[3,10,12,29–31]. Leen et al. [9] classified GLUT-1DS into three clinical phenotypes based on an analysis of genetically-confirmed 57 cases: (1) classic clinical phenotype: accounting for 85% of all cases, this group was classified into an early-onset type (onset at 2 years of age or younger) and delayed type (older than 2 years of age); (2) atypical phenotype: accounting for 15%, this group was characterized by mental retardation and paroxysmal kinesigenic choreoathetosis (paroxysmal exertion-induced dyskinesia) and the absence of epilepsy; and (3) adult phenotype with only subtle symptoms. Moreover, *SLC2A1* gene mutations were also found in various cases with established epileptic as well as neurological syndromes, such as early-onset absence epilepsy, myoclonic-astatic epilepsy, and alternating hemiplegia syndrome, underscoring the diversity of the clinical manifestations of GLUT-1DS [10–12,30]. In our study, 31 cases or 94% of all cases were subclassified into classical phenotype, while the remaining two cases, one was a child and another was his mother, were subclassified into atypical phenotype and adult phenotype, respectively. It was difficult to refer to the details of epileptic phenotypes in order to determine whether established epileptic syndromes were included in our study because of the limitation of the questionnaire study. However, as far as the GLUT-1DS diagnosis is concerned, the presence of the characteristic neurological and neurocognitive abnormalities, in addition to the epileptic seizures regardless of seizure types, can help to distinguish GLUT-1DS from specific epileptic syndromes.

Our study included two families comprising 4 cases. Familial cases with autosomal dominant and recessive transmission have recently been described and included a parent or siblings having the same *SLC2A1* mutation to a proband who exhibited the typical clinical characteristics, but almost no clinical symptoms or mild symptoms [9,20,30,32]. Hashimoto et al. already reported the details of the two families included in the present study, in which both families included an affected parent and sibling and mild clinical symptoms in the mother were undiagnosed until the proband was diagnosed [20].

EEG findings have frequently been examined in children with GLUT-1DS because epileptic seizures have been identified as the primary symptoms in most patients [18]. In this study, the slowing of background activity, epileptic focal spike discharge, especially from the frontal region, and generalized 2.5–4 Hz spike–wave discharges were detected from early childhood and most frequently during the adolescent and later period. The epileptic EEG abnormality was generally infrequent during infancy and increased in frequency after early childhood. However, the most important EEG finding was the improvement observed in both epileptic abnormalities and background activities by the consumption of food or administration of a glucose injection [18,25]. The EEG examination can be performed on an outpatient basis such that the first EEG can be recorded in the morning after a whole night of fasting and the second EEG can be performed 30 min after breakfast, thereby showing marked EEG improvements and providing important information for an accurate diagnosis of GLUT-1DS.

Neuroimaging abnormalities including cerebral atrophy, myelination delay, and high-signal foci in the subcortical white matter (T2-weighted fluid-attenuated inversion recovery imaging) were reported; however, none of these were specific to this syndrome by themselves. In contrast, the relatively enhanced accumulation of glucose by the basal ganglia and reduced uptake of glucose by the thalamus on FDG-PET are considered to be very specific to GLUT-1DS [33]. However, FDG-PET scanning is not highly recommended when attempting to diagnose GLUT-1DS because it is expensive and its usefulness in early diagnosis has not been proven.

Heterozygous *de novo* mutations in the *SLC2A1* gene (1p35-31:3, OMIM 138140) have been detected in approximately 70%–80% of patients with GLUT-1DS, and causes this syndrome due to haploinsufficiency [8,9,34]. Genetic mutations were not detected in 10%–20% of cases, which is consistent with our results. Five patients in the present study including 4 without identifiable *SLC2A1* gene mutations were clinically diagnosed with GLUT-1DS based on their clinical symptoms and low CSF glucose levels as well as good responses to KD. A previous study speculated that potential disease mechanisms in patients without mutations in the coding regions of the *SLC2A1* gene could be posttranscriptional modifications such as alternative splicing, defects in N-glycosylation, GLUT-1 trafficking and GLUT-1 assembly, which affect the GLUT-1 function[35]. Patients with a missense mutation frequently exhibit milder clinical symptoms; however, no clear genotype-phenotype relationship has yet been established [9]. The results of this study also showed that patients with a missense mutation had better mental outcomes, later onset age of the first symptoms, and milder C/B ratio. This study included 3 cases of GLUT-1DS with *SLC2A1* missense mutations who did not exhibit the low uptake of 3-*O*-methyl-D-glucose by erythrocytes. Recent study demonstrated that the specific type of mutation alter GLUT confirmation and asymmetrically affects glucose flux across the cell by perturbing efflux (glucose release in CSF) than influx (uptake of glucose) [36]. A diversity of phenotypes was observed among patients with the same mutation and even within autosomal dominant family members, suggesting a complex onset pathomechanism. The results of our study were also consistent with findings reported in the US and Europe.

In conclusion, this study demonstrated that unexplained paroxysmal abnormal eye movements, apneic/cyanotic attacks, and convulsive seizures in infancy in combination with complex movement disorders (ataxia,

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dystonia/dyskinesia, and motor paralysis) in early childhood and thereafter were important clinical manifestations for suspected GLUT-1DS. An early CSF study is recommended for these patients and the early introduction of KD or ketone milk is important to prevent irreversible brain dysfunction due to the chronic depletion of glucose in the brain. Further studies are warranted to identify key clinical and laboratory information that will lead to a CSF study and accurate diagnosis of GLUT-1DS at as early an age as possible. We need a more accumulation of GLUT-1DS cases whose early clinical and EEG information can be obtained in details to make an accurate diagnostic and treatment guideline. We also need to confirm whether the early introduction of KD can improve brain function and prevent secondary brain disorders.

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Original article

Carnitine–acylcarnitine translocase deficiency: Two neonatal cases with common splicing mutation and *in vitro* bezafibrate response

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Abstract

Background: Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of acute encephalopathy- or myopathy-like illness. Carnitine–acylcarnitine translocase (CACT) deficiency is a rare FAO disorder, which represent an energy production insufficiency during prolonged fasting, febrile illness, or increased muscular activity. CACT deficiency is caused by mutations of the *SLC25A20* gene. Most patients developed severe metabolic decompensation in the neonatal period and died in infancy despite aggressive treatment.

Patients and methods: We herein report the clinical findings of two unrelated cases of CACT deficiency with mutation confirmation, and *in vitro* bezafibrate responses using *in vitro* probe acylcarnitine (IVP) assay. Patients 1 and 2 are products of nonconsanguineous parents. Both patients developed cardiac arrest at day 3 of life but survived the initial events. Their blood chemistry revealed hypoglycemia and metabolic acidosis. The acylcarnitine profiles in both patients demonstrated increased long-chain acylcarnitines, suggesting CACT or carnitine palmitoyltransferase-2 (CPT2) deficiency.

Results: The mutation analysis identified homozygous IVS2-10T>G in the *SLC25A20* gene in both patients, confirming the diagnosis of CACT deficiency. The IVP assay revealed increased C16, C16:1, but decreased C2 with improvement by bezafibrate in the cultured fibroblasts. The short-term clinical trial of bezafibrate in Patient 1 did not show clinical improvement, and died after starting the trial for 6 months.

Conclusion: This splicing mutation has been identified in other Asian populations indicating a possible founder effect. IVP assay of cultured fibroblasts could determine a response to bezafibrate treatment. A long-term clinical trial of more enrolled patients is required for evaluation of this therapy.

