ELSEVIER

Three Novel SURF-1 Mutations in Japanese Patients With Leigh Syndrome

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Leigh syndrome, a severe neurodegenerative disorder, commonly is associated with cytochrome c oxidase deficiency. Recent studies in white patients indicate that SURF-1 gene mutations can cause Leigh syndrome associated with cytochrome *c* oxidase deficiency. When we measured cytochrome *c* oxidase activity in cultured lymphoblastoid cells from our Japanese patients with typical Leigh syndrome, three patients demonstrated cytochrome *c* oxidase deficiency. Three novel mutations of the SURF-1 gene were identified in two of these three patients with cytochrome *c* oxidase deficiency. All mutations predicted loss of function of the SURF-1 protein; in both patients' cells, cytochrome *c* oxidase activity was decreased to less than 20% of the control mean. These results indicate that cultured lymphoblastoid cells are useful for elucidating the etiology of Leigh syndrome, and that loss of function of the SURF-1 gene product can be responsible for Leigh syndrome associated with severe cytochrome *c* oxidase deficiency in Japanese patients.

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Introduction

Leigh syndrome (MIM 516060) is a progressive neurodegenerative disorder of infancy and childhood characterized by the presence of symmetric necrotizing lesions in the brainstem, basal ganglia, thalamus, and spinal cord. Clinical presentation and course vary among patients, frequently including recurrent episodes of vomiting, generalized muscle hypotonia, psychomotor retardation, respiratory abnormalities, and signs of brainstem or basal ganglia dysfunction. Several defects of mitochondrial enzyme complexes result in Leigh syndrome, including pyruvate dehydrogenase complex (EC 1.2.4.1) [1,2] and respiratory chain enzyme complex I, II, and IV [3]. A generalized defect in complex IV (cytochrome *c* oxidase; EC 1.9.3.1) is inherited as an autosomal-recessive trait and is the most common biochemical abnormality associated with Leigh syndrome.

Recent studies in white patients identified mutations in the SURF-1 gene that caused cytochrome *c* oxidase deficiency associated with Leigh syndrome (LS^{COX-}) [4]. Complementation analysis using fibroblasts from LS^{COX-} patients indicated that the SURF-1 gene is located on chromosome 9p34 and that transfer of SURF-1 cDNA to fibroblasts from patients restored cytochrome *c* oxidase activity [5]. We investigated SURF-1 mutations in cultured lymphoblastoid cells from three Japanese LS^{COX-} patients.

Case Reports

Patient 1

The patient manifested normal development until 10 months of age when he developed motor retardation, muscle hypotonia, and recurrent episodes of vomiting. Metabolic acidosis was demonstrated and blood and cerebrospinal fluid lactate concentrations (2.6 and 6.8 mM), and pyruvate concentrations (0.13 and 0.29 mM) were elevated. He was diagnosed with Leigh syndrome because T_2-weighted magnetic resonance images (MRI) revealed bilateral areas of high intensity in the putamen and cerebral peduncle. His condition remained relatively stable, although death occurred suddenly at 2 years and 3 months of age from respiratory failure. Postmortem examination was not permitted.

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Table 1. Activities of respiratory chain enzyme in cultured lymphoblastoid cells

Patient	Complex I + III	Complex II + III	Complex IV
1	30.9	12.8	18.4 ± 2.4 (3)*
2	28.5	10.2	6.6 ± 2 (4)*
3	33.5	15.7	44.8 ± 5.4 (4)**
Controls (8)	36.6 ± 10.2	15.5 ± 6.1	93.8 ± 15.8

Values are means ± S.D. of (n) determinations or a single determination.
* $P < 0.001$, ** $P < 0.01$ vs controls.

Patient 2

Born at term after a normal pregnancy and delivery, the patient demonstrated normal development in the first year of life. After a febrile illness at 14 months of age, he could not maintain a sitting position, and he developed a breathing pattern with episodically prolonged inspirations. At 20 months of age, his family noticed cyanosis during sleeping. He was admitted to the hospital for evaluation. On admission, blood concentrations of lactate (4.7 mM) and pyruvate (0.29 mM) were elevated. The concentration of lactate and pyruvate in the cerebrospinal fluid were elevated, being 4.8 and 0.21 mM, respectively. Amino acid analysis of plasma revealed an elevated alanine level at 925 μmol/L (normal range = 230-490). Symmetric hypodensities on computed tomography and hyperintensity lesions on T_2-weighted MRI were evident in the bilateral basal ganglia. He was diagnosed with Leigh syndrome.

Patient 3

A female weighing 2,826 gm at birth was born at 38 weeks of gestation but subsequently was admitted to the hospital for 10 days because of failure to thrive. At 4 months of age she cried and turned cyanotic after feeding. She was admitted to the hospital for examination. Biochemical determinations revealed metabolic acidosis and elevations of blood and cerebrospinal fluid lactate (2.6 and 4.5 mM) and blood pyruvate (0.13 mM). At 5 months of age, she had an episode of hematemesis resulting in shock and manifested respiratory insufficiency. T_2-weighted MRI revealed a bilateral area of high intensity in basal ganglia. She was diagnosed with Leigh syndrome on the basis of clinical findings, biochemical studies, and MRI data.

Methods

Lymphoblastoid cells were established from these three patients diagnosed with Leigh syndrome using methods previously described [6]. Mitochondria were isolated from lymphoblastoid cells by a modification of the method of Glerum et al. [7]. Lymphoblastoid cells (4-8 × 10^7 cells; approximately 5-10 mg of protein) were washed twice with phosphate-buffered saline and resuspended in 10 mL of medium A (0.27 mol/L mannitol, 0.1 mM EDTA, 0.05% bovine serum albumin, and 10 mM Tris-HCl at pH 7.3). The preparation was centrifuged at 500*g* for 2 minutes, and the resulting pellet was suspended in 2 mL of medium A. After cells were homogenized in a glass and Teflon homogenizer, the homogenate was mixed with an equal volume of medium A and centrifuged at 770*g* for 7 minutes. The resulting supernatant was further centrifuged at 1,100*g* for 7 minutes, yielding a crude mitochondria preparation (the pellet; approximately 0.3-0.6 mg of protein). This preparation was resuspended in approximately 200 μL of medium B (2.5 mol/L sucrose, 1 mM EDTA, and 20 mM Tris-HCl at pH 7.4). The mitochondria were sonicated on ice for 30 seconds. Aliquots of this preparation were used for assays of respiratory chain enzyme activities. Activities of the following respiratory chain components were measured: rotenone-sensitive NADH-cytochrome *c* reductase (complex I and III) by the method of Sottocasa et al. [8]; succinate-cytochrome *c* reductase (complex II and III) by the method of Stumph et al. [9]; and cytochrome c oxidase (complex IV) by the method of Wharton et al. [10]. The protein concentration in samples was measured by the method of Lowry et al. [11], with bovine serum albumin used as a standard. Pyruvate dehydrogenase complex activity was determined by a radioisotopic assay [6]. Harvested cells were incubated for 30 minutes at 37°C in phosphate-buffered saline containing 5 mM sodium dichloroacetate to activate pyruvate dehydrogenase complex. Biochemical data were analyzed by Student's *t* test. Differences of *P*-value less than 0.05 were accepted as statistically significant.

In total DNA prepared from lymphoblastoid cells, we performed analysis for point mutations associated with Leigh syndrome at nucleotide positions 3243, 8344, 8993, and 9176. Polymerase chain reaction was performed. After amplification, polymerase chain reaction products were digested with restriction enzymes, as reported previously [12,13]. Total DNA was used as a template to amplify exons 1 through 9 of the SURF-1 gene with intronic primers described by Tiranti et al. [4,14]. The sequence of these polymerase chain reaction products was determined in

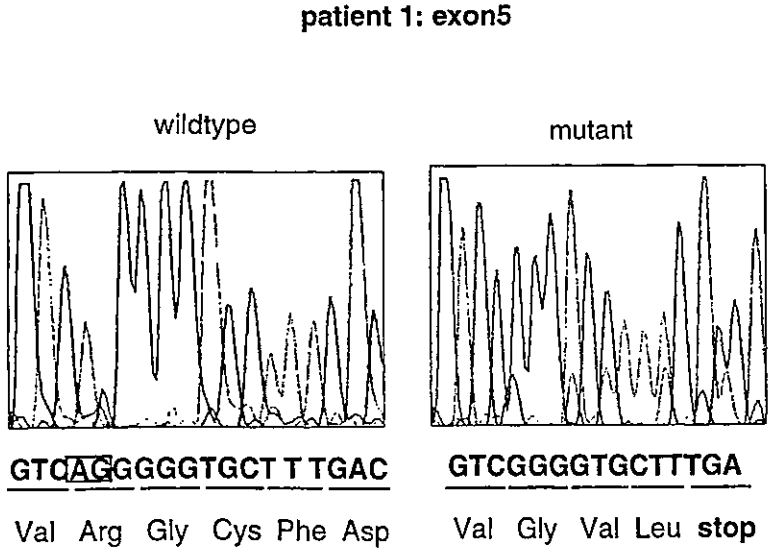

Figure 1. Mutation of the SURF-1 gene is detected by direct sequence analysis. Wild type of sequences are also presented. Mutation 367del AG is found in exon 5 of patient 1. Deleted AG is boxed. 367del AG mutation is homozygous.

