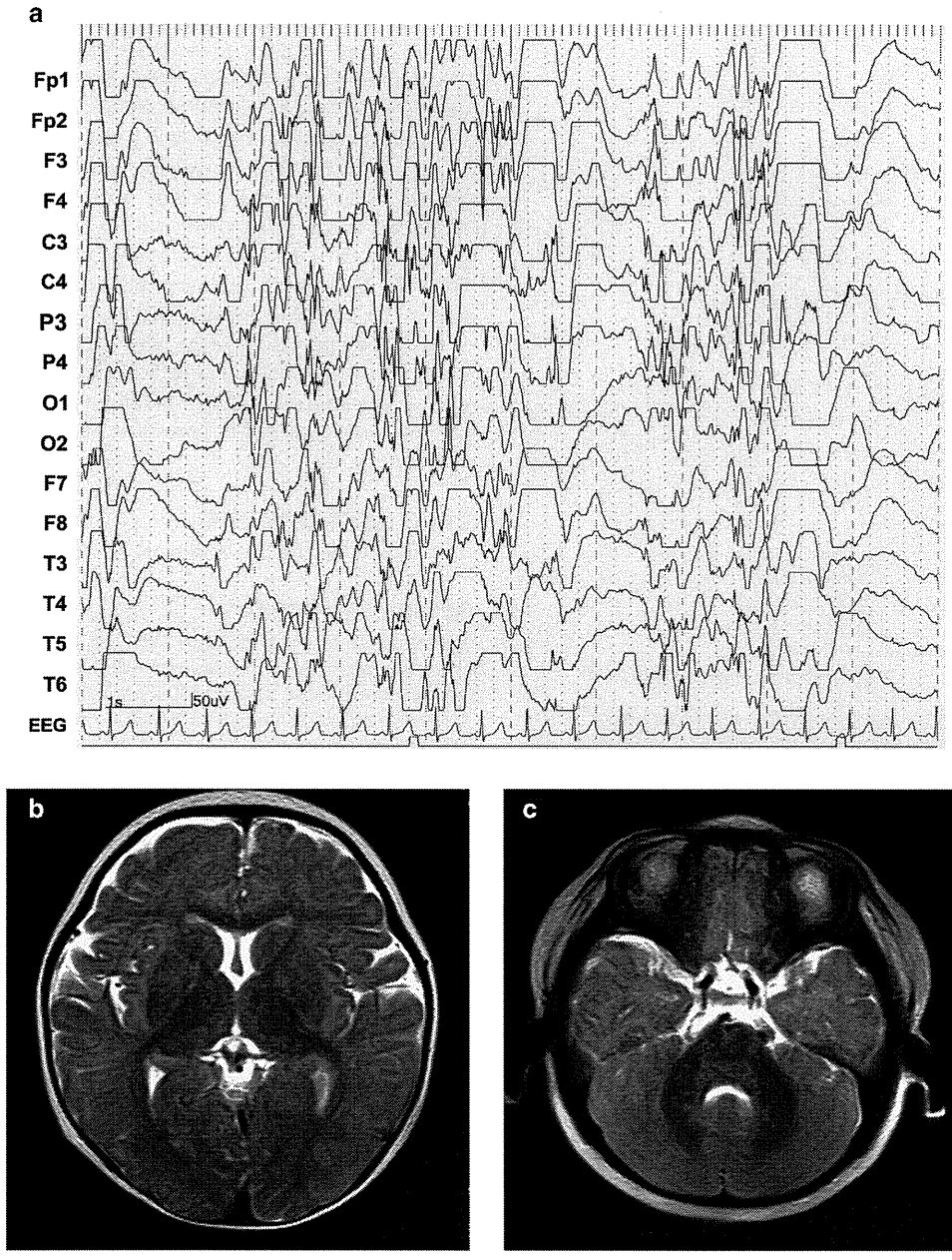

Figure 1 Electroencephalogram (EEG) and brain magnetic resonance imaging in the patient at 7 months of age. (**a**) Interictal EEG showed high-amplitude multifocal spikes with irregular slow waves consistent with a finding of hypsarrhythmia. T2-weighted axial images through the basal ganglia (**b**) and the cerebellum (**c**) showed no abnormalities.

