Conclusion Primary bile acid treatment using chenodeoxycholic acid was effective for these patients treated in early infancy before the late stage of chronic cholestatic liver dysfunction.

Abbreviations

5β-reductase	3-oxo-Δ^4-steroid 5β-reductase
TBA	total bile acids
GGT	γ-glutamyltransferase
3β-HSD	3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase
ALT	alanine aminotransferase
GC-MS	gas chromatography–mass spectrometry
CDCA	chenodeoxycholic acid
AST	aspartate aminotransferase
UDCA	ursodeoxycholic acid
Cr	creatinine
CMV	cytomegalovirus
CA	cholic acid

Introduction

In 1988, Clayton et al reported that in pediatric patients with severe liver disease such as cholestatic disease with cirrhosis and fumarylactoacetase deficiency, the major unusual bile acids detected were 3-oxo-Δ^4 bile acids, such as 7α-hydroxy- and 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acids. These patients had secondary 3-oxo-Δ^4-steroid 5β-reductase (5β-reductase) deficiency. At about the same time, primary 5β-reductase deficiency was described (Setchell et al 1988). This inborn error of bile acid synthesis is very rare and shows autosomal recessive inheritance. Main findings in 5β-reductase deficiency are normal or slightly elevated concentrations of total bile acids (TBA) and γ-glutamyltransferase (GGT) in serum; elevated conjugated bilirubin and alanine aminotransferase (ALT); and fatty stools. During synthesis of bile acids from cholesterol in the classic pathway, decreased activity of 5β-reductase enzymes reduces synthesis of primary bile acids and increases synthesis of 3-oxo-Δ^4 bile and allobile acids. However, no analysis of the 5β-reductase gene was reported in Setchell's patient.

The most common inborn error of bile acid synthesis is 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase (3β-HSD) deficiency, which has a clinical presentation similar to that of 5β-reductase deficiency: neonatal cholestatic jaundice, elevated transaminases but normal GGT, and evidence of fat-soluble vitamin malabsorption (Subramaniam et al 2010). Moreover, 3-oxo-Δ^4 bile acids can be detected as major urinary bile acids in primary 5β-reductase deficiency and secondary deficiency, such as in neonatal hemochromatosis (Sumazaki et al 1997; Lemonde et al 2003; Ueki et al 2009; Clayton 2011).

Lemonde et al (2003) and Gonzales et al (2004) reported *SRD5B1* (*AKR1D1*) gene analysis in 5β-reductase deficiency involving chromosome 7q32-33. In these two reports describing five patients, mutations in the *SRD5B1* gene were identified in all, including three homozygous mutations and two compound heterozygous mutations. Recently we reported two heterozygous mutations in patients diagnosed with 5β-reductase deficiency by bile acid analysis using gas chromatography–mass spectrometry (GC-MS) (Ueki et al 2009).

Here we report two Japanese infants, a 6-month-old boy, and a 9-month-old girl, with 5β-reductase deficiency resulting from a heterozygous mutation of *SRD5B1* in one and compound heterozygous mutations of the same gene in the other. We examined the clinical effect of oral chenodeoxycholic acid (CDCA) treatment in these two patients.

Patients and methods

Patient report

Patient 1 A male Japanese infant was delivered at 37 weeks gestational age after an uneventful pregnancy. His parents were not consanguineous and were healthy, without liver disease. At birth, he showed abdominal distension and metabolic acidosis. Abdominal radiography and computed tomography disclosed free air. We performed exploratory laparotomy at Saitama Medical University Hospital at 2 days after birth for suspected gastrointestinal perforation. We intraoperatively diagnosed the patient with meconium peritonitis from jejunal perforation associated with neonatal intussusception.

He soon developed jaundice, which was present until 6 months of age. We carried out technetium-99 m (^{99m}Tc)-DISIDA cholescintigraphy at 2 months and liver biopsy at 3 months. Cholescintigraphy failed to show intestinal radioactivity, while liver microscopic findings included giant cell transformation and wide fibrotic bands at portal areas (Fig. 1).

Laboratory results included serum aspartate aminotransferase (AST) concentration of 344 U/L (normal, <37); ALT, 441 I/L (<31); and total/direct bilirubin, 4.4/3.4 mg/dL (<1.2/0.4). GGT concentration in serum was 46 U/L (<52). We did not examine serum TBA at admission, but when we suspected an inborn error of bile acid synthesis from the results above, we analyzed serum and urine bile acids using GC-MS (Table 1). Results of the bile acid analysis suggested 5β-reductase deficiency, and the patient was given ursodeoxycholic acid (UDCA) treatment.

Patient 2 A female Japanese infant with a birth weight of 2832 g was delivered without complications by spontaneous vaginal delivery at a gestational age of 38 weeks after an

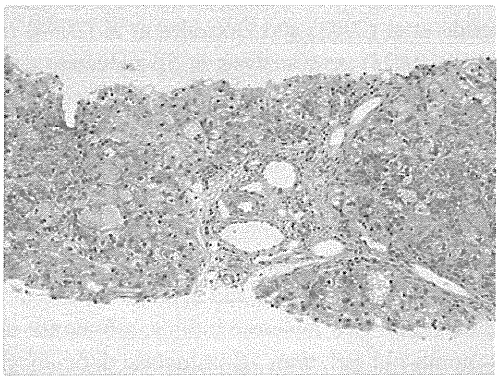


Fig. 1 Liver pathologic findings of patient 1. A liver biopsy specimen from patient 1 at 3 months showed lobular disarray resulting from extensive giant cell transformation and wide fibrotic bands in portal areas (Azan stain; magnification, x100)

uneventful pregnancy, her mother's first. The parents were not consanguineous and were healthy, without liver disease.

Progressive jaundice became apparent in the infant at 3 months. At 6 months, the patient was referred to Kyushu University Hospital because of jaundice and liver dysfunction.

On physical examination, growth and development were within the normal range. No dysmorphic features were present. Hepatomegaly and jaundice were noted. Neurologic findings were normal. Stools were gray. Initial laboratory results included serum concentrations of AST, 315 U/L; ALT, 229 U/L; alkaline phosphatase, 2,717 U/L (115 to 359); total/direct bilirubin, 6.3/3.6 mg/dL; albumin, 4.8 g/dL (4.0 to 5.0); total cholesterol, 216 mg/dL (128 to 219); prothrombin time, 14.8 s (10.0 to 13.5); and blood ammonia, 66 μg/dL (<66). Serum GGT was 61 U/L and serum TBA, 5.2 μmol/L (<10). Other causes of liver disease such as autoimmune hepatitis, chronic viral hepatitis, and other metabolic conditions were excluded by appropriate investigations. Abdominal ultrasonography showed a visible gallbladder and hepatomegaly; no choledochal cyst, bile duct dilation, or ascites was demonstrated. Serial technetium-99 m (^{99m}Tc)-DIS-IDA cholescintigraphy indicated that tracer entered the intestine. We suspected an inborn error of bile acid synthesis from the results above and analyzed serum and urine bile acids by GC-MS at 9 months of age (Table 1). Based on results of bile acid analysis, we suspected 5β-reductase deficiency.

Table 1 Bile acid analysis using gas chromatography-mass spectroscopy in two patients with 3-oxo-Δ^4-steroid 5β-reductase deficiency

	Patient 1 (6 months old)	Patient 2 (9 months old)	Infants with cholestasis[a] (n=2)	Normal infants[b] (n=4)
Serum (μmol/L)				
Cholic acid	n.d.	n.d.	200.1–209.7	0.2–1.8
Chenodeoxycholic acid	0.1	n.d.	55.1–56.8	1.2–5.3
Deoxycholic acid	0.2	n.d.	n.d.	n.d.
Lithocholic acid	n.d.	n.d.	n.d.	n.d.
Ursodeoxycholic acid	1.5	n.d.	0.5–8.6	n.d.
Allo-cholic acid	n.d.	n.d.	0.2–0.8	n.d.
Allo-chenodeoxycholic acid	0.9	n.d.	n.d.	n.d.
7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid	0.3	14.4	n.d.–3.3	n.d.
7α-Hydroxy-3-oxo-4-cholen-24-oic acid	1.7	10.7	n.d.	n.d.
Others	0.6	2.3	6.6–1.1	n.d.–0.9
Total bile acids	5.3	27.4	265.1–277.7	1.4–6.2
Urine (μmol/mmol Cr)				
Cholic acid	0.2	n.d.	10.1–29.4	0.03–0.3
Chenodeoxycholic acid	n.d.	n.d.	1.0–18.1	0.01–0.1
Deoxycholic acid	0.1	n.d.	n.d.–0.2	n.d.–0.02
Lithocholic acid	n.d.	n.d.	n.d.–0.1	n.d.
Ursodeoxycholic acid	0.9	n.d.	0.1–17.4	n.d.–0.02
Allo-cholic acid	0.8	n.d.	0.1–0.3	n.d.–0.05
Allo-chenodeoxycholic acid	n.d.	n.d.	n.d.	n.d.
7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid	121.1	37.5	10.2–27.1	n.d.
7α-Hydroxy-3-oxo-4-cholen-24-oic acid	2.8	21.4	1.0–1.2	n.d.
Others	n.d.	0.2	3.0–23.4	0.04–2.2
Total bile acids	125.9	59.1	25.7–116.9	0.1–2.6

[a] progressive familial intrahepatic cholestasis type 1 (2 and 11 months old); [b] 2, 5, 6, and 9 months old

n.d. not detected

Hepatitis C virus antibody and hepatitis B virus s antigen in the two patients were not detected, and autoimmune hepatitis was ruled out by negative anti-nuclear antibody test results, immunoglobulin G (420 mg/dL), and, in patient, liver histologic findings. In patient 1, progressive familial intrahepatic cholestasis was ruled out by bile acid analysis using GC-MS.

Qualitative and quantitative bile acid analysis

Serum and urine samples were collected and stored at −25 °C until analysis. Concentrations of individual bile acids in the urine were corrected for creatinine (Cr) concentration and expressed as μmol/mmol of Cr.

After we synthesized certain specific unusual bile acids, such as 3β-hydroxy-Δ^5 (Tohma et al 1986), 3-oxo-Δ^4 (Leppik 1983) and allo-bile acids (Ueki et al 2009) frequently seen in inborn errors of bile acid synthesis, we routinely analyzed bile acids in patient urine and serum by GC-MS using selected ion monitoring of the characteristic fragments of the methyl ester-dimethylethylsilyl ether-methoxime derivatives of bile acids as described previously (Kimura et al 1999), after enzymatic hydrolysis (choloylglycine hydrolase 30 units) and solvolysis (sulfatase 150 units; Sigma Chemical, St. Louis, MO).

The two patients in this study had bile acids in serum and urine analyzed using GC-MS on admission and during bile acid treatment.

Liver functional tests, bile acid analysis, and urinary steroid analysis before and after CDCA treatment suspension

After informed consent, we stopped CDCA treatment for 1 month in patient 1. We examined AST, ALT, serum and urinary bile acids, as well as urinary steroids, before and during the CDCA treatment suspension. We measured 5α- and 5β- metabolites of both cortisol and cortisone, such as tetrahydrometabolites including THFs (5α-tetrahydrocortisol, 5β-tetrahydrocortisol) and THEs (5α-tetrahydrocortisone, 5β-tetrahydrocortisone) by GC-MS using selected ion monitoring (Homma et al 2003).

In patient 2, we could not obtain consent for CDCA treatment suspension from the patient's parents. We did examine urinary steroids during CDCA treatment.