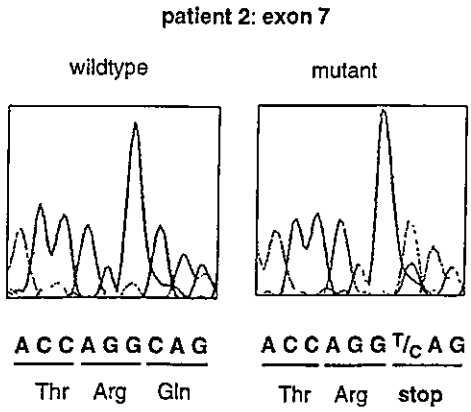

Figure 2. Mutation of the SURF-1 gene is detected by direct sequence analysis. Wild type of sequences are also presented. C640T mutation is evident in exon 7 of patient 2. C640T mutation is heterozygous.

an ABI Prism 377 autosequencer, using a DNA sequencing kit for dye terminator cycle sequencing kit (Applied Biosystems, Norwalk, CT).

Results

Enzyme assays in cultured lymphoblastoid cells from three patients with Leigh syndrome demonstrated decreased cytochrome *c* oxidase activity, ranging from 7-48% of normal control activity (Table 1). The cells possessed pyruvate dehydrogenase complex activity in the normal range (data not shown).

Polymerase chain reaction-restriction fragment length polymorphism screening revealed that the patients had no mitochondrial DNA point mutation. We identified one homozygous mutation in exon 5 of Patient 1 (367AG del) (Fig 1) and one heterozygous mutation in each of exon 7 (C640 torr) (Fig 2) and exon 8 (802del 26bp insGG) of Patient 2 (Fig 3). We performed subcloning analysis with exons 7-9 of Patient 2 and determined that each mutation was the opposite from the other allele (data not shown). No mutation of the SURF-1 gene was found in Patient 3.

Discussion

Mutations in the SURF-1 gene have recently been demonstrated to be a cause of Leigh syndrome associated with cytochrome *c* oxidase deficiency [5]. Most mutations were identified in white patients with cytochrome *c* oxidase defects expressed in skin fibroblasts or in muscle. In our study, cytochrome *c* oxidase deficiency was detected in cultured lymphoblastoid cells from three Japanese patients with typical Leigh syndrome. Three novel pathogenic mutations in the SURF-1 gene were evident in two of the three patients with LS^{COX-}, confirming that cultured lymphoblastoid cells were useful for elucidating the specific etiology of Leigh syndrome. Further, loss of function of the SURF-1 gene product was responsible for LS^{COX-} in these two Japanese patients.

Sequencing of the nine SURF-1 exons and of the intron-exon junctions identified three novel pathogenic mutations predicting a truncated SURF-1 protein in two independent pedigrees with no consanguineous parents. The homozygous frameshift mutation in exon 5 (367AG del) created a stop codon 10 bases downstream. The heterozygous mutation in exon 7 (C640T) converted the codon CAG for glutamine at position 214 into a stop

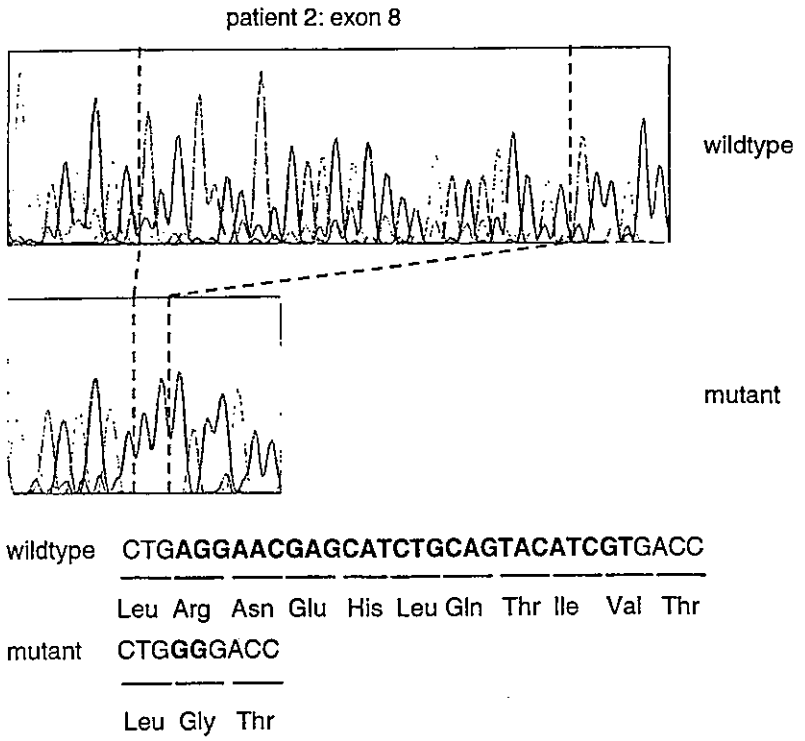

Figure 3. Mutation of the SURF-1 gene is detected by using subcloning analysis. Wild type of sequences are also depicted. Mutation 802del 26bp insGG is evident in exon 8 of patient 2. Mutation 802del 26bp insGG is heterozygous.

Table 2. SURF-1 mutations in LS^{COX-} patients

Location	Mutation	Hetero/Homo	Amino Acid Changes	Cases*
Exon1	37ins17	Homo	Frameshift	1
Exon2	74G>A	Hetero	Stop	1
Intron3	240+1G>T	Hetero	Splice	1
Intron3	240+1G>T	Homo	Splice	1
Exon4	244C>T	Homo	Stop	1
Exon4	312del10,insAT	Hetero	Frameshift	12
Exon4	312del10,insAT	Homo	Frameshift	3
Intron4	323+2T>C	Hetero	Splice	1
Exon5	**367delAG**	**Homo**	**Frameshift**	**1**
Exon5	370G>A	Hetero	Missense	1
Exon5	371G>A	Hetero	Missense	1
Intron5	515+2T>G	Hetero	Splice	1
Intron5	516−1delAG	Homo	Splice	1
Exon6	550delAG	Hetero	Frameshift	2
Exon6	552delG	Homo	Frameshift	2
Exon6	574insCTGC	Hetero	Frameshift	3
Exon6	574insCTGC	Homo	Frameshift	1
Exon6	587insCAGG	Hetero	Frameshift	2
Intron6	588+1delG	Hetero	Splice	1
Intron6	588+1G>A	Hetero	Splice	1
Exon7	**640C>T**	**Hetero**	**Stop**	**1**
Exon7	604G>C	Hetero	Missense	1
Exon7	688C>T	Hetero	Stop	2
Exon7	688C>T	Homo	Stop	1
Exon7	737T>C	Hetero	Missense	1
Exon7	751C>T	Hetero	Stop	2
Exon7	751C>T	Homo	Stop	1
Intron7	752-3C>G	Hetero	Splice	1
Exon8	758del2CA	Homo	Frameshift	1
Exon8	772delCC	Hetero	Frameshift	1
Exon8	790delAG	Hetero	Frameshift	1
Exon8	**802del26bp insGG**	**Hetero**	**Deletion**	**1**
Exon8	808G>T	Hetero	Stop	1
Exon8	814delCT	Hetero	Frameshift	1
Exon8	820T>G	Hetero	Missense	1
Exon9	845delCT	Hetero	Frameshift	9
Exon9	845delCT	Homo	Frameshift	2
Exon9	868insT	Homo	Frameshift	2

Novel mutations are shown in bold.
* From References [4], [5], and [14] to [18].

Abbreviations:
Hetero = Heterozygous
Homo = Homozygous

codon (TAG); exon 8 lost eight amino acids with the other heterozygous mutation (802del 26bp insGG). These mutations would greatly change the structure and predict loss of function of the SURF-1 protein. No mutation of the SURF-1 gene was found in the third patient. The two patients with SURF-1 mutations had severely decreased cytochrome *c* oxidase activity (less than 20% of the control mean in lymphoblastoid cells), whereas the third patient without SURF-1 mutation demonstrated a milder decrease, to 48% of control mean, as presented in Table 1. The latter patient, then, presumably had a different cause of cytochrome *c* oxidase deficiency than a SURF-1 abnormality. Further studies are necessary to determine the gene at fault in this patient.

