used an allograft from a heterozygous living donor. We describe the clinical and laboratory features of our patient together with the hepatic and renal histopathological findings and the results of bile acid and *CYP7B1* gene analyses.

PATIENTS AND METHODS

Clinical Course and Laboratory Findings

A female Japanese infant with a birth weight of 3004 g was delivered by spontaneous vaginal delivery without complications at a gestational age of 39 weeks. The mother was a primigravida, and the pregnancy was uneventful. The parents were not consanguineous; both were healthy without evidence of liver disease. In particular, her mother had no pruritus, jaundice, or abnormal results for routine tests of liver function during her pregnancy.

The infant developed progressive jaundice by the age of 5 months. At 6 months, she was referred to Juntendo University Hospital with jaundice and hepatic dysfunction.

According to a physical examination, her growth and development were within normal limits (height = 66.1 cm, weight = 7.8 kg). No dysmorphic features were present. Jaundice and hepatosplenomegaly were noted. No abnormal neurological signs were elicited. Her stool was yellowish. The initial results of the laboratory tests included the following: a serum aspartate aminotransferase level of 803 U/L (normal < 37 U/L), an alanine aminotransferase level of 345 U/L (normal < 43 U/L), an alkaline phosphatase level of 4334 U/L (normal = 110-348 U/L), a total bilirubin level of 13.1 mg/dL (normal < 1.2 mg/dL), a direct bilirubin level of 7.7 mg/dL (normal < 0.3 mg/dL), an albumin level of 3.1 g/dL (normal = 4.0-5.2 g/dL), a prothrombin time of 26.0 seconds (normal = 11.1-15.1 seconds), and a blood ammonia level of 95 μg/dL (normal < 70 μg/dL). The serum 25-hydroxy vitamin D level was <5 ng/mL (normal = 7-41 ng/mL). The levels of other vitamins, including vitamins A, E, and K, were not assayed. The serum γ-glutamyltransferase level was 36 U/L (normal < 75 U/L), the total cholesterol level was 171 mg/dL (normal = 150-219 mg/dL), and the serum total bile acid (TBA) level was 6.5 μmol/L (normal < 10 μmol/L). The results of a complete blood count were within normal limits. Specific liver diseases such as autoimmune hepatitis and chronic viral hepatitis and other metabolic defects were excluded by the appropriate investigations. Abdominal ultrasonography revealed a visible gallbladder and hepatosplenomegaly; no choledochal cysts, no dilation of bile ducts, and no ascites were demonstrated. Contrast-enhanced computed tomography showed polycystic changes in the kidneys (Fig. 1A). There was radiological evidence of rickets. Serial technetium-99m diisopropyl iminodiacetic acid cholescintigraphy showed that the tracer had entered the intestine.

The patient's initial management included the administration of medium-chain triglycerides, ursodeoxycholic acid (UDCA; 16 mg/kg/day), fat-soluble vitamins, and infusions of fresh frozen plasma. After 61 days of these treatments, her liver function tests had deteriorated further (aspartate aminotransferase level = 568 U/L, alanine aminotransferase level = 232 U/L, alkaline phosphatase level = 2205 U/L, total bilirubin level = 14.3 mg/dL, direct bilirubin level = 9.1 mg/dL, albumin level = 3.7 g/dL, prothrombin time = 20.9 seconds, γ-glutamyltransferase level = 24 U/L, and TBA level = 54.4 μmol/L). At the age of 8 months, the patient developed decompensated hepatocellular failure, and she was referred to the National Center for Child Health and Development for an LT assessment. The patient underwent liver biopsy and kidney biopsy. Living donor liver transplantation (LDLT) was performed; her mother was the donor.

Progressive familial intrahepatic cholestasis types 1 and 2, which are characterized by low serum γ-glutamyltransferase levels, were excluded for our patient because her serum TBA levels were not elevated before her treatment with UDCA.

Qualitative and Quantitative Analyses of Bile Acids

Serum and urine samples were collected and stored at −25°C until they were analyzed. The concentrations of individual bile acids in her urine were corrected for the creatinine concentration and were expressed as micromoles per millimole of creatinine.

After the synthesis of positive control samples for rare bile acids that occur in patients with inborn errors of bile acid synthesis (eg, 3β-hydroxy-Δ^5 bile acid,[4] 3-oxo-Δ^4 bile acid,[5] and allo-bile acids[5]), we analyzed the bile acids in her serum and urine with gas chromatography/mass spectrometry and with selected ion monitoring of characteristic fragments of methyl ester/dimethylethylsilyl ether/methoxime bile acid derivatives, as described previously.[5] The samples were prepared for gas chromatography/mass spectrometry analysis by enzymatic hydrolysis (30 U of cholylglycine hydrolase) and solvolysis (150 U of sulfatase; Sigma Chemical, St. Louis, MO). *N*-Acetylglucosamine was not used.

Genetic Analysis

Informed parental consent was obtained, and analyses of the cholesterol 7α-hydroxylase gene [cytochrome P450 7A1 (*CYP7A1*)] and the *CYP7B1* gene were undertaken with the DNA of peripheral blood lymphocytes from the patient, the patient's parents, and 103 healthy controls. Polymerase chain reaction (PCR) primers were designed to amplify fragments containing the exon coding regions of the *CYP7A1* and *CYP7B1* genes.[6,7] DNA fragments, which included all coding regions of the *CYP7A1* and *CYP7B1* genes, were amplified with PCR.

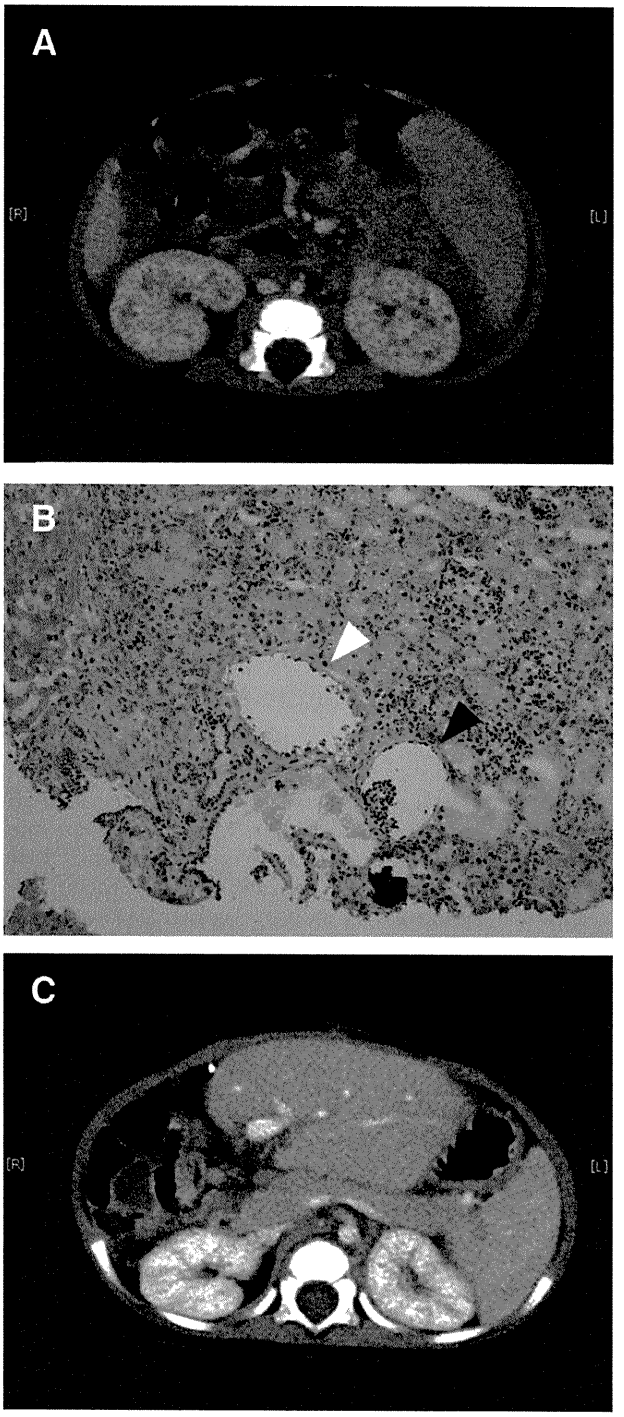

Figure 1. Polycystic kidneys in the patient with an oxysterol 7α-hydroxylase deficiency. (A) Bilateral polycystic kidneys were detected with contrast-enhanced computed tomography. (B) Microscopy of the kidneys revealed glomerular microcysts associated with atrophic glomeruli, cystic dilation of Bowman's capsule (black arrowhead), and renal tubular dilatation (white arrowhead; hematoxylin and eosin, original magnification ×400). (C) The resolution of the polycystic changes in the kidneys was demonstrated with contrast-enhanced computed tomography after LDLT.

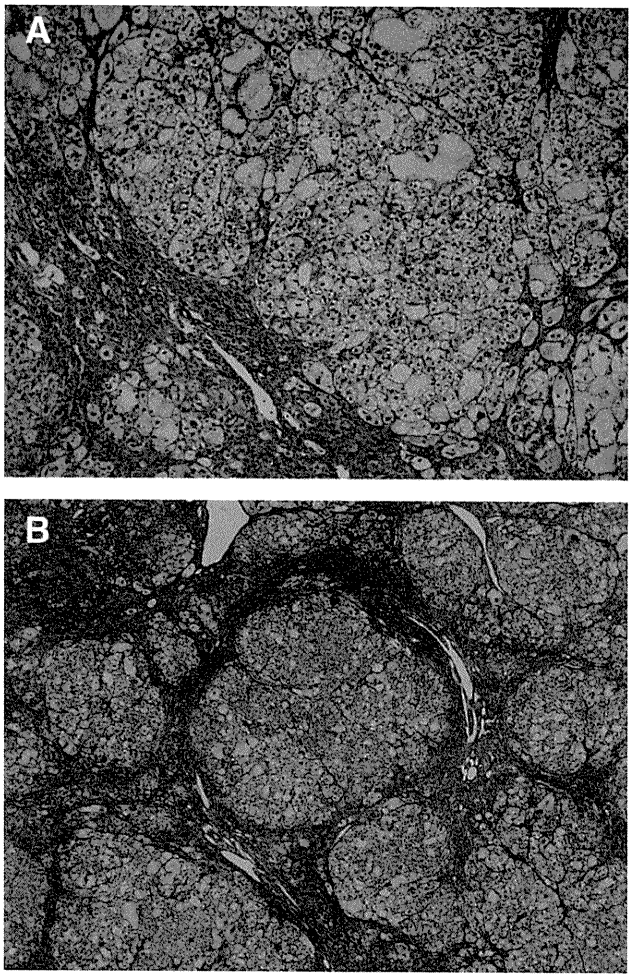

Figure 2. Hepatic pathology at the age of 8 months. A histological examination found features of neonatal hepatitis, which included giant cell transformation of hepatocytes and bridging fibrosis [hematoxylin and eosin, original magnification (A) ×100 or (B) ×40].