Keywords: CACT deficiency; *SLC25A20* mutation; IVP assay; Bezafibrate

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1. Introduction

Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of neuromuscular symptoms as well as acute encephalopathy or even sudden death. In particular, the carnitine cycle is important in energy-producing pathway for cardiac and skeletal muscle and for preventing from hypoglycemia especially during prolonged fasting or increased muscular exercise. Carnitine–acylcarnitine translocase (CACT, EC 2.3.1.21) is one of the enzymes in the carnitine cycle, which catalyzes the transfer of the long-chain fatty acylcarnitines across the inner mitochondrial membrane in exchange of free carnitine. CACT deficiency (OMIM 212138) was first described in 1992 [1]. It is an autosomal-recessive disease caused by mutations of the *SLC25A20* gene located in chromosome 3p21.31 [2]. The gene consists of 9 exons and encodes protein comprising 301 amino acids [3]. CACT deficiency is a very rare disorder with so far as approximately 30 patients have been described, and accounted for 10% of patients with FAO disorders in French population [4]. However, it might be a common FAO disorder in some East Asian countries such as Hong Kong with the estimated incidence of 1 in 60,000 live births, and accounted for 33% of patients with FAO disorders [5]. Most patients develop neonatal-onset encephalopathy with nonketotic hypoglycemia, hyperammonemia, and hypothermia, or sudden death from cardiac arrhythmias. Cardiomyopathy and hepatic dysfunction may be the associated complications. CACT deficiency could be detected by elevations of C16 and C18 acylcarnitines, and low free carnitine in acylcarnitine profiles. However, the same profile could be found in neonatal carnitine palmitoyltransferase-2 (CPT2) deficiency. Therefore, confirmation of diagnosis requires CACT enzyme assay or molecular analysis of the *SLC25A20* gene [6]. Treatment includes intravenous glucose for acute decompensation, and avoidance of long fasting with frequent meals. Long-chain fatty acids may be restricted in diet, but medium-chain triglyceride (MCT) oil is supplemented instead. Carnitine therapy is still controversial. Despite aggressive treatment, most patients still died in infancy [7]. However, there have been some patients who received early treatment with good outcomes [8,9]. Novel therapy for FAOD using bezafibrate, which is a hypolipedimic drug acting as a peroxisome proliferator-activated receptor (PPAR) agonist has been reported. The clinical trials of bezafibrate showed clinical improvement in adult patients with CPT2 deficiency [10], and a child with glutaric acidemia type 2 (GA2) [11]. *In vitro* probe acylcarnitine (IVP) assay can be used to evaluate FAO disorders [12], and determine the effect of bezafibrate [13]. We herein report the clinical findings of two unrelated cases with neonatal-onset CACT deficiency, and *in vitro* bezafibrate response using the IVP assay.

2. Patients and methods

2.1. Patients

2.1.1. Case 1

This patient was the first child of possibly consanguineous parents from the southern province of Thailand. He was born at 37 weeks of gestation with birth weight of 2460 g (25th percentile), length 48 cm (3rd percentile), and head circumference 30 cm (<3rd percentile). He developed hypothermia at 10 h of age. Sepsis was suspected, but the patient rapidly responded to rewarming treatment. However, after rooming-in with the mother, he developed hypothermia again. At 60 h after birth, he had cardiac arrest. On physical examination, no abnormalities were found. Serum glucose was 1.2 mmol/L and acetoacetate was 0 mmol/L. Venous blood pH was 7.24 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 471 μmol/L (normal, <110 μmol/L). There were mildly elevated liver enzymes aspartate aminotransferase (AST) (97 U/L; normal, 0–32) and alanine aminotransferase (ALT) (78 U/L; normal, 0–33). Serum creatine kinase was 4439 U/L (normal, <190). He had a good response to treatment with intravenous glucose administration. Urine organic acids were unremarkable. A dried blood spot acylcarnitine profile by tandem mass spectrometry (MS/MS) showed free carnitine (C0), 5.26 μM (10–60); C16-acylcarnitine, 14.14 μM (0.6–7); C18-acylcarnitine, 2.71 μM (0.15–2.1); C18:1-acylcarnitine, 4.3 μM (0.3–3.2); and a (C16 + C18)/C0 ratio, 3.21 (0.007–0.5). The profile was consistent with CPT2 or CACT deficiency. The patient has been treated with a modular medical formula, which has been composed of modified fats (long-chain fatty acid restriction along with supplementation of 83% of fat as medium-chain triglyceride oil), protein, maltodextrins, minerals, and fat-, and water-soluble vitamins. L-Carnitine at a daily dosage of 100–150 mg/kg has been supplemented. Thereafter, he has had several episodes of hypoglycemia, hyperammonemia, and metabolic acidosis following infections. At 8 months of age, he developed cholestasis and hepatomegaly. At 9 months of age, an echocardiogram revealed hypertrophic cardiomyopathy. At the age of 15 months, he had mild developmental delay and generalized hypotonia. He could stand with support, put block in cup, and say one word. Then he had a metabolic crisis, and developed generalized weakness. After he recovered from encephalopathy, neurologic examination revealed normal cranial nerves, muscle weakness (grade 3/5), and decreased muscle tone and deep tendon reflexes (1+) in all extremities. A brain computed tomography scan was normal. Serum creatine kinase was elevated (1419 U/L). A nerve conduction study showed no evidence of demyelination. He had been ventilator-dependent since then. At 2½ years of

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age, he had several complications including chronic liver disease, upper gastrointestinal bleeding, and osteoporosis. He died at the age of 2 years and 8 months from upper gastrointestinal bleeding and metabolic decompensation.

2.1.2. Case 2

The patient was the first child of nonconsanguineous parents. She was born at 35 weeks of gestation with a birth weight of 2.3 kg (50th percentile), length 44 cm (25th percentile), and head circumference 30 cm (10th percentile). At 2 days after birth, she developed lethargy, poor feeding, and cardiac arrest. Blood glucose was 0.56 mmol/L. She responded to cardiac resuscitation and intravenous glucose infusion. Serum acetoacetate was 0 mmol/L. Venous blood pH was 7.39 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 157 μmol/L (normal, <110 μmol/L). There were elevated liver enzymes AST (638 U/L; normal, 0–32) and ALT (83 U/L; normal, 0–33). Plasma lactate dehydrogenase (LDH) was 522 U/L (normal, 240–480). An echocardiogram revealed no cardiomyopathy. A dried blood spot acylcarnitine profile by MS/MS analysis showed C0, 13.8 μM (10–60); C16-acylcanitine, 15 μM (0.6–7); C18-acylcarnitine, 4.3 μM (0.15–2.1); C18:1-acylcarnitine, 5.9 μM (0.3–3.2); and a (C16 + C18)/C0 ratio, 1.4 (0.007–0.5). The profile was consistent with either CPT2 or CACT deficiency. The patient had been treated with a high-MCT formula (Portagen®, Mead Johnson Nutritionals), and 100 mg/kg/day of L-carnitine. At 1 month of age, she developed anemia from Hb AE Bart's disease – a thalassemia intermedia resulting from the interaction between α-thalassemia and heterozygous Hb E, which required monthly blood transfusion. At the age of 4 months, she had poor feeding and cardiac arrest. Blood glucose was 0.5 mmol/L. The patient died without any response to resuscitation. An autopsy revealed left ventricular hypertrophy, micro/macrovesicular steatosis of the liver with focal areas of bridging fibrosis, and abnormal lipid accumulation in skeletal muscles and the proximal renal tubules.

2.2. Materials and methods

This study was approved by the Siriraj Institutional Review Board. The written informed consents for the mutation analysis, IVP assay, and bezafibrate trial were obtained from the parents. Genomic DNA was extracted from leukocytes. Mutation analyses of the *CPT2* and *SLC25A20* genes were performed in case 1, and only *SLC25A20* gene in case 2. All coding exons and their flanking intron sequences (up to 20 bases for both sides) of the *CPT2* and *SLC25A20* genes were PCR-amplified and directly sequenced according to the previously described method [14]. The IVP assay was performed using the skin fibroblasts in the absence and presence of bezafibrate according to the previously described method [11].

3. Results

3.1. Mutation analysis and IVP assay

Mutation analysis of the *SLC25A20* gene identified homozygous c.199-10T>G (IVS2-10T>G) mutation in both patients, and heterozygous mutation in their parents (Fig. 1). Mutation analysis of the *CPT2* gene revealed no pathogenic mutation in Case 1. The IVP assay profiles revealed increased C16, C16:1 acylcarnitines, and decreased C2 (acetylcarnitine) indicating a typical pattern of CPT2 or CACT deficiency, with substantial reduction of long-chain acylcarnitines by the presence of bezafibrate in the cultured fibroblasts from both patients (Fig. 2). However, C2 acylcarnitine did not increase as expected.