Mutations in the SURF-1 gene reported to date are presented in Table 2. Most of these predict loss of function of the SURF-1 protein from frameshift or nonsense mutations, but missense mutations have been described in few patients with typical Leigh syndrome. These data suggest that missense mutations might account for milder or less generalized clinical and biochemical phenotypes observed in both infant and adult patients with Leigh syndrome. One particular mutation (exon 4 del 10ins2) was reported in 15 patients, but this mutation has not been observed among Japanese patients. No obvious correlation between position and type of mutation and clinical phenotype was observed. All of the LS^{COX-} patients with mutations in the SURF-1 gene had an early onset (0-16 months after birth) and progressive psychomotor regression. Of 24 patients, 18 (75%) died at a young age (3-14 years of age) [14-18].

SURF-1 is the first nuclear gene to be an etiologic agent of respiratory chain defects causing Leigh syndrome. Most of reported SURF-1 mutations are loss of function, predicting a truncated protein product, as indicated in Table 2. Western blot analysis with anti-SURF-1 antibodies demonstrated a specific 30-kDa protein in control cells but no protein in patient cells with LS^{COX-} [19]. Steady-state levels of both nuclear- and mitochondrial-encoded cytochrome *c* oxidase subunits were also markedly reduced in patient cells, consistent with a failure to assemble or maintain a normal amount of the cytochrome *c* oxidase complex [19]. Four intermediates in the assembly of cytochrome *c* oxidase were identified recently. The absence of the SURF-1 protein causes an accumulation of early intermediates (COX I and COX I + COX IV subunit) before the assembly of the COX II subunit [20]. Further studies are necessary to establish the SURF-1 protein interacts directly or indirectly with other cytochrome *c* oxidase subunits during the assembly process.

Molecular investigation for SURF-1 mutations can be used to diagnose LS^{COX-} and provide a simple and accurate test for prenatal diagnosis. In addition, these studies will further the understanding of SURF-1 function in cytochrome *c* oxidase assembly, which ultimately may lead to effective therapy for patients with LS^{COX-}.

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Type II citrullinaemia (citrin deficiency) in a neonate with hypergalactosaemia detected by mass screening

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Summary: Type II citrullinaemia (CTLN2) is an adult- or late childhood-onset liver disease characterized by a liver-specific defect in argininosuccinate synthetase protein. The enzyme abnormality is caused by deficiency of the protein citrin, which is encoded by the *SLC25A13* gene. Until now, however, few cases with *SLC25A13* mutations have been reported in children with liver disease. We describe an infant who presented with neonatal hepatitis in association with hypergalactosaemia detected by neonatal mass screening. DNA analysis of *SLC25A13* revealed that the patient was homozygous for a IVS11+1G>A mutation. This case suggests that *SLC25A13* mutant should be suspected in neonatal patients with hypergalactosaemia of unknown cause.

Citrullinaemia is an autosomal recessive disease caused by deficiency of argininosuccinate synthetase (ASS; EC 6.3.4.5). Two forms of this disease are recognized (Saheki et al 1987): classical or types I and III citrullinaemia (CTLN1; McKusick 215700) and adult-onset type II citrullinaemia (CTLN2; McKusick 603471). Most patients with CTLN1 follow a severe course, with symptoms evident from birth and with death during the neonatal period in more than half of cases. CTLN1 is caused by mutations in the ASS gene on chromosome 9q34 (Kobayashi et al 1995), and the enzyme defect is found in all tissues and cells in which ASS is expressed. CTLN2 is characterized by liver-specific ASS deficiency with no abnormalities in the ASS gene (Kobayashi et al 1993). The most characteristic feature of CTLN2

is the late onset of serious and recurring symptoms. Ages at clinical onset in patients reported to date have ranged from 11 to 72 years (Kobayashi et al 1997). The *SLC25A13* gene was recently identified on chromosome 7q21.3, with five different DNA sequence alterations at this locus (Kobayashi et al 1999) that account for all mutations found in CTLN2 patients examined thus far. *SLC25A13*, which is expressed abundantly in the liver, encodes citrin, a calcium-binding mitochondrial carrier protein whose precise function is unknown. Until now, few cases with *SLC25A13* mutation have been reported among children (Ohura et al 2001; Tazawa et al 2001; Tomomasa et al 2001). We describe an infant with *SLC25A13* mutation associated with hypergalactosaemia detected by neonatal mass screening.

SUBJECTS AND METHODS

Patient: A girl weighing 2192 g at birth was born at 37 weeks of gestation. She was referred to the Tokushima University Hospital at the age of 30 days because of hypergalactosaemia detected by neonatal mass screening at 25 days of age and confirmed at 29 days (Paigen method, > 20 mg/dl; Beutler method, normal). The patient's parents were unrelated. Initially breast-fed, the infant was changed to lactose-free formula at 29 days because of hypergalactosaemia. No cataracts or jaundice were evident on admission, but she developed appetite loss and poor weight gain.

Methods: Blood galactose was estimated by the Paigen method; hereditary galactosaemia was excluded by assays of galactose-metabolizing enzyme activities in red blood cells (Mizoguchi et al 2001). Amino acid analysis was performed using an L8800 high-speed amino acid analyser (Hitachi; Tokyo).

DNA diagnosis of mutation: The procedure for DNA diagnosis of *SLC25A13* mutations was previously reported in detail (Kobayashi et al 1999; Ohura et al 2001). Polymerase chain reaction (PCR) amplification was peformed for detection of the IVS11+1G>A mutation, using the primer set Ex-11F (5′-CAGCTTTGACTGTTTTAAGAAAGT-3′ and IVS-11Bm2 (5′-AGGTATTGAGCATGTGGCACTG-3′. The underlined G is a mismatched base creating a cleavage site for the *Sau*IIIA1 restriction enzyme that serves to detect mutant genes. PCR products were digested with *Sau*IIIA1, and fragments were separated on a 3% agarose gel.

RESULTS

Clinical data on admission: Blood galactose concentrations in the patient exceeded 20 mg/dl at ages of 25 and 29 days, but had fallen markedly by 30 days because of feeding with lactose-free formula. The patient's laboratory data on admission are presented in Table 1. Hyperammonaemia, hypoproteinaemia, elevation of total bile acids, and mild liver dysfunction were evident. Plasma amino acid analysis showed hyperaminoacidaemia including a citrulline concentration of 1354 μmol/L

(41 times the upper limit of the normal range); methionine, 757 μmol/L (17 times); tyrosine, 722 μmol/L (8 times); ornithine, 271 μmol/L (2 times); lysine, 814 μmol/L (4 times); arginine, 648 μmol/L (6 times); threonine, 1273 μmol/L (6 times); serine, 469 μmol/L (3 times); phenylalanine, 708 μmol/L (9 times); histidine, 233 μmol/L (3 times); and proline, 612 μmol/L (2 times) (Table 1). Other amino acid concentrations were within or near the normal range. Activities of galactokinae, galactose-1-phosphate uridyltransferase, and UDPgalactose-4-epimerase were within the normal range (Table 2).

Ultrasonography: No portosystemic venous shunt, including congenital shunts between the portal system and left renal vein, were evident in our patient.

Table 1. Serum biochemical data in an infant with *SLC25A13* mutation

	Days of age						
Parameter	*30*	*46*	*57*	*86*	*152*	*223*	*Normal range*
Galactose (mg/dl)	1.5[a]	0.7[a]	17.4	0.7[a]	0.5	0.3	< 2.0
Ammonia (μg/dl)	158	77	58	45	57	39	12–66
Aspartate aminotransferase (IU/L)	124	42	140	61	51	37	10–35
Alanine aminotransferase (IU/L)	64	38	86	43	38	30	5–40
Total bile acids (μmol/L)	177	51	168	94	7.0	6.4	2.9–11
Total protein (g/dl)	4.4	4.9	4.6	5.6	7.1	7.1	6.5–8.2
Citrulline (μmol/L)	1354	157	526	64	33	42	15–33
Methionine (μmol/L)	757	1009	885	701	37	42	17–44
Tyrosine (μmol/L)	722	303	124	107	137	90	38–88
Ornithine (μmol/L)	271	246	381	131	66	81	40–116
Lysine (μmol/L)	814	516	440	242	202	183	105–223
Arginine (μmol/L)	648	277	90	141	97	101	42–114
Threonine (μmol/L)	1273	427	1196	219	247	191	71–206
Serine (μmol/L)	469	280	379	156	96	113	77–159

[a]Feeding was with lactose-free formula at the time point indicated

Table 2 Galactose-metabolizing enzyme activities[a] in red blood cells from an infant with *SLC25A13* mutation

	Galactokinase	*Galactose-1-phosphate uridyltransferase*	*UDPgalactose 4-epimerase*
Patient	3.59	22.2	23.7
Controls	3.66 ± 1.32	26.1 ± 4.2	13.3 ± 2.5

[a]Activities are expressed as nmol/h per mg Hb

Clinical course: Feeding with lactose-free formula resulted in a favourable course including good appetite and good weight gain. Abnormal aminograms persisted for a few further months but then improved. The patient was changed to a formula containing lactose for 3 days as a challenge test, beginning at the age of 56 days. Serum galactose (8.6 mg/dl at 56 days, 17.4 mg/dl at 57 days and 14.5 mg/dl at 58 days), citrulline and total bile acids again increased (Table 1). Appetite loss and poor weight gain also were noted, but serum ammonia did not increase.