After enzymatic processing with ExoSAP-IT (USB Corp., Cleveland, OH), the direct sequencing of the amplified PCR products was undertaken with the DTCS quick-start kit (Beckman Coulter, Inc., Fullerton, CA) according to the manufacturer's protocol; the primers were the same as those used for PCR amplification. The sequencing reaction product was analyzed electrophoretically with the SEQ2000XL analyzer (Beckman Coulter, Inc., Brea, CA).

Two putative mutations were found in the patient. Subsequently, the patient's parents and 103 healthy individuals were screened for these 2 mutations by a direct sequence analysis or by the digestion of the appropriate PCR fragment with a restriction enzyme.

RESULTS

Hepatic and Renal Pathology

At the time of LT, the patient's liver weighed 541 g; it was atrophic and had irregular surface contours. A

TABLE 1. Initial, Pre-LT, and Post-LT Analyses of Bile Acids During UDCA Treatment

	Initial Analysis: 6 Months Old	Analysis Before LT: 8 Months Old	Analysis After LT: 9 Months Old	Analysis After LT: 28 Months Old
Serum (μmol/L)				
CA	ND	1.8	7.6	—
CDCA	1.1	9.5	2.1	—
Deoxycholic acid	0.2	ND	ND	—
UDCA	21.2	34.0	6.5	—
3β-Hydroxy-5-cholen-24-oic acid	7.3*	ND	ND	—
Polyhydroxylated bile acids	ND	0.1	ND	—
TBAs	29.8	45.4	16.2	—
Urine (μmol/mmol of creatinine)				
CA	3.5	5.5	0.2	0.2
CDCA	1.3	0.7	Trace	Trace
Deoxycholic acid	0.1	ND	ND	ND
Lithocholic acid	0.1	ND	ND	ND
UDCA	59.2	88.1	1.3	1.3
3β-Hydroxy-5-cholen-24-oic acid	41.7†	4.4‡	ND	ND
Polyhydroxylated bile acids	1.5	3.0	0.1	0.1
Allo-bile acids	0.1	0.1	ND	ND
Unsaturated ketonic bile acids	2.0	7.3	ND	ND
Other unsaturated bile acids	0.2	0.7	ND	ND
TBAs	109.7	109.8	1.6	1.6

NOTE: A dash indicates that the test was not performed.
*The percentage of TBAs was 24.5%, and the percentage with UDCA excluded from the denominator was 84.9%.
†The percentage of TBAs was 38.0%, and the percentage with UDCA excluded from the denominator was 82.9%.
‡The percentage of TBAs was 4.0%, and the percentage with UDCA excluded from the denominator was 20.3%.

liver biopsy sample showed changes consistent with micronodular cirrhosis; there were wide bands of fibrous tissue, marked lobular disarray, and frequent giant cell transformation (Fig. 2A,B). A renal biopsy sample revealed glomerular microcysts (Fig. 1B).

Clinical Course After LDLT

After an uneventful postoperative course, the patient was discharged on postoperative day 43; at this time, her therapy comprised oral tacrolimus, a corticosteroid, and UDCA. Subsequently, after 20 months of follow-up when the patient was 29 months old, her treatment consisted of only oral tacrolimus (0.07 mg/kg/day) and UDCA (5.8 mg/kg/day). At that time, the results of routine liver function tests were normal, and her growth and development were satisfactory (height = 89.1 cm, weight = 13.8 kg).

After LDLT, the renal cysts (Fig. 1C) and the rachitic bone lesions were resolved.

Biochemical Identification of an Inborn Error of Bile Acid Synthesis

An analysis of the bile acids present in her serum and urine during UDCA therapy when the patient was 6 months old detected large amounts of the rare bile acid 3β-hydroxy-5-cholen-24-oic acid (Table 1). In addition, small amounts of other uncommon bile acids, such as 3β-dihydroxy-Δ^5 bile acids, allo-bile acids, and 3-oxo-Δ^4 bile acids, were also detected in her urine. Common bile acids [eg, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid, and lithocholic acid] were absent or were detected only in small amounts in her serum and urine. 3β-Hydroxy-5-cholen-24-oic acid accounted for 84.9% and 82.9% of TBAs (with UDCA excluded) in her serum and urine, respectively. In the absence of a standard sample for 3β-hydroxy-5-cholen-27-oic acid, this bile acid could not be accurately measured in samples from the patient.

The concentrations of common bile acids in her serum and urine at 8 months were higher than those at 6 months. The concentration of TBAs in her serum was also higher at 8 months. The concentration of 3β-hydroxy-5-cholen-24-oic acid in her urine was lower at 8 months, but the concentrations of other uncommon bile acids were higher (Table 1). After LDLT, the concentrations of bile acids in her serum and urine tended to normalize; in particular, uncommon bile acids could not be detected in her serum or urine (Table 1). The results of analyses of bile acids in specimens from the parents (before LDLT) are shown in Table 2.

Identification of Defects in the *CYP7B1* Gene

Two heterozygous mutations of the *CYP7B1* gene (but no mutations of the *CYP7A1* gene) were identified. One heterozygous mutation (R112X) was found in

TABLE 2. Analysis of the Parents' Bile Acids Before LDLT

	Father	Mother (Donor)
Serum (μmol/L)		
CA	—	ND
CDCA	—	0.5
Deoxycholic acid	—	0.4
Lithocholic acid	—	ND
UDCA	—	ND
3β-Hydroxy-5-cholen-24-oic acid	—	ND
TBAs	—	0.9
Urine (μmol/mmol of creatinine)		
CA	Trace	0.2
CDCA	Trace	Trace
Deoxycholic acid	Trace	Trace
Lithocholic acid	ND	ND
UDCA	Trace	Trace
3β-Hydroxy-5-cholen-24-oic acid	ND	ND
Polyhydroxylated bile acids	ND	Trace
Other unsaturated bile acids	ND	ND
TBAs	Trace	0.2

NOTE: A dash indicates that the test was not performed.

exon 3 at nucleotide 538; R112X is a previously reported C-to-T substitution that changes arginine (CGA) to a stop codon (TGA) at amino acid position 112.[3] The other heterozygous mutation (R417C), which has not been reported previously, is a C-to-T substitution in exon 6 at nucleotide 1453; it results in a substitution of cysteine (TGT) for arginine (CGT) at amino acid position 417. R112X was detected in the father but was absent in the mother and 103 healthy controls. R417C was detected in the mother but was absent in the father and 103 healthy controls.

The previously cited nucleotide numbers indicating the positions of the individual mutations are based on GenBank accession number NM_004820.

DISCUSSION

Our patient manifested clinical and laboratory features that are associated with an oxysterol 7α-hydroxylase deficiency: progressive jaundice beginning soon after birth, hepatomegaly, conjugated hyperbilirubinemia unaccompanied by pruritus, an absence of normal TBA concentrations in serum (measured enzymatically with 3α-hydroxysteroid dehydrogenase), normal serum γ-glutamyltransferase levels, and progressive intrahepatic cholestasis associated with severe hepatic fibrosis.[2,3] High levels of 3β-monohydroxy-Δ^5 bile acids in her serum and urine and a compound heterozygous mutation in the *CYP7B1* gene were detected. She underwent LDLT, after which the features of cholestatic liver disease were resolved.

Macroscopically, the surface of the excised liver was dark brown and was characterized by many large nodules. The patient's liver was found to have the following microscopic features: giant cell transformation of hepatocytes (Fig. 2A); consistent and prominent portal zone inflammation; periportal fibrosis, which had progressed to micronodular cirrhosis associated with bile ductular proliferation by 8 months (Fig. 2B); and bile plugs in a few cholangioles and hepatocytes but not in interlobular ducts. These findings agreed with those reviewed by Bove et al.[8] Interestingly, polycystic changes in the kidneys were demonstrated by computed tomography; such changes also occur in patients with Zellweger syndrome.[9] Her renal function was normal despite the cystic renal lesions (Fig. 1A). A microscopic examination of her kidneys revealed glomerular microcysts associated with atrophic glomeruli, cystic dilation of Bowman's capsule, and renal tubular dilation (Fig. 1B). The cause of these renal changes has not been established; they may have arisen because of renal toxicity induced by certain bile acids (especially monohydroxy bile acids such as 3β-hydroxy-5-cholen-24-oic acid).

After LDLT, the levels of uncommon toxic bile acids such as 3β-monohydroxy-Δ^5 bile acids decreased, presumably because of the increased activity of oxysterol 7α-hydroxylase, and the polycystic changes in the kidneys were resolved.