3.2. Clinical trial of bezafibrate

We started a clinical trial of bezafibrate in case 1 at age of 2 years and 2 months, after the IVP assay which

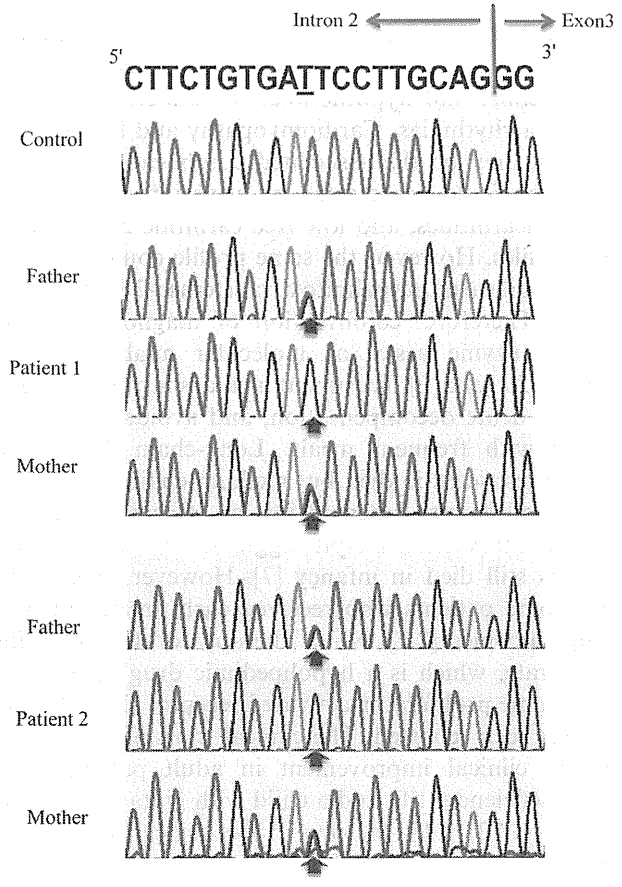

Fig. 1. The reference DNA sequence of an intron 2/exon 3 boundary of the *SLC25A20* gene, and the IVS2-10T>G mutation identified in both patients and their parents denoted by black arrows and the underlined letter.

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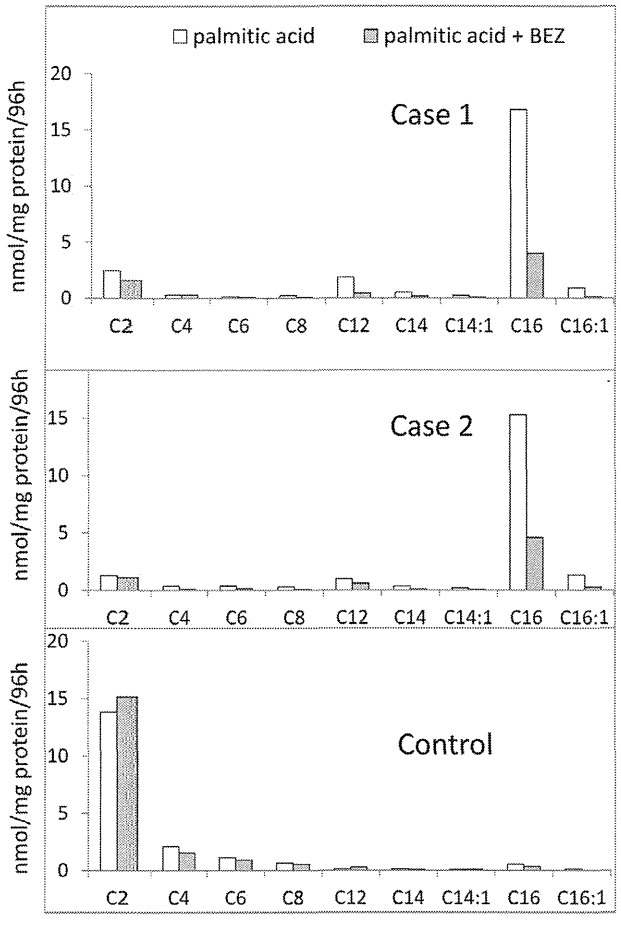

Fig. 2. Acylcarnitine profiles of IVP assay in the presence and absence of bezafibrate (BEZ) of cases 1, 2, and normal control respectively. Unit of vertical lines, nmol/mg protein of acylcarnitines (ACs); the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C12, C14, C14:1, C16, and C16:1. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars.

showed some improvement in acylcarnitine profiles with bezafibrate. We used a dosage of 17–25 mg/kg/day as previously described [11]. Monitoring of liver functions, lactate dehydrogenase (LDH), creatine kinase (CK), and lipid profiles showed no adverse effects of bezafibrate. A short-term evaluation, after 6 months of the trial, did not show clinical improvement except for slightly increased back muscle strength noted by the mother. An echocardiography showed stable but no improvement in a left ventricular mass index. Acylcarnitine profiles in dried blood spots and other biochemical parameters did not show improvement (data not shown). Case 2 died before a clinical trial was considered.

4. Discussion

We report 2 unrelated cases of CACT deficiency with molecular confirmation first identified in Thailand. The c.199-10T>G (IVS2-10T>G) nucleotide change was the most prevalent mutation and identified in 14 out of 76 mutant alleles [15]. This mutation was homozygously identified in three Vietnamese and three Chinese patients. In the present study, in spite that two families had no consanguineous history, both patients were also a homozygotes of the c.199-10T>G mutation. In Japan, three CACT deficient patients have been described. Among them the same mutation was identified heterozygously in only one patient [14]. We propose that this mutation is a founder mutation in Asian populations. Clinical history of the three Chinese patients with homozygous c.199-10T>G mutation were reported [16]. All of them developed cardiac arrest within two days of age, as well as our two patients. Hence the phenotype of homozygotes of c.199-10T>G mutation is severe. This mutation was suggested to reside at a consensus lariat branch point sequence resulting in skipping of exons 3 and 4 or exon 3 alone, which leads to truncation of the protein [17].

Although our cases 1 and 2 were homozygotes of the same mutation, Case 1 survived until 2 years and 8 months and Case 2 died at 4 months of age. Several factors might attribute to their different clinical outcomes: (1) Thalassemia disease in case 2 which required repeated blood transfusions might affect cardiac functions by chronic hypoxia, iron overload, or decreased carnitine [18]; (2) differences in possible modifier genes such as *SLC25A29* gene (CACT-like, CACL) which has palmitoyl-carnitine transporting activity [19]; and (3) different formulas using in our cases, one is a synthetic modular formula and the other is a commercial formula. However, the rationale of both special formulas for diet therapy is a reduction in long-chain fatty acids together with supplementation of medium-chain triglyceride oil to be a caloric source shunting an obstruction of long-chain fatty acid β-oxidation.

Although increased FAO flux induced by bezafibrate was clearly shown in fibroblasts only from patients with mild phenotypes of FAO disorders, increased mRNA expression after bezafibrate exposure also occurred in cell lines from patients with severe phenotypes [20]. This could explain *in vitro* response to bezafibrate observed in fibroblasts of patient 1 and 2. Despite the severe genotype leading to barely detectable enzyme activity [21], we believe that there should be some FAO flux which could be enhanced by bezafibrate in these patients. Our hypothesis is if there is entirely absent FAO flux in these patients, they should have anomalies like those found in a lethal neonatal form of CPT2 deficiency or GA2 [22], even though there has been no report of such findings in CACT deficiency. To our knowledge, patient 1 is the first case of neonatal-onset CACT deficiency who underwent a clinical trial of bezafibrate after showing an *in vitro* response by IVP assay. However, no beneficial short-term effect was shown. This might indicate the irreversible damage of the affected organs esp. the cardiac and skeletal muscles, and liver. Moreover, the difference between the *in vitro* and *in vivo* responses is

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probably due to the difference of bezafibrate concentration used in the IVP assay (400 μmol/L) and typical concentrations obtained in patients on bezafibrate therapy (50–200 μmol/L) [23]. Another possible reason is inadequate acetyl-CoA production despite bezafibrate treatment. This hypothesis is supported by persistently low C2 acylcarnitines in IVP assays of our cases and a previous case with CACT deficiency [11]. Moreover, C16 acylcarnitine did not decrease to the control level after bezafibrate treatment. Overall, although some improvement of acylcarnitine profile was shown in the patient 1 and 2's fibroblasts in IVP assay with bezafibrate, the effect of bezafibrate was less than those in fibroblasts from patients with mild forms of FAO disorders [11,24]. Hence clinical improvement in this patient was thought to be limited. Since CACT-deficient patients who developed metabolic decompensation in early neonatal period had poor prognosis with routine management [7], we decided to use bezafibrate treatment in patient 1. He survived until two years of age with bezafibrate treatment. However, it is uncertain whether this longer survival owed to the effect of bezafibrate treatment or not, since no apparent improvement of clinical laboratory data was obtained.

In conclusion, CACT deficiency may be a common FAO disorder in East Asian populations probably from a founder effect. IVP assay of fibroblasts could determine a response to bezafibrate treatment. A long-term clinical trial and more enrolled patients are required for evaluation of this therapy.