Genetic analysis

With informed consent, *SRD5B1* gene analysis was performed using genomic DNA from peripheral lymphocytes from the two patients, their parents, and 50 healthy individuals. DNA fragments spanning the nine coding regions and exon-intron junctions of the *SRD5B1* gene were amplified by polymerase chain reaction (PCR) using Gene Taq (Nippon Gene, Toyama, Japan) and nine sets of primers to obtain DNA fragments of optimal length for direct sequence analysis (Ueki et al 2009).

After enzymatic processing with ExoSAP-IT (USB, Cleveland, OH), direct sequencing of amplified PCR products was carried out with a DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's protocol, using the same primers as for PCR amplification. The sequencing reaction product was analyzed electrophoretically using an SEQ2000XL analyzer (Beckman Coulter).

After the three putative mutations were found in the patients, their parents and 50 healthy individuals were screened for these three mutations by direct sequence analysis.

All studies were undertaken with permission from the Ethical Committee of Kurume University School of Medicine.

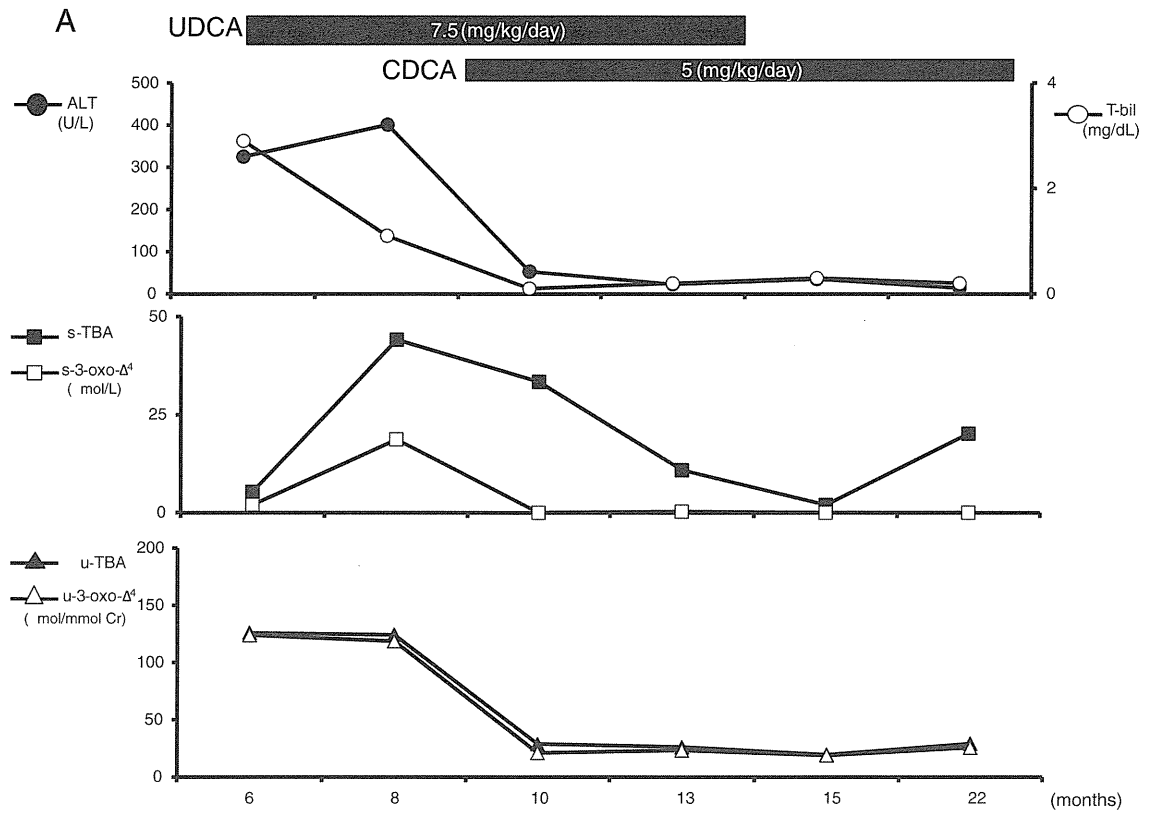
Results

Biochemical identification of an inborn error of bile acid synthesis

Patient 1 had jaundice since the early neonatal period, especially after surgery. Patient condition stabilized with UDCA (7.5 mg/kg/day) treatment. UDCA treatment was effective against liver dysfunction, such as elevated ALT and total bilirubin. After diagnosis, we added CDCA (5 mg/kg/day) to UDCA treatment. Liver dysfunction resolved completely with combined UDCA (7.5 mg/kg/day) and CDCA (5 mg/kg/day) treatment (Fig. 2a). Serum total bilirubin concentration decreased to the normal range, followed by a decrease in ALT. While concentrations of 3-oxo-Δ^4 bile acids in serum and urine were not reduced by UDCA (7.5 mg/kg/day) treatment, combined UDCA (7.5 mg/kg/day) and CDCA (5 mg/kg/day) treatment, gradually decreased serum 3-oxo-Δ^4 bile acids to undetectable amounts (less than 1 μmol/L). Urinary 3-oxo-Δ^4 bile acids decreased after serum fluctuation. Since UDCA treatment was stopped at 14 months of age, liver function and bile acid profiles in serum and urine have been stable with CDCA (5 mg/kg/day) monotherapy.

Importantly, during CDCA treatment suspension for 1 month, values for total/direct bilirubin, AST, and ALT remained in the normal ranges (Table 2).

Fig. 2 Clinical course of patient 1 (**a**) and patient 2 (**b**). Responses of serum alanine aminotransferase (ALT; *filled circles*), total bilirubin (T Bil; *open circles*), serum total bile acids (s-TBA; *filled squares*), serum 3-oxo-Δ^4 bile acids (s-3-oxo-Δ^4; *open squares*), urine total bile acids (u-TBA; *filled triangles*), and urine 3-oxo-Δ^4 bile acids (u-3-oxo-Δ^4; *open triangles*) to treatment with combined ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) treatment or CDCA mono-treatment are shown


A
UDCA
7.5 (mg/kg/day)
CDCA
5 (mg/kg/day)
ALT (U/L)
T-bil (mg/dL)
s-TBA
s-3-oxo-Δ4 (mol/L)
u-TBA
u-3-oxo-Δ4 (mol/mmol Cr)
(months)

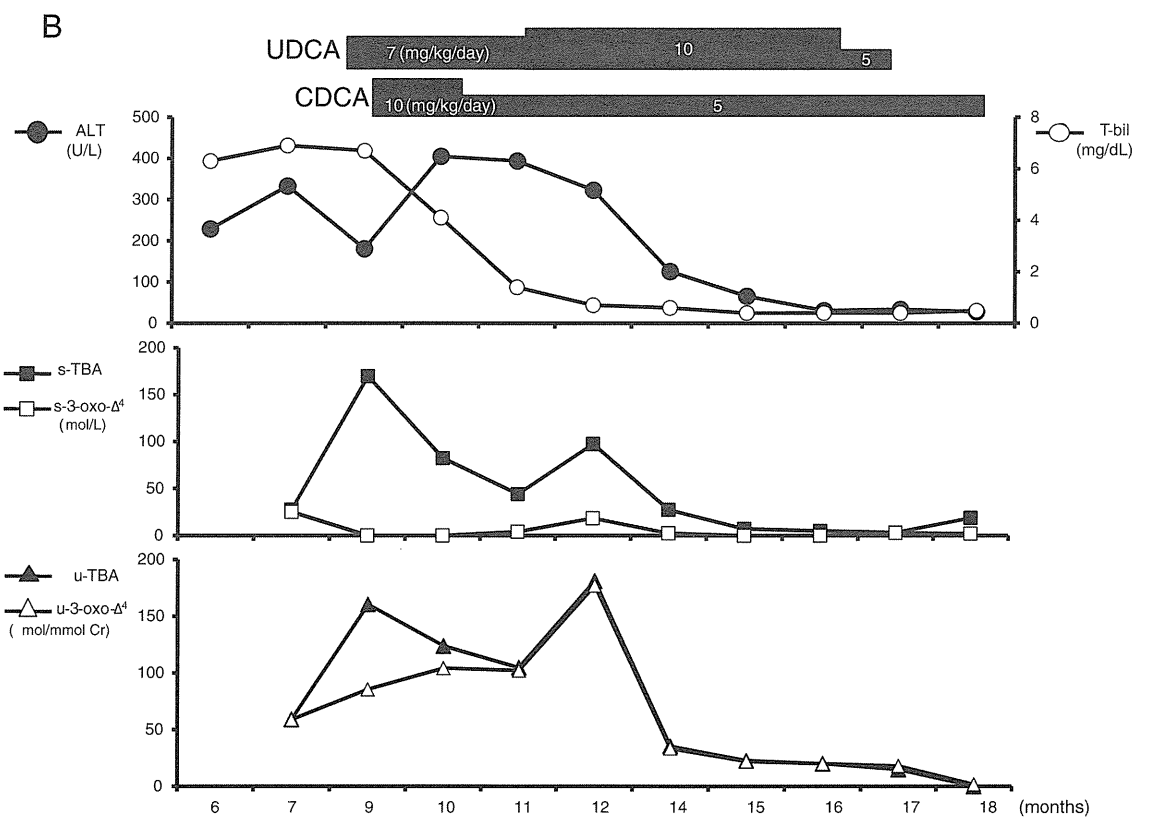

B
UDCA
7 (mg/kg/day)
10
5
CDCA
10 (mg/kg/day)
5
ALT (U/L)
T-bil (mg/dL)
s-TBA
s-3-oxo-Δ4 (mol/L)
u-TBA
u-3-oxo-Δ4 (mol/mmol Cr)
(months)

Table 2 Liver function tests and bile acid analysis in patient 1 before and during CDCA treatment suspension

	Before CDCA treatment suspension	During CDCA treatment suspension	
		After 2 weeks	After 4 weeks
Total bilirubin (mg/dL)	0.2	0.2	0.3
Direct bilirubin (mg/dL)	0.1	0.1	0.1
AST (U/L)	29	29	28
ALT (U/L)	13	12	12
Serum (μmol/L)			
Usual bile acids	23.8	5.2	1.5
Allo-bile acids	1.1 (4.2 %)	1.6 (18.9 %)	1.4 (29.9 %)
3-Oxo Δ^4 bile acids	1.4 (5.2 %)	1.7 (19.7 %)	1.6 (34.3 %)
Other bile acids	0.1	0.1	0.1
Total bile acids	26.4	8.6	4.7
Urine (μmol/mmol Cr)			
Usual bile acids	1.73	0.51	0.24
Allo-bile acids	0.30	0.17	0.23
3-Oxo Δ^4 bile acids	37.08 (93.6 %)	30.58 (96.4)	33.66 (97.4 %)
Other bile acids	0.50	0.46	0.44
Total bile acids	39.62	31.72	34.57

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *Usual bile acids* cholic acid + chenodeoxycholic acid + ursodeoxycholic acid + deoxycholic acid + lithocholic acid; Allo-bile acids, allo-cholic acid + allo-chenodeoxycholic acid, *3-Oxo Δ^4 bile acids* 7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid + 7α-Hydroxy-3-oxo-4-cholen-24-oic acid

Patient 2 began UDCA (7 mg/kg/day) treatment at 8 months of age without improvement of clinical symptoms or liver function. After diagnosis, we added CDCA (10 mg/kg/day) to UDCA treatment at 9 months of age. Liver dysfunction, ALT and serum TBA increased, but 3-oxo-Δ^4 bile acids in serum decreased. Nearly all serum TBA was CDCA, while concentrations of TBA and 3-oxo-Δ^4 bile acids in urine were increased (Fig. 2b). We then decreased the dose of CDCA from 10 to 5 mg/kg/day and increased the dose of UDCA from 7 to 10 mg/kg/day. This treatment was effective against liver dysfunction. Serum concentrations of total bilirubin decreased to the normal range, followed by a decrease in ALT as occurred with patient 1. Concentrations of TBA and 3-oxo-Δ^4 bile acids in serum and urine gradually decreased. Clinical course, liver function parameters, and bile acid profiles in serum and urine gradually improved with CDCA monotherapy at 17 months of age. When serum ALT and TBA in serum and urine became elevated, we detected elevated serum titers of anti-cytomegalovirus (CMV) antibodies (IgM normal, under 0.34 at 6 months of age; but rising to 1.27 at 11 months). Serum IgG antibodies at 6 months of age were under 2.0 (normal), rising to 28.0 at 11 months.