After a return to lactose-free formula, weight gain soon improved. By the age of 3 months, all biochemical abnormalities had improved, and these normalized by the age of 5 months. Re-challenge with lactose at 152 days did not increase galactose, citrulline, or total bile acids. Follow-up has been maintained for 9 months; no developmental delay or neurological abnormalities are evident.

DNA diagnosis: The principal clinical features in our present case were abnormal aminograms showing citrulline concentrations more than 41 times beyond the upper limit of normal. Suspecting that these abnormalities might be related to CTLN2, we obtained informed consent from the patient's parents to perform genetic diagnosis. DNA analysis of the *SLC25A13* gene showed that our patient was homozygous for a known IVS11+1G>A mutation (Kobayashi et al 1999; Yasuda et al 2000), while her sister was heterozygous for this mutation (Figure 1). This mutation was confirmed by direct sequencing of genomic DNA from the patient (data not shown). This mutation is a G>A substitution at the 5′ end of intron 11, resulting in abnormal splicing and deletion of exon 11 in mRNA. This causes a loss of 53 amino acids within the first hydrophilic loop between the first and second predicted transmembrane domains of citrin (Kobayashi et al 1999).

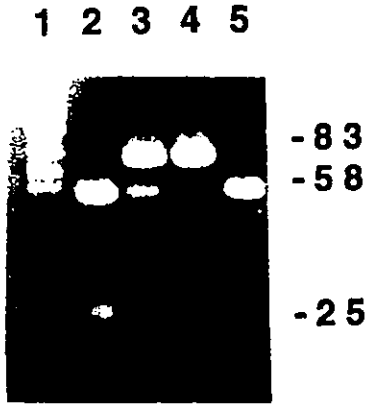

Figure 1 Identification of the *SLC25A13* mutation. Lane 1 contains molecular markers; lanes 2–5 represent samples from the patient, her sister, a normal control, and a homozygous control, respectively. The polymerase chain reaction product was 83 bp long, and was cut into 58 bp and 25 bp bands by *Sau*IIIA1 digestion in the patient and her sister. The patient was homozygous for the IVS11+1G>A mutation, while her sister was heterozygous

Discussion

We successfully treated clinical and biochemical abnormalities in an infant with *SLC25A13* mutation who presented with hypergalactosaemia detected by neonatal mass screening. Clinical features of our patient were similar to those of recently reported infants with *SLC25A13* mutation who presented with intrahepatic cholestasis in the neonatal period (Ohura et al 2001; Tazawa et al 2001; Tomomasa et al 2001), except that our patient had hyperammonaemia on admission. Despite initial improvement with lactose-free formula, hypergalactosaemia, hypercitrullinaemia and liver dysfunction in our patient were worsened by a lactose challenge at 56 days. Lactose-free formula was required until the age of 5 months.

Elevation of methionine, tyrosine or galactose in serum has been reported in liver diseases such as the neonatal hepatitis syndrome (Balistreri 1985). The aminogram of our patient showed elevation of not only serum methionine and tyrosine but also citrulline, lysine, threonine and arginine. In an analysis of serum amino acids in CTLN2 patients, Saheki and colleagues (1986) noted that serum arginine concentrations were higher than in controls and correlated significantly with serum citrulline. In addition, serum concentrations of alanine, glycine and branched-chain amino acids were lower than in controls, while that of threonine was somewhat higher. This biochemical profile is considered unique to CTLN2 and is similar to laboratory findings in our patient with *SLC25A13* mutation. We suspect that hypergalactosaemia, hypercitrullinaemia and liver dysfunction were secondary abnormalities caused by citrin deficiency and these findings depend on ageing. Despite initial improvement with lactose-free formula in our patient with citrin deficiency, hypergalactosaemia, hypercitrullinaemia and liver dysfunction were worsened by a lactose challenge at 56 days, but re-challenging with lactose at 152 days did not worsen these findings. Therefore, in our patient galactose was an important factor for some metabolisms in an early infant, but this was not important at the age of 5 months. The abnormal metabolism caused by citrin deficiency might have been changed during 5 months. In fact, all biochemical abnormalities of patients with *SLC25A13* mutation who presented with intrahepatic cholestasis in the neonatal period normalized by the age of 12 months without special therapy (Ohura et al 2001; Tazawa et al 2001; Tomomasa et al 2001). Elucidation of the function of citrin should permit a fuller understanding of these phenomena.

CTLN2 has been diagnosed exclusively in Japan, where its prevalence has been reported to be approximately 1 in 100 000 (Kobayashi et al 1993). However, molecular analysis of *SLC25A13* suggests that the actual prevalence of the disease is likely to be higher, about 1 in 20 000 (Kobayashi et al 1999; Yasuda et al 2000). We presume that some individuals with *SLC25A13* mutation in both alleles may not present symptoms during life or may receive an erroneous or nonspecific diagnosis such as neonatal hepatitis. A variety of liver diseases may have some relationship to CTLN2, and various phenomena may develop in the neonatal period as described in this study. In 31 patients with galactosaemia detected by neonatal mass screening in Hiroshima, 6 cases were reported as having an unknown cause (Mizoguchi et al 2001). Some of these neonates might in fact have *SLC25A13*

mutations. Our patient is now 9 months old, and laboratory data including serum galactose, ammonia, the aminogram and total bile acids are within the normal range. Regular follow-up of this patient will need to be maintained to detect any possible emergence of symptoms of CTLN2.

In summary, we describe an infant who manifested symptoms of citrin deficiency. Identification of the *SLC25A13* gene provides a powerful tool for detecting citrin deficiency in children with neonatal hepatitis and for clarifying the mechanisms underlying abnormalities found in CTLN2 patients. Making a diagnosis of *SLC25A13* mutations before onset of typical CTLN2 symptoms is extremely important; the patient may sustain severe brain damage during their first attack of hyperammonaemia. Neonatal screening for hypergalactosaemia provides an important opportunity for diagnosis of CTLN2 in infancy.

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OBSERVATIONS

Association Between Vitamin D Receptor Genotype and Age of Onset in Juvenile Japanese Patients With Type 1 Diabetes

1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$] not only regulates calcium metabolism but also modulates the immune system. Some reports have suggested that 1,25$(OH)_2D_3$ helps to prevent the development of type 1 diabetes. The association between the vitamin D receptor (VDR) genotype and susceptibility to type 1 diabetes has been examined, but a definitive conclusion has not yet been reached (1,2). We examined the VDR genotype in juvenile Japanese patients with type 1 diabetes.

A total of 108 diabetic patients (41 boys and 67 girls, age of onset 0.4–18 years with a median age of 8.9) and 120 nonrelated nondiabetic subjects were studied. Three polymorphic restriction fragment–length polymorphisms (RFLPs), i.e., Fok I, *Apa*I, and *Taq*I, were genotyped by PCR-RFLP method. The genotype or allele frequencies were compared statistically by the χ^2 test. The significance of differences in each genotype for the age of onset was tested with the Mann-Whitney *U* test.

Among the patients, the FF (n = 50) and tt (n = 5) genotypes were found relatively frequently, and aa (n = 46) was infrequent compared with those in control subjects, but these differences were not statistically significant (P = 0.14, 0.18, and 0.38 for FF, tt, and aa genotypes, respectively). There was also no significant difference in the allele frequency of each polymorphism, although the incidence of the F allele tended to be higher in the diabetic patients (P = 0.051). Concerning the age of onset of diabetes, patients with the ff genotype (n = 12, median 5.2 years, range 1.7–11.0) were significantly younger at onset than those with FF (n = 50, 9.7 years, 0.4–15.9, P = 0.01) or Ff (n = 46, 8.9 years, 0.9–18.0, P = 0.03). No significant association was observed between the *Taq*I or *Apa*I genotype and the age of onset.