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Case report

Early replacement therapy in a first Japanese case with autosomal recessive guanosine triphosphate cyclohydrolase I deficiency with a novel point mutation

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Abstract

Autosomal recessive guanosine triphosphate cyclohydrolase I (GTPCH) deficiency is an inborn error of tetrahydrobiopterin (BH4) synthesis from GTP. GTPCH deficiency causes severe reduction of BH4, resulting in hyperphenylalaninemia (HPA) and decreased dopamine and serotonin synthesis. Without treatment, a patient with GTPCH deficiency develops complex neurological dysfunctions, including dystonia and developmental delays. The first Japanese patient with GTPCH deficiency was discovered by HPA during asymptomatic newborn screening. The phenylalanine level at the age of 5 days was 1273 μmol/L (cutoff value, 180.0 μmol/L). The high serum phenylalanine level was decreased to normal after adequate BH4 oral supplementation. Serum and urinary pteridine examination revealed very low levels of neopterin and biopterin. Sequence analysis of *GCH1* revealed compound heterozygous point mutations, including a novel point mutation (p.R235W). Replacement therapy with BH4 and L-dopa/carbidopa were started at the age of 1 month, and 5-hydroxytryptophan (5-HTP) was started at the age of 5 months. At 10 months of age, the patient showed slight dystonia but no obvious developmental delay. Cerebrospinal fluid should be examined to determine the appropriate dosage of supplement drugs. In conclusion, it is important to control the serum phenylalanine level and perform early replacement of neurotransmitters to prevent neurological dysfunction.

Keywords: Autosomal recessive guanosine triphosphate cyclohydrolase I (GTPCH); Tetrahydrobiopterin (BH4); Hyperphenylalaninemia; Early replacement therapy

1. Introduction

Tetrahydrobiopterin (BH4) is an essential cofactor in the enzymatic hydroxylation of phenylalanine, tyrosine, and tryptophan. The BH4 loading test is performed to distinguish BH4 deficiency from hyperphenylalaninemia (HPA) during newborn screening. BH4 deficiency causes HPA and decreased production of the neurotransmitters dopamine and serotonin. Five types of enzyme deficiencies have been reported in BH4 deficiency: guanosine triphosphate cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS), sepiapterin reductase (SR), dihydropteridine reductase (DHPR), and pterin-4a-carbinolamine dehydratase (PCD) [1]. GTPCH deficiency is an error of BH4 synthesis. *GCH1*, the gene

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symbol of GTPCH, is located on chromosome 14q22.1-q22.2 and comprises six exons. GTPCH deficiency has autosomal dominant and autosomal recessive forms. The autosomal dominant (AD) form is known as dopa-responsive dystonia (DRD, Segawa disease), whereas the autosomal recessive (AR) form results in swallowing difficulties, truncal hypotonia, seizures, mental retardation, and developmental delays. The residual GTPCH enzyme activity is thought to be the cause of clinical severity between the AD and the AR form. The AR form is so rare that only 17 cases are listed in the BIOMDB Database [1], and no case has been reported in Japan. The combination of BH4 replacement and neurotransmitter precursor supplementation of both L-dopa/carbidopa and 5-hydroxytryptophan (5-HTP) is a common therapeutic approach.

We experienced the first Japanese case of autosomal recessive GTPCH deficiency with HPA during newborn screening. We herein describe the clinical symptoms and treatments with a review of previous reports.

2. Case report

The patient was the first child of healthy, nonconsanguineous parents. He was born in the 40th week of pregnancy by spontaneous delivery (birth weight, 2744 g; birth height, 50 cm). At the age of 11 days, the patient was hospitalized because of HPA detected by newborn screening. The phenylalanine level measured by Guthrie test at the age of 5 days was 1273 μmol/L (cutoff value, 180.0 μmol/L). There were no abnormalities on physical or neurological examinations. Laboratory examinations showed HPA. The serum phenylalanine level was 2206 μmol/L (reference interval, 61.2 ± 14 μmol/L). A BH4 loading test with 10 mg/kg of sapropterin hydrochloride was performed. The serum phenylalanine level was decreased to normal 8 h after adequate BH4 oral supplementation (Fig. 1). Serum and urinary pteridine examination revealed very low levels of neopterin and biopterin. The serum levels of neopterin and biopterin were 5.76 nM (reference interval, 33.8 ± 4.9 nM) and 3.31 nM (reference interval, 15.0 ± 1.6 nM), respectively. The urinary level of neopterin and biopterin were 0.14 mmol/mol creat. (reference interval, 2.09 ± 0.52 mmol/mol creat.) and 0.64 mmol/mol creat. (reference interval, 1.08 ± 0.36 mmol/mol creat.). The cerebrospinal fluid (CSF) concentration of 5-hydroxyindoleacetic acid (5-HIAA) was 114 nmol/L (reference interval, 746 ± 207 nmol/L), and that of homovanillic acid (HVA) was 21 nmol/L (reference interval, 1083 ± 339 nmol/L) (Table 1); both were reduced.

Molecular genetic analysis of *GCH1* revealed compound heterozygous point mutations in exon 5 of *GCH1* (p.R184H) and exon 6 of *GCH1* (p.R235W) (Fig. 2). His father was heterozygous for p.R184H, and his mother was heterozygous for p.R235W. The

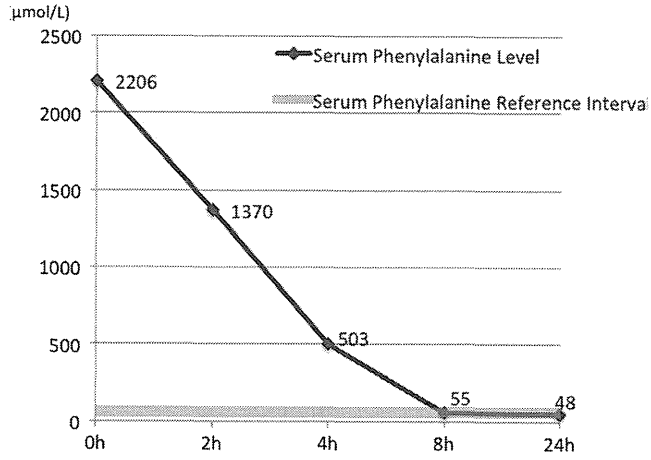

Fig. 1. Response of serum phenylalanine level by oral BH4 loading. Serum phenylalanine level was decreased to reference interval (61.2 ± 14 μmol/L) at the 8 h after BH_4 supplement.

p.R235W has not been previously reported in patients with GTPCH deficiency.

At the age of 1 month, treatments with L-dopa/carbidopa and BH4 were started, with initial doses of 2 and 5 mg/kg/day, respectively. Mild dystonia appeared at the age of 3 months. It was slightly improved by increasing the dose of L-dopa/carbidopa supplementation. Because 5-HTP is not an approved medicine in Japan, we began supplemental therapy with 5-HTP at the age of 5 months (4 mg/kg/day) after approval by our hospital ethics committee. We adjusted the dose of BH4 according to the level of serum phenylalanine, L-dopa/carbidopa according to the clinical symptom of dystonia and serum prolactin and CSF HVA levels, and 5-HTP according to the CSF 5-HIAA level. At 8 months of age, a brain MRI was normal, and the CSF concentrations of 5-HIAA and HVA were improved (5-HIAA, 143 nmol/L; HVA, 403 nmol/L). In the most recent examination at 10 months old, he still had slight dystonia but no obvious developmental delay (sitting without support and playing toy with babbling) under treatment with BH4 (5 mg/kg/day), L-dopa/carbidopa (16 mg/kg/day), and 5-HTP (4 mg/kg/day).

3. Discussion

We herein report the first Japanese case of autosomal recessive GTPCH deficiency detected by newborn screening and presenting as HPA. We identified a novel point mutation of *GCH1*. The patient had no family history, and his parents were asymptomatic carriers. Although five *GCH1* mutations (p.Q110X (exon 1), p.R184H (exon 5), p.M213T (exon 6), p.M211V (exon 6), and p.M211I (exon 6)) have been reported in patients with HPA (BIOMDB Database) [2], there seem to be no obvious phenotype or genotype correlations.

Table 1
Clinical features and therapies for autosomal recessive GTPCH deficiency with HPA.