The level of 3β-hydroxy-5-cholen-24-oic acid as a percentage of TBAs and the absolute concentration of this bile acid in her serum and urine decreased between the first and second analyses of the bile acids (the interval was only 2 months; see Table 1). The changes indicated that the main pathway for bile acid metabolism had changed from the acidic pathway to the classic pathway; the activity of cholesterol 7α hydroxylase gradually increased from the late neonatal period to late infancy, even though the C_{27} bile acid present in patients with this disease, a 3β-monohydroxy-Δ^5-C_{27} bile acid,[2] is a high-affinity ligand for farnesoid X receptor (FXR; Fig. 3).[10] We suggest that the level of activity of cholesterol 7α-hydroxylase in this patient (ie, a low level or none) is consistent with previous physiological observations establishing that the activity of cholesterol 7α-hydroxylase is low or absent in the fetal and early neonatal periods.[11,12]

Progressive liver disease in patients with this condition may be exacerbated by the accumulation of 3β-hydroxy-5-cholen-24-oic acid; our patient had developed cirrhosis by the age of 8 months. Clearly, there is a need for a novel treatment that promotes the excretion of toxic monohydroxy bile acids such as 3β-monohydroxy-Δ^5 bile acids. Currently, orthotopic LT is the only therapeutic option. Any new treatment ideally would prevent the development of cirrhosis after the diagnosis is made in the early neonatal period. An early diagnosis would be facilitated by screening for inborn errors of bile acid synthesis: urine samples would be subjected to liquid secondary ionization mass spectrometry.[13]

Primary bile acid therapy with CA, CDCA, or both is effective in the treatment of inborn errors of bile acid synthesis.[14,15] In our patient, however, *CYP7A1* enzyme activity in the classic pathway would have

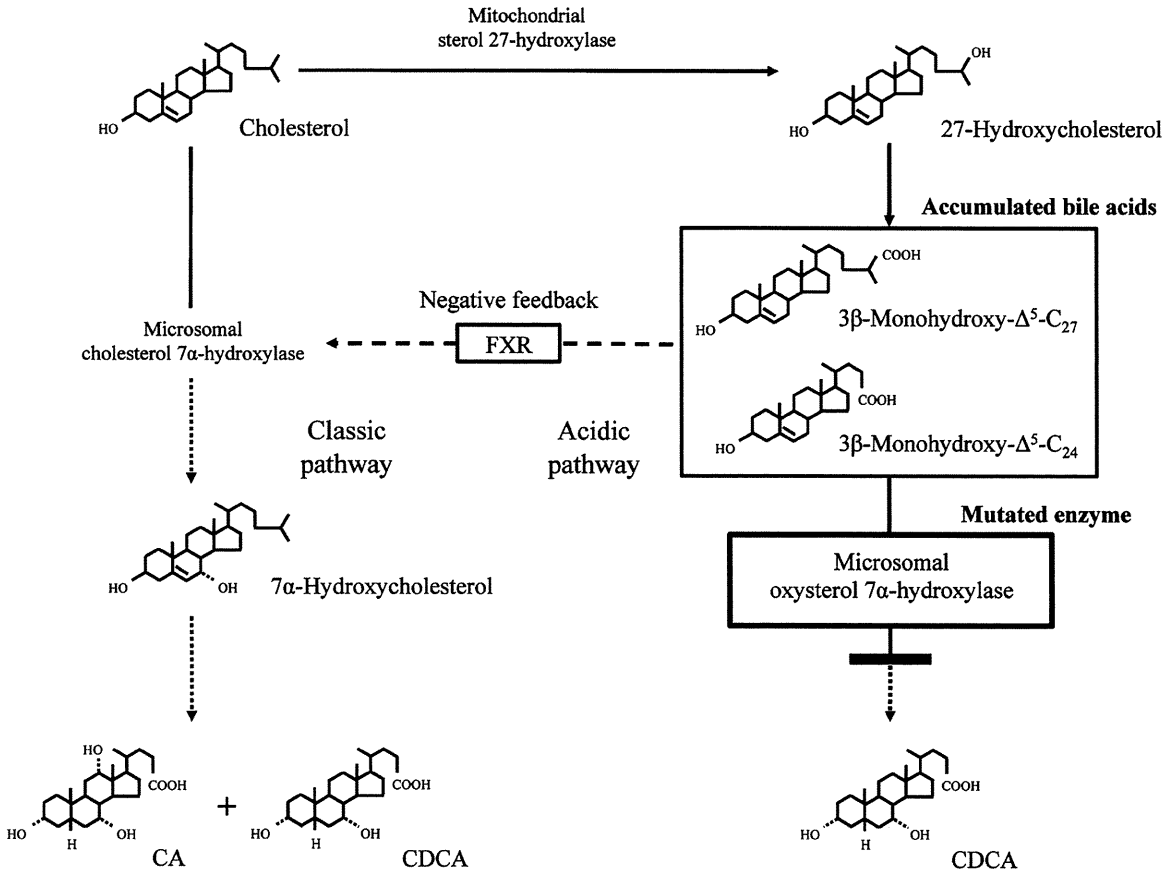

Figure 3. Flow chart showing the effects of an oxysterol 7α-hydroxylase deficiency. The reduced synthesis of primary bile acids from cholesterol in the classic pathway is associated with the increased synthesis of 3β-monohydroxy-Δ^5 bile acids in the acidic pathway. The 3β-monohydroxy-Δ^5 bile acids (especially 3β-monohydroxy-Δ^5-C_{27} acid), which have a high affinity for FXR, lead to reduced cholesterol 7α-hydroxylase activity. However, the activity of cholesterol 7α-hydroxylase may increase from the late neonatal period to late infancy when the main pathway of bile acid synthesis changes from the acidic pathway to the classic pathway.

been suppressed via FXR by therapeutic doses of primary bile acids; consequently, toxic intermediates of bile acid synthesis such as 3β-monohydroxy-Δ^5-C_{27} bile acid in the acidic pathway would have accumulated. We suggest that this patient may have benefited at an early stage from mild suppression of *CYP7A1* induced by low-dose primary bile acid therapy. Because our patient was diagnosed at a late stage, primary bile acid therapy was not attempted.

The human *CYP7B1* gene contains 6 coding exons that correspond to 506 amino acids; so far, 2 distinct mutations that result in an oxysterol 7α-hydroxylase deficiency have been reported.[2,3] Our patient had 2 heterozygous mutations, R112X and R417C, in the *CYP7B1* gene. The previously reported R112X heterozygous nonsense mutation in exon 3 was identified in the father but not in the mother or in control subjects. The novel R417C heterozygous missense mutation in exon 6 was identified in the mother but not in the father or in control subjects. The patient received 1 allele containing the R112X mutation from the father and another allele containing R417C from the mother. Thus, the patient was a compound heterozygote for the *CYP7B1* gene.

Screening for the potentially informative mutation R417C was undertaken for 103 healthy individuals, but this mutation was absent in all of them. Moreover, the R417C mutation was predicted to probably have an adverse effect (score = 1.000) by an analysis with Polymorphism Phenotyping version 2.[16] Accordingly, we believe that the R417C mutation may have contributed to a loss of function of oxysterol 7α-hydroxylase in our patient.

LT is an established treatment for patients with heritable metabolic disorders. For our patient, we obtained an allograft from her mother. After LDLT, all abnormal effects of chronic cholestatic liver disease were gradually resolved. When an appropriate parent is available as the donor, LDLT represents an effective treatment option for pediatric patients with heritable metabolic disorders.[17] In pediatric patients with autosomal recessive disorders, the parent who serves as the donor is almost always a heterozygote. Morioka et al.[17] confirmed that transplantation from

heterozygous donors does not have a negative impact on either the donor or the recipient. However, when the effects of the disease are less hepatospecific than the effects in our patient, additional treatment may be necessary to optimize the outcome after LDLT.

In conclusion, we have reported the first Japanese patient with an oxysterol 7α-hydroxylase deficiency and compound heterozygous mutations (R112X and R417C) in the *CYP7B1* gene. LT with an allograft obtained from a heterozygous living donor was followed by the resolution of the manifestations of the disease.

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Original Article

Maternal and fetal circulation of unusual bile acids: A pilot study

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Abstract ***Background***: Large amounts of unusual bile acids are synthesized by the fetal liver in late gestation. These compounds are mostly transferred from fetus to mother, although some are excreted into the amniotic fluid. We investigated the role of placental transfer of bile acids in fetal bile acid metabolism, particularly with respect to the unusual bile acids (1β-hydroxylated and ketonic bile acids).

Methods: We measured concentrations of bile acids in umbilical cord blood and urine of newborn infants, and in perinatal maternal serum and urine, using gas chromatography-mass spectrometry. Serum and urine specimens from healthy non-pregnant women were used as controls.

Results: In newborn infants at delivery, cord blood and urine contained mostly primary and 1β-hydroxylated bile acids, respectively. We also detected large amounts of ketonic bile acids in their urine, and the urinary concentration of total bile acids was elevated. Main maternal bile acids at 30 and 35 weeks of gestation and at delivery were 1β-hydroxylated bile acids. After delivery, main bile acids changed from 1β-hydroxylated bile acids to primary bile acids ($P < 0.03$), which also predominated in healthy non-pregnant women.

Conclusion: Fetally synthesized unusual bile acids were transported from fetus to mother. Pregnant women appear to excrete these bile acids into the urine, lowering both fetal and maternal serum bile acid concentrations.

Key words fetal bile acid, ketonic bile acid, perinatal bile acid metabolism, placenta.

A variety of unusual bile acids have been identified in fluids such as urine, amniotic fluid, meconium, and biliary bile from the human fetus and newborn.[1–6] These findings suggest the existence of an altered pathway of bile acid metabolism in the fetal liver resembling the pathway in livers of patients with cholestasis. Large amounts of unusual bile acids are synthesized by the fetal liver late in gestation. These compounds are mostly transferred from fetus to mother,[7] with some being excreted into amniotic fluid.[4]

Our study objective was to examine in detail the qualitative and quantitative bile acid composition of serum and urine from healthy pregnant women obtained at different times during late gestation and within the first month after delivery, comparing the findings with serum and urine bile acid composition in healthy non-pregnant women. We also analyzed bile acids in umbilical cord blood and urine of newborn infants at delivery.

We suspected that placental transport of bile acids from fetus to mother relates importantly to aspects of fetal bile acid metabolism, such as synthesis of unusual bile acids like 1β-hydroxylated and unsaturated ketonic bile acids. To test this hypothesis, we compared concentrations of bile acids in umbilical cord blood and urine of newborn infants to concentrations in maternal serum and urine. Analysis of these data elucidated important aspects of perinatal bile acid metabolism in late gestation.

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Methods

Study design

This pilot study was intended to demonstrate placental bile acid transfer between fetus and mother. We determined serum and urinary bile acid composition in five healthy pregnant women at intervals from the gestational ages of 30 weeks to 1 month after delivery, as well as umbilical cord blood and urinary bile acid composition in their five newborn infants. The data were compared with serum and urinary bile acid composition of healthy non-pregnant women. Informed consent for observation and analysis was obtained from each pregnant and non-pregnant woman studied. The Kurume University Ethics Committee approved the study protocol for this human research at Kurume University School of Medicine.