After contracting a fever at the age of 1 year and 10 months, her seizures were controlled using a combination therapy of topiramate, valproic acid, clobazam and vitamin B1. However, the patient still could not speak any meaningful words at 4 years and 9 months of age but could walk with support; she had a developmental quotient of 13. At this age she still showed stereotypic hand movements as well as autistic features such as deficits in communication, hyperactivity and excitability.

RESULTS AND DISCUSSION

G-banded karyotyping was normal (46,XX). No pathological copy number aberrations were detected by the 2.7M Array (Affymetrix, Santa Clara, CA, USA). Whole exome sequencing of the patient and her parents was performed. Genomic DNA of blood leukocytes was captured using the SeqCap EZ Exome Library v2.0 (Roche NimbleGen, Madison, WI, USA), and sequenced with on HiSeq2000 (Illumina, San Diego, CA, USA). Variants were detected as previously described.[6] Variants with a Phred-like consensus quality score of >100 were considered as candidate variants. We found a total of four *de novo* candidate variants, in which two mutations were further validated as *de novo* by Sanger sequencing: *SELPLG* NM_001206609.1: c.794C>T (p.Thr265Met) and *TBL1XR1* NM_024665.4:c.209G>A (p.Gly70Asp). The other two variants were transmitted from her mother, demonstrating that the two variants were falsely uncalled in the mother. Neither of the two *de novo* mutations was found in the 6500 National Heart, Lung, and Blood Institute exomes nor in our

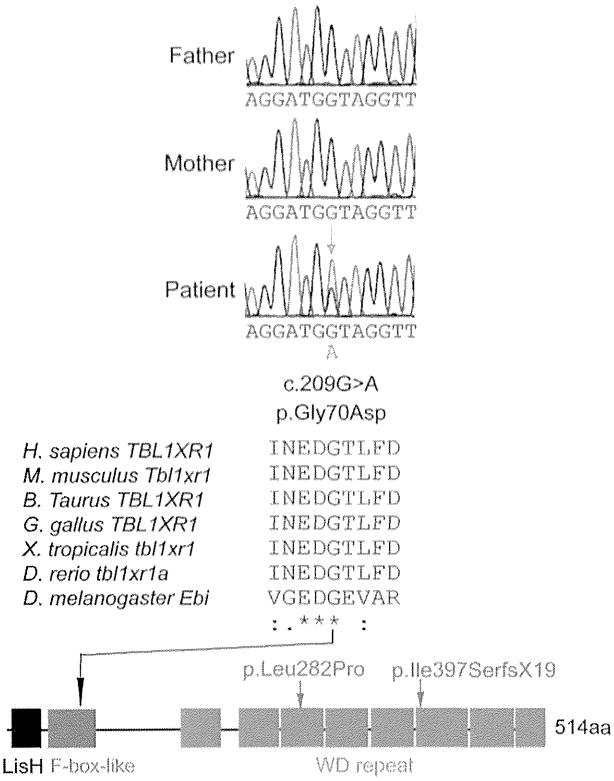

Figure 2 *De novo TBL1XR1* mutation. The c.209G>A (p.Gly70Asp) mutation occurred *de novo* at an evolutionarily conserved amino acid in an F-box-like domain. Multiple amino-acid sequences of TBL1XR1 proteins were aligned with tools available on the CLUSTALW web site. Two previously reported mutations (p.Leu282Pro and p.Ile397SefsX19) are highlighted in red. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

575 in-house control exomes. Both mutations were predicted as damaging by SIFT and PolyPhen2. However, MutationTaster classified only p.Gly70Asp in *TBL1XR1* as damaging whereas p.Thr265Met in *SELPLG* was predicted as a polymorphism. We found no recessive mutations in known early-onset epileptic encephalopathy genes including *SLC25A22*, *PNPO*, *PNKP* and *PLCB1*.[7] All experimental protocols used were approved by the Institutional Review Board of Yokohama City University School of Medicine.