Report	Our case	Blau et al. [3]	Bandmann et al. [4]	Matalon et al. [5]
Age at diagnosis	1 mo	5 mo	4.5 mo	6 mo
GCH1 mutation	R184H/R235 W	M211I/M211I	M211 V/M211 V	R184H/R184H
Serum phenylalanine level (μmol/L) (reference interval: 61.2 ± 14 μmol/L)	2206	1488	3500	>2400
CSF 5-HIAA Level (nmol/L) (reference interval: 746 ± 207 nmol/L)	114	92	Normal	Unknown
CSF HVA level (nmol/L) (reference interval: 1083 ± 339 nmol/L)	20.9	50	Decreased level	Unknown
Age at therapy	1 mo	9 mo	Unknown	Unknown
Therapy & initial dose	L-dopa/carbidopa: 2 mg/kg/day BH4: 5 mg/kg/day 5-HTP: 4 mg/kg/day	L-dopa/carbidopa: 5.8 mg/kg/day BH4: 3.5 mg/kg/day 5-HTP: 3 mg/kg/day	L-dopa/carbidopa: unknown dose BH4: unknown dose 5-HTP: unknown dose	L-dopa/carbidopa: unknown dose BH4: unknown dose 5-HTP: unknown dose
Symptoms before therapy	1 mo: no symptoms	Generalized hypotonia, dystonia	Feeding problems	0 mo: feeding problems 6 mo: delayed in development 2 y: be unable to walk, seizure, choreoayhetosis
Symptoms after therapy	3 mo: slight dystonia	15 mo: slight axial hypotonia	5 y: learning difficulties, moving disorder (stiffening when patient is tired or upset)	Improved the choreoayhetosis
	10 mo: no mental retardation, slight dystonia	33 mo: slight mental retardation		10 y: died

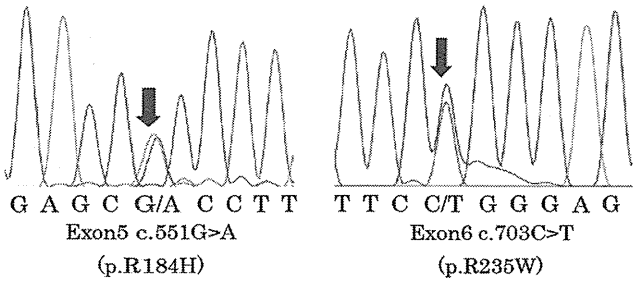

Fig. 2. Gene analysis of *GCH1*. Sequence analysis revealed the compound heterozygous mutation, G-to-A transition (c.551G>A) in exon 5 causing an amino acid substitution of p.R184H and C-to-T transition (c.703C>T) in exon 6 causing an amino acid substitution of p.R235W.

Table 1 shows a comparison of our case with previous reports on autosomal recessive GTPCH deficiency with HPA. The age at diagnosis was 3–6 months in previous reports. Feeding problems, hypotonia, and dystonia were often observed before replacement therapy. After replacement therapy with BH4, L-dopa/carbidopa, and 5-HTP was started, the feeding problems, hypotonia, and dystonia were mildly improved.

We began replacement therapy from the age of 1 month, earlier than previous reports [3–5]. Mild dystonia appeared at the age of 3 months. We then increased the dose of L-dopa/carbidopa. The dose of L-dopa/carbidopa should be adjusted according to the level of HVA in the CSF. However, CSF sampling is invasive and difficult to perform frequently. The serum prolactin level is reportedly a more sensitive and pre-symptomatic marker than is the CSF level of HVA with respect to guiding the drug adjustment of L-dopa/carbidopa in patients with PTPS deficiency [6]. Although the serum prolactin level immediately decreased to the normal range after L-dopa/carbidopa supplementation, the CSF HVA level remained low, and dystonia was not completely improved. Moreover, an increase in the 5-HTP dose may be required because of a low level of CSF 5-HIAA. We consider that scheduled CSF sampling is necessary for adjustment of drugs to prevent development of neurological symptoms.

In the most recent examination at 10 months old, the patient showed normal psychological development. Blau et al. reported that slight mental retardation remained at the age of 33 months [3]. The other cases also had neurological impairments such as speech delays, learning difficulties, and moving disorders past the age of 3 years [4]. These reports indicate that mild mental retardation and/or learning difficulties may remain. Early diagnosis and starting medical treatment during early infancy are key to prevention of mental impairment.

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Changes of lipoproteins in phenylalanine hydroxylase-deficient children during the first year of life

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ABSTRACT

Background: Influence of hyperphenylalaninemia on lipoproteins in early life remains unclear.
Methods: We enrolled 24 phenylalanine hydroxylase (PAH)-deficient children who were classified into a phenylketonuria (PKU) group ($n = 12$) lacking PAH activity and a benign hyperphenylalaninemia (HPA) group ($n = 12$) having partial PAH activity, and their 11 non-affected siblings. We measured serum total-cholesterol, low-density lipoprotein (LDL)-cholesterol, and high-density lipoprotein (HDL)-cholesterol levels together with apolipoproteins for the first year of life, and compared them with those of 30 age-matched healthy controls.
Results: The affected groups invariably had lower cholesterol levels than non-affected groups. At birth, HDL-cholesterol decrease was greatest and predominated over the LDL-cholesterol decrease: total cholesterol, 28/36% decrease to the control level in HPA/PKU; HDL-cholesterol, 33/51%; LDL-cholesterol, 20/28%. At 3 months, the opposite changes were observed: total cholesterol, 16/28%; HDL-cholesterol, 13/23%; LDL-cholesterol, 16/33%. At 12 months, LDL were still significantly lower in both groups (8/18%, $p < .05$ and .001), although HDL was significantly decreased only in the PKU group (15%, $p < .05$). Apolipoprotein A-I/A-II and B changed respectively in accordance with HDL-cholesterol and LDL-cholesterol changes. Despite similar phenylalanine levels, the PKU group invariably had lower cholesterol concentrations than the HPA group had.
Conclusion: Irrespective of phenylalanine concentrations, lipoprotein synthesis in PAH-deficient children, particularly in PKU children, was suppressed in early life.

1. Introduction

Phenylketonuria (PKU: OMIM, 261600) is an autosomal recessive disorder caused by hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) deficiency. Adverse effects of hyperphenylalaninemia along with brain or neuron-related metabolic changes such as changed neurotransmitters have been increasingly documented [1–5].

Unless young PKU children receive phenylalanine-restricted diets, they develop convulsions, developmental delay, and mental retardation. However, the metabolic and nutritional effects of PKU and phenylalanine-restricted diets have been understood insufficiently to date.

Experimentally, suppressed cholesterol production in PKU has been suggested [6–8]. Clinically, Shulpis and his colleagues reported that PKU children adherent to phenylalanine-restricted diets with low cholesterol contents showed decreased low-density lipoprotein (LDL) cholesterol concentrations [9]. However, our recent studies for adult PKU demonstrated that, irrespective of diet or serum phenylalanine concentrations, total-cholesterol and LDL-cholesterol concentrations were, to some degree, decreased in them [10,11]. Changes

Abbreviations: PKU, phenylketonuria; PAH, phenylalanine hydroxylase.

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of cholesterol-derived metabolites functioning as bioactive substances such as oxysterols and vitamin D were also reported [10,11].

Considering that young children achieve rapid growth and development using various nutrients, including lipids, suppressed cholesterol production might be worrisome. However, reports of studies assessing the actual influence of PAH deficiency on lipoprotein metabolism in early life are rare.

In this study, to ascertain the influence of PAH deficiency on lipoprotein metabolism in early life, we performed chronological analyses of lipoprotein profiles in patients with PAH deficiency during the first year of life.

2. Subjects and methods

2.1. Subjects and sample collection

We enrolled 8 PKU patients (4 female, 4 male) at birth and 16 PKU patients (8 female, 8 male) after mass screening at around 5 days of age (Table 1). For the 24 affected children, the diagnoses of phenylalanine hydroxylase (PAH) deficiency were made by the analysis of dihydropteridine reductase activity in erythrocytes, biopterin loading test, and/or pteridine analysis in urine [1]. Patients were all found to have hyperphenylalaninemia by mass screening at around 5 days of age. They were classified into 2 groups (a PKU group comprising 12 children (female/male, 6/6) and a benign hyperphenylalaninemia (HPA) group comprising 12 children (female/male, 6/6) groups) according to the serum phenylalanine (phe) concentrations that had been determined repeatedly before the initiation of phenylalanine-restricted diets at the ages of 10–24 days. The PKU group included those children with serum fasting phenylalanine concentration of >600 μmol/l on normal formula or breast milk. The HPA group included the concentration of <600 μmol/l [1]. Soon after the diagnoses, the former patients strictly received restricted diets with phenylalanine-free milk, whereas the latter patients received mildly restricted diets. Consequently, both affected groups showed similar phenylalanine concentrations for the first years of life (Table 1).

In the same period, 11 non-PKU siblings (sibling group, 6 female and 5 male) and 30 healthy children (healthy control group, 16 female and 14 male) were enrolled. The data of the latter group were partially presented in our previous report [12].

2.2. Study design

For the affected patients and their non-affected siblings, we measured serum concentrations of total cholesterol, triglycerides (TG), LDL-cholesterol and high-density lipoprotein (HDL)-cholesterol together with serum phenylalanine concentration at birth, and at 3 months and 12 months of age. Simultaneously, we measured apolipoprotein (apo) A-I, A-II, B, and E. Their parameter concentrations at every age were compared with the respective controls' concentrations at the same ages. The time-to-time changes of parameters in each group were evaluated statistically.