Sample collection

Serum and urine samples were collected from five healthy pregnant women (mean age, 30 years; range, 21–40) whose serum

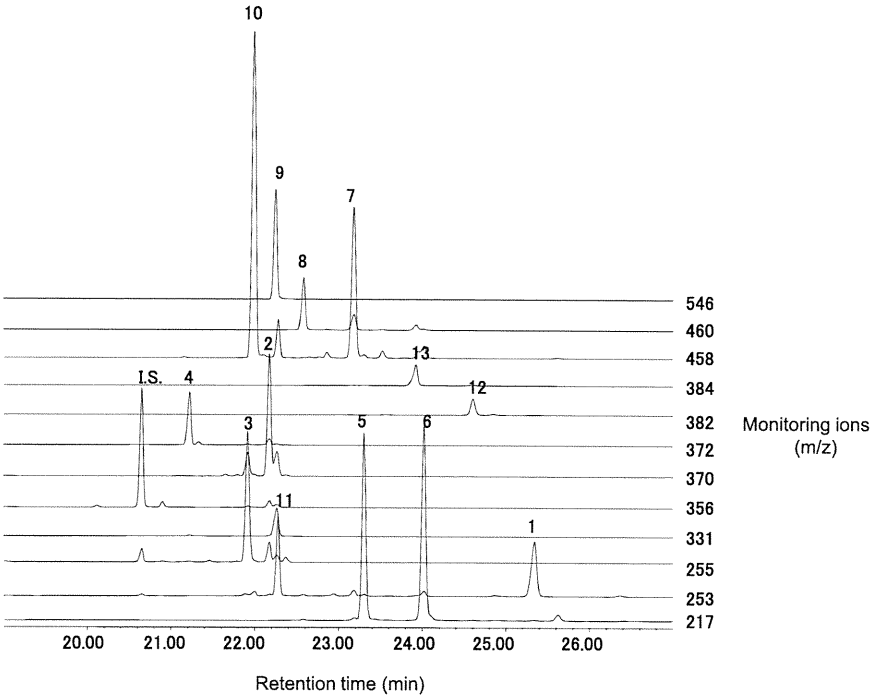

Fig. 1 A typical ion gas chromatography-mass spectrometry chromatogram of methyl ester-trimethylsilyl ether derivatives of reference bile acids. Peak numbers and compounds are the same as in Table 1.

and urine were sampled serially at 30 weeks of gestation, 35 weeks of gestation, at delivery, 7 days after delivery, and 1 month after delivery. Serum and urine samples were also obtained from five healthy non-pregnant women (mean age, 30 years; range, 29–31). Serum and urine samples were obtained before breakfast from both pregnant and non-pregnant women. We also obtained umbilical cord blood and urine samples from the five newborn infants at delivery (four male and one female; mean gestational age, 39.8 weeks [range, 39.1–40.5]; mean birthweight, 3105 g [range, 2714–3562 g]). Each sample was stored at –25°C until assay. Concentrations of individual bile acids in urine from each subject were corrected for creatinine (Cr) concentration and expressed as μmol/mmol of Cr, while concentrations of individual bile acids in serum from each subject were expressed as μmol/L.

Materials and reagents

The following bile acids were synthesized as described previously:[8–10] 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 1β,3α,7α-trihydroxy-5β-cholan-24-oic acid; 3β,7α,12α-trihydroxy-5-cholen-24-oic acid; 3β,7α-dihydroxy-5-cholen-24-oic acid; 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid; and 7α-hydroxy-3-oxo-4-cholen-24-oic acid. Cholic, chenodeoxycholic, deoxycholic, lithocholic, hyocholic, ursodeoxycholic, and 3β-hydroxy-5-cholen-24-oic acids were obtained from Sigma Chemical (St. Louis, MO, USA).

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) was performed with a Hewlett-Packard 5972A instrument (Hewlett-Packard Japan, Tokyo, Japan) using an HP-5MS gas chromatographic column (30 m × 0.25 mm I.D.; film thickness, 0.25 μm; and a fused silica capillary column bound with methylsilicon from J & W Scientific, Folsom, CA, USA) with column temperature programmed to increase from 170°C to 230°C at 10°C/min and from 230°C to 300°C at 5°C/min. Helium was used as the carrier gas at a flow rate of 45 cm/s. Mass spectra were recorded at an ionization energy of 70 eV with an ion source temperature of 300°C. Figure 1 shows a chromatogram obtained by selected ion monitoring of the characteristic fragments of the methyl ester-trimethylsilyl ether (Me-TMS) derivatives of a mixture of reference bile acids.

Derivatization of bile acids for GC-MS analysis

Bile acids were extracted from the solution using a Bond Elut C18 cartridge (3 mL, Varian, Harbor City, CA, USA). The cartridge was washed with water (5 mL), and bile acids were eluted with ethanol (5 mL). After evaporation of solvents, the residue was dissolved in 1 mL of 90% aqueous ethanol. The solution was applied to a piperidinohydroxypropyl dextran gel column (Shimadzu, Kyoto, Japan; 30 m × 6 mm I.D.), which had been equilibrated with 90% aqueous ethanol. After the column was washed with 90% ethanol (4 mL) to remove neutral compounds, bile acids were eluted with 0.1 M acetic acid in 90% ethanol (5 mL). Following evaporation, purified bile acids were derivatized to methyl esters, using diazomethane at room temperature for 10 min. After removal of excess regent, the trimethylsilyl ether was obtained by heating the residue with 30 μL of dimethylethylsilylimidazole (Tokyo Kasei, Tokyo, Japan) at 60°C for 40 min.

Table 1 Gas chromatography-mass spectrometric data for methyl ester-trimetylsilyl ether derivatives of bile acids

No.	Bile acid	Base peak (m/z)	Fragment ions (m/z)
Common bile acids			
1.	Cholic acid	253†	343, 368
2.	Chenodeoxycholic acid	370†	255, 355
3.	Deoxycholic acid	255†	370, 460
4.	Lithocholic acid	215	257, 372†
1β- and 6α-hydroxylated bile acids			
5.	1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid	217†	251, 366
6.	1β,3α,7α-Trihydroxy-5β-cholan-24-oic acid	217†	368, 458
7.	Hyocholic acid	458†	147, 369
Isomerized 7β-hydroxylated bile acid			
8.	Ursodeoxycholic acid	460†	255, 370
3β-Hydroxyl-Δ^5-bile acids			
9.	3β,7α,12α-Trihydroxy-5-cholen-24-oic acid	546†	209
10.	3β,7α-Dihydroxy-5-cholen-24-oic acid	458†	209
11.	3β-Hydroxy-5-cholen-24-oic acid	129	249, 331†
Unsaturated ketonic bile acids			
12.	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid	382†	267, 472
13.	7α-Hydroxy-3-oxo-4-cholen-24-oic acid	384†	459, 474
IS.	3α,7α-Dihydroxy-24-nor-5β-cholan-23-oic acid	431	356†

†Fragment ions used for selected ion monitoring. IS, internal standard.

The resulting preparation was applied to a silica gel column (30 m × 6 mm I.D.) and eluted with n-hexane/ethyl acetate (3:1 by volume). Derivatized bile acids were recovered from the first 5 mL of effluent, and the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in n-hexane (50 μL), and an aliquot of 1 μL was injected into the GC-MS system.

We obtained calibration curves for determination of bile acids by plotting peak area ratios corresponding to the monitored internal standard versus the amount of each bile acid. A linear correlation was obtained ($r > 0.976$) over a range of 1.5–10 ng for each bile acid.[11] In particular, polyhydroxylated bile acids showed a good correlation, but using our method, 7α,12α-dihydroxy-3-oxo-4,6-cholen-24-oic and 7α-hydroxy-3-oxo-4,6-cholen-24-oic acids were produced from 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic, and 7α-hydroxy-3-oxo-4-cholen-24-oic acids during preparation of the sample. We therefore believe that 3-oxo-bile acids were derivatized to methoximes after addition of the internal standard to the sample.[11]

Analysis of bile acids

Samples of human biologic fluids were prepared routinely for GC-MS analysis as described in our previous reports.[11–15] Briefly, an internal standard (3α,7α-dihydroxy-24-nor-5β-cholan-23-oic acid, 2 μg) was added to 0.5 mL of sample. Conjugated bile acids were extracted from solution using a Bond Elut C18 cartridge as described above. After solvents had evaporated, the residue was subjected to enzymatic hydrolysis by 150 U of sulfatase type H-1 from *Helix pomatia* (Sigma Chemical) in 200 μL of 0.05 M sodium acetate buffer (pH 5.6) with 200 μL of 0.6 mM dithiothreitol, 200 μL of 0.05 M ethylenediaminetetraacetic acid, and 100 mL of distilled water, at 37°C for 12 h. Resulting unconjugated bile acids were re-extracted with a Bond Elut C18 cartridge. The cartridge was washed with 5 mL of distilled water and eluted with 5 mL of 90% ethanol. Unconjugated bile acids were extracted with piperidinohydroxypropyl dextran gel, eluted with 5 mL of 0.1 M acetic acid in 90% ethanol, and converted to Me-TMS derivatives for GC-MS analysis.

Identification and quantitation of individual bile acids

GC-MS data for individual bile acids are summarized in Table 1, including their characteristic fragment ions and relative abundance.

Statistical analysis

Data are reported as the mean ± SD. One-way ANOVA was used to determine the significance of differences between groups. Comparisons of categorical data between groups were made with the Aspin–Welch *t*-test. A *P*-value less than 0.05 was accepted as indicating statistical significance.

Results

In the serum of pregnant and healthy non-pregnant women (Table 2), main bile acids relative to total serum bile acids included primary (cholic and chenodeoxycholic) and secondary (deoxycholic and lithocholic) bile acids. We noted no change in the ratio of main to total bile acids between pre- and post-delivery samples. Serum concentrations of total bile acids also did not change.