We could not find evidence of liver cirrhosis such as splenomegaly or prominent abdominal veins in either patient during this study.

Results of serum and urinary bile acid analysis and urinary steroid analysis before and during CDCA treatment suspension for 1 month

In patient 1, the main usual bile acid in serum was CDCA during CDCA treatment. During CDCA treatment suspension for 1 month, small amounts of allo- and ketonic bile acids were detected in serum, as at the time of initiation of CDCA treatment. As the month continued, however, percentages of allo- and ketonic bile acids among total bile acids gradually increased from 4.2 and 5.2 % to 29.9 and 34.3 %, respectively (Table 2). On the other hand, values and percentages of allo- and ketonic bile acids in urine did not change from those present during CDCA treatment.

In the two patients, excretion of 5β metabolites, such as 5β-tetrahydrocortisol and 5β-tetrahydrocortisone, was sharply reduced (Table 3). Overall alteration in cortisol and cortisone metabolism was evident from high 5α/5β ratios (Table 3).

Identification of *SRD5B1* gene defects

We identified one previously unreported mutation in one patient and two previously reported mutations in the other.

Patient 1 A single novel heterozygous mutation was found in exon 7, at nucleotide number 866, representing a G-to-A substitution causing an amino acid change from arginine at position 266 to glutamine (R266Q). The mutation was detected in heterozygous form in the patient's mother, but was absent in the father and controls.

Patient 2 Two previously reported heterozygous mutations were found. One was detected in exon 6, at nucleotide number 737, representing a G-to-A substitution causing an amino acid change from glycine at position 223 to glutamic acid (G223E). G223E was detected in heterozygous form in the patient's father, but was absent in the mother and 50 healthy individuals. The other mutation was detected in exon 7, at nucleotide number 850, representing a C-to-T substitution causing an amino acid change from arginine at position 261 to cysteine (R261C). R261C was detected in heterozygous form in the patient's mother, but was absent in the father and controls.

The above nucleotide numbers indicating positions of individual mutations are based on GenBank accession no. NM_0059892.

Table 3 Urinary steroid analysis in patient 1 during CDCA treatment and CDCA treatment suspension, and in patient 2 during CDCA treatment

Patient 1 (3 years of age, male)			
	During CDCA treatment	During CDCA treatment suspension (after 4 weeks)	Normal range*
5αTHF (mg/g Cr)	1.62	1.08	0.95–3.97
5βTHF (mg/g Cr)	0.01	<0.01	0.91–4.41
5αTHE (mg/g Cr)	0.37	0.19	0.12–0.53
5βTHE (mg/g Cr)	0.03	0.02	1.61–6.36
5α/5βTHF	231	270	0.79–2.31
5α/5βTHE	12.4	8.07	0.05–0.18
Patient 2 (2 years of age, female)			
	During CDCA treatment	Normal range**	
5αTHF (mg/g Cr)	2.86	1.04–2.35	
5βTHF (mg/g Cr)	0.03	0.97–1.65	
5αTHE (mg/g Cr)	0.39	0.11–0.30	
5βTHE (mg/g Cr)	0.18	2.32–5.88	
5α/5βTHF	110	1.04–1.70	
5α/5βTHE	2.14	0.04–0.07	

THF tetrahydrocortisol, *THE* tetrahydrocortisone

*normal range, from 2 to 4 years old, male, $n=10$, 24-h urine samples, 2.5–97.5th percentile

**normal range, from 2 to 4 years old, female, $n=10$, 24-h urine samples, 2.5–97.5th percentile

Discussion

In our two patients with 5β-reductase deficiency and *SRD5B1* mutation, CDCA treatment was effective in improving liver function and reducing unsaturated ketonic bile acids in serum and urine. Oral bile acid treatment such as cholic acid (CA) is safe and effective for treating most common inborn errors of bile acid synthesis, including 5β-reductase deficiency (Gonzales et al 2009). CA treatment may be superior to alternatives because CA activates negative feedback regulation of bile acid synthesis to inhibit production of hepatotoxic metabolites more effectively than CDCA, and is not itself hepatotoxic. However, CA is not available for clinical use in Japan. In a previous report (Clayton et al 1996), combined CA and CDCA were used together to treat 5β-reductase deficiency. We found that oral bile acid treatment using CDCA monotherapy could be effective for patients with 5β-reductase deficiency. However, assessment of CDCA monotherapy ultimately may require longer follow-up.

In bile acid analysis results during combined UDCA and CDCA treatment, exogenous UDCA was detected more in urine than in serum, while exogenous CDCA was detected more in serum than in urine (data not shown). We believe that after transport from hepatocyte to blood by hepatic multidrug resistance-related protein (MRP) 4 in the hepatocytic basolateral membrane, hydrophilic UDCA may undergo preferential renal excretion mediated by renal MRP 4 (Wagner and Trauner 2005; Marschall et al 2005; Stapelbroek et al 2010). Unsaturated ketonic bile acids – 3-oxo-Δ^4 bile acids – were detected more in urine than in serum; these are transported across the basolateral membrane into the blood via MRP3 (Yamaguchi et al 2010). Then 3-oxo-Δ^4 bile acids are excreted directly into urine by a bile acid transporter. As a result, increased urinary 3-oxo-Δ^4 bile acids may be detected in this disease.

Even with normal liver function, we detected 3-oxo-Δ^4 bile acids in serum and especially in urine. We speculate that the negative feedback induced by 5 mg/kg/day of CDCA may be incomplete, but liver function might worsen from a higher CDCA dose. A CDCA dose such as 10 mg/kg/day may provide very effective negative feedback at cholesterol 7α-hydroxylase via the farnesoid X receptor (Gonzales et al 2009). Patients so treated require close follow-up. Liver function tests, analysis of bile acids in serum and urine, determination of fibrosis markers such as type IV collagen 7 s domain, and especially liver biopsy are desirable. However, changes in urinary 3-oxo-Δ^4 bile acids may not always indicate stage of disease; since we analyze bile acids using single serum and urine specimens, we cannot eliminate diet-related variability. Moreover, some liver diseases reduce activity of 5β-reductase, including metabolic disorders, viral infections (Clayton et al 1988; Kimura et al 1998), and drug-induced hepatitis. Therefore, neonatal viral infections such as CMV infection as well as medication effects must be considered. We suspected that 3-oxo-Δ^4 bile acids in serum and urine from our patient 2 were increased by intrinsic hepatotoxicity of CDCA (Fig. 2b) and/or effects of CMV hepatitis.

In our patients with 5β-reductase deficiency, time needed to normalize laboratory data during oral bile acid treatment using combined UDCA and CDCA treatment or CDCA monotherapy has been longer than for patients with 3β-HSD deficiency (Yamato et al 2001; Mizuochi et al 2010). We recommended that patients with 5β-reductase deficiency begin oral bile acid treatment in early infancy, before

deficiency causes chronic cholestatic liver dysfunction by as early as 10 months (Gonzales et al 2004; Ueki et al 2009).

In patient 1, results of serum liver function tests such as total bilirubin and ALT did not change during or after CDCA treatment suspension, but percentages of allo- and ketonic bile acids among total bile acids in serum gradually increased (Table 2). Also, patients 1 and 2 showed sharply reduced production of the 5β metabolites 5β-tetrahydrocortisol and 5β-tetrahydrocortisone (Table 3), as reported by Palermo et al (2008). Therefore, we believe that patient 1, with a heterozygous mutation, had a 5β-reductase deficiency. Like deficient patients reported by Palermo et al (2008) and Ueki et al (2009), our patient 1 showed no change in liver function, even though results of bile acid analysis in serum and urine when CDCA was suspended were the same as those at diagnosis of 5β-reductase deficiency. The basis of this pathophysiologic state is unknown, but alleviation of cholestatic liver disease by early primary bile acid administration appeared to preserve liver function. We suspect that activity of hepatocytic MRP3 may be low during early infancy, permitting cholestatic liver disease to arise from hepatocytic accumulation of unsaturated ketonic bile acids such as 3-oxo-Δ^4 bile acids in hepatocytes. With maturation, activity of hepatocytic MRP3 normally increases, favoring transport of 3-oxo-Δ^4 bile acids across the basolateral membrane into the blood (Yamaguchi et al 2010) and ultimately the urine—presumably avoiding late-infancy liver damage.

The human *SRD5B1* gene contains nine coding exons corresponding to 326 amino acids; so far, seven distinct mutations causing 5β-reductase deficiency have been reported (Lemonde et al 2003; Gonzales et al 2004; Ueki et al 2009). Transmission of this deficiency is considered autosomal recessive. Here we describe genetic analysis of the *SRD5B1* gene in two patients with 5β-reductase deficiency, identifying one new mutation (R266Q) and two previously reported mutations (G223E and R261C) (Gonzales et al 2004; Ueki et al 2009; Drury et al 2010). Screening for R266Q was undertaken in 50 healthy individuals, proving absent in all. Moreover, an adverse effect from the mutation was predicted by Polymorphism Phenotyping software (version 2; Adzhubei et al 2010; score, 0.995). We believe that the heterozygous R266Q mutation likely contributed to loss of 5β-reductase function in patient 1, although we still suspect that he may have an unknown mutation in another allele—making him a compound heterozygote. Unfortunately we could not determine expression of 5β-reductase protein in patient 1 because no frozen liver sample had not been retained. Patient 2 had two heterozygous mutations (G223E/R261C), receiving one G223E mutation from each parents making her a compound heterozygote for the *SRD5B1* gene.

In conclusion, we diagnosed two patients with 5β-reductase deficiency, one with heterozygous and the other with compound heterozygous mutation in the *SRD5B1* gene. Primary bile acid treatment in early infancy using CDCA (5 mg/kg/day) proved effective in both of these patients.

Conflict of interest None.