For all groups, blood samples were obtained from cord veins at birth, or cubitus veins in the morning (at AM 7:30–8:00) before milk or meal intakes following 5–6 h and 7–9 h fasting, respectively, at the ages of 3–4 and 12 months. Serum lipid and apolipoprotein concentrations, and serum phenylalanine concentration were determined within 12 h of sample collection. The protocol was approved by the ethical committees of participating institutions. Written informed consent was obtained from the parents of all subjects.

2.3. Determination of serum lipids and apoproteins

Serum concentrations of TC and TG were determined using enzymatic methods with commercial kits (Kyowa Medex Co. Ltd.). Then HDL-C was measured using 13% polyethylene glycol (PEG 300; Wako Pure Chemical Industries Ltd.). LDL-C was measured using enzyme immunoassay with a commercial kit (LDL-C, Daiichi Pure Chemicals Co. Ltd.).

The Apo A-I, A-II, B, and E concentrations were measured using turbidimetric immunoassay with commercial kits (Daiichi).

Table 1
Changes in serum lipids and apolipoproteins during the first year of life.

	PKU ($n = 12$)	HPA ($n = 12$)	Non-affected siblings ($n = 11$)	Controls ($n = 30$)
M/F	6/6	6/6	6/5	14/16
Gestation pd. (week)	38–41	38–41	39–41	38–41
Birth weight (g)	2745–3210	2690–3150	2667–3333	2689–3465
Phe ranges (μmol/l)				
At birth	292 (30)[a,d,#]	123 (24)[a,##]	56 (15)	55 (17)
10–24 days	1352 (249)[a,d]	399 (97)[a]	75 (14)	73 (14)
3–4 M	189 (33)[a]	201 (46)[a]	69 (10)	63 (11)
12 M	211 (49)[a]	189 (38)[a]	72 (12)	66 (11)
TC (mg/dl)				
At birth	44 (3)[b,#]	50 (3)[##]	66 (8)	69 (14)
3–4 M	108 (14)[a,e]	127 (14)[b]	150 (13)	151 (20)
12 M	126 (17)[b,e]	138 (14)[c]	159 (14)	156 (23)
TC (mg/dl)				
At birth	44 (3)[b,#]	50 (3)[##]	66 (8)	69 (14)
3–4 M	108 (14)[a,e]	127 (14)[b]	150 (13)	151 (20)
12 M	126 (17)[b,e]	138 (14)[c]	159 (14)	156 (23)
TG (mg/dl)				
At birth	26 (6)[#]	29 (6)[##]	28 (9)	25 (22)
3–4 M	99 (9)[c]	97 (15)	91 (12)	88 (32)
12 M	95 (16)[c]	85 (9)	89 (20)	80 (29)
LDL-C (mg/dl)				
At birth	18 (2)[c,#]	20 (3)[##]	26 (6)	25 (8)
3–4 M	49 (11)[a,e]	61 (11)[c]	73 (10)	73 (16)
12 M	62 (13)[a,e]	70 (11)[c]	78 (10)	76 (19)
HDL-C (mg/dl)				
At birth	19 (2)[a,#]	26 (3)[c,##]	36 (8)	39 (13)
3–4 M	41 (5)[b,f]	46 (8)[c]	53 (5)	53 (9)
12 M	45 (4)[b,f]	51 (5)	54 (5)	53 (10)
Apo A-I (mg/dl)				
At birth	44 (3)[a,#]	59 (4)[c,##]	84 (10)	89 (15)
3–4 M	93 (8)[b,f]	109 (17)[c]	117 (9)	120 (21)
12 M	106 (11)[c]	112 (11)	120 (7)	119 (22)
Apo A-II (mg/dl)				
At birth	8 (4)[a,#]	12 (4)[c,##]	18 (4)	20 (3)
3–4 M	20 (3)[b,f]	23 (5)[c]	24 (6)	25 (5)
12 M	22 (2)[c]	24 (4)	25 (3)	25 (7)
Apo B (mg/dl)				
At birth	14 (1)[c,#]	17 (1)[##]	20 (5)	19 (5)
3–4 M	51 (9)[a,e]	64 (10)[b]	74 (11)	75 (17)
12 M	63 (14)[a,f]	72 (11)[c]	79 (11)	78 (19)
Apo E (mg/dl)				
At birth	3.1 (0.5)[c,#]	3.6 (0.4)[##]	4.4 (0.7)	4.9 (1.5)
3–4 M	3.9 (0.6)[c]	4.3 (0.9)[c]	5.0 (0.7)	4.8 (1.2)
12 M	4.3 (0.7)[c]	4.4 (0.9)	4.7 (0.9)	4.7 (1.6)

Data of lipids and apolipoproteins are presented as mean (SE).
PKU, phenylketonuria; HPA, benign hyperphenylalaninemia; Non-affected Sib, healthy sibling; Phe, serum phenylalanine: TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol.
[#] Blood samples were collected from 4 of the 12 PKU patients.
[##] Blood samples were collected from 4 of the 12 HPA patients.
[a] $p < .001$ vs. age-matched controls.
[b] $p < .01$ vs. age-matched controls.
[c] $p < .05$ vs. age-matched controls.
[d] $p < .001$ vs. HPA patients.
[e] $p < .05$ vs. HPA patients.

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2.3.1. Statistic analyses

Differences between the 2 groups were estimated at birth, at 3 months and at 12 months of age using 2-sided Student *t*-tests. For each group, the time-to-time changes of parameter concentrations were estimated repeatedly using ANOVA tests. Any p value < .05 was regarded as significant.

3. Results

3.1. Serum phenylalanine concentrations in affected groups and non-affected groups

At birth, we were able to determine serum phenylalanine concentrations for 4 of 12 PKU children, 4 of 12 HPA children and 11 non-affected sibling children, and compared them with those of 30 healthy control children (Table 1). Phenylalanine concentrations of the affected children, particularly the PKU children, were overtly high compared to those of non-affected siblings and the control children. Of the 24 affected children, 16 were enrolled after mass screening at around 5 days of age, although the 30 healthy control members never dropped out during this study.

At 10–19 days of age when both the 24 affected children took breast milk or normal formula containing phenylalanine, as did 11 non-affected siblings and 30 control children, the 12 PKU children exhibited extremely high phenylalanine concentrations greatly in excess of 600 μmol/l. 12 HPA children also exhibited high phenylalanine concentrations, but the values never exceeded 600 μmol/l (Table 1).

Thereafter, the affected children adhered to phenylalanine-restricted diets. Most of them achieved the recommended phenylalanine concentrations. However, those concentrations were still higher than those of non-affected children (Table 1).

3.2. Serum lipid concentrations in affected groups and non-affected groups

At birth, total-cholesterol and HDL-cholesterol concentrations in the affected group were decreased greatly compared to those of the control group. The percentage values of lipid decreases to the control group's concentrations were the following: total-cholesterol, 28 (HPA group)–36% (PKU group); HDL-cholesterol, 49–67%. Consequently, HDL-cholesterol in the PKU group was much lower than those of non-affected children (p < .001 vs. healthy controls). The Apo A-I and A-II concentrations were also greatly decreased in the affected children, as was the HDL-cholesterol concentration (p < .001 vs. healthy controls). The decreases of LDL-cholesterol and apo B concentrations were also considerably less in PKU children (about 28% decrease, p < .05), but such decreases were not statistically significant for HPA children (about 20% decrease, p > .05). In addition, apo E concentrations were decreased in PKU children (about 40% decrease, p < .05) and HPA children (about 30% decrease, p < .05).

At 3 months, the HDL-cholesterol, apo A-I and A-II in the affected 2 groups were increased about 2-fold. The changes were greater (p < .001) than those in the non-affected 2 groups, which showed about a 1.5-fold increase (p < .01) (Table 1 and Fig. 1). The LDL-cholesterol and apo B concentrations were 2.5-fold to 3-fold increased in all groups. HDL-cholesterol, apo A-I and A-II concentrations in the affected 2 groups were still significantly lower than those in the non-affected children: PKU group, p < .01 vs. healthy control group; HPA group, p < .05. LDL-cholesterol and apo B concentrations in the affected groups remained considerably lower than those in non-affected 2 groups (PKU group, p < .001; HPA group, p < .01). Furthermore, apo E concentrations in the affected groups were significantly lower than those in non-affected groups (p < .05). In contrast, the TG concentration was not different between these four groups.