On the other hand, in urine from pregnant women (Table 3), the main bile acids among total bile acids were 1β-hydroxylated bile acids at 30 and 35 weeks' gestational age ($P < 0.03$) and at delivery. Main bile acids relative to total bile acids changed after delivery from 1β-hydroxylated bile acids to primary bile acids, the main urinary bile acids detected in healthy non-pregnant women. Relative to total bile acids, unsaturated ketonic bile acids made up a higher percentage after delivery and in healthy non-pregnant women than before delivery and at delivery. The

Table 2 Bile acid composition of serum in women before and after delivery

Bile acid (Compound number in Table 1)	Gestational age 30 weeks ($n = 5$)	Gestational age 35 weeks ($n = 5$)	At delivery ($n = 5$)	7 Days after delivery ($n = 5$)	1 Month after delivery ($n = 5$)	Healthy non-pregnant women ($n = 5$)
Primary bile acids (1,2)	59.88 ± 12.17 [47.0–76.3]	68.20 ± 12.44 [52.9–83.7]	58.82 ± 9.84 [47.3–68.5]	69.50 ± 8.38 [62.4–78.6]	55.28 ± 7.39 [44.4–64.4]	64.82 ± 16.73 [36.9–80.1]
Secondary bile acids (3,4)	23.86 ± 15.33 [1.7–37.2]	18.52 ± 13.09 [2.4–32.8]	20.80 ± 15.63 [7.4–43.0]	20.64 ± 8.74 [13.4–32.1]	25.38 ± 14.11 [9.1–42.9]	19.30 ± 21.85 [1.3–55.0]
1β-Hydroxylated bile acids (5,6)	2.30 ± 1.08 [1.2–4.0]	1.66 ± 1.09 [0.3–3.3]	1.68 ± 0.45 [1.1–2.3]	1.94 ± 2.28 [0.6–6.0]	1.76 ± 0.96 [0.5–2.7]	3.70 ± 1.42 [1.8–5.0]
Hyocholic acid (7)	0.84 ± 0.20 [0.6–1.1]	0.72 ± 0.26 [0.3–1.0]	3.70 ± 6.32 [0.7–15.0]	0.70 ± 0.58 [0.2–1.7]	0.70 ± 0.34 [0.3–0.8]	Trace
Ursodeoxycholic acid (8)	10.60 ± 5.51 [3.7–18.8]	9.12 ± 4.39 [3.7–13.6]	9.90 ± 7.99 [4.1–23.4]	4.98 ± 2.54 [0.7–6.8]	14.78 ± 12.34 [5.7–35.2]	11.76 ± 8.74 [2.5–23.3]
3β-Hydroxylated bile acids (9–11)	2.48 ± 1.25 [1.3–4.5]	1.90 ± 1.25 [0.4–3.8]	1.62 ± 0.46 [1.0–2.1]	2.22 ± 2.57 [0.7–6.8]	2.10 ± 1.17 [0.6–3.4]	0.42 ± 0.18 [0.2–0.6]
3-Oxo-Δ^4-bile acids (12,13)	Trace	Trace	Trace	Trace	Trace	Trace
Total bile acids (μmol/L)	3.69 ± 1.56 [1.8–6.0]	7.38 ± 7.64 [2.1–20.8]	5.44 ± 1.92 [3.7–8.4]	6.65 ± 3.80 [1.2–11.8]	5.93 ± 4.97 [2.6–14.5]	8.82 ± 1.35 [4.8–15.4]

All data are mean ± SD (% of total bile acids). [ ], sample range.

Table 3 Bile acid composition of urine in women before and after delivery

Bile acid (Compound number in Table 1)	Gestational age 30 weeks ($n = 5$)	Gestational age 35 weeks ($n = 5$)	At delivery ($n = 5$)	7 Days after delivery ($n = 5$)	1 Month after delivery ($n = 5$)	Healthy non-pregnant women ($n = 5$)
Primary bile acids (1,2)	10.98 ± 9.18 [5.6–27.3]	12.80 ± 7.63 [5.1–25.0]	14.72 ± 7.69 [2.7–21.7]	29.80 ± 17.39 [12.5–50.0]	19.64 ± 8.52 [10.0–28.6]	25.40 ± 12.39 [11.1–38.5]
Secondary bile acids (3,4)	15.04 ± 10.11 [4.0–30.8]	16.62 ± 7.32 [8.8–27.3]	15.44 ± 16.23 [3.3–43.5]	18.11 ± 10.78 [6.3–30.8]	20.52 ± 8.04 [11.1–28.6]	10.86 ± 13.86 [trace – 33.3]
1β-Hydroxylated bile acids (5,6)	50.44 ± 13.28†,‡ [27.3–60.0]	46.50 ± 10.02§ [29.2–54.5]	44.36 ± 17.69 [19.6–67.6]	19.42 ± 6.83 [11.5–25.0]	16.22 ± 8.38 [7.1–28.6]	17.38 ± 10.71 [9.5–33.3]
Hyocholic acid (7)	5.42 ± 7.29 [trace – 18.2]	6.94 ± 8.10 [trace – 20.8]	8.58 ± 6.09 [2.7–16.7]	7.53 ± 9.46 [trace –8.3]	9.40 ± 7.82 [trace 20.0]	5.38 ± 3.23 [trace – 7.7]
Ursodeoxycholic acid (8)	6.18 ± 4.61 [trace – 12.0]	6.68 ± 5.69 [trace – 14.7]	5.14 ± 3.65 [0.9–10.0]	6.46 ± 4.75 [trace – 18.8]	16.92 ± 14.53 [trace – 38.9]	14.20 ± 10.98 [trace – 23.1]
3β-Hydroxylated bile acids (9–11)	5.26 ± 3.37 [trace – 8.3]	3.20 ± 3.38 [trace – 7.7]	3.64 ± 2.57 [1.7–8.1]	7.68 ± 8.85 [trace – 18.8]	5.98 ± 6.27 [trace – 14.3]	6.06 ± 4.09 [trace – 11.1]
3-Oxo-15-Δ^4-bile acids (12,13)	8.28 ± 0.52 [7.7–9.1]	7.36 ± 2.44 [4.2–10.3]	8.14 ± 1.99 [5.9–10.9]	13.50 ± 7.98 [7.7–25.0]	11.36 ± 3.06 [7.1–14.3]	10.20 ± 7.63 [6.7–23.1]
Total bile acids (μmol/mmol Cr)	0.29 ± 0.14 [0.1–0.5]	0.47 ± 0.47 [0.1–0.4]	0.56 ± 0.33 [0.3–1.1]	0.17 ± 0.09 [0.1–0.3]	0.11 ± 0.05 [0.1–0.2]	0.14 ± 0.04 [0.1–0.2]

All data are mean ± SD (% of total bile acids). [ ], sample range. †$P < 0.05$ vs 7 days after delivery. ‡$P < 0.03$ vs 1 month after delivery, and healthy non-pregnant women. §$P < 0.05$ vs 1 month after delivery.

Table 4 Bile acid composition of umbilical cord blood and urine in newborn infants

Bile acid (Compound number in Table 1)	Umbilical cord (n = 5)	Urine (n = 5)
Primary bile acids (1,2)	70.38 ± 13.02 [51.2–86.2]	5.30 ± 2.25 [2.3–8.3]
Secondary bile acids (3,4)	5.26 ± 3.46 [2.4–10.3]	1.08 ± 0.67 [trace –1.7]
1β-Hydroxylated bile acids (5,6)	2.34 ± 0.72 [1.4–3.4]	66.44 ± 14.81† [62.1–80.3]
Hyocholic acid (7)	5.92 ± 9.28 [0.9–22.5]	4.72 ± 2.25 [2.3–8.3]
Ursodeoxycholic acid (8)	7.50 ± 6.22 [4.2–18.6]	0.76 ± 0.87 [trace –2.1]
3β-Hydroxylated bile acids (9–11)	2.46 ± 1.26 [1.0–4.4]	4.34 ± 1.49 [2.9–6.4]
3-Oxo-Δ^4-bile acids (12,13)	1.36 ± 2.38 [trace –5.6]	16.56 ± 14.45 [6.3–40.3]
Total bile acids	3.82 ± 1.35 (μmol/L) [2.1–5.7]	2.14 ± 0.97‡ (μmol/mmolCr) [1.0–3.5]

All data are mean ± SD (% of total bile acids); [], sample range. †$P < 0.001$ vs 7 days and 1 month after delivery, and healthy non-pregnant women in Table 3. ‡$P < 0.001$ vs 30 and 35 weeks of gestation, at delivery, 7 days and 1 month after delivery, and healthy non-pregnant women in Table 3.

concentration of total bile acids excreted was higher before delivery and at delivery than after delivery or in healthy non-pregnant women.

In newborn infants (Table 4), main bile acids in umbilical cord blood and urine were primary and 1β-hydroxylated bile acids, respectively. We also detected large amounts of unsaturated ketonic bile acids in urine, and the concentration of urinary total bile acids was elevated compared to pregnant and healthy non-pregnant women.

Discussion

We previously reported that the fetus synthesized large amounts of unusual bile acids, such as 1β-hydroxylated and unsaturated ketonic bile acids, and that these compounds were conveyed from fetus to mother by placental transfer. We also suggested that pregnant women may excrete large amounts of bile acids into the urine to control the serum concentration of bile acids in the fetus.[16] Moreover, during the perinatal period, unusual bile acids probably facilitate bile acid excretion, and bile acid metabolism in both mothers and infants changes significantly after birth.[7]

In this study, the serum bile acid composition in women before and after delivery showed primary bile acids as the main bile acids, and the serum concentration of total bile acids was essentially the same in each group during this study. As before, the overall findings indicated that the large amounts of bile acids excreted into the urine by pregnant women would act to control the serum concentration of bile acids in both fetus and mother.[16] Specifically, the proportion of 1β-hydroxylated bile acids as well as total bile acids were increased in urine from pregnant women between 30 weeks of gestational age and delivery. These increased urinary bile acids reflect the presence of subclinical intrahepatic cholestasis resulting from pregnancy. Bile acids in urine obtained from pregnant women before delivery, including mainly 1β-hydroxylated bile acids, is significantly higher than amounts seen in mothers after delivery. Also, total urinary bile acid concentrations before delivery and at delivery were higher than after delivery. Further, in urine of women after delivery, the main bile acids consisted of primary bile acids. The changes in urinary bile acids during pregnancy may facilitate excretion of fetal bile acids by the maternal kidney.

The main bile acids in umbilical cord blood consisted of primary bile acids, while the main bile acid composition in urine from newborn infants consisted of 1β-hydroxylated and unsaturated ketonic bile acids; the urinary data are in agreement with our previous reports.[5,6,14,15] Also interesting is the finding that unsaturated ketonic bile acids in umbilical cord blood were increased more than in maternal blood. This finding supports placental transfer of unsaturated ketonic bile acid from fetus to mother. These toxic bile acids were not only excreted into amniotic fluid by fetal urine but additionally into maternal urine after placental transfer to the mother.

A high concentration of unsaturated ketonic bile acids (3-oxo-Δ^4 bile acids) in urine has been associated with deficiency in, or reduction of, 3-oxo-Δ^4-steroid 5β-reductase (5β-reductase) activity, an enzyme that catalyzes conversion of 3-oxo-Δ^4 C27 sterol intermediates to 3-oxo-5β products in the classical pathway for primary bile acid synthesis.[17] In this study, we detected large amounts of 3-oxo-Δ^4 bile acids in the urine of pregnant women and newborn infants. This condition reflects normal development of bile acid metabolism, including the initial immaturity of hepatic enzymes, such as 5β-reductase, in fetuses and newborn infants. At 1 month after delivery and in healthy non-pregnant women, we detected only a small amount of 3-oxo-Δ^4 bile acids in urine, probably arising from actions of bacterial flora.[18]

Polyhydroxylated bile acids, such as 1β-hydroxylated bile acids, and 3-oxo-Δ^4 bile acids, are excreted readily in the urine after these fetally synthesized bile acids are transported from hepatocytes to the sinusoids by multidrug resistance-associated protein 3, which is localized within hepatocytes along the basolateral membrane.[19] Once these bile acids reach the kidney via the circulation, they are rapidly excreted into the urine.