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Oxysterol changes along with cholesterol and vitamin D changes in adult phenylketonuric patients diagnosed by newborn mass-screening

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ABSTRACT

Background: Phenylketonuria (PKU) possibly leads to hypocholesterolemia and lowered vitamin D (VD) status. Metabolism of oxysterols linking with those of cholesterol and VD has never been examined in PKU.
Methods: Blood oxysterols along with blood phenylalanine, lipids and VD were examined for 33 PKU adults aged 21–38 years and 20 age-matched healthy controls.
Results: Total- and low-density cholesterols, and 25-hydroxy VD_3 were decreased significantly in the PKU group (cholesterols, 10% decrease; 25-hydroxy VD_3 35% decrease vs. the control group). 24S-hydroxycholesterol (24S-OHC) eliminating brain cholesterol, and 27-OHC and 7α-hydroxycholesterol (7α-OHC) representing peripheral and hepatic cholesterol elimination, respectively, were significantly decreased in PKU group: 24S-OHC, 25% decrease, $p<.01$; 27-OHC and 7α-OHC, 35–40% decrease, $p<.001$. 7β-Hydroxycholesterol (7β-OHC) reflecting oxidative stress was increased significantly in PKU group ($p<.05$). 7α-OHC and 27-OHC levels in PKU group always showed similar values, regardless of other parameters while the 24S-OHC and 7β-OHC levels decreased and increased, respectively, showing significant correlations with phenylalanine level ($p<.005$). 27-OHC level showed a significant positive correlation with the 25-hydroxy VD_3 level in this group ($p<.001$).
Conclusion: Blood oxysterol changes predominate over blood cholesterol changes and influence on VD status in adult PKU patients.

1. Introduction

Phenylketonuria (PKU: OMIM, 261600) is an autosomal recessive disorder caused by deficiency of hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1). Diverse unfavorable effects of hyperphenylalaninemia on the central nervous system in children have been well described [1–5]. Unless phenylalanine intake is restricted, they develop convulsions, developmental delay, and mental retardation. Additionally, evidence that high plasma phenylalanine concentrations possibly cause behavioral

Abbreviations: PKU, phenylketonuria; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; VD, vitamin D; PTH, parathyroid hormone; 7α-OHC, 7α-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; 24S-OHC, 24S-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol.

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abnormalities, cognitive impairment, and emotional disturbance in adults has been reported increasingly [6,7].

Aside from the clinical presentations, various metabolic changes in PKU, particularly changes related to neurological manifestations, have been studied [1–5]. Among them, the most frequently explored are the low productions of neurotransmitters such as serotonin, dopamine and catecholamine, together with disturbance in the transport systems [1–5]. In addition, several studies have demonstrated the possibility that enhanced oxidative stress is, in part, associated with neurological manifestations in PKU [8–12]. Probably, multiple metabolic factors are associated with the clinical presentations of the disease. Nevertheless, information related to the biochemistry in PKU remains insufficient.

Oxysterols are metabolites originated from cholesterol, and some oxysterols have been implicated in the pathophysiology of neurological disorders [13–18]. Oxysterols are also metabolically connected to vitamin D (VD) (Fig. 1). In PKU, cholesterol and VD productions have

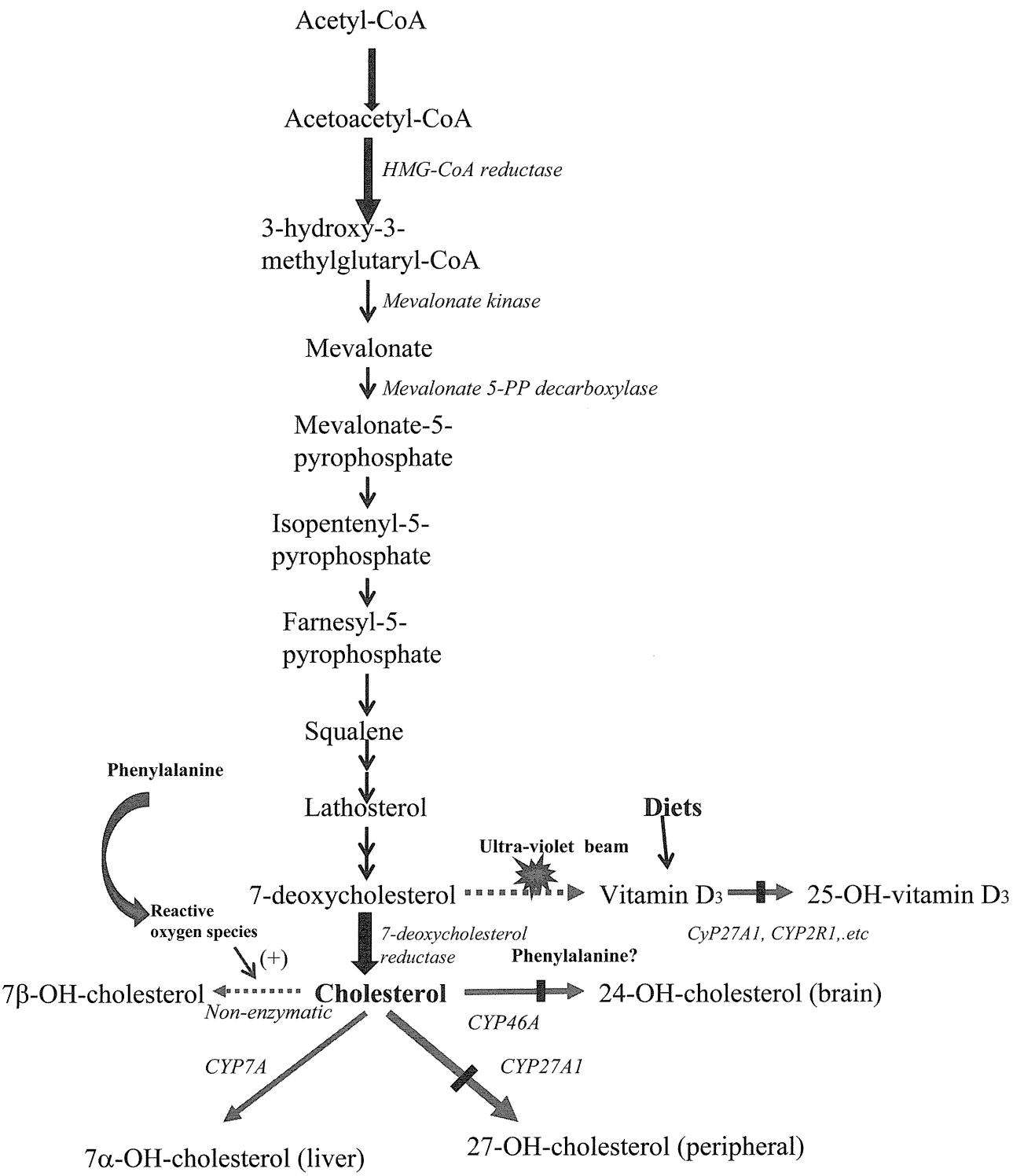

Fig. 1. Metabolic map illustrating cholesterol, oxysterol and vitamin D productions.

been suggested to be suppressed, although the mechanisms remain unclear [1,19–26]. These backgrounds of PKU encouraged us to investigate oxysterols in PKU.

In this report, we describe blood oxysterols associated to phenylalanine, lipids, and VD in adult PKU patients.

2. Subjects and methods

2.1. Subjects and sample collection

We enrolled 33 PKU patients (18 female, 15 male) with ages ranging between 21 and 38 years (mean ± SD, 28.1 ± 5.1 years, Table 1). Phenylalanine hydroxylase (PAH) deficiency was diagnosed 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. After the diagnoses, they exclusively received phenylalanine-restricted diets. After the age of 20 years, restrictions of phenylalanine differed greatly among patients. Some patients continued to receive strictly restricted diets. Others received mildly restricted or less restricted diets. Results showed that plasma phenylalanine levels differed greatly among individuals, but intra-individual changes were small. As control subjects, we enrolled 20 healthy volunteers (10 female, 10 male) with ages of 21–38 years old (mean ± SD, 28.9 ± 4.5 years).

Table 1
Characteristics of 33 phenylketonuric patients and 20 healthy age-matched controls.

	Healthy controls	PKU patients
Age (y)	28.9 ± 4.5	28.9 ± 5.3
Number (M/F)	22 (10/10)	33 (15/18)
BMI (kg/m^2)	23.7 ± 2.2	23.1 ± 1.9
Dietary intake	2234 ± 261	1890 ± 265[c]
Energy (kcal/day)		
Protein (g/day)	80 ± 15	71 ± 17[c]
Natural protein (g/day)	66 ± 14	50 ± 13[d]
Fat (g/day)	57 ± 13	55 ± 21
Calcium (mg/day)	1170 ± 387	1094 ± 339
Vitamin D	120 ± 23	107 ± 21
Phenylalanine (μmol/l)	30 ± 15	1019 ± 380[d]
TP (g/dl)	7.5 ± 0.3	7.2 ± 0.3
Albumin (g/dl)	4.6 ± 0.2	4.3 ± 0.2
Urate (mg/dl)	4.7 ± 0.5	4.5 ± 0.5
Creatinine (mg/dl)	0.4 ± 0.1	0.4 ± 0.1
ALT (IU/l)	11 ± 3	9 ± 2
AST (IU/l)	18 ± 4	18 ± 3
TC (mg/dl)	173 ± 26	155 ± 24[b]
TG (mg/dl)	82 ± 23	81 ± 20
LDL-C (mg/dl)	99 ± 18	87 ± 20[b]
HDL-C (mg/dl)	57 ± 7	54 ± 11
Oxysterol		
7α-(OH) cholesterol (ng/ml)	47.6 ± 21.9	30.9 ± 17.1[b]
7β-(OH) cholesterol (ng/ml)	10.3 ± 3.0	13.5 ± 8.7[b]
24S-(OH) cholesterol (ng/ml)	49.3 ± 9.9	37.6 ± 6.8[b]
27-(OH) cholesterol (ng/ml)	94.8 ± 26.4	55.4 ± 14.1[c]
Markers for cholesterol synthesis and absorption		
Lathosterol (μg//ml)	1.37 ± 0.52	1.05 ± 0.33[b]
Campesterol(μg//ml)	2.33 ± 1.29	2.57 ± 1.11
Sitosterol (μg//ml)	2.31 ± 0.80	2.20 ± 0.73
7-deoxycholesterol (μg//ml)	0.23 ± 0.08	0.16 ± 0.05[b]
Bone markers [a]		
25-(OH) vitamin D_3 (ng/ml)	28.9 ± 2.3	19.9 ± 2.1[d]
1.25-$(OH)_2$ vitamin D_3 (pg/ml)	40.7 ± 2.7	55.5 ± 3.7[d]
Intact PTH (pg/dl)	32.5 ± 3.5	37.1 ± 2.4[b]

Samples were collected approximately during the same time period.
Data are presented as mean ± SD.
BMI, body mass index; TP, total protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; PTH, parathyroid hormone.
[a] The sunlight exposure was similar between the 2 groups.
[b] $p<.05$.
[c] $p<.01$.
[d] $p<.001$ vs. controls.

2.2. Study design

For PKU patients and healthy controls, we tested serum levels of oxysterols together with serum levels of phenylalanine and lipids such as total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol. Serum VD_3 and intact parathyroid hormone (PTH) levels were also examined [23].

Regarding oxysterols, we examined 7-alpha-hydroxycholesterol (7α-OHC) and 27-hydroxycholesterol (27-OHC) as major precursors of bile acids reflecting the whole body cholesterol elimination, and 24S-hydroxycholesterol (24S-OHC) representing brain cholesterol elimination (Fig. 1) [13–16]. Further, we examined 7β-hydroxycholesterol (7β-OHC) reflecting the status of oxidative stress [19]. Among these oxysterols, 24S-OHC is the only one produced in the brain [13–18]. Blood was drawn from a peripheral vein in the morning after overnight fasting. The protocol was approved by the relevant institutional review boards. Parents of all patients provided written informed consent before the start of the study.