At 12 months, increases of LDL-cholesterol and apo B in the affected groups were greater than those in the non-affected groups showing no significant changes. Such changes were significant (p < .01 for PKU group and p < .05 for HPA group) (Table 1 and Fig. 1). Increases of HDL-cholesterol and apo As in the affected groups were also significant (p < .05), although such significant increases were not observed in non-affected groups. Consequently, differences in lipid and apo concentrations between the affected and non-affected groups became smaller. Nevertheless, total cholesterol and LDL-cholesterol concentrations, and apo B concentrations in the affected were still lower than non-affected groups: PKU group, p < .01 for total-cholesterol concentration and p < .001 for LDL-cholesterol and apo B concentrations: HPA group, p < .05 for total-cholesterol and LDL-cholesterol concentrations and apo B concentration. The PKU group, but not the HPA group showed significantly lower HDL-cholesterol, apo A-I and A-II concentrations: p < .01 for HDL-cholesterol concentration and p < .05 for apo A-I and A-II concentrations. Apo E concentration remained low in the PKU group, but not the HPA group.

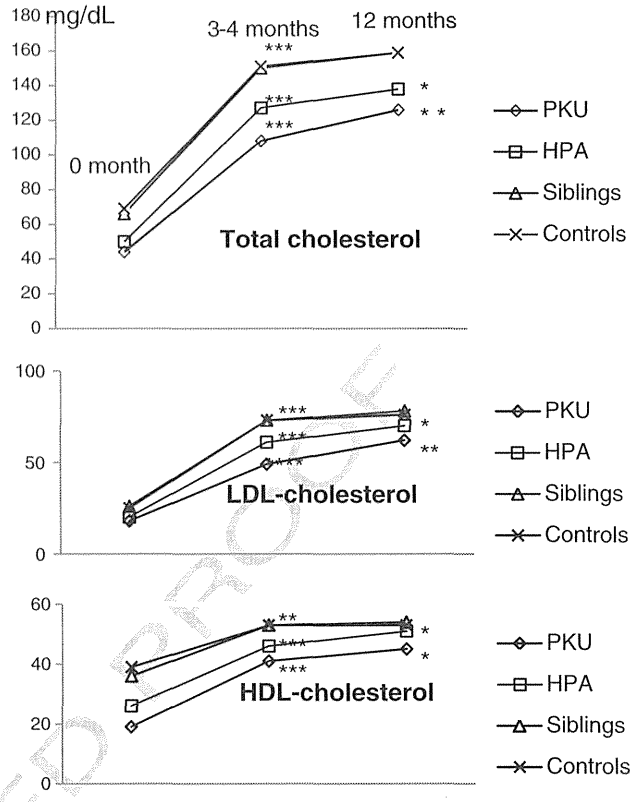

Fig. 1. Lipid changes in respective groups during the observation period. PKU, phenylketonuria; HPA, benign hyperphenylalaninemia, LDL-cholesterol, low-density lipoprotein cholesterol; HDL-cholesterol, high-density lipoprotein cholesterol. *p < .05, **p < .01, ***p < .001 vs. pre-values.

4. Discussion

We performed chronological analyses for lipoprotein profiles in children with PAH deficiency for the first year of life. This report is the first describing details of lipoprotein metabolism in PKU patients of early life.

Greater influences of PAH on HDL-cholesterol and LDL-cholesterol concentrations in early life than in later life were found. The results also show that the magnitude of the influence on lipoprotein metabolism differs by the clinical phenotypes, but not by the serum phenylalanine concentration. The change of lipoprotein profile in the PKU group lacking PAH activity was always significantly greater than that in the HPA group having partial PAH activity, even though their phenylalanine concentrations were mutually comparable.

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Repeated comparisons of lipoprotein profiles between affected and non-affected groups revealed that the influence of PAH deficiency on the lipoprotein metabolism changes during the first year of life: At birth, the decreases of HDL-cholesterol and the 2 major apolipoproteins on HDL particles, apo A-I and apo A-II, predominated over the decreases of LDL-cholesterol and apo B on LDL particles. In contrast, at 3 months, the decreases of LDL-cholesterol and apo B predominated over the decreases of HDL-cholesterol, apo A-I and apo A-II concentrations. At 12 months, decreases of LDL-cholesterol and apo B were decreased to some degree in the 2 affected groups, whereas HDL-cholesterol and apo A-I/A-II were decreased significantly only in PKU groups. Consequently, suppression patterns of lipids were not consistent during the first year of life.

In PKU, cholesterol production and production of cholesterol-derived metabolites such as oxysterols and vitamin D have been suggested to be suppressed [6–11]. Our recent study also showed that total-cholesterol and LDL-cholesterol concentrations were decreased about 10% compared to the respective control concentrations, irrespective of the serum phenylalanine concentration, in PKU adults [10,11]. In contrast, the decrease of HDL-cholesterol was minute in them. Decrease of LDL-cholesterol at 12 months of age in the PKU group was comparable to that in adult PKU, but no decrease of HDL-cholesterol in the PKU group was observed in PKU adults.

As mechanisms of suppressed cholesterol production in PKU, reduced activities of 2 key enzymes for cholesterol production, 3-hydroxy-3methylglutaryl-CoA reductase and mevalonate-5-pyrophosphate decarboxylase have been suggested [6–8]. Our recent study showed that oxysterols reflecting cholesterol production – lanosterol and lathosterol – were decreased in adult PKU [11]. In contrast, campesterol and sitosterol as markers for cholesterol absorption from the intestine remained unchanged in them. If this is a case in neonate and infant with PAH deficiency, their liver PAH activity but not serum phenylalanine concentration might be deeply associated with the suppression of cholesterol production.

The lipoprotein profile in fetuses differs greatly from that in adults for the reason that lipoprotein synthesis is low because of immature hepatic function and the absence of intestinal lipid absorption [12,13]. The major plasma lipoprotein in fetuses is HDL, whereas that in adults is LDL. Furthermore, HDL particles in the fetus contain much apo E. Lipoprotein metabolism changes drastically for the first year after birth. In this context, it is plausible that the impact of PAH deficiency on lipoprotein metabolism differs according to age. This study showed that the apo E concentration was, to some degree, decreased in the affected groups, in particular at birth. We were able to infer that this decrease of apo E was, to considerable degrees, attributable to the suppressed HDL synthesis.

Consequently, substantial and considerable decreases of lipoprotein production in PAH deficiency of early life were demonstrated. Nevertheless, the actual shortcomings of the lipoprotein production suppression in neonates and infants remain to be elucidated. Cholesterol is transformed to bioactive oxysterols and vitamin D [14–22]. Moreover, crucial roles of brain-related oxysterols such as 24S-hydroxycholesterol in the brain have been suggested [16–18].

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RESEARCH LETTER

Hypogonadotropic hypogonadism in a female patient previously diagnosed as having waardenburg syndrome due to a sox10 mutation

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Introduction

Hypogonadotropic hypogonadism (HH) is a clinically and genetically heterogeneous condition that can be associated with several additional clinical features such as anosmia, cleft palate, and hearing loss [1]. HH with anosmia is referred to as Kallmann syndrome (KS). More than 20 genes are known to underlie HH and/or KS, although mutations in these genes account for only a minor portion of the etiology of HH/KS [1–4]. In 2013, Pingault et al. identified *SOX10* mutations in seven patients with KS [5]. Furthermore, Pingault et al. found that genetic knockout of *Sox10* disrupted migration of GnRH cells in murine fetuses [5]. Subsequently, Vaaralahti et al. identified an additional KS patient with a *SOX10* mutation [6]. These results indicate that *SOX10* mutations constitute rare genetic causes of KS. Currently, *SOX10* is known as one of the causative genes of Waardenburg syndrome (WS), a rare genetic disorder characterized by hearing loss and hypopigmentation in the skin, hair, and eye [7]. Indeed, hearing impairment with or without gray/white hair was found in most of the KS cases reported by Pingault et al. and Vaaralahti et al. [5, 6]. However, detailed clinical assessment of the *SOX10* mutation-positive patients and functional assays of the *SOX10* mutants remain fragmentary. Thus, genetic links between HH/KS and WS have not been fully established. Here, we performed molecular and clinical analyses of a previously reported patient with WS due to a frameshift mutation in *SOX10*.

Patient

The patient was first described in 2008 as an infant with WS-type 2 (WS without dystopia canthorum) [8]. Shortly after birth, she presented with hypopigmented irides and a

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piece of white forelock. Dystopia canthorum, broad nasal root, limb anomaly, and Hirschsprung disease were absent. Ophthalmologic examinations revealed bilateral ocular albinism with hypopigmented fundus and hypochromic iris. Auditory brainstem response indicated bilateral sensorineural deafness. The patient underwent cochlear implantation at 2.6 years of age. Direct sequence analysis of WS causative genes (*SOX10, PAX3*, *MITF* and *SNAI2*) identified a heterozygous mutation in *SOX10* (c.506delC, p.P169fsX117) and excluded mutations in the other genes [8]. Until nine years of age, her growth followed the −1.0 standard deviation (SD) growth curve of Japanese female population. Thereafter, the SD scores for height and height velocity gradually decreased.