In conclusion, the fetus synthesizes unusual bile acids, such as 1β-hydroxylated and unsaturated ketonic bile acids, which are then transported from fetus to mother. Maternal urinary excretion of these bile acids maintains low concentrations of serum bile acids in both fetus and mother.

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原　著

肝障害・難治性下痢を契機に発見されたミトコンドリア呼吸鎖Ⅰ異常症

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要　旨

症例は7か月男児，在胎27週1日，出生体重786g，Apgar score：6点/8点にて出生した．日齢60に壊死性腸炎を発症し人工肛門を造設，残存小腸は約50cmであった．成分栄養剤で経腸栄養していたが，水様性下痢が持続した．体重増加は高カロリー輸液（TPN）に依存していた．日齢132に人工肛門閉鎖術を施行し，残存小腸は約35cmとなった．徐々に胆汁うっ滞・肝障害が進行し，TPNの合併症と考えられたため，日齢182 TPNを中止した．しかし，各種治療に反応せず，TPN中止後の体重増加は不良であった．日齢231に肝生検を行い，酵素診断とBN-PAGEにより，ミトコンドリア呼吸鎖複合体Ⅰ欠損症（ComplexⅠ欠損）と診断した．

本症例では，TPNによる肝障害や腸管切除に伴う水様性下痢を考え，診断にたどり着くまで時間を要した．血清乳酸値の上昇やアシドーシスを認めない場合でも肝障害，難治性下痢の原因として本症を鑑別におくことは重要と考えられた．

キーワード：ミトコンドリア呼吸鎖異常症，肝不全，難治性下痢

緒　言

ミトコンドリア呼吸鎖異常症は約5,000人に1人の頻度で発症する疾患で，あらゆる年齢に発症し，その症状は多彩である[1]．本邦におけるミトコンドリア呼吸鎖異常症の診断は，臨床所見，組織所見，ミトコンドリアDNAの遺伝子検索を中心にして行われてきた．数年前よりミトコンドリア呼吸鎖複合体酵素活性の測定が行われるようになり，ミトコンドリア呼吸鎖異常症の報告例も散見されている[2][3]．しかし，本邦におけるミトコンドリア呼吸鎖異常症の報告例はまだ少ない．ComplexⅠ欠損症は臨床的に脳卒中様症状を伴うミトコンドリア脳筋症（Mitochondrial myopathy，Encephalopathy，Lactic Acidosis，Stroke-like episodes；MELAS）を呈するものが多く，難治性下痢を呈する症例は少ない[4][5]．本症例は難治性下痢・肝障害を伴った興味深い症例であったため報告する．

症　例

7か月，男児

主訴：持続する下痢，黄疸

周産期歴：母は初産婦．在胎25週2日，切迫早産のため近医に入院．在胎26週0日，発熱，炎症反応上昇を認めたため，羊膜絨毛膜炎（CAM）を考え抗菌薬の投与，子宮収縮抑制を開始したが変動一過性徐脈が頻発し，在胎27週1日，緊急帝王切開術となった．

現病歴：在胎27週1日，出生体重786g（－1.5SD），Apgar score 6点（1分値）/8点（5分値）緊急帝王切開にて出生．呼吸状態は良好で，nasal-DPAP（酸素投与なし）により管理可能であった．早産低出生体重児の管理目的にてNICU入院となった．

家族歴：肝疾患や中枢神経疾患の家族歴なし．血族結婚なし．

臨床経過（図1）：日齢1より母乳栄養を開始．胃残が多いため日齢9に上部・下部消化管造影を施行したが器質的異常は認めなかった．日齢60に壊死性腸炎（necrotizing enterocolitis；NEC）を合併し，絶食，抗生剤およびγ-グロブリンで加療した．日齢65にNECによる腸管穿孔のため小腸部分切除（切除小腸約38cm，残存小腸約50cm，回盲弁あり）と人工肛門造設を施行した．術後はエレンタールPにて経腸栄養を再開したが，水様性下痢が持続し，体重増加は高カロリー輸液（TPN）に依存していた．輸液は最高糖濃度20%，アミノ酸（プレアミンP）3.0g/kg/day，脂肪製剤（イントラリポス20%）2.5g/kg/dayまで投与していた．

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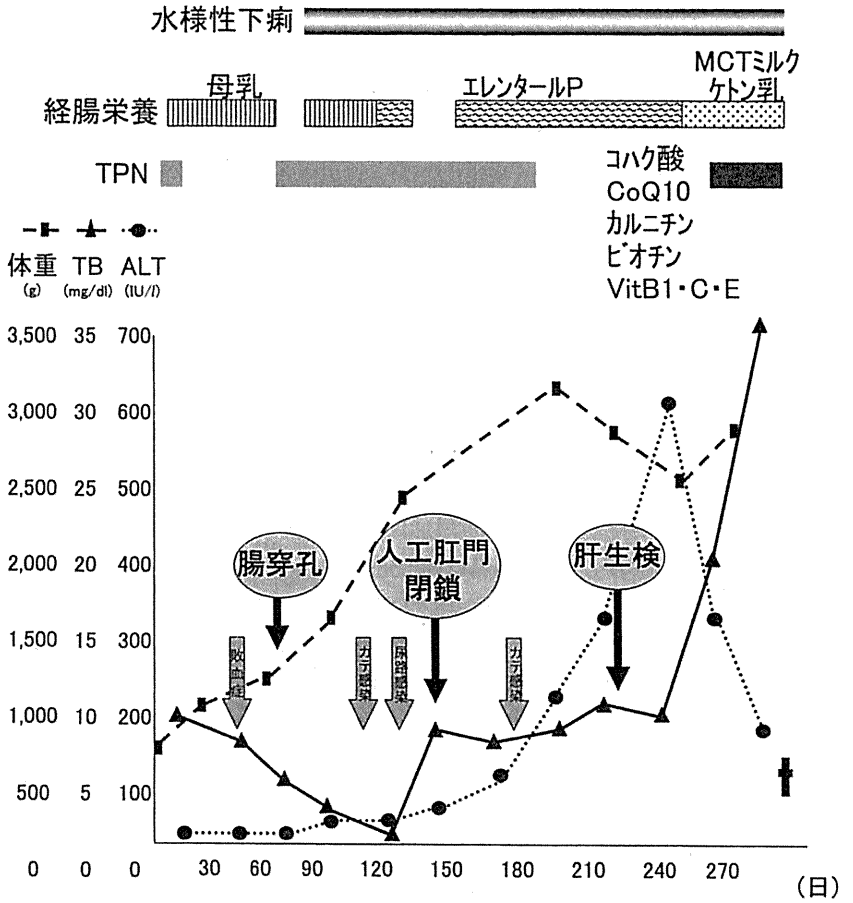

図1　臨床経過

糖濃度は経腸栄養量に応じて14%までを適宜変更していた．日齢132人工肛門の腸管脱出と絞扼が著明となり，人工肛門閉鎖術を施行（拡張腸管を約15cm切除，残存小腸約35cm，回盲弁あり）．その後も水様性下痢が持続し，TPNを継続していたが，徐々に胆汁うっ滞・肝障害・血小板減少・凝固障害が進行し，日齢182 TPNによる肝硬変と判断しTPNを中止した．TPN中止後も水様性下痢は持続し，シンバイオティックス，グルタミン，食物繊維，止痢剤などの加療に不応で，体重増加は不良であった．短腸症候群や長期TPNによる肝障害以外の原因検索を行った．

身体所見（生後7か月）：意識清明で，大泉門は5mm×5mm，平坦．胸部は呼吸音清，心音整，心雑音は認めず．腹部は軟で膨満し，肝を4cm触知，脾を1cm触知した．皮膚は黄染あり，下腹部に皮下静脈怒張を認めた．出血斑は認めず．筋力低下や痙攣は認めず．

黄疸と肝脾腫，腹壁静脈怒張から，肝硬変を示唆する所見であった．

検査所見（表1）：血液一般では血小板減少を認めた(PLT 9.7万/mm^3)．肝機能検査ではビリルビン，トランスアミナーゼ，胆道系酵素，総胆汁酸の上昇を認めた(AST 410IU/*l*，ALT 289IU/*l*，LDH 308IU/*l*，ALP 1,799IU/*l*，TB 14.4mg/dl，DB 9.2mg/dl，γ-GTP 84IU/*l*，総胆汁酸112μmol/*l*)．低蛋白血症，腎機能障害，電解質異常は認めなかった．凝固機能検査ではプロトロンビン時間，ヘパプラスチンテスト等の低下を認めた(PT 56%，ヘパプラスチンテスト41%，フィブリノーゲン102mg/dl，ATIII 40%)．代謝性アシドーシス，乳酸，アンモニア上昇など先天代謝異常を疑う所見は認めなかった．

肝線維化マーカーは高値で(ヒアルロン酸745ng/*l*，P-III-P 5.0U/ml，VI型コラーゲン7S 16mg/ml)，胆汁うっ滞・肝硬変を示唆する所見であった．ウイルス学的検査ではサイトメガロウイルスIgMが陽性であったが，白血球中のDNAは陰性で，活動性ではなく既感染が考えられた．

骨髄検査は特記すべき所見を認めなかった．血清・尿中アミノ酸分析，尿中胆汁酸分析，尿中有機酸分析などを行ったが，いずれも特異所見は得られなかった．

便検査では培養検査を数回行い有意菌の検出は認めなかった．便浸透圧は319mOsm/kg/H_2O，Na 5mEq/*l*以下，K 15.8mEq/*l*であった．便中に脂肪やでんぷんが検出され吸収不良がみられた．絶食中も水様性下痢であったが，検査上は浸透圧性下痢の所見であった．