SELPLG encodes P-selectin glycoprotein ligand 1, for which a knock-out mouse study showed that *Selplg* is required for leukocyte adhesion and rolling.[8,9] No neurological abnormalities were reported, suggesting that the *SELPLG* mutation is less likely to be involved in the phenotype of this patient.

TBL1XR1, also denoted as *TBLR1*, is required for β-catenin–Tcf-mediated Wnt signaling.[1,2] Mutations in *TCF4*, an essential mediator of Wnt signaling, have been shown to cause Pitt–Hopkins Syndrome, which presents with severe intellectual disability, seizures and stereotypic movements.[10,11] This suggests that the β-catenin–Tcf-mediated Wnt pathway of signaling is essential for normal brain function. Moreover, two *de novo TBL1XR1* mutations (p.Leu282Pro and p.Ile397SerfsX19) were found in 2 of 2446 patients with autism spectrum disorders.[4,5] In our case, the p.Gly70Asp mutation occurred in an evolutionarily conserved amino acid within an F-box-like domain (Figure 2). Indeed, the F-box-like domain of TBLR1 (TBL1XR1) is essential for a high affinity interaction between TBL1XR1 and SMRT, a co-repressor of nuclear hormone receptors.[3] This implies that p.Gly70Asp may affect this interaction. Therefore, the evidence suggests that the p.Gly70Asp mutation may cause a West syndrome phenotype with Rett-like and autistic features.

The role of *TBL1XR1* mutations was also investigated in 280 epileptic patients. High-resolution melting analysis revealed that three rare missense variants (p.Ala116Ser, p.Gly405Glu and p.Asn407Ser) were present in three patients. Since they were predicted as benign by PolyPhen-2, these mutations are unlikely to be pathogenic. These data suggest that *TBL1XR1* mutations are rarely involved in epileptic patients.

In conclusion, we describe a Japanese girl with a *de novo TBL1XR1* mutation that is predicted as pathogenic. Our report suggests that the clinical spectrum of *TBL1XR1* mutations includes autistic features as a core phenotype, as well as presenting with West syndrome and Rett-like features.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Clinical Observations

Fever of Unknown Origin as the Initial Manifestation of Valproate-Induced Fanconi Syndrome

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ABSTRACT

BACKGROUND: Valproate-induced Fanconi syndrome is a rare adverse effect of valproate. Severely disabled patients who require tube feeding are reported to be susceptible to valproate-induced Fanconi syndrome. Although most patients with valproate-induced Fanconi syndrome are asymptomatic and detected incidentally with findings such as hypophosphatemia, hypouricemia, increased urinary β2-microglobulin, and generalized hyperaminoaciduria, clinical symptoms such as bone fracture, fever, tachypnea, and edema have been reported. **PATIENT DESCRIPTION:** This 15-year-old, severely disabled, tube-fed, male patient with cytochrome oxidase deficiency had taken valproate for 3 years when he developed fever for 3 weeks. Hypophosphatemia, hypouricemia, hypokalemia, increased urinary β2-microglobulin, and generalized hyperaminoaciduria, as well as hypocarnitinemia, were found, indicating that he had Fanconi syndrome. Valproate was the most likely cause of Fanconi syndrome in this patient. After discontinuation of valproate, the fever resolved immediately, and the laboratory findings normalized. **CONCLUSION:** Valproate-induced Fanconi syndrome should be considered when individuals taking valproate develop fever of unknown origin.

Keywords: Fanconi syndrome, valproate, fever of unknown origin, side effects, valproate-induced Fanconi syndrome

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Introduction

Fanconi syndrome is a generalized dysfunction of the proximal renal tubules that causes urinary excretion of amino acids, glucose, phosphate, bicarbonate, uric acid, and other substances. Valproate (VPA)-induced Fanconi syndrome is a rare adverse effect of VPA.[1] Several case reports have shown that severely disabled, tube-fed patients are vulnerable to VPA-induced Fanconi syndrome.[2,3] Most patients with VPA-induced Fanconi syndrome are diagnosed during routine or incidental laboratory examinations without any obvious symptoms.[2] The case of a severely disabled, tube-fed patient with cytochrome oxidase deficiency who presented with fever of unknown origin that was most likely caused by VPA-induced Fanconi syndrome is presented.