The ff genotype has been reported to be associated with a lower expression of VDR mRNA and reduced inhibition of phytohemagglutinin-stimulated growth of peripheral blood mononuclear cells. Thus, the Fok I genotype may influence the rate of the progression of insulitis by modifying the autoimmune process, which may have led to the significant difference in the age of onset. The relatively high frequency of the F allele in diabetic patients, which has also been found in Japanese adult diabetic patients (2), is apparently inconsistent with this observation. A possible explanation is that the impact of Fok I polymorphism may not be strong enough to prevent the progression of autoimmune insulitis into overt diabetes and thus does not influence susceptibility to the disease itself.

In conclusion, VDR gene polymorphism does not appear to have a strong enough impact to clearly influence susceptibility to the disease itself, but Fok I polymorphism might influence the age of onset of diabetes in juvenile Japanese diabetic patients.

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The Cost of Self-Monitoring of Blood Glucose Is an Important Factor Limiting Glycemic Control in Diabetic Patients

Maintenance of near normoglycemia can delay or prevent microvascular complications, but it cannot be carried out without a program of patient education, including self-monitoring of blood glucose (SMBG) (1,2). Motivation toward SMBG depends on several ill-defined factors, and there is no consensus on the effectiveness of SMBG in diabetes management (3–6).

We undertook a single-blinded, control-matched, longitudinal study of patients with insulin-requiring diabetes (n = 62) to examine barriers to SMBG and determine whether eliminating the cost barrier would increase SMBG frequency and glycemic control. Eligibility criteria were insulin treatment with at least two injections/day for at least 1 year (1), HbA_{1c} >120% of upper limit of normal (2), and recent diabetes education (3). The patients completed questionnaires reporting their habitual SMBG frequency, perceived barriers to SMBG, monthly income, and any private health insurance plans to verify coverage for glucometer reagents. They were randomly assigned in a patient-blinded fashion to two groups of 31 patients each, matched for age, sex, education, income, private health insurance coverage, diabetes type, diabetes duration, number of years on insulin, habitual SMBG frequency, random blood glucose, HbA_{1c}, and number of daily insulin injections. They were asked to participate in the study over a period of 12 months, with second monthly visits to the research nurse, and they were instructed in the use of the glucometer DEX (Bayer, Etobicoke, Canada), but they were not

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Diagnosis and molecular analysis of three male patients with thiamine-responsive pyruvate dehydrogenase complex deficiency

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Abstract

Pyruvate dehydrogenase complex (PDHC) deficiency is a major cause of congenital lactic acidemia in children. PDHC catalyzes the thiamine-dependent decarboxylation of pyruvate. Thiamine treatment was effective for some patients with PDHC deficiency. We reexamined 30 patients with congenital lactic acidemia of unknown origin who had normal PDHC activity in their cultured fibroblasts using a routine assay with a high (0.4 mM) thiamine pyrophosphate (TPP) concentration. We measured the activity of PDHC in the presence of a low (1×10^{-4} mM) TPP concentration, and analyzed for mutations in the E1α subunit gene. Three males had low PDHC activity in the presence of 1×10^{-4} mM TPP. The DNA sequence of these three patients' X-linked E1α subunit revealed a substitution of alanine for valine at position 71 (V71A) in exon 3, phenylalanine for cysteine at position 101 (C101F) in exon 4, and glycine for arginine at position 263 (R263G) in exon 8, respectively. Thiamine treatment was effective in these three patients. Therefore, they had a thiamine-responsive PDHC deficiency due to a point mutation in the E1α subunit gene. PDHC activity should be measured at a low TPP concentration to detect thiamine-responsive PDHC deficiency so that thiamine treatment can be initiated as soon as possible.

Keywords: Lactic acidemia; Pyruvate dehydrogenase complex deficiency; E1α subunit; Thiamine pyrophosphate; Thiamine treatment

1. Introduction

The human pyruvate dehydrogenase complex (PDHC) catalyzes the thiamine-dependent decarboxylation of pyruvate. It plays an important role in the energy metabolism of the cell since it is an essential and rate-limiting enzyme connecting glycolysis with the tricarboxylic acid cycle and oxidative phosphorylation. Defects in PDHC are an important cause of primary lactic acidosis. Clinical symptoms can vary considerably in patients with PDHC deficiencies [1]. The diagnosis is usually established by the measurement of PDHC activity, and the analysis is most commonly performed on cultured skin fibroblasts from the patients [1,2]. The majority of PDHC deficiencies result from mutations in the X-linked E1 α subunit gene [3]. The E1 component also contains a thiamine pyrophosphate (TPP) binding site that is shared by the α and β subunits [1]. In this study, we describe the diagnosis and molecular analysis of three male patients with thiamine-responsive PDHC deficiency, which underscores the need to perform the assay for PDHC with low TPP concentrations.

2. Materials and methods

2.1. Patients

2.1.1. Patient 1

The clinical features and biochemical studies in this patient have been described by Kinoshita et al. [4]. Briefly, when he was 6 months old, he suddenly developed weakness of the extremities while suffering from a respiratory infection. At 16 months, he developed pneumonia and marked generalized muscle hypotonia and weakness, and was placed on mechanical ventilation. The plasma lactate and pyruvate levels were elevated to 4.48 mM (Normal values: 0.37–1.65 mM) and 0.55 mM (Normal values: 0.03–0.10 mM), respectively. The muscle weakness improved gradually, and he could walk again 2 months after the attack. He experienced recurrent episodes of ataxic gait, tremors, and drowsiness two to four times per year,

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Table 1
PDHC activity in cultured skin fibroblasts

	PDHC (nmol /min/mg protein)[a]	
	1×10^{-4}mM TPP	0.4 mM TPP
Case 1	0.64±0.12* (8)	1.90
Case 2	0.14±0.04* (3)	2.25
Case 3	0.39±0.18* (3)	1.73
Lactic acidemia (27)	2.03±0.28	2.29±0.29
	(1.58–2.63)	(1.83–2.85)
Controls	2.29±0.49 (10)	2.32±0.62 (16)
	(1.57–3.52)	(1.65–3.85)

PDHC: pyruvate dehydrogenase complex; TPP: thiamine pyrophosphate.
[a] DCA-activated PDHC. Values are the means±SD of (n) determinations or the result of a single determination.
* $p<0.001$ vs. controls.

coincident with febrile illnesses. During these attacks, the intravenous administration of thiamine led to a rapid clinical improvement. With the oral administration of 500 mg/day of thiamine, the neurologic symptoms including weakness during infections disappeared, compared with two to four episodes per year before continuous thiamine therapy. At remission, the plasma lactate and pyruvate levels were decreased to 1.20 and 0.13 mM, respectively. The administration of 500 mg/day of thiamine to this patient was effective in preventing further episodic neurologic attacks. He has continued oral thiamine treatment till now, and shows normal development at the age of 11 years.

2.1.2. Patient 2

The clinical features and biochemical studies in this patient have been described by Di Rocco et al. [5]. Briefly, when he was 5 years and 6 months old, the patient's weight and height were 3 SD below normal; he also suffered from severe psychomotor retardation and peripheral neuropathy. Abnormal signals in the basal ganglia bilaterally on MRI were consistent with Leigh syndrome. The plasma lactate and pyruvate levels were elevated to 4.2 and 0.46 mM, respectively. He was begun on thiamine treatment. After several weeks, he was able to walk without support, his muscle weakness and ataxia decreased and he no longer developed metabolic derangements during febrile illnesses. When he was 8 years old, after 2 years of thiamine treatment (200 mg/day), his weight and height corresponded to the 25th and 10th percentile, respectively. The plasma lactate and pyruvate levels were decreased to 2.7 and 0.21 mM, respectively. Currently, he has mild hypotonia and hyporeflexia and can walk without support; a Brunet–Lezine test showed a mental age of 6 years.

2.1.3. Patient 3

This patient was a 9-year-old boy, the first child of healthy, unrelated parents; his three sisters were healthy. He was clinically normal at birth and during the first year of life. At the age of 18 months, he developed an ataxic gait and muscle weakness after suffering from a respiratory infection, and was admitted to the hospital for evaluation. Metabolic acidosis developed and his plasma lactate concentrations ranged between 5.0 and 6.0 mM, and his pyruvate concentrations were between 0.44 and 0.98 mM with lactate/pyruvate ratios between 6.1 and 11.4. The cerebrospinal fluid lactate concentration was 7.8 mM with a normal lactate/pyruvate ratio. An MRI of the brain showed hyperintense signals on the T2 weighted images in the basal ganglia bilaterally, consistent with Leigh syndrome. He received 400 mg/day of thiamine. After thiamine therapy, he was able to walk without support, his muscle weakness and ataxia decreased, and he no longer developed metabolic acidosis during febrile illnesses. He was admitted to a local elementary school.