2.3. Determination of serum lipid, VD_3 and intact PTH

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

Serum 1.25-hydroxy VD_3 and 25-hydroxy VD_3 levels were determined, respectively, using RIA kits from Immunodiagnostic Systems Holdings plc (Boldon, UK) and Diasorin, Inc. (Stillwater, MN). Serum intact PTH level was determined using a radioimmunoassay (RIA) kit from the Nichols Institute (Quest Diagnostics, Geneva, Switzerland).

2.4. Assays for determination of oxysterols blood levels

Oxysterol concentrations in plasma samples were determined using the GC–MS method described previously with some modification [27].

Briefly, 300 μl of plasma in a glass tubes containing 150 ng of 27-hydroxycholesterol-26, 26, 26, 27, 27-D_5 (27d_5-OHC) as an internal standard was saponificated with 2 ml of 10 mol/l potassium hydroxide and 5 ml of methanol at room temperature for 1 h. After adjusting pH to 7.0 with 50% H_3PO_4, 5 ml of H_2O was added to the mixture; then oxysterols were extracted twice with 10 ml of *n*-hexane. The *n*-hexane layer was loaded immediately on the Bond Elut SI cartridge (Agilent Technologies Inc., California), which was pre-conditioned using *n*-hexane. The large excess amount of cholesterol was removed by washing with 2 ml of *n*-hexane and 10% of ethyl acetate with *n*-hexane.

Oxysterols retained onto the cartridge were eluted with 2 ml of ethyl acetate. After dryness of the eluate, the collected oxysterols were derivatized with trimethylsilyl (TMS) reagent. The derivatized oxysterols were re-suspended in 50 μl of *n*-hexane for GC–MS analysis. GC–MS analysis was conducted (GCMS-QP2010 plus; Shimadzu Corp., Kyoto) using a capillary column (30 m × 0.25 mm, 0.25 μm thickness, DB-5 ms; GL Sciences Inc., Tokyo) connected to AOC20i automatic sample injector and AOC20S autosampler. The oven temperature program was set as follows: 180 °C for 1 min, 20 °C/min to 250 °C and then 5 °C/min to 300 °C where the temperature was kept for 15 min. Helium was used as a carrier gas, and the flow rate was set at 0.96 ml/min. Injection was performed in the splitless mode and the injector temperature was kept at 230 °C. The MS ion source temperature and interface temperature were 250 °C. Samples were injected 5 μl for analysis of oxysterols.

The mass spectrometer was operated in the selected ion monitoring mode. A few ions were detected simultaneously. The ions used for analysis (m/z) and typical retention times (min) for the compounds

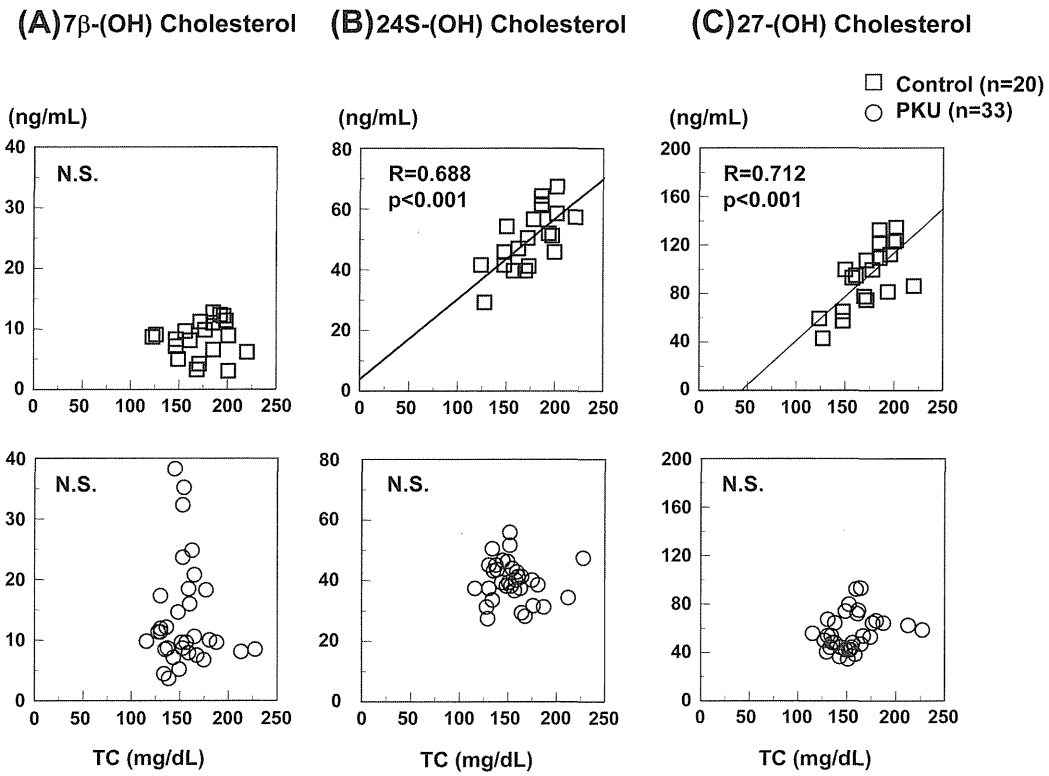

Fig. 2. Scatter graphs of oxysterol levels against total cholesterol level in control and PKU groups. TC, total cholesterol.

were the following: 7α-OHC, 456, 15.5; 7β-OHC, 456, 17.4; 24S-OHC, 413, 20.1; 27-OHC, 131, 20.6. Using this system, intra-assay and inter-assay CVs were within 10 and 15%, respectively.

2.5. Statistic analyses

Differences between values of patients and those of controls were estimated using Student's *t*-test. The relation between each pair of parameters was estimated using Pearson's correlation test. All results for which $p \leq .05$ were inferred as statistically significant.

3. Results

3.1. Daily nutrition and liver function in PKU patients compared with controls

Average daily energy and protein intakes in PKU patients were lower than those in controls, but the average fat and calcium and vitamin D intakes were not different. Between these two groups, no significant difference in age, body mass index, and liver function test was found (Table 1).

3.2. Lipid levels in PKU patients compared with controls

Total- and LDL-cholesterol levels in PKU patients were significantly lower than those in the controls ($p = 0.03$). The mean values were nine-tenths of the control values. Triglycerides and HDL-cholesterol levels were not significantly different (Table 1).

3.3. V D and intact PTH levels in PKU patients compared with controls

25-Hydroxy VD_3 level was significantly decreased in the PKU group ($p = 0.0008$) and the mean value was about two-thirds of that in the control value. On the contrary, 1.25-hydroxy VD_3 and intact parathyroid hormone levels were 1.4- and 1.15-folds increased, respectively, and showed significant differences with respective control levels; 1.25-hydroxy VD_3, $p = 0.0007$; intact PTH, $p = 0.04$ (Table 1).

3.4. Oxysterol levels in PKU patients compared with controls

7α-OHC, 24S-OHC, and 27-OHC levels in PKU group were significantly lower than those in the control group (Table 1). The mean values were about two-thirds, four- fifths and three-fifths for the respective control values. In contrast, the 7β-OHC level in PKU group was significantly higher than that in the control group (Table 1).

3.5. Correlations between oxysterols and lipids in controls and PKU patients

In the control group, 27-OHC and 24-OHC levels but not other oxysterols showed significant positive correlations with the total cholesterol level (Fig. 2; upper panels). In PKU group, no such significant correlation was found (Fig. 2: lower panels).

3.6. Correlations between oxysterols and phenylalanine in PKU patients

7β-OHC and 24S-OHC levels but not 27-OHC and 7α-OHC levels showed significant correlations with phenylalanine levels (7β-OHC, $p = 0.004$: 24S-OHC, $p = 0.0008$) (Fig. 3A and B). Among the PKU patients with phenylalanine levels exceeding 650–700 μmol/l, the 7β-OHC and 24S-OHC levels were out of the normal ranges at high rates. However, their 27-OHC levels were under a normal range at high rates, irrespective of the phenylalanine level (Fig. 3C).

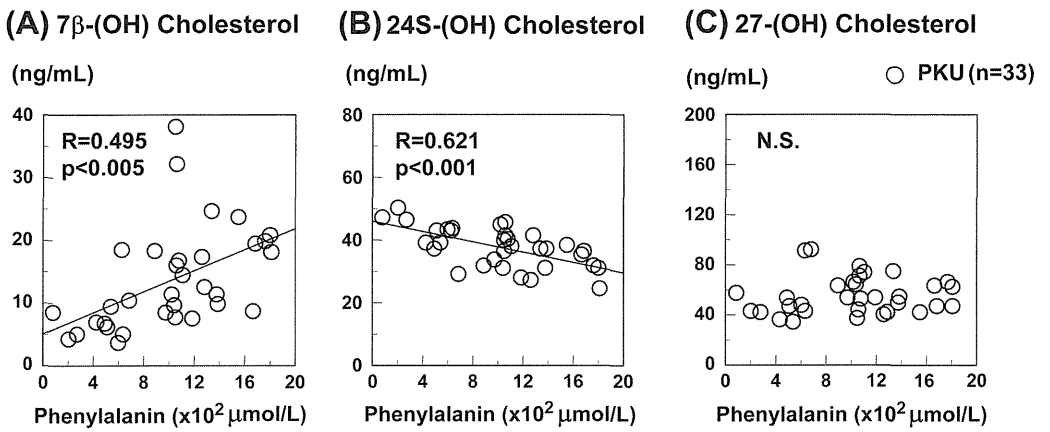

Fig. 3. Scatter graphs of oxysterol levels against phenylalanine level in the PKU group.

3.7. Correlations between oxysterols and VD in PKU patients

27-OHC level showed a significant positive correlation with 25-hydroxy VD_3 level but not with 1.25-hydroxy VD_3 and intact PTH levels (Fig. 4). Other oxysterols never showed significant correlations with bone parameters.

4. Discussion

This study demonstrated changes of blood oxysterols that predominated over those of total- and LDL-cholesterols in adult PKU. Blood levels of 24S-OHC, 27-OHC, and 7α-OHC were considerably decreased in adult PKU patients. On the contrary, 7β-OHC was considerably increased.

The control group exhibited significant correlations between 27-OHC, total- and LDL-cholesterol levels. However, the PKU group lacked such cholesterol–oxysterol correlation. In the PKU group, 24S-OHC and 7β-OHC levels were correlated significantly with the phenylalanine level, suggesting that plasma phenylalanine, to some degrees, influences on 24S-OHC and 7β-OHC metabolisms, although not directly. In this affected group, 27-OHC showed a significant positive correlation with 25-hydroxy VD_3 as a representative marker for VD status.

Aside from distinct hyperphenylalaninemia, cholesterol synthesis might be suppressed in PKU [1,20–22]. Reduced activities of two key enzymes for cholesterol synthesis, 3-hydroxy-3methylglutaryl-CoA reductase and mevalonate-5-pyrophosphate decarboxylase, have been suggested in PKU (Fig. 1) [20–22]. In the PKU group, total- and LDL-cholesterol levels were about 10% decreased as compared to the respective control levels, consistent with such precedent reports. Plasma levels of 7-dehydrocholesterol (7-DC) and lathosterol as cholesterol precursors were about 20% decreased in PKU group while cholesterol absorption markers such as campesterol and sitosterol remained unchanged (Table 1). Oxysterols are oriented from cholesterol, and, therefore, it would be likely that productions of oxysterols such as 7α-OHC, 27-OHC and 24S-OHC might be influenced by cholesterol synthesis.