表1　検査結果

血液一般		凝固機能	
WBC	13,300 /mm³	PT	56 %
Hb	11.9 g/dl	APTT	46 秒
PLT	9.7 万 /mm³	HPT	41 %
		Fibrinogen	102 mg/dl
血液生化学		ATIII	40 %
AST	410 IU/l		
ALT	289 IU/l	血液ガス（静脈血）	
LDH	308 IU/l	pH	7.387
TB	14.4 mg/dl	pCO_2	37.3 mmHg
DB	9.2 mg/dl	HCO_3^-	21.9 mmol/l
γ-GTP	84 IU/l	BE	−2.3 mmol/l
TBA	112 μmol/l	Lac	2.2 mmol/l
TP	5.7 g/dl		
Alb	3.7 g/dl	ウイルス学的検査	
BUN	17 mg/dl	CMV IgM	(+)
Cr	0.16 mg/dl	CMV IgG	(+)
Na	138 mEq/l	DNA	<2.0×10 copies/10^6 cells
K	4.0 mEq/l	HSV IgM	(−)
Cl	106 mEq/l	風疹 IgM	(−)
NH_3	48 μg/dl	EBV VCA-IgM	(−)
乳酸	11.3 mg/dl	パルボウイルス B19 IgM	(−)
ピルビン酸	0.79 mg/dl	HBs 抗原	(−)
Fe	73 μg/dl	HCV 抗体	(−)
フェリチン	176 ng/ml	HTLV-1 抗体	(−)
トランスフェリン	140 mg/dl	HIV 抗体	(−)
Cu	64 μg/dl		
抗核抗体	<40 倍	代謝学的検査	
セルロプラスミン	17 mg/dl	極長鎖脂肪酸分析	
α_1-アンチトリプシン	142 mg/dl	アシルカルニチン分析	
酸性フォスファターゼ	37.4 IU/l	血中アミノ酸分析	
総カルニチン	16.5 mmol/l	尿中アミノ酸分析	
ACE	16 U/l	尿中脂肪酸分析	
ヒアルロン酸	745 ng/ml	尿中有機酸分析	
P-III-P	5.0 U/ml	いずれも特記すべき所見なし	
IV 型コラーゲン 7S	16 ng/ml		

以上の臨床経過と検査所見より肝硬変徴候と機能的短腸症候群と診断した．肝臓と腸管の2臓器の障害が存在すると判断し，ミトコンドリア呼吸鎖異常症の鑑別のために，日齢231に肝生検を施行した．

肝生検組織所見（日齢231）：光顕・HE染色（図2A，B）では，胆汁栓や小葉辺縁での細胆管の増生，肝細胞壊死，肝細胞のballooning，偽小葉の形成を認めた．電顕（図2C）所見では，肝細胞中のミトコンドリア数の増加とミトコンドリアの腫大を認めた．

呼吸鎖複合体酵素活性，イムノブロット解析及び免疫染色：日齢231に肝生検時に肝・大腸組織材料を，剖検時に肝・心筋組織材料を採取した．

呼吸鎖複合体（I〜IV）の酵素活性は，生検材料のホモジネート液600g分の上清を用いて分析した[4)6)]．それぞれの複合体酵素活性は，12人の健康コントロールの平均値と比較したパーセント値と，ミトコンドリア酵素のマーカーであるcitrate synthase（CS），あるいは複合体IIに対するパーセント値で算出した．

肝組織中のComplex Iの発現レベルについては，イムノブロットによるBlue-Native電気泳動法（BN-PAGE）により解析した[7)8)]．ウェスタンブロットはComplex Iは30kDサブユニット，Co IIは70kDサブユニット，Co IIIはコアサブユニット，Co IVはCOX 1サブユニットのそれぞれに対する特異的抗体を用いて行った．大腸粘膜におけるComplex Iの発現については免疫染色を行った[2)]．Complex Iは30kDサブユニット，Co IIは70kDサブユニットに対するモノクローナル抗体を一次抗体として用いた．

ミトコンドリア呼吸鎖複合体酵素活性（表2）：肝生検時，剖検時の肝組織を用いた酵素診断ではいずれも

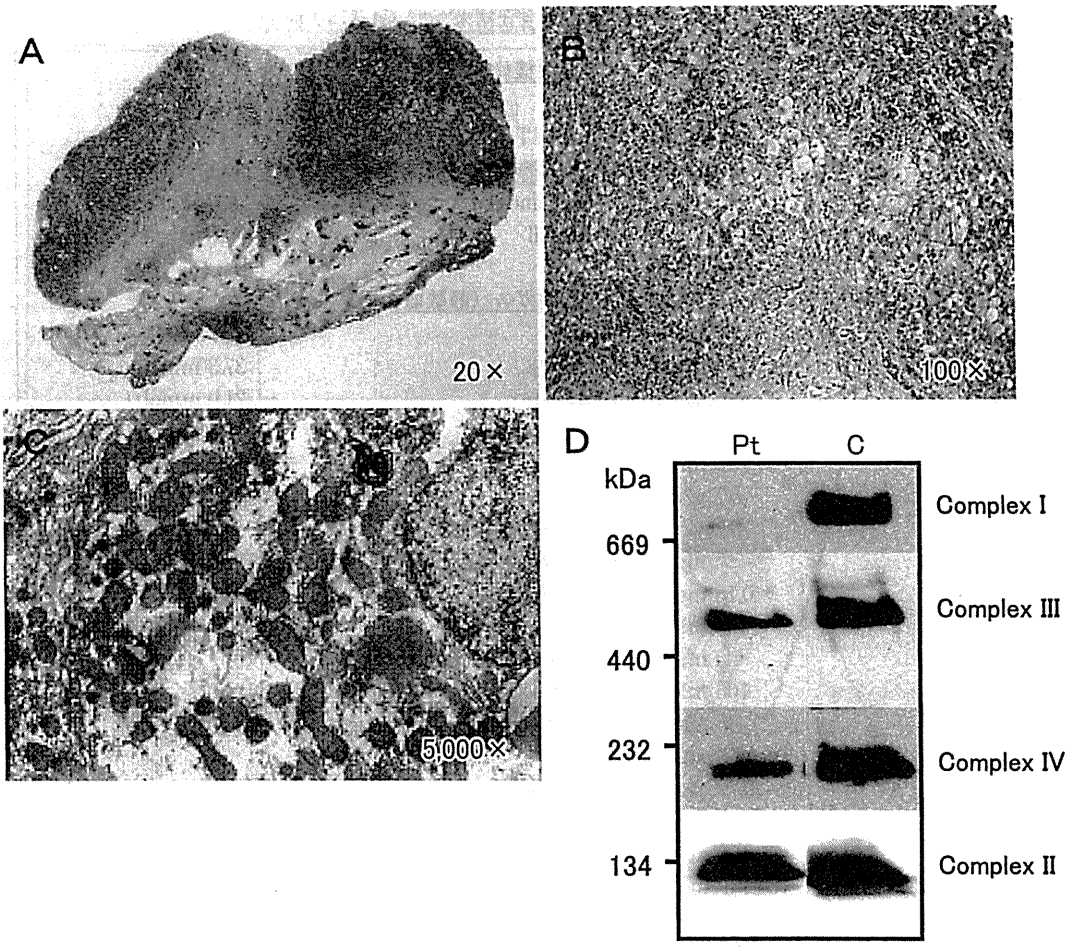

図2　肝臓組織所見（光顕・電顕・BN-PAGE解析・免疫染色）
日齢231時点での肝生検組織所見．A，B（光顕所見）：胆汁栓や小葉辺縁での細胆管の増生が見られ，肝細胞壊死，ballooningを認めた．また偽小葉の形成も認められた．C（電顕所見）：肝細胞中のミトコンドリア数の増加とミトコンドリアの腫大を認めた．D（肝臓を用いたBN-PAGE解析）：Pt患児，C control：Complex Iのシグナルが明らかに低下していた．

Complex Iの酵素活性の低下を認めた．剖検時の心筋組織を用いた酵素診断ではミトコンドリア呼吸鎖複合体酵素活性の低下は認めなかった．

BN-PAGE解析：肝臓を用いたBN-PAGE解析ではComplex Iのシグナルが明らかに低下していた（図2D）．

回腸組織所見（日齢132）：光顕所見では，異常所見は認めなかった(データ未提示)．Complex I/II免疫染色（図3A）では，Complex Iシグナルの低下を部分的に認めた．

大腸組織所見（日齢231）：光顕所見では，異常所見は認めなかった（データ未提示）．また免疫染色（図3D）でも，Complex Iの発現を認めた．

以上の結果より，肝臓でのComplex I活性低下を証明し得たため，Complex I欠損症と確定診断した．回腸のComplex Iシグナル低下を部分的に認めた．一方で大腸でのComplex I抗原の明らかな欠損は認めなかった．

診断確定後，ミトコンドリア呼吸鎖を補助する目的で，MCTミルク・ケトン乳へ変更し，CoQ10，コハク酸，カルニチン，ビオチンなどビタミン製剤の強化を行った．肝小腸同時移植が国内では極めて困難だったため，小腸機能改善後肝移植を目指した．しかし，治療変更後も症状は改善せず，水様性下痢が持続，肝不全も進行し，日齢299に永眠した．

考　察

ミトコンドリア呼吸鎖異常症は最も頻度の高い先天代謝異常症で，頻度は出生5,000人に1人と言われている[1]．その障害臓器は多様であるため，様々な症状を呈する．ミトコンドリア呼吸鎖異常症の特性として，ミトコンドリア遺伝子と核遺伝子の双方の異常に影響されるため病因遺伝子の検索が難しい．そこで本疾患の診断には遺伝学的アプローチだけなく，生化学的ア

表2　ミトコンドリア呼吸鎖酵素活性

肝生検時（生後7か月時）

Liver	Co I	Co II	Co III	Co IV	CS
% of normal	**34.2**	87.5	75.1	80.1	181.1
CS ratio (%)	**18.6**	48.0	40.8	43.3	
Co II ratio (%)	**38.6**		85.2	90.1	

剖検時（生後9か月時）

Liver	Co I	Co II	Co III	Co IV	CS
% of normal	**14.1**	64.5	85.7	95.7	107.7
CS ratio (%)		59.5	78.3	87.0	
Co II ratio (%)	**21.7**		131.9	146.1	

Heart	Co I	Co II	Co III	Co IV	CS
% of normal	103.3	95.4	90.8	63.7	72.3
CS ratio (%)	136.3	132.7	122.6	85.0	
Co II ratio (%)	91.7		84.6	58.1	

Co I；Complex I, Co II；Complex II, Co III；Complex III, Co IV；Complex IV, CS；citrate synthase, 太字は活性の低下を示す.

プローチとしてミトコンドリア呼吸鎖複合体酵素活性の測定が数年前より本邦で実施され，診断例が散見されている[2)3)].