2.2. Enzymatic assay

The PDHC activity in cultured skin fibroblasts from 30 patients with congenital lactic acidemia was assayed using two different concentrations of TPP (0.4 and 1×10^{-4} mM) after the activation of PDHC using sodium dichloroacetate (DCA) as previously described [6,7]. Skin fibroblasts were cultured in Eagle's MEM medium (Nissui, Tokyo) containing 3×10^{-3} mM thiamine–HCl. The concentration of TPP in normal human blood is 1×10^{-4} mM [8]. The protein concentrations were measured by the method of

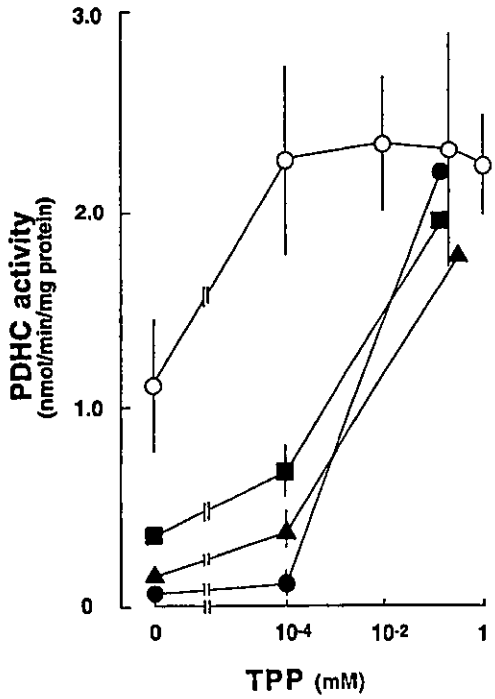

Fig. 1. Effect of thiamine pyrophosphate (TPP) on the activity of sodium dichloroacetate (DCA)-activated pyruvate dehydrogenase complex (PDHC) in cultured skin fibroblasts from normal subjects and three patients with lactic aidemia. The activity of PDHC in cultured skin fibroblasts is shown at various concentrations of TPP. O, Normal controls; ■, patient 1; ●, patient 2; ▲, patient 3.

Lowry et al. [9]. The statistical analysis of the biochemical data was performed using Student's *t*-test. A level of $p<0.05$ was accepted as statistically significant.

2.3. Molecular analysis of the PDHC-E1α gene

Mutation analysis of the E1α gene, a major cause of PDHC deficiency, was performed using genomic DNA from cultured skin fibroblasts. For the genetic analysis of the 11 exons of the E1α gene, the individual exons were amplified using primer pairs and conditions as described previously [10]. These fragments, used to detect SSCP, were sequenced directly using appropriate amplification primers. SSCP analysis was performed with the GeneGel Exel kit (Amersham Pharmacia Biotech UK, Bucks, UK). Sequence analysis was performed using an automated ABI 377 autosequencer with a dye-terminator kit (Applied Biosystems, Norwalk, CT).

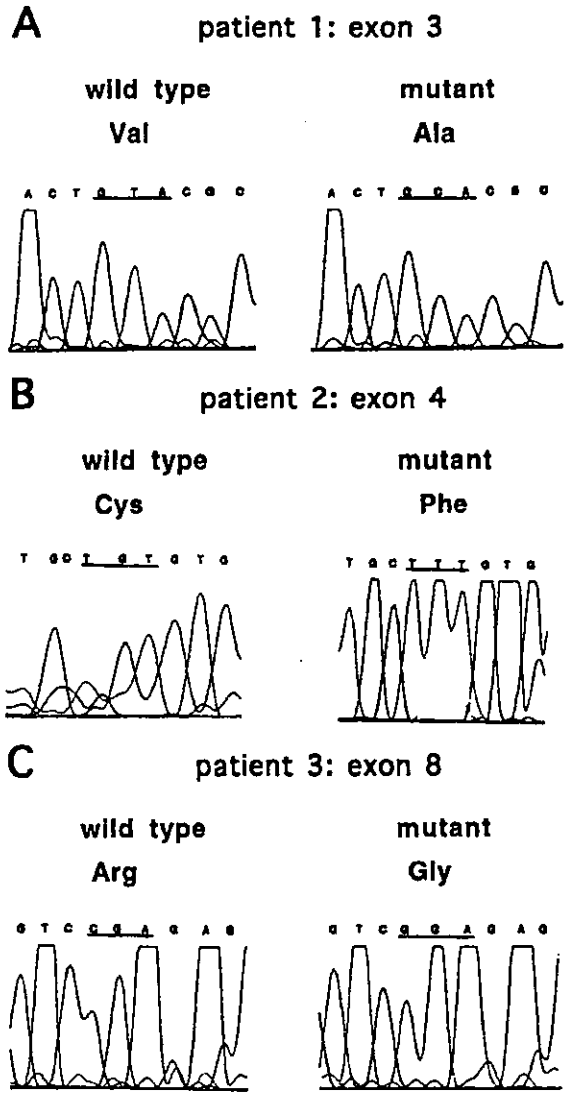

Fig. 2. Amplified genomic fragments for the 11 exons of the E1α subunit gene from three patients were sequenced and analyzed. (A) The genomic sequence for exon 3 of patient 1 shows a T→C point mutation at nucleotide 212, resulting in a substitution of alanine for varine at position 71 (V71A). (B) The genomic sequence for exon 4 of patient 2 shows a G→T point mutation at nucleotide 302, resulting in a substitution of phenylalanine for cysteine at position 101 (C101F). (C) The genomic sequence for exon 8 of patient 3 shows a C→G point mutation at nucleotide 787, resulting in a substitution of glycine for arginine at position 263 (R263G).

3. Results

3.1. PDHC activity

We reexamined 30 patients with congenital lactic acidemia who had normal PDHC activity in their cultured fibroblasts in the presence of a high (0.4 mM) TPP concentration. Three young males among these 30 patients had low PDHC activity in the presence of 1×10^{-4} mM TPP as shown in Table 1. We investigated the affinities of normal and mutant PDHC for TPP. As shown in Fig. 1, normal cells showed approximately 50% of maximal PDHC activity in the absence of TPP, and the addition of 1×10^{-4} mM TPP restored full activity, with no further change in PDHC activity at higher concentrations of TPP. However, cells from the three thiamine-responsive patients showed significantly lower PDHC activity at the 0 and 1×10^{-4} mM TPP concentration. The addition of a high concentration (0.4 mM) of TPP restored to normal PDHC activity. Therefore, the PDHC deficiency in these three patients was likely due to a decreased affinity of PDHC for TPP.

3.2. Mutation analysis of the E1α subunit gene

DNA sequence analysis of these three patients' X-linked E1α subunit revealed a T→C point mutation at nucleotide 212, resulting in the substitution of alanine for valine at position 71 (V71A) in exon 3 of patient 1, a G→T point mutation at nucleotide 302 resulting in phenylalanine for cysteine at position 101 (C101F) in exon 4 of patient 2, and a C→G point mutation at nucleotide 787 resulting in glycine for arginine at position 263 (R263G) in exon 8 of patient 3 (Fig. 2). No other mutation was found in the E1α subunit gene in these three patients.

4. Discussion

We previously reported a patient with thiamine-responsive PDHC deficiency due to a missense mutation (R263G) in the E1α subunit gene [11]. The PDHC activity in the cultured lymphoblastoid cells of this patient increased exponentially with TPP concentrations above 1×10^{-4} mM and was restored to within the normal range by the addition of a high concentration (0.4 mM) of TPP [11]. PDHC deficiency is generally diagnosed by measuring the

activity of PDHC in the presence of a high concentration of TPP (0.1–0.7 mM) [6,12–15]. Therefore, this type of thiamine-responsive PDHC deficiency may not be diagnosed solely on the basis of the measurement of PDHC activity in cultured skin fibroblasts or lymphoblastoid cells in the presence of a high TPP concentration.

In this study, we reexamined 30 patients with congenital lactic acidemia of unknown origin by using cultured skin fibroblasts. Three young males in this group had low PDHC activity in the presence of 1×10^{-4} mM TPP and a point mutation in the E1α subunit gene. Their clinical symptoms were improved by treatment with a high dose of thiamine. Clinical thiamine responsiveness in our three patients was confirmed by in vitro studies showing a thiamine-responsive functional defect in cultured skin fibroblasts. The PDHC activity of these three patients was within the normal range in the presence of a high concentration of TPP (0.4 mM). Therefore, in order to diagnose this type of thiamine-responsive PDHC deficiency and to prevent a diagnostic error, it is important to measure the activity of PDHC in the presence of a low (1×10^{-4} mM) as well as a high TPP concentration. Furthermore, patient 1 shows normal development at the age of 11 years but patient 2 showed severe psychomotor retardation and peripheral neuropathy at the age of 5 years and 6 months. Patient 3 showed middle level as compared with patient 2. The clinical presentation in these three patients is correlated with PDHC activities in the presence of 1×10^{-4} mM TPP as shown in Table 1.