24S-OHC is the brain specific cholesterol elimination product made by the neuronal specific cholesterol 24-hydroxylase (CYP46A) [13,28]. Reduced plasma concentration was found as a consequence of neurodegeneration [13,15,28]. However, it is possible that plasma concentration might rise concomitantly with increased brain cholesterol turnover. It has been shown that plasma cholesterol never enter into brain, passing through blood–brain barrier, and that suppression of cholesterol synthesis possibly lead to the decrease of plasma 24S-OHC [13,17]. So, the decrease of 24S-OHC in PKU group might be, in part, attributable to the reduced cholesterol synthesis in the brain and/or disturbed conversion from cholesterol to 24S-OHC.

Recent studies have shown the effects of 24S-OHC to prevent amyloid plaque formation as seen in Alzheimer disease and to promote growth of brain cells [29–32]. Probably, 24-OHC has several crucial biological functions in the neurological system. Therefore, we will pursuit the neurological consequence of long-standing decrease of 24-OHC in adult PKU.

27-OHC is a product of CYP27A, which expression is abundant in peripheral tissues but rather poor in the liver, [13,33–35]. This oxysterol is the first step of cholesterol elimination via acidic bile acids pathway

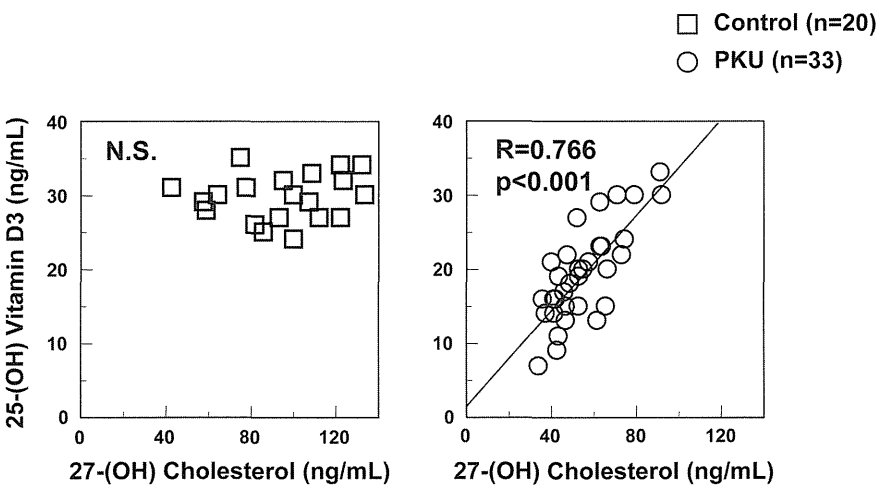

Fig. 4. Scatter graph of 25-hydroxy vitamin D_3 level against 27-hydroxycholesterol level in the PKU group.

[13,29–35]. Unlike cholesterol, 27-OHC passes through blood–brain barrier easily and enters into the brain. A positive correlation between cholesterol and 27-OHC level in the circulation, together with considerable amount of 27-OHC uptake by the human brain from the circulation, has been shown [36,37].

The decrease of 27-OHC was prominent and disproportionate to the decrease of total- and LDL-cholesterol in PKU patients. The 27-OHC level was always two-thirds of control level against the lipid and phenylalanine levels. The data obtained from the control group, together with earlier reports, support that blood 27-OHC level shows a positive correlation with blood total-and/or LDL-cholesterol levels [13,36,37]. Contradictorily, it has been shown that the effect of statins inhibiting 3-hydroxy-3methylglutaryl-CoA reductase on blood 27-OHC is often obscure even though the decrease of cholesterol level is clear [18]. On the other hand, Björkhem et al. reported that blood 27-OHC level greatly increased in Smith–Lemli–Opits syndrome (OMIM, 270400) manifesting poor cholesterol synthesis together with accumulation of 7-deoxycholesterol (7-DC) due to 7-DC reductase deficiency (EC 1. 3. 1. 21) (Fig. 1) [17].

At this time, we do not have a convincing explanation for a distinct decrease of blood 27-OHC independent of blood total-and/or LDL-cholesterol levels. Our recent study showed that vitamin D_3, which is originated from 7-DC, was also decreased in disproportion to the total- and LDL-cholesterol levels in PKU [23]. This study showed that 27-OHC level is strongly correlated with 25-hydroxy VD_3 level in PKU patients. Considering that both 27-OHC and 25-hydroxy VD_3 productions are greatly regulated by CYP27A activity, it is likely that CYP27A activity is suppressed in them (Fig. 1) [38].

The decrease of 7α-OHC, a product of 7α-hydroxylase (CYP7A) in the liver, also predominated over the decreases of total- and LDL-cholesterol, exhibiting no correlation with phenylalanine level. 7α-Hydroxylase (CYP7A) plays a central role in the bile acid synthesis and cholesterol elimination in the liver [33,35]. We inferred that the considerable decrease of 7α-OHC might reflect liver cholesterol pool.

7β-OHC production is promoted by oxidative stress. Therefore, this oxysterol is regarded as a parameter for oxidative stress [13,9]. 7β-OHC was increased in PKU patients. Particularly patients having a phenylalanine level of more than 650–700 μmol/l often exhibited high levels (Fig. 2). Our recent study provides evidence that oxidative stress is enhanced in PKU according to the phenylalanine level [10].

Thus, this study suggested changes of oxysterol together with those of cholesterol metabolism and VD status. However, to gain a better understanding of cholesterol and oxysterol metabolism in PKU, more comprehensive studies covering key enzymes should be necessary.

In summary, this study showed variable changes in oxysterols in adult PKU patients. To identify the unfavorable effects of altered oxysterol metabolism on the nervous system and the bone will be our future project.

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Bezafibrate can be a new treatment option for mitochondrial fatty acid oxidation disorders: Evaluation by in vitro probe acylcarnitine assay

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ABSTRACT

Background: The number of patients with mitochondrial fatty acid oxidation (FAO) disorders is recently becoming larger with the spread of newborn mass screening. Despite the advances in metabolic and molecular characterization of FAO disorders, the therapeutic studies are still limited. It was reported recently that bezafibrate (BEZ), an agonist of peroxisome proliferating activator receptor (PPAR), can restore FAO activity in cells from carnitine palmitoyltransferase-2 (CPT2) and very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiencies as well as clinical symptoms in the adult patients.
Methods: In this study, the therapeutic effect of BEZ was determined by in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry on various FAO disorders. The clinical trial of BEZ treatment for a boy with the intermediate form of glutaric acidemia type 2 (GA2) was also performed.
Results: The effect of BEZ was proven in cells from various FAO disorders including GA2, deficiencies of VLCAD, medium-chain acyl-CoA dehydrogenase, CPT2, carnitine acylcarnitine translocase and trifunctional protein, by the IVP assay. The aberrantly elevated long- or medium-chain acylcarnitines that are characteristic for each FAO disorder were clearly corrected by the presence of BEZ (0.4 mmol/L) in culture medium. Moreover, daily administration of BEZ in a 2-year-old boy with GA2 dramatically improved his motor and cognitive skills, accompanied by sustained reduction of C4, C8, C10 and C12 acylcarnitines in blood, and normalized the urinary organic acid profile. No major adverse effects have been observed.
Conclusion: These results indicate that BEZ could be a new treatment option for FAO disorders.

1. Introduction

Mitochondrial β-oxidation (FAO) is an essential energy producing pathway, particularly during the reduced energy supply from carbohydrate due to prolonged starvation or low caloric intake during infection, diarrhea or febrile illness. A number of FAO disorders have been recognized with the spread of tandem mass spectrometry (MS/MS) in the field of study of inborn metabolic disease as well as neonatal mass screening [1,2]. Many of them show episodic attacks like lethargy, acute encephalopathy or even sudden death due to energy production insufficiency.

It is considered that the FAO system consists of the following four groups : 1) carnitine cycle, which activates long-chain fatty acids for undergoing β-oxidation, including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 or -2 (CPT1 or CPT2, respectively, EC 2.3.1.21), or carnitine acylcarnitine translocase (CACT, EC 2.3.1.21); 2) long-chain FAO, whose enzymes are connected to the mitochondrial inner membrane, including very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.99.13) deficiency, and trifunctional protein (TFP, EC 1.1.1.211 and EC 2.3.1.16); 3) medium-chain FAO, whose enzymes are located in the mitochondrial matrix, including medium- and short-chain acyl-CoA dehydrogenases (MCAD, EC 1.3.99.3 and SCAD, EC 1.3.8.1) respectively), enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, or medium- and short-chain 3-ketothiolase (MCKAT and SCKAT, respectively); and 4) electron transfer system, from the dehydrogenases to respiratory chain, including electron transferring flavoprotein (ETF, EC 1.5.8.2) and ETF dehydrogenase (ETFDH, EC 1.5.5.1) [3–5].

Clinical features of FAO disorders can be roughly divided into the following three types: 1) severe form (neonatal form): patients present life-threatening illness with profound hypoglycemia, liver failure or hyperammonemia, and are often fatal in early infancy; 2) intermediate

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form (juvenile form): patients have intermittent episodic attacks like lethargy, encephalopathy, or even sudden death often onset in infancy or young childhood; 3) mild form (myopathic form): the patients may often show late onset after school ages or adulthood with episodes of hypotonia, myalgia, lethargy, myopathy-like symptoms, or liver dysfunction [6].

In vitro probe acylcarnitine profiling (IVP) assay was developed to evaluate FAO disorders recently [7,8]. Acylcarnitine (AC) profiles in the special culture medium as below after incubating with fatty acids as substrates are determined by MS/MS. Bezafibrate (BEZ) is a hypolipidemic drug, which is an agonist of peroxisome proliferating activator receptor (PPAR), and is claimed to act for induction of several FAO enzymes [9–11].

In this study, the effect of BEZ on various FAO disorders was evaluated using the IVP assay. Furthermore, we report an in vivo trial of BEZ on a boy with the intermediate form of GA2, presenting dramatic improvement with BEZ.

2. Materials and methods

2.1. Subjects and skin fibroblasts

Fibroblasts from 10 Japanese children with FAO disorders, one each of severe and intermediate forms of GA2, 2 each of severe and myopathic (mild) forms of VLCAD deficiency, one each of deficiencies of MCAD, CPT2, CACT, and TFP as well as 6 controls (healthy volunteers, passages 3 to 16) were used. The clinical types and genotypes are shown in Table 1. The child with MCAD deficiency was detected in a newborn mass screening and non-symptomatic, while one with the intermediate form of CPT2 deficiency had liver dysfunction in infancy. The child with the intermediate form of CACT deficiency had two life-threatening episodes in infancy, and after that no episodes were noted with normal development [12]. The child with TFP deficiency had an episode of liver failure in infancy, and then intermittent episodes of myalgia or hypotonia particularly following infection.

The clinical types and genotypes are shown in Table 1. In all cases, at least one allele has missense mutation, although the other alleles had missense or truncated mutations. In CACT deficiency (case 9), a missense mutation in an initiation codon (c.3G>A) in SLC25A29 was detected, but this could harbor a residual activity (Fukao et al., unpublished data).

Table 1
Clinical types and genotypes of patients with mitochondrial fatty acid oxidation disorders investigated.