本児は肝障害，難治性慢性下痢のため原因検索を行ったところ，生検肝での酵素活性及びBN-PAGE解析にてミトコンドリア呼吸鎖複合体I欠損症（Complex I欠損症）と診断しえた．Complex Iは電子伝達系の最初の酵素でありエネルギー産生に寄与しているが，呼吸鎖の中で最も複雑な構造をしており，ミトコンドリアDNAにコードされた7個のサブユニットと核DNAにコードされた40以上のサブユニットから構成されている[4)]．Complex I欠損症では，本児に認められたように肝障害・肝不全や消化吸収障害，子宮内発育不全のほかに，精神発達遅滞，筋力低下，心筋症，腎不全，難聴，視覚障害などが認められる[4)]．一般的にミトコンドリア呼吸鎖異常症では高乳酸血症や特異的な筋組織所見を認めるが，Complex I欠損症では38%で乳酸値は正常，62%で正常筋組織であったと報告されている[4)]．本児においても，高乳酸血症や筋力低下など筋症状の出現はなく，村山ら[2)]の報告例でも乳酸値は正常であった．

本症例は壊死性腸炎によって2回の腸管切除を行い，残存小腸が約35cmとなった．そのため短腸症候群による胆汁酸吸収低下や胃酸分泌過多，脂肪吸収障害が慢性下痢の原因と判断していた．文献的には回盲弁を有する短腸症候群において，Goulet ら[9)]は残存小腸が40cm以上では100%，40cm以下でも86%の症例がTPNから離脱すると報告し，Dorney ら[10)]は残存小腸が25cm以上でTPNからの離脱が可能であると報告している．本症例は残存小腸が約35cmあり，多くの症例では短腸症候群に陥らない長さであるが，結果として短腸症候群になったということは，腸管でのComplex Iの発現があるにもかかわらず，その機能に異常を来していた可能性が考えられる．回腸粘膜のComplex I免疫染色の結果，Complex Iシグナルの部分的な低下が判明した．残存腸管長に比して下痢が持続した原因の一つとして，回腸でのComplex I欠損が関与している可能性が示唆された．

肝障害に関しては，長期経静脈栄養による胆汁うっ滞性肝障害，加えてカテーテル感染など種々の感染による悪化と考えていた．肝障害はTPNを行っている超低出生体重児で約50%，極低出生体重児では約10%に認められ，胆汁うっ滞は経静脈栄養児の約23%，60日を超えると80%，3か月以上だと90%の児に認められるが[11)]，一般的には胆汁うっ滞性肝硬変に至る症例は少ないとされる[11)]．

経静脈栄養による肝障害の原因としては，未熟性，経腸栄養の欠如，敗血症の反復，腸内細菌の異常増殖，カロリー過多など様々な要因が報告されている[11)]．非アルコール性脂肪肝炎（NASH）やアルコール性肝障害など種々の肝疾患の病態として，ミトコンドリア機能障害によるReactive Oxygen Species（ROS）の過剰産生が誘引となって肝細胞死を引き起こすことが考えられている[12)]．一方，壊死性腸炎の機序の一つとして酸化ストレスの関与が報告されている[13)]．すなわちROSという酸化ストレスがミトコンドリア機能不全を惹起し，腸管上皮細胞のアポトーシスを誘導することで壊死性腸炎を引き起こすとされる．このように，いずれ

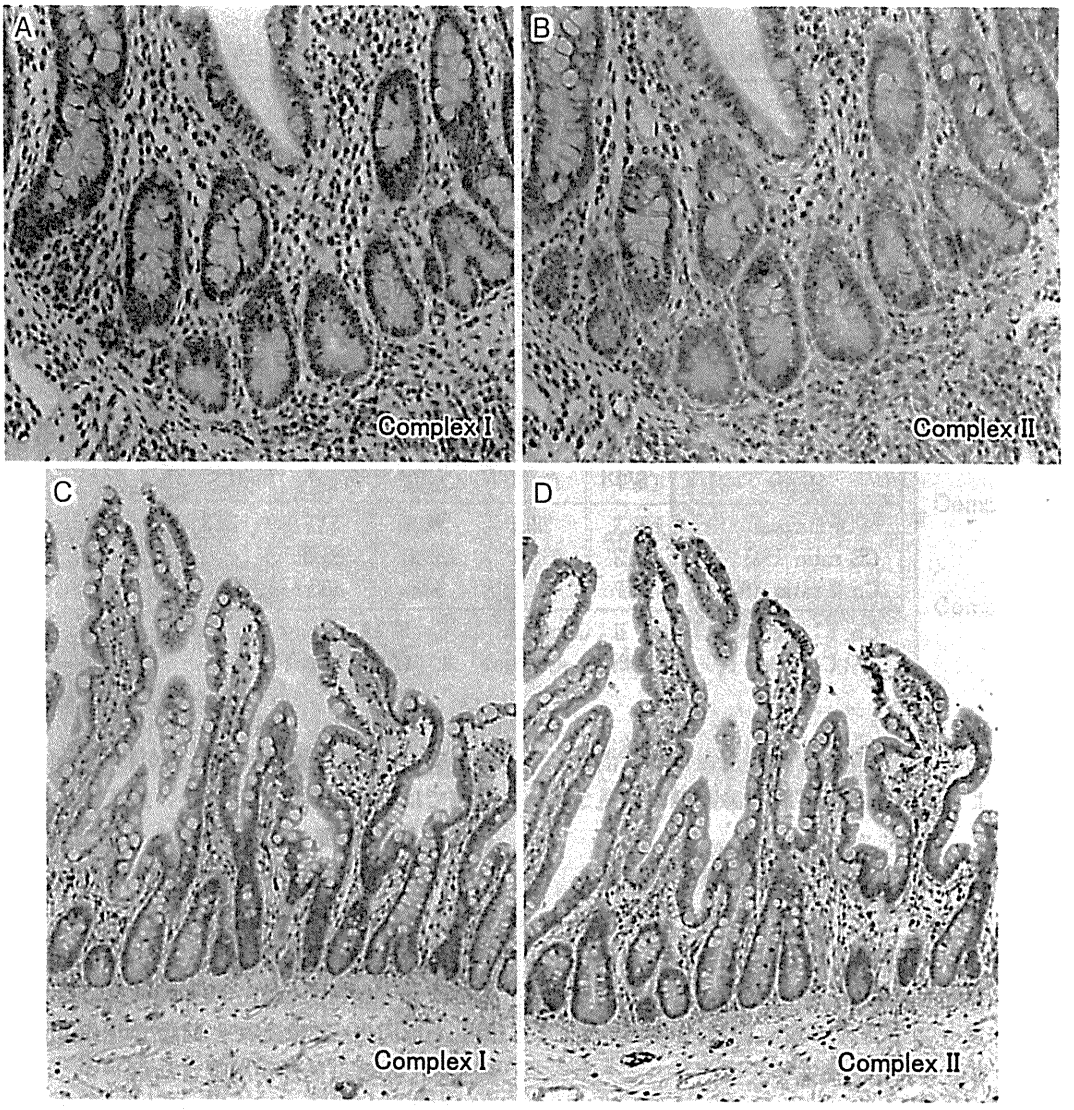

図3　回腸・大腸免疫染色所見（Complex I/II 免疫染色）
Complex I/II 免疫染色所見．A, B：回腸粘膜で Complex I シグナルが低下していた．C, D：大腸粘膜で Complex I/II の発現を認めた．

の疾患においてもミトコンドリア機能障害に伴う酸化ストレス亢進が重要であると言える．Caenorhabiditis elegans[14]や神経細胞[15]を用いた研究報告において，Complex I の活性低下により ROS 産生を増加させることが示されており，本症例では Complex I 欠損による活性低下が ROS 産生亢進を招き，壊死性腸炎の発症や急激な肝障害の進行に関与したと考えられた．

本症例ではもともと短腸症候群による下痢と TPN に伴う肝障害があったと考える．しかし，前述したように残存小腸の長さからは短腸症候群に陥いることは少ないと考えられるにもかかわらず，短腸症候群による慢性下痢を発症したことで，何らかの背景疾患を疑った．短腸症候群により TPN から離脱困難となったが，TPN 開始から胆汁うっ滞が明確になるまでに約3か月経過しており，肝障害の原因としては一般的な TPN によるものであることは否定できない．一方，Complex I 欠損症の発症時期としても，生直後から13歳までと様々であるが，平均生後8か月頃に発症すると言われている[4]．本症例もほぼ同時期に発症し，精査を行い診断にいたることができた．前述したようにミトコンドリア機能障害は肝障害の増悪因子と考えられるので，Complex I 欠損症の存在が短腸症候群による下痢と TPN に伴う肝障害の遷延・重症化に関与した可能性は否定できない．短腸症候群や TPN に伴う肝障害が重症化する症例の中には Complex I 欠損症を含むミトコンドリア呼吸鎖異常症が潜在する可能性が示唆されたので，このような症例に対しては積極的にミトコンドリア呼吸鎖異常症を診断していく必要があると考えられた．

結　論

本症例では残存小腸が約35cmであったにもかかわらず水様性下痢が持続し，肝障害の急激な悪化を認めた．TPNによる肝障害や腸管切除に伴う短腸症候群による病態を念頭において治療したものの反応せず，さらなる精査を進めたところComplex I欠損症の診断に至ることができた．血清乳酸値の上昇やアシドーシスを認めない場合でも肝障害，難治性下痢の原因として本症を鑑別におくことは重要と考えられた．またその診断には，障害臓器の生検材料によるミトコンドリア呼吸鎖複合体酵素活性の測定が有用であった．

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Mitochondrial Respiratory Chain Complex I Deficiency Manifested by Liver Damage and Intractable Diarrhea : Case Report

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A male infant, born at 27 weeks and 1 day of gestation with a birth weight of 786g with 6 at 1 min and 9 at 5 min of Apgar score, developed necrotizing enterocolitis, and colostomy was performed at 60 days old. The residual small intestine was about 50cm. Although he was treated by enteral nutrition using elemental diet, he developed persistent watery diarrhea. His weight gain was mainly dependent on total parenteral nutrition (TPN). On day 132 the colostomy was closed, and residual small intestine became about 35cm. TPN was stopped on day 182 because his cholestasis and liver dysfunction were exacerbated. After the discontinuation of TPN, weight gain became poor despite various treatments. Liver biopsy was performed on day 231. The findings of enzyme activity analysis and BN-PAGE led to the diagnosis of mitochondrial respiratory chain complex I deficiency.

In this case, we initially assumed that liver dysfunction was caused by TPN and watery diarrhea by bowel resection. Therefore, the actual diagnosis was delayed. Our case suggests that patient with unexplained liver dysfunction and watery diarrhea should consider mitochondrial respiratory chain complex disorders even if without elevated lactate level and acidosis.