Normal cells showed half of maximal PDHC activity in the absence of TPP as shown in Fig. 1. The reasons are as follows. The PDHC activities are measured using skin fibroblasts cultured in medium containing 3×10^{-3} mM thiamine–HCl and the assay is performed after the additions of various concentration of TPP (0–0.4 mM) to the assay mixture containing homogenized skin fibroblasts. Thus, this assay mixture contains TPP binding to PDHC of fibroblasts during culturing skin fibroblasts in medium containing 3×10^{-3} mM thiamine–HCl. Therefore, the PDHC activity in the absence of TPP depends on the volume of TPP binding to PDHC of fibroblasts during the culture. As the PDHC in normal subjects is tightly binding to TPP, it showed half of maximal PDHC activity in the absence of TPP. However, because of a decreased affinity of PDHC for TPP in three patients with thiamine-responsive PDHC deficiency, the assay mixture of these three patients contains little TPP binding to PDHC of fibroblasts during the culture. Therefore, the PDHC activities in these three patients in the absence of TPP were decreased as compared with normal controls.

The V71A mutation in exon 3 of patient 1 and the C101F mutation in exon 4 of patient 2 were novel [3]. The R263G mutation in exon 8 of patient 3 had previously been identified in 11 patients [3,11,16,17]. A comparison of the PDH E1α amino acid sequence from a variety of different sources (human sperm-specific form, pig heart, rat liver, and mouse liver) shows that the amino acids 71, 101, and 263 are valine, cysteine, and arginine, respectively [18–21]. The V71A mutation must be a de novo mutation because it was not found in the genomic DNA of the patient's mother. Unfortunately, it has not been possible to analyze the mothers of patients 2 and 3. We therefore do not know if these are new or inherited mutations. We believe that the V71A and C101F mutations were the cause of their disease: aside from the presence of an appropriate defect in thiamine-responsive PDHC activities, no other mutations were found in the E1α subunit gene, these two amino acids (valine and cysteine) were conserved in E1α amino acid sequence from various species, and these two mutations were not present in the genomic DNA from 50 unrelated controls. The R263G mutation is the most common E1α mutation reported to date [3,11]. All patients were affected by a slowly progressive neurologic disorder. Two patients with the R263G mutation were improved by treatment with a high dose of thiamine [11,17]. Among the patients with PDHC deficiency responding to thiamine therapy, five mutations have been reported previously: H44R, R88S, G89S, R263G, and V389fs [7,11,16,22]. Four mutations including that in our patient 1 (H44R, V71A, R88S, G89S) were in exon 3. Therefore, exon 3 in the E1α subunit appears to be important in thiamine-responsive PDHC deficiency.

No effective therapy for congenital lactic acidemia has been established. In this study, clinical symptoms in our three patients were improved by treatment with a high dose of thiamine. However, the clinical improvement for patient 2 was not complete in spite of large doses of thiamine. Thiamine therapy for this patient was only started at age 5 years after the development of severe neurological deterioration. Therefore, thiamine therapy for patients with thiamine-responsive PDHC deficiency should be started as early as possible. In patient 2, if thiamine therapy had been started at an early stage of neurological involvement, it might have had a greater effect. Clinical thiamine responsiveness in our three patients was confirmed by in vitro studies showing a thiamine-responsive functional defect in cultured skin fibroblasts. The PDHC activity in three patients showed maximal catalytic activity in the presence of a high concentration of TPP and after DCA treatment in cultured cells. These data suggested that the concomitant administration of a high dose of thiamine and DCA may be required to achieve maximal PDHC activity in patients with thiamine-responsive PDHC deficiency, especially in patients with an acidotic crisis.

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Thiamine-responsive pyruvate dehydrogenase deficiency in two patients caused by a point mutation (F205L and L216F) within the thiamine pyrophosphate binding region

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Abstract

The human pyruvate dehydrogenase complex (PDHC) catalyzes the thiamine-dependent decarboxylation of pyruvate. Thiamine treatment is very effective for some patients with PDHC deficiency. Among these patients, five mutations of the pyruvate dehydrogenase (E_1)α subunit have been reported previously: H44R, R88S, G89S, R263G, and V389fs. All five mutations are in a region outside the thiamine pyrophosphate (TPP)-binding region of the $E_1\alpha$ subunit. We report the biochemical and molecular analysis of two patients with clinically thiamine-responsive lactic acidemia. The PDHC activity was assayed using two different concentrations of TPP. These two patients displayed very low PDHC activity in the presence of a low (1×10^{-4} mM) TPP concentration, but their PDHC activity significantly increased at a high (0.4 mM) TPP concentration. Therefore, the PDHC deficiency in these two patients was due to a decreased affinity of PDHC for TPP. Treatment of both patients with thiamine resulted in a reduction in the serum lactate concentration and clinical improvement, suggesting that these two patients have a thiamine-responsive PDHC deficiency. The DNA sequence of these two male patients' X-linked $E_1\alpha$ subunit revealed a point mutation (F205L and L216F) within the TPP-binding region in exon 7.

Keywords: Lactic acidemia; Pyruvate dehydrogenase complex deficiency; $E_1\alpha$ subunit; Thiamine pyrophosphate; Thiamine treatment; TPP binding region

1. Introduction

The human pyruvate dehydrogenase complex (PDHC) catalyzes the thiamine-dependent decarboxylation of pyruvate, and plays an important role in energy metabolism of the cell since it is an essential and rate-limiting enzyme connecting glycolysis with the tricarboxylic acid cycle and oxidative phosphorylation. Defects in PDHC are a common cause of primary lactic acidemia. Clinical symptoms can vary considerably in patients with PDHC deficiencies [1]. Thiamine treatment is very effective for some patients with PDHC deficiency. The majority of PDHC deficiencies result from mutations in the X-linked pyruvate dehydrogenase (E_1)α subunit gene [2,3]. The E1 component also contains a thiamine pyrophosphate (TPP) binding site that is shared by the α and β subunits [1,2]. In this study, we describe two male patients with a point mutation (F205L and L216F) within the TPP-binding region of the $E_1\alpha$ subunit.

2. Materials and methods

2.1. Patients

2.1.1. Patient 1

A boy weighing 1464 g at birth was born at 34 weeks of gestation. At 10 months of age, he suddenly developed marked generalized muscle hypotonia and weakness, while suffering from examthema subitum. He was admitted to the hospital for evaluation. On admission, his blood lactate concentrations ranged between 8.1 and 6.9 mM, and his pyruvate concentrations between 1.1 and 0.76 mM with lactate/pyruvate ratios between 7.4 and 9.2. The concentrations of lactate and pyruvate in the cerebrospinal fluid

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(CSF) were significantly elevated at 8.2 and 1.2 mM, respectively. Amino acid analysis of his plasma showed an elevated alanine concentration of 1497 μmol/l (normal range, 320 to 480). He responded to 100 mg/day of thiamine. His blood lactate and pyruvate concentrations decreased to 2.7 and 0.4 mM, respectively. After treatment with a high dose of thiamine, the muscle weakness improved, and he was able to walk. He no longer developed generalized muscle hypotonia and weakness during febrile illness, and his condition remained relatively stable at the age of 2 years and 10 months. CT and MRI scans of the patient's brain showed no abnormalities.

2.1.2. Patient 2

The second patient was an 8-year-old boy, the second child of healthy unrelated parents; his sister was healthy. He was clinically normal at birth and during his first 2 years of life. At 24 months, he developed an ataxic gait and muscle weakness during a febrile illness, and he could not walk. He was admitted to the hospital for evaluation. On admission, his blood lactate (6.2 mM) to blood pyruvate (0.6 mM) ratio was 10.3. The concentration of lactate and pyruvate in the CSF were elevated at 5.1 and 0.64 mM, respectively. An MRI of the brain showed a bilateral hypointense signal on the T1 weighted images in the basal ganglia. He received 60 mg/day of thiamine. After the start of thiamine therapy, his blood lactate and pyruvate concentrations decreased to the normal range, and he was able to walk without support. His muscle weakness and ataxia decreased and he did not develop metabolic acidosis during febrile illnesses. The abnormality on the brain MRI improved. He showed normal development with only a slight gait disturbance after the age of 6 years and was admitted to an elementary school.