Disease & case No.	Phenotype	Gene	Genotype, nucleotides (amino acids)	
			Allele 1	Allele 2
GA2				
1 (B)	Severe	*ETFA*	c.799G>A (G267R)	c.7C>T (R3X)
2 (C)	Intermediate	*ETFDH*	c.1217G>A (S406N)	c.1675C>T (R559X)
VLCAD deficiency				
3 (D)	Severe	*ACADV*	c.553G>A (G185S)	IVS9+1g>c
4 (E)	Severe	*ACADV*	c.454G>A (G152S)	c.997insT (A333fsX358)
5 (F)	Myopathic	*ACADV*	c.790A>G (K264E)	c.997insT (A333fsX358)
6 (G)	Myopathic	*ACADV*	c.1144A>C (K382Q)	c.1339G>A (G447R)
MCAD deficiency				
7 (H)	Non-symptomatic	*ACADM*	c.134A>G (Q45R)	c.449delCTGA (T150fsX153)
CPT2 deficiency				
8 (I)	Intermediate	*CPT2*	c.151A>G (R51G)	c.520G>A (E174K)
CACT deficiency				
9 (J)	Intermediate	*SLC25A29*	c.3G>A (M1I)	IVS4+1g>t
TFP deficiency				
10 (K)	Intermediate	*HADHB*	c.739C>T (R247C)	c.817delG (D273fsX292)

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; GA2, glutaric acidemia type 2; VLCAD, very-long-chain acyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase-2; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase. Case 2 (C) is a boy with GA2 who underwent the clinical trial of BEZ. Non-symptomatic case 7 (H) was detected in the newborn mass screening. Severe, intermediate, and myopathic forms are mentioned in the text. (B) to (K) correspond to those of Fig. 1.

2.2. In vitro probe assay with BEZ

Fibroblasts were cultured in 75 cm^2 flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO_2/95% air incubator [13].

Fibroblasts harvested by trypsinization were seeded onto 6-well microplates (35 mm i.d., Iwaki, Japan) with the fresh above medium (2 mL/per well) until they reached confluence. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of experimental substrate (experimental medium). The experimental medium is MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabeled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, in the presence or absence of BEZ (0.4 mmol/L; Sigma). AC profiles in the culture medium were analyzed after 96 h. The experiments for each case were performed in triplicate.

2.3. Quantitative acylcarnitine analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA) as described previously [13]. Briefly, methanol (200 μL) including an isotopically-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of the supernatant from culture medium. The portions were placed on ice for 30 min, and centrifuged at 1000×*g* for 10 min. Then, 150 μL of the supernatant was dried under a nitrogen stream, and butyl-derivatized with 50 μL of 3N n-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80% acetonitrile:water (4:1 v/v). The ACs in 10 μL of the resultant aliquots were analyzed using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. The AC concentrations are expressed as nmol/mg protein.

2.4. Organic acid analysis using GC/MS

Urinary organic acids were analyzed according to the previous method [14]. Briefly, 40 μg of tropate (IS-2) and 20 μg each of heptadecanoate (IS-1) and tetracosane (C24) as internal standards were added to a urine specimen containing 0.2 mg creatinine. The samples were oxime-derivatized, and solvent extracted with ethylacetate, and trimethylsilylated (TMS-derivatization). The resultant aliquots were subjected to GC/MS (Shimadzu GC/MS QP2010 Plus, Kyoto, Japan), with a DB-5 column of 0.25 mm I.D×30 m, 1 μm film thickness (J&W, Folsom, CA). The temperature program was from 100 °C to 290 °C at a rate of 4 °C/min).

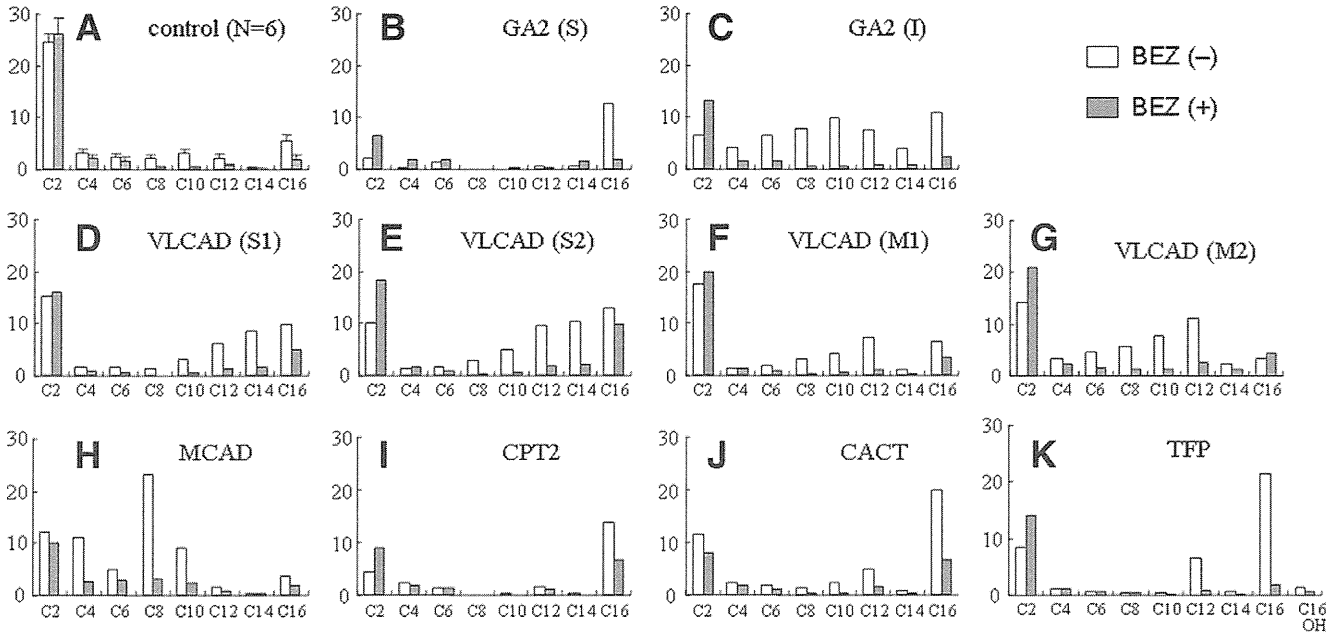

Fig. 1. Acylcarnitine profiles of in vitro probe assay in the presence and absence of bezafibrate. A, normal control; B, severe form of GA2; C, intermediate form of GA2 (the boy who underwent the clinical trial) (S and I, the clinically severe and intermittent form, respectively); D and E, severe form of VLCAD deficiency (S1 and S2, two cases, respectively); F and G: myopathic (mild) form of VLCAD deficiency (M1 and M2, two cases, respectively); H, I, J, and K: deficiencies of MCAD, CPT, CACT, and TFP, respectively. Unit of vertical lines, nmol /mg protein of acylcarnitines; the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C10, C12, C14, C16, and C16-OH. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars. In control (A), the mean plus SD values of 6 controls are shown.

2.5. BEZ trial on a child with the intermittent form of GA2

A Japanese boy with GA2 was detected in the newborn mass screening using MS/MS, and had no special symptoms in infancy with therapies of special formula and carnitine (approximately100 mg/kg/day, div. 3). After 1 year of age, however, he sometimes experienced episodes of hypotonia or lethargy following infection, and muscle weakness, often falling. At the age 2 years and 1 month, he was hospitalized for 2 and a half months, because of infection and lethargy, receiving treatments including artificial respiration to repeated aspiration pneumonia and unconsciousness in intensive care unit (ICU). At discharge, he could not walk alone, and could speak only a few words. So, his family consulted us, and strongly expressed a desire for any new therapies that might help their son.

Thereafter, under the approval by the ethical committee of Shimane University, we started a clinical trial of BEZ, continuing the dietary and carnitine therapies as before, since 2 years and 9 months of his age. His body weight ranged from 12 to 14 kg during the treatment, and 200 to 300 mg/day (approximately 17 to 25 mg/kg/day, div. 3) of BEZ was used in the trial. BEZ was purchased from Kissei Co Ltd, Tokyo, Japan. The study had no potential conflicts of interest (COI) to the authors.

3. Results

3.1. Effects of BEZ on FAO disorders by IVP assay

The AC profiles in the culture medium of fibroblasts from various FAO disorders in the presence and absence of BEZ are illustrated in Fig. 1. In control cells, C2 (acetylcarnitine) is the only prominent peak, and many of ACs further decreased in the presence of BEZ (Fig. 1A).

In the severe form of GA2 (Fig. 1B/S), C16 was apparently decreased, and C2 increased in the presence of BEZ, while C16 was extremely high before BEZ addition. The increase of C2 may indicate the acceleration of FAO, namely an increase of acetyl-CoA production. In the intermediate form of GA2 (Fig. 1C/I), all elevated ACs clearly decreased and normalized in the presence of BEZ, although broad ranges of ACs from C4 to C16 were extremely high before adding BEZ. This patient is the case 3 in Table 1, who underwent the clinical trial of BEZ treatment as illustrated in Fig. 2.

In 2 cases of the severe form of VLCAD deficiency (Figs. 1D/S1, and 1E/S2), elevation of C14 and C16 was larger, compared with that in 2 cases of the mild form (Figs. 1F/M1, and 1G/M2). The elevated ACs

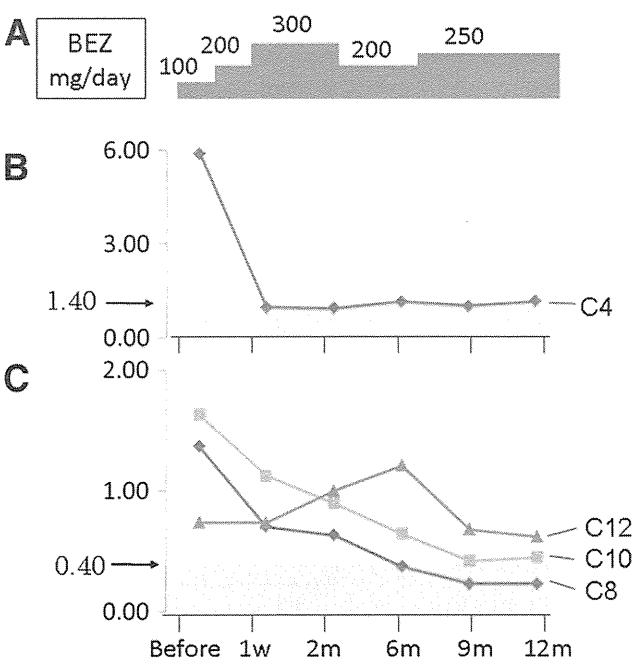

Fig. 2. Bezafibrate administration and changes in blood acylcarnitines. A, dose of bezafibrate, mg/day (approximately 17 to 25 mg/kg/day, div. 3); B, change of C4 acylcarnitine; C, changes in C8, C10, and C12. Arrows with the 1.40 and 0.40 indicate the cutoff values of blood acylcarnitines. Unit of acylcarnitine is nmol/mg protein.

Table 2
Time course of biochemical findings after initiation of bezafibrate administration.

	(Unit)	Before	After the start of BEZ treatment					Reference value*
			1w	2 m	6 m	9 m	12 m	
AST	(IU/L)	47	35	44	43	26	42	10–38
ALT	(IU/L)	27	17	22	24	20	21	5–40
LDH	(IU/L)	448	426	392	384	341	371	100–215
CK	(IU/L)	496	185	187	324	174	207	36–216
TChol	(mg/dL)	161	127	117	141	127	140	150–219

* : used in Shimane University Hospital. Abbreviations: AST, aspartate amino transferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; and TChol, total cholesterol.

such as C10, C12, C14, or C16 in both the severe and mild forms apparently decreased in the presence of BEZ.