Patient Description

This 15-year-old boy was born at 41 weeks' gestation with a birth weight of 3270 g. His Apgar score was 3 at 1 minute and 6 at 5 minutes. He developed spastic tetraplegia and needed tube feeding. At age 4 months, he presented with infantile spasms. He was diagnosed with probable Leigh syndrome because of high lactate levels in the blood (39.6 mg/dL) and cerebrospinal fluid (34.5 mg/dL), as well as high-intensity signals in bilateral basal ganglia and thalami on T2-weighted magnetic resonance imaging at age 2 years. Pyruvate dehydrogenase complex activities in the lymphocytes and respiratory chain complex activities in the muscle, as well as histopathology of the skeletal muscle, were normal. Screening for known mitochondrial DNA mutations was negative. Seizure control was poor, and he had been on VPA and carbamazepine without l-carnitine supplementation from age 12 years. Two

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months before his admission at age 15 years, the VPA dose was increased (34 mg/kg/day) because of seizure deterioration, and l-carnitine (6 mg/kg/day) was added as a supplement to prevent secondary carnitine deficiency.

He was admitted to our hospital because of high-grade fever (39.1°C) that had lasted for 3 days. Bronchial pneumonia was suspected, and cefotaxime was administered. However, the fever persisted for 3 weeks. His body weight decreased from 16.7 kg at admission to 14.8 kg during the 3 weeks despite sufficient water (1800 mL/day) and caloric intake (1500 kcal/day). The following laboratory examinations were normal: white blood cell count, C-reactive protein, serological tests for viral or mycoplasma infection, antinuclear antibodies, rheumatoid factor, thyroid hormones, lymphocyte stimulation test for VPA, and bacterial and mycotic cultures. However, the erythrocyte sedimentation rate was high (90 mm/hr). Whole-body [F^{18}]-fluorodeoxyglucose positron emission tomography scan did not reveal any findings indicating focal inflammation. On the other hand, hypophosphatemia (1.3 mg/dL; normal 2.6-6.3 mg/dL), hypokalemia (3.3 mEq/L; normal 3.6-5.2 mEq/L), and hypouricemia (1.6 mg/dL; normal 2.0-7.0 mg/dL) were found, indicating the possibility of proximal renal tubular dysfunction. Urinalysis showed proteinuria, glycosuria, elevated β2-microglobulin (4460 μg/L; normal <230 μg/L), and generalized hyperaminoaciduria. These results confirmed Fanconi syndrome. In addition, hypocarnitinemia (19.0 μmol/L) was found despite carnitine supplementation, indicating secondary carnitine deficiency due either to VPA or Fanconi syndrome, or both. VPA was discontinued at 18 days from the start of fever. Four days later, the high fever resolved, and he gained 1 kg of weight. After the fever resolved, the l-carnitine supplement was increased (40 mg/kg/day) to treat hypocarnitinemia. Two months later, laboratory findings associated with Fanconi syndrome were normal. After recovery, a skin biopsy revealed cytochrome oxidase deficiency. The patient was not re-challenged with VPA, and no recurrence of Fanconi syndrome has been evident for more than 2 years.

Discussion

Fanconi syndrome is characterized by a general dysfunction of proximal renal tubules that causes urinary excretion of amino acids, glucose, phosphate, bicarbonate, uric acid, and other substances.[4] Recently, VPA-induced Fanconi syndrome has been recognized mostly in severely disabled patients.[2,3] The fever of unknown origin and weight loss in the present patient were likely the manifestations of VPA-induced Fanconi syndrome due to (1) the patient's vulnerability to this condition due to his diagnosis of cytochrome oxidase deficiency and severe disability requiring tube feeding, and (2) the fact that fever of unknown origin, weight loss, and laboratory findings indicative of Fanconi syndrome normalized after discontinuation of VPA.