At 12.9 years of age, she revisited our clinic because of delayed puberty. Physical examination revealed mild short stature (−2.1 SD) and a lack of pubertal signs (breast and pubic hair, Tanner stage 1) (Figure S1). A smell test using intravenous injection of combined vitamins (Alinamin, Takeda Pharmaceutical Co. Ltd., Japan) [9] induced no response. Other standard smell tests such as UPSIT were not performed. Her bone age was delayed (∼11 years of age). Endocrine studies revealed a low level of estradiol (E_2), together with apparently normal gonadotropin levels at the baseline and after GnRH stimulation (Table 1). The blood values of other hormones were grossly normal; whereas the TSH response to TRH was blunted, normal levels of thyroid hormones suggested preserved thyroid function [10, 11]. Low E_2 levels and normal gonadotropin levels were also observed in examinations performed at 13.8 and 14.1 years of age (Table 1). Brain magnetic resonance imaging (MRI) was not performed, because her cochlear implants contained magnetic components. From 14.1 years of age, she received E_2 supplementation therapy, which successfully induced breast budding and improved height growth (Figure S1). To confirm the genetic basis of HH in this patient, we performed further molecular analyses.

Methods

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining informed consent. Mutation screening was carried out for the coding regions of 20 causative genes for HH/KS: *CHD7*, *FGF8*, *FGFR1*, *FSHB*, *GNRH1*, *GNRHR*, *HS6ST1*, *KAL1*, *KISS1*, *KISS1R*, *LEP*, *LEPR*, *LHB*, *NELF*, *PROK2*, *PROKR2*, *SEMA3A*, *TAC3*, *TACR3*, and *WDR11* [2, 4]. Nucleotide alterations were determined by the Haloplex system (Agilent Technologies, Palo Alto, CA, USA) on a MiSeq sequencer (Illumina, San Diego, CA, USA). Genome-wide copy-number analysis was performed by oligoarray-based comparative genomic hybridization using an array-based catalog CGH (4 × 180 k format, catalog number G4449A; Agilent Technologies).

In vitro reporter assays for the *SOX10* mutation were carried out by a previously reported method with slight modifications [12, 13]. An expression vector for wildtype *SOX10* was purchased from Kazusa DNA Research Institute (Kisarazu, Chiba, Japan). An expression vector for the mutant *SOX10* was generated by site-directed mutagenesis. A luciferase reporter vector containing the *MITF* promoter sequence (–2253 to +97 bp) and an expression vector for *PAX3* [12, 13] were kindly provided by Dr. Bondurand and Professor Goossens. Transient transfection was performed using HEK293 cells seeded in 24-well plates (1.0×10^5 cells/well) and Lipofectamine 2000 Reagent (Life technologies, Carlsbad, CA, USA), with the expression vectors (20 ng/well or 40 ng/well), the luciferase reporter vector (10 ng/well), and a pCMV-PRL internal control vector (5 ng/well; Promega, Madison, WI, USA). As controls for the expression vectors, an empty counterpart vector (HaloTag vector, Promega) was transfected. At 48 h after transfection, the cells were harvested and subjected to luciferase analysis using the dual luciferase reporter assay system and GloMax Luminometer (Promega). Luciferase assays were also performed with co-expression of a *PAX3* expression vector (20 ng/well). These experiments were carried out in triplicate within a single experiment and the experiment was repeated four times. Statistical significance was determined by the *t* test.

To predict the pathogenicity of the *SOX10* mutation, we performed direct sequencing of *SOX10* for the samples obtained from the clinically normal parents of the patient. In this experiment, we used previously described primers [8].

Results

Mutation screening excluded mutations in other HH/KS-associated genes. Comparative genomic hybridization analysis detected no pathogenic copy-number alterations. The mutant SOX10 protein barely transactivated the *MITF* promoter and exerted no dominant-negative effect on wildtype SOX10 (Figure S2). The *SOX10* mutation was not detected in the parental samples.

Discussion

Herein, we report a female patient who developed HH. Although standard smell tests and brain MRI were not performed for the patient, the lack of response to

Table 1 Endocrine data of the patient

Hormone	Stimulus (dosage)	Patient		Reference values[a]	
		Baseline	Peak	Baseline	Peak
At 12.9 years of age					
LH (mIU/ml)	GnRH (100 μg)[b]	1.0	11.8	0.4–4.1	8.5–15.5
FSH (mIU/ml)	GnRH (100 μg)[b]	2.6	13.2	4.8–10.4	8.3–20.0
Estradiol (pg/ml)		<10		<10–144	
GH (ng/ml)	Insulin (3 U)[b]	3.74	**4.83**[c]	0.3–33.1	>6.0
GH (ng/ml)	Arginine (18 g)[d]	4.2	9.7	0.3–33.1	>6.0
Prolactin (ng/ml)	TRH (350 μg)[b]	25.2	43.1	1.2–13.2	2.4–52.8
TSH (μIU/ml)	TRH (350 μg)[b]	1.64	**5.08**	0.32–4.0	10–35
ACTH (pg/ml)	Insulin (3 U)[b]	55.8	88.4	5.2–38.8	10.4–116.4
Cortisol (μg/dl)	Insulin (3 U)[b]	25.2	29.8	3.0–12.0	6.0–36.0
IGF-1 (ng/ml)		205		206–731	
Free T_3 (pg/ml)		3.94		2.43–4.48	
Free T_4 (ng/dl)		1.05		0.98–1.90	
At 13.8 years of age					
LH (mIU/ml)	GnRH (100 μg)[b]	1.0	12.3	0.2–2.1	1.7–5.0
FSH (mIU/ml)	GnRH (100 μg)[b]	2.6	11.6	0.6–3.4	1.4–11.5
Estradiol (pg/ml)		**<10**		12–162	
At 14.1 years of age					
LH (mIU/ml)	GnRH (100 μg)[b]	0.6	10.0	0.2–2.1	1.7–5.0
FSH (mIU/ml)	GnRH (100 μg)[b]	2.5	10.5	0.6–3.4	1.4–11.5
Estradiol (pg/ml)	hMG (150 U)[e]	**<10**	430	13–174	300–1000

The conversion factors to the SI unit: LH, 1.0 (IU/liter); FSH, 1.0 (IU/liter); GH, 1.0 (μg/liter); prolactin, 43.48 (pmol/liter); TSH, 1.0 (mIU/liter); ACTH, 0.22 (pmol/liter); cortisol, 27.59 (nmol/liter), estradiol, 3.671 (pmol/liter); free T_3, 1.536 (pmol/liter); free T_4, 0.1287 (pmol/liter)

Hormone values below the reference range are boldfaced

[a] Reference values in age-matched Japanese females [10, 11]

[b] GnRH, insulin, and TRH i.v.; blood sampling at 0, 30, 60, 90, and 120 min

[c] Low GH may be due to insufficient hypoglycemic stimulation

[d] Arginine i.v.; blood sampling at 0, 30, 60, 90, and 120 min

[e] hMG i.m. for 4 consecutive days; blood sampling on days 1 and 4

intravenous injection of combined vitamins indicated impaired olfactory function. In infancy, the patient was diagnosed as having WS due to a *SOX10* mutation [8]. In the present study, we performed further molecular analysis of the patient and excluded mutations in other HH/KS-associated genes and copy-number alterations in the genome. Furthermore, we confirmed that the *SOX10* mutation is not shared by the clinically normal parents and that the mutant SOX10 has impaired transactivating activity for the *MITF* promoter. These results indicate that the phenotype of this patient results from the *SOX10* mutation. However, we cannot exclude the possibility that the patient has an additional mutation in a hitherto unknown HH/KS-causative gene, because several unidentified genes seem to underlie HH/KS [2–4]. To date, hypogonadism is known as a relatively rare complication in patients with WS due to *SOX10* mutation/deletion [14]. Furthermore, hearing loss was observed in most of the previously reported patients with KS and *SOX10* mutations [5, 6], although hypopigmentation in the eye or skin were not described in these individuals. Our data, together with the previous findings, indicate that *SOX10* mutations can lead to various developmental defects including an overlapping phenotype of HH/KS and WS. Therefore, thorough clinical evaluations including hormonal assessment should be performed for WS patients with *SOX10* mutations, because subnormal gonadotropin secretion may account for a certain fraction of such patients.

It is worth mentioning that our patient showed normal gonadotropin responses to GnRH stimulation and a normal estrogen response to human menopausal gonadotropin stimulation. Thus, hypothalamic dysfunction appears to be the primary lesion of this patient. These results are consistent with the previously proposed notion that SOX10