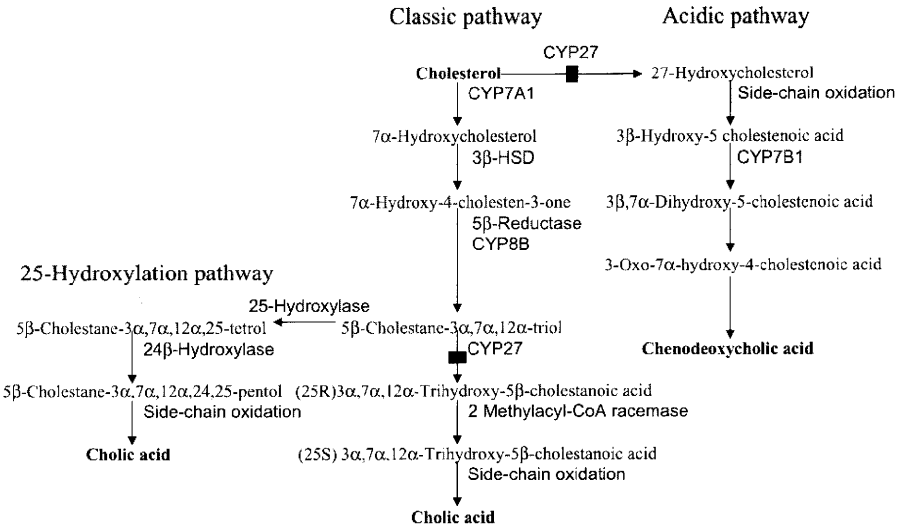


**Fig. 6** Metabolic pathways for the synthesis of primary bile acids including the classic pathway, acidic pathway, and 25-hydroxylation pathway. CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP27, cholesterol 27-hydroxylase; CYP8B, cholesterol 12α-hydroxylase; 3β-HSD, 3β-hydroxy-$\Delta^5$-$C_{27}$-steroid dehydrogenase/isomerase; 5β-reductase, 3-oxo-$\Delta^4$-steroid 5β-reductase.

after birth, from about 10% to trace amounts until about 6 months of age, when a slight increase was noted.

### *Other bile acids*

We detected very small amounts of other bile acids, such as unsaturated 3β-hydroxylated bile acids, hyocholic and ursodeoxycholic acids, accounting for less than 5% of total urinary bile acids (data not shown).

## Discussion

This investigation of urinary excretion of bile acids in 10 infants born at gestational age between 25 and 33 weeks indicated low total bile acid/Cr ratios during the first 2 to 3 weeks after birth. This may reflect limited bile acid synthesis by the liver, limited excretion of bile acids into the urine because of immaturity of renal function, or both. Thereafter, urinary excretion of total bile acids relative to that of Cr increased during the 2 to 5 months following birth, as bile acid synthesis in the liver increased at a time of persistent immaturity of transport systems contributing to the enterohepatic circulation, such as the bile salt exceed pump (BSEP), the organic anion transporting polypeptide (OATP), and the $Na^+$ taurocholate cotransporting polypeptide (NTCP).[16] In rats, RNA and protein expression for NTCP, OATP, and BSEP have been found to require several weeks to increase to equal adult expression1[17–19]; this delayed development of hepatobiliary organic anion transport systems has been linked with physiological cholestasis occurring at birth, in which the serum total bile acid concentration in healthy full-term infants significantly exceeds that seen in children older than 1 year.[20–22] As the enterohepatic circulation matures, urinary excretion of bile acids then gradually decreases (Fig. 2). Duration of physiological cholestasis is clearly longer in preterm infants than in full-term infants. In our view, persistent physiological cholestasis may be a sign of reduced mitochondrial function consequent to hypoxia with acidosis arising from respiratory dysfunction, representing a secondary mitochondrial disorder.[23]

Among usual bile acids, the principal bile acid detected was cholic acid, with usual bile acids initially accounting for 20% to 30% of total bile acids prior to a subsequent gradual decrease. If the main pathway of fetal and perinatal bile acid synthesis is the acidic pathway, large amounts of chenodeoxycholic acid should be detected (Fig. 6).[24–26] On the other hand, we believe the cholic acid presently detected to be derived from the 25-hydroxylation pathway (Fig. 6).[27–29] Because the preterm infants studied may have had low activity of mitochondrial cholesterol 27 hydroxylase reflecting respiratory dysfunction at birth, that is, RDS, the 25 hydroxylation pathway may have been a particularly important pathway of fetal and neonatal bile acid synthesis in these preterm infants.[30,31] Clayton *et al.*[32] recently suggested that early in life, side-chain cleavage in bile acid formation might proceed via the 25-hydroxylation pathway, but further studies are needed to identify the exact site of the underlying defect. Alternatively, cholic acid may have a maternal origin with transfer across the placenta,[33] then undergoing preferential excretion in the urine at the expense of chenodeoxycholic acid because a trihydroxylated bile acid is more hydrophilic than a dihydroxylated bile acid. This could account for larger amounts of urinary cholic acid than chenodeoxycholic acid detected in this study. In adults, the 25-hydroxylation pathway accounts for less than 5% of bile acid synthesis,[27] but perinatal synthesis in preterm infants may be more dependent upon the 25-hydroxylation pathway.