A search of the PubMed database and the Japan Medical Abstract Society website for articles using the keywords "Fanconi syndrome" and "valproate" or "valproic acid" identified 20 reports of 49 patients (Table).[1-20] In these, sex and age were described in 37 patients (19 males and 18 females), with ages ranging from 2 to 32 years (median age, 8 years). As previously reported,[3] a high percentage of Japanese patients (36 of 49 patients) was evident. Among the 49 patients identified, 47 were described as being severely disabled (n = 42) or not (n = 5); in addition, feeding was described in 41 of the 49 patients, with 36 reported as being tube-fed. The duration of VPA treatment ranged from 3 months to 21 years (median, 4 years), and the VPA blood levels ranged from 21 to 141 μg/mL (median, 77.6 μg/mL). When VPA was discontinued, 45 of 47 patients recovered completely from VPA-induced Fanconi syndrome.

The duration needed for recovery ranged from 1 week to 18 months (median, 4 months). Two patients developed renal failure or continuing proteinuria despite the discontinuation of VPA.[6,13] Thus, the clinical course of VPA-induced Fanconi syndrome in the present patient was similar to the previous reports. Among the 13 patients in whom serum carnitine levels or carnitine supplementation was described, 3 patients had hypocarnitinemia,[11,16,19] 1 patient had a normal carnitine level,[15] and 9 patients with no description of serum carnitine levels had carnitine supplementation.[12,13,17,18] None of the patients whose serum carnitine levels were described had fever of unknown origin. Thus, there was no apparent association between hypocarnitinemia and prolonged fever, as appeared in the present patient. Furthermore, the weight loss and elevated erythrocyte sedimentation rate found in the present patient were not described in the other reported patients with VPA-induced Fanconi syndrome.

Overall, in 19 of the 49 reported patients, VPA-induced Fanconi syndrome was found on routine or incidental laboratory examinations without any obvious symptoms. In the remaining 30 patients, the initial clinical manifestations led to the diagnosis: 11 had fracture, 9 had fever, 3 had tachypnea, 2 had edema, 2 had weakness, 1 had anorexia, abdominal pain, and myopathy-like symptoms, 1 had hypertension, and 1 had fatigue and confusion. Among the 9 patients with fever,[8,9,14,15,20] 2 had prolonged fever described as fever of unknown origin.[8] In the remaining 7 patients, VPA-induced Fanconi syndrome was diagnosed and treated before the fever became prolonged.

In the present case, it took time to suspect VPA-induced Fanconi syndrome because fever is a common symptom in severely disabled patients. After infection was ruled out, more time was taken to rule out various other causes. The present case report, therefore, suggests the importance of considering VPA-induced Fanconi syndrome in severely disabled patients on VPA who develop prolonged fever and of measuring serum phosphate, uric acid, and electrolyte levels, as well as urinalysis including β2-microglobulin. The present case also suggested that supplementation with less than 10 mg/kg of carnitine is insufficient in these patients.

Although the precise pathogenic mechanism of VPA-induced Fanconi syndrome remains unknown, a mitochondrial abnormality in the proximal renal tubules is a typical finding of drug-induced Fanconi syndrome. There are at least three hypotheses for the pathogenesis of the renal tubular dysfunction. The first is an inhibition of β-oxidation in the mitochondria of the proximal renal tubules either directly by VPA or indirectly by the secondary carnitine deficiency caused by VPA.[1,5] The second is tubulo-interstitial nephritis (TIN) caused by hypersensitivity to VPA or a direct toxic effect of VPA.[5,8] The third is increased oxidative stress due to the VPA-induced decrease in plasma glutathione peroxidase activity, which causes mitochondrial dysfunction in the tubules.[1] The mechanisms of fever in VPA-induced Fanconi syndrome are unknown. Only two reported patients had prolonged fever of unknown origin, and TIN was found in one patient.[8] Because TIN can cause fever and weight loss,[8] fever of unknown origin and weight loss in the present patient might have been caused by TIN, but it could not be confirmed without renal biopsy. Another possibility is