2.2. Enzymatic assays

Lymphoblastoid cell lines derived from two patients with clinically thiamine-responsive lactic acidemia were established and cultured in RPMI 1640 medium (Nissui, Tokyo) containing 3×10^{-3} mM thiamine-HCl as previously described [4]. The activities of the PDHC and E1 in cultured lymphoblastoid cells were assayed at two different concentrations of TPP (0.4 and 1×10^{-4} mM) [4]. The concentration of TPP in normal human blood is 1×10^{-4} mM [5]. Protein concentrations were measured by the method of Lowry et al. [6] with bovine serum albumin as a standard.

2.3. Assay of rate of [1-^{14}C]pyruvate decarboxylation

The rate of [1-^{14}C]pyruvate decarboxylation was measured by a modification of the method of Naito et al. [7]. Briefly, intact lymphoblastoid cells were harvested, washed, and suspended with PBS. After preincubation with or without thiamine-HCl (4 mM) for 60 min, the cells were incubated in 0.2 ml of PBS containing 2.5 mM [1-^{14}C]pyruvate (0.1 mCi/mmol) in closed vessels at 37 °C for 30 min.

2.4. Molecular analysis of the PDHC-$E_1\alpha$ gene

The great majority of PDHC deficiencies result from mutations in the $E_1\alpha$ subunit gene [3]. Mutation analysis of the $E_1\alpha$ gene was performed using genomic DNA from cultured lymphoblastoid cells. For genetic analysis of the 11 exons of the $E_1\alpha$ gene, individual exons of the gene were amplified using primer pairs and conditions as described previously [8]. These fragments, used to detect single-strand conformation polymorphisms (SSCP), were sequenced directly using appropriate amplification primers. SSCP analysis was performed using the GeneGel Exel kit (Amersham Pharmacia Biotech UK Ltd., Bucks, UK). Sequence analysis was performed by an automated ABI 377 autosequencer using a dye-terminator kit (Applied Biosystems, Norwalk, CT).

3. Statistical analysis

Statistical analysis of the biochemical data was performed using Student's *t*-test. A value of $P<0.05$ was accepted as statistically significant.

4. Results

4.1. Activities of PDHC

Two thiamine-responsive patients showed apparently low activity of PDHC in the presence of low (1×10^{-4} mM) TPP, as shown in Table 1, but their PDHC activities significantly increased at a high (0.4 mM) TPP concentration. Most strikingly, the PDHC activity of patient 2 increased to within the normal range. The mother of patient 2 also had a low activity of PDHC under low TPP concentrations, but the PDHC activity was normal at a high TPP concentration. Therefore, the PDHC deficiency in these two patients and the mother of patient 2 was due to a decreased affinity of PDHC for TPP.

Table 1
Activities of PDHC in cultured lymphoblastoid cells

	PDHC (nmol/min/mg protein)[a]	
	With 1×10^{-4} mM TPP	With 0.4 mM TPP
Patient 1	0.05 ± 0.02* (4)	2.65 ± 0.38** (4)0
Patient 2	1.82 ± 0.11* (4)	3.24 ± 0.33 (4)
Mother of Patient 2	2.34 ± 0.21* (3)	4.34
Controls	3.99 ± 0.31 (6)	4.01 ± 0.66 (10)

PDHC: pyruvate dehydrogenase complex; TPP: thiamine pyrophosphate.
[a] DCA-activated PDHC values are the means ± S.D. of (*n*) determinations or the result of a single determination.
* $P<0.001$ vs. controls.
** $P<0.01$ vs. controls.

4.2. Affinity of PDHC for TPP

We investigated the affinities of normal and mutant PDHC for TPP. As shown in Fig. 1, normal cells showed approximately 50% of maximal PDHC activity in the absence of TPP, and the addition of 1×10^{-5} mM TPP restored full activity, with no further change in PDHC activity at higher concentrations of TPP. However, cells from the two thiamine-responsive patients showed significantly lower PDHC activity at the 1×10^{-3} mM TPP concentration. The addition of a high concentration (0.4 mM) of TPP restored activity to 66% and 81%, respectively, of the maximal PDHC activity of normal cells.

4.3. Affinity of E1 for TPP

To investigate the affinity of E1 for TPP, we measured the activities of DCA-activated E1 in the presence of 0.4 and 1×10^{-4} mM TPP (Table 2). There was no difference in the E1 activity of normal controls in the presence of 0.4 and 1×10^{-4} mM TPP. In contrast, the activity of DCA-activated E1 of the two patients was significantly lower in the presence of 1×10^{-4} mM TPP, and the addition of a high TPP concentration (0.4 mM) increased the average activity to 36% and 64%, respectively, of the controls. Thus, the E1 of these two patients also showed decreased affinity for TPP.

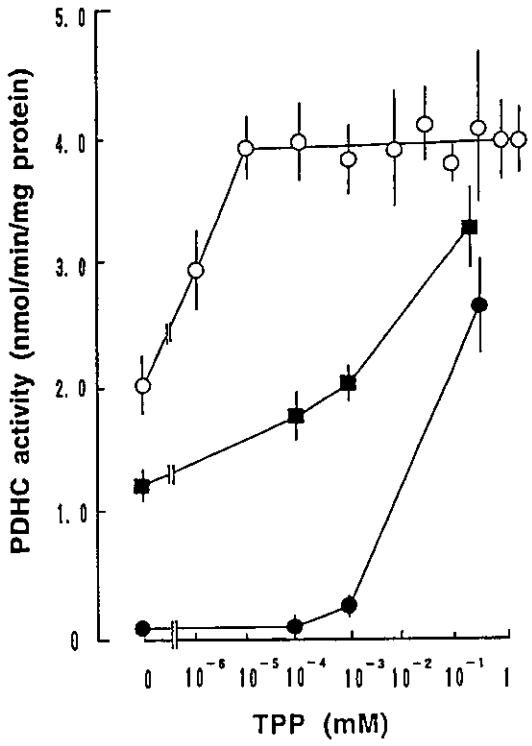

Fig. 1. Effect of TPP on the activity of DCA-activated PDHC in cultured lymphoblastoid cells from normal subjects and two patients with thiamine-responsive lactic acidemia. The activity of PDHC in cultured lymphoblastoid cells is shown at various concentrations of TPP. O, normal controls; ●, patient 1; ■, patient 2.

Table 2
Activities of E1 in cultured lymphoblastoid cells

	E1 activity (nmol/h per mg protein)[a]	
	With 1×10^{-4} mM TPP	With 0.4 mM TPP
Patient 1	0.23 ± 0.13 * (3)	1.41 ± 0.51 ** (3)
Patient 2	0.61 ± 0.27 * (3)	2.54 ± 0.30 (3)
Controls	3.17 ± 0.19 (5)	3.95 ± 1.06 (7)

E1: pyruvate dehydrogenase; TPP: thiamine pyrophosphate.
[a] DCA-activated E1 values are the means ± S.D. of (*n*) determinations or the result of a single determination.
* $P<0.001$ vs. controls.
** $P<0.01$ vs. controls.

4.4. Rate of [1-^{14}C]pyruvate decarboxylation in intact lymphoblastoid cells

During incubation without thiamine-HCl, intact lymphoblastoid cells from the two patients with thiamine-responsive PDHC deficiency showed decreased decarboxylation rates of 25% and 22%, respectively, of the controls. However, during incubation with 4 mM thiamine-HCl, the decarboxylation rate of these two patients increased markedly to 45% and 49%, respectively, of the controls. In contrast, the decarboxylation rate of the normal controls scarcely increased (Table 3). Thus, pyruvate decarboxylation by intact lymphoblastoid cells from these two patients were also thiamine-responsive.

4.5. Mutation analysis of the $E_1\alpha$ subunit

DNA sequence analysis of the two patients' X-linked $E_1\alpha$ subunit revealed a substitution within the TPP-binding region in exon 7 of the $E_1\alpha$ subunit. The genomic sequence of patient 1 had a C → G point mutation at nucleotide 615, resulting in the substitution of leucine for phenylalanine at position 205 (F205L), and the genomic sequence of patient 2 had an A → C point mutation at nucleotide 648, resulting in the substitution of phenylalanine for leucine at position 216 (L216F) as shown in Fig. 2. The genomic sequence of the mother of patient 2 yielded A and C at nucleotide 648,

Table 3
Rate of [1-^{14}C]pyruvate decarboxylation in intact lymphoblastoid cells

	Pyruvate decarboxylation (nmol/h/mg protein)	
	Without thiamine-HCl	With thiamine-HCl
Patient 1	19.9 ± 6.3 * (4)	35.9 ± 5.3 * (4)
Patient 2	17.2 ± 4.0 * (3)	38.9 ± 5.2 * (4)
Controls	78.6 ± 7.2 (4)	80.0 ± 5.8 (3)

HCl: hydrochloride.
Values are the means ± S.D. of (*n*) determinations or the result of a single determination.
* $P<0.001$ vs. controls.