Relatively large amounts of 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid represented the major polyhydroxylated bile acid in the urine during this study. Formation of this bile acid is probably linked to mechanisms of bile salt excretion in healthy infants with physiological cholestasis, and possibly more so in preterm infants. Large amounts of 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid persisted for longer than 6 months in

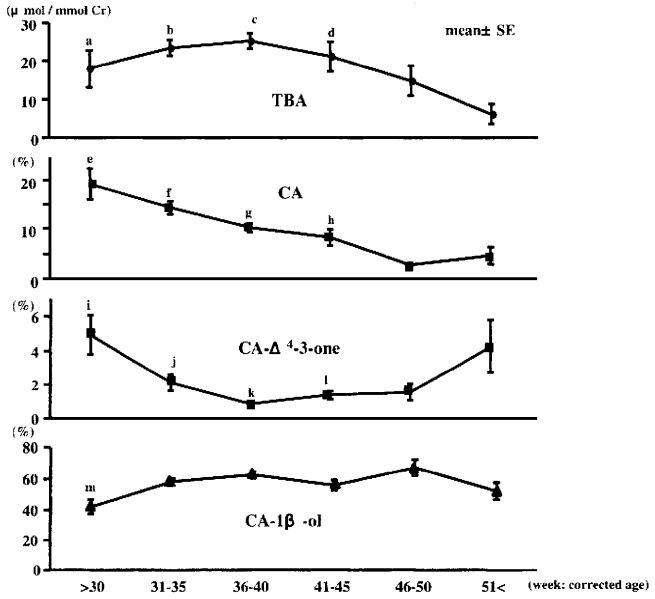


**Fig. 7** Developmental pattern of urinary excretion of bile acids in preterm infants. TBA, total bile acids (filled circle); CA, cholic acid (open squares); CA-$\Delta^4$-3-one, 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid (filled squares); and CA-1β-ol, 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (open triangles). Percentages (%) refer to the fraction of each bile acid in relation to TBA. [a] $P <$ 0.05 vs 36 to 40 weeks. [b] $P < 0.05$ vs >51 weeks. [c] $P < 0.01$ vs >51 weeks. [d] $P < 0.01$ vs >51 weeks. [e] $P < 0.01$ vs 36 to 40 weeks, 41 to 45 weeks, 46 to 50 weeks, and 51< weeks. [f] $P < 0.05$ vs 36 to 40 weeks; $P < 0.01$ vs 41 to 45 weeks, and 51< weeks; $P < 0.001$ vs 46 to 50 weeks. [g] $P < 0.01$ vs 46 to 50 weeks; $P < 0.05$ vs 51< weeks. [h] $P < 0.05$ vs 46 to 50 weeks. [i] $P < 0.05$ vs 31 to 35 weeks, and 41 to 50 weeks; $P < 0.01$ vs 36 to 40 weeks. [j] $P < 0.05$ vs 51< weeks. [k] $P$ $< 0.05$ vs 41 to 45 weeks, and 46 to 50 weeks. [l] $P < 0.05$ vs 51< weeks. [m] $P < 0.01$ vs 31 to 35 weeks, and 46 to 50 weeks; $P < 0.001$ vs 36 to 40 weeks; $P < 0.05$ vs 41 to 45 weeks. Cr, creatinine.

preterm infants, in contrast to about 3 months in healthy full-term infants.[11] Accordingly, physiological cholestasis can persist in preterm infants for more than 6 months postnatally, more than twice as long as in full-term healthy infants (Fig. 2).

We detected trace amounts of unsaturated ketonic and 3β-hydroxy-$\Delta^5$-bile acids, such as 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic and 3β-hydroxy-5-cholen-24-oic acids, in the urine of the preterm infants. Full-term infants have been found to excrete large amounts of 3-oxo-$\Delta^4$ bile acids in urine because activity of 3-oxo-$\Delta^4$-steroid 5β-reductase in the normal "classic" pathway for primary bile acid synthesis is relatively low during the early neonatal period.[7,8] Accordingly, our results in preterm infants differed from the normal developmental sequence of bile acid metabolism. The major pathway of bile acid metabolism in the fetus and newborn infant is the acidic pathway