In MCAD deficiency (Fig. 1H), the AC peaks of C4 to C10 were significant, but in the presence of BEZ, these AC peaks were almost normalized. In cases of CPT2 deficiency (Fig. 1I), CACT deficiency (Fig. 1J) and TFP deficiency (Fig. 1K), the extremely high AC peaks of C16 and/or C12 apparently decreased to an almost normal level, in the presence of BEZ.

3.2. Clinical trial of BEZ to a GA2 patient

Since the start of BEZ treatment, his motor and social development, and languages remarkably improved, and no metabolic episodes were noted. He became able to walk alone, showed improved muscle strength, and could speak markedly more words in a few weeks. Furthermore, several months later, he could ride a kid's tricycle by himself, although his intellectual ability was on the borderline for entrance into a kindergarten. For at least 1 year of the administration, no adverse effects of BEZ such as hypolipidemia or rhabdomyolysis have been observed.

The routine laboratory data such as blood AST, ALT, LDH or CK were in normal or subnormal ranges as shown in Table 2, showing stable levels of each test, although these laboratory data had sometimes fluctuated, in particular, when his condition was unstable before the initiation of BEZ. For example, during the stay in the ICU at the age of 2 years, the maximum levels of AST, ALT, LDH or CK were 1450 IU/L, 825 IU/L, 5200 IU/L, or 10,750 IU/L, respectively. The maximum level of blood ammonia at the ICU was 126 μg/dL, while no significant elevation was observed after that. Hypoglycemic attacks have not been noted.

BEZ is a hypolipidemic drug, and we have paid attention to the blood level of Cholesterol (TChol), because of the potential adverse effects. The dose of BEZ was 100 mg/day for the first 3 days, 200 mg/day for 4 days, and 300 mg/day for 2 months, respectively, as shown in Fig. 2A. At 2 months after starting BEZ of 300 mg/day, TChol level was a bit low, 117 mg/dL. Since then the dose has been lowered to 200 or 250 mg/day, and the TChol level has ranged between around 130 to 150 mg/mL, as shown in Table 2.

The changes in the AC levels of C4, C8, C10, and C12 are illustrated in Figs. 2B and C, respectively. All the increased ACs returned to approximately normal levels with the administration of BEZ after several months. In particular, C4 decreased to the normal range within a few weeks. Urinary organic acid analysis showed remarkable increases of ethylmalonate, methylsuccinate, adipate, 2-hydroxyglutarate, hexanoylglycine, suberate, and suberylglycine, before the BEZ treatment as shown in Fig. 3. The abnormalities in urinary organic acids were markedly corrected as early as 2 weeks after the initiation of BEZ therapy. The profile was almost normal but for a slight increase of ethylmalonate, and/or hexanoylglycine as illustrated in Fig. 3B.

4. Discussion

The treatments for FAO disorders have generally been described as follows: 1) avoiding a "long fasting": it prevents the increased requirement of fuel from FAO; 2) early infusion of glucose: it should be performed during the metabolic stress resulting from infection, diarrhea or overexercise, to prevent hypercatabolism; 3) carnitine therapy: it may be effective in many cases, although controversy

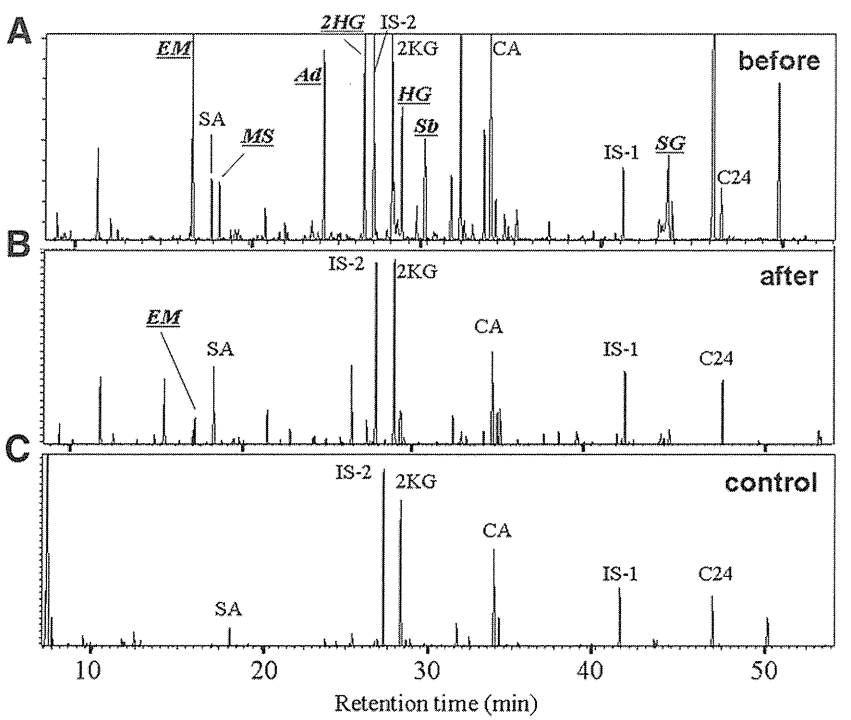

Fig. 3. Urinary organic acid profiles before and after bezafibrate administration. A, The total ion chromatogram (GC/MS) of urinary organic acids just before the start of BEZ; B, One year after the treatment; C, Normal control. Abbreviations: IS-2, IS-1 and C24 are tropate, heptadecanoate, and tetracosane, respectively, as internal standards; EM, ethylmalonate; SA, succinate; MS, methylsuccinate; Ad, adipate; 2HG, 2-hydroxyglutarate; 2KG, 2-ketoglutarate; HG, hexanoylglycine; Sb, suberate; CA, citrate; SG, suberylglycine. Metabolites judged as abnormal are shown in bold letters underlined.

remains in some cases; and 4) dietary therapy, including high carbohydrate/low lipid diet: Dietary restriction in FAO disorders may be less strict [15–18].

In this study, we demonstrated the effect of BEZ on various FAO disorders at both in vitro and in vivo levels. It was indicated by the IVP assay that FAO capacity was corrected by BEZ in various FAO disorders, and a clinical trial of BEZ in a boy with the intermediate form of GA2 showed a favorable consequence. Bastin, Djourdi and their colleagues reported the potential effect of BEZ for FAO disorders showing the increase of enzyme activity and mRNA production in several FAO enzymes from normal individuals, or reduced ACs in cells from VLCAD deficiency by the IVP assay using stable isotope-labeled palmitate [19]. Furthermore, they are performing a clinical trial on adult cases of mild form of CPT2 deficiency [20,21]. We should continue to pay attention to potential adverse effects of BEZ, including hypolipidemia or rhabdomyolysis, although such signs have never seen up to now.

We used the IVP assay to investigate the effect of BEZ in the other FAO disorders including GA2, deficiencies of MCAD, CACT, and TFP as well as CPT2 or VLCAD deficiencies. The beneficial effect of BEZ was clearly demonstrated in all these cases tested in this study, which included the clinically intermediate or severe forms as well as the mild form, having missense mutation of at least one allele. However, it is not yet clear whether the effect of BEZ is due to induction of mutant enzyme itself, or due to stimulation of the other FAO enzymes. If the effect is due to the latter mechanism, BEZ could potentially induce a "high pressure" on the FAO pathway, even resulting in devastating outcomes. We should further investigate the effect on the other severe forms of FAO disorders, the relation with the genotypes, or the dose dependency.

BEZ is an agonist of PPAR, which facilitates transcription of genes encoding FAO enzymes, and subsequently induces FAO enzyme production. Eventually, it can be considered to correct the FAO capacity in FAO disorders. Recently, it was reported that resveratrol which is a natural polyphenol and an activator of Sirtuin 1, is also expected to be a novel treatment option for FAO disorders [22]. The effect of resveratrol on FAO capacity can also be evaluated by the IVP assay like this study.

In conclusion, BEZ could be a new promising treatment option for FAO disorders. Many of patients with FAO disorders, particularly children with the milder form or adult cases, are intellectually normal, and their life prognosis is favorable if they can be prevented from severe episodes like encephalopathy. Symptoms or severity of FAO disorders are very heterogeneous depending on the disease, genetic background or lifestyle. Additional clinical studies of BEZ treatment will be essential for confirmation of its safety and practical utility.

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Brief Communication

Clinical and molecular aspects of Japanese children with medium chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

We report the outcome of 16 Japanese patients with medium chain acyl-CoA dehydrogenase deficiency. Of them, 7 patients were diagnosed after metabolic crisis, while 9 were detected in the asymptomatic condition. Of the 7 symptomatic cases, 1 died suddenly, and 4 cases had delayed development. All 9 patients identified by neonatal or sibling screening remained healthy. Of 14 mutations identified, 10 were unique for Japanese, and 4 were previously reported in other nationalities. Presymptomatic detection including neonatal screening obviously improves quality of life of Japanese patients, probably regardless of the genotypes.

1. Introduction

Medium chain acyl-CoA dehydrogenase deficiency (MCADD) (MIM #201450) is an autosomal recessive inherited metabolic disorder of mitochondrial fatty acid oxidation. The number of MCADD patients has recently become larger in Japan with the spread of acylcarnitine analysis using tandem mass spectrometry (MS/MS). The disease frequency was estimated to be approximately 1:100,000 in Japan according to a newborn screening pilot study of 1.57 millions babies (unpublished report). Clinical symptoms of MCADD are heterogeneous, ranging from asymptomatic to severe handicaps followed by metabolic crisis or sudden unexpected death (SUD) [1,2]. Approximately 20% of previously undiagnosed patients die during their first metabolic decompensation [3–7]. Blood acylcarnitine, urinary organic acid analyses, MCAD activity and mutation analyses are major tools for diagnosis of MCADD. A common c.985A>G mutation has been reported in 80–90% of Caucasian patients [8–16] while c.449–452delCTGA mutation was identified in 45% of mutant alleles in Japanese patients with MCADD [17]. In recent years, the detection incidence of the presymptomatic patients with MCADD has increased since the neonatal mass screening was expanded in Japan. However, there are few reports of the outcomes of the Japanese patients. Herein, we report the relation of clinical onsets, genotypes and outcomes of 16 Japanese children with MCADD, and 4 heterozygote carriers, which were analyzed in Shimane University.

2. Subjects and methods

2.1. Subjects

Sixteen Japanese patients with MCADD from 15 unrelated families, including previously reported 9 cases [17], and 4 carriers were studied (Table 1). The patients were analyzed for confirmation of diagnosis in Shimane University from 2001 to 2011. Of them, 8 (cases 8 to 16) were identified by neonatal mass screening, 7 (cases 1 to 7) were diagnosed after metabolic crisis, and 1 was detected by sibling screening. Cases 2 and 8 were siblings, and cases 19 and 20 were parents of case 16. Diagnosis of the patients was confirmed by urinary organic acid, blood acylcarnitine and mutation analyses.

2.2. Mass spectrometric analysis

Acylcarnitines in blood spots on filter paper were analyzed by a method standardized for neonatal mass screening using MS/MS, an API 3000 instrument (Applied Biosystems, Foster City, CA, USA) [8,18]. Urinary organic acids were analyzed using the solvent extraction method by the QP 2010 capillary GC/MS system (Shimadzu Co., Ltd., Kyoto, Japan) [19]. The determination of test values was assessed using reference values set at the Shimane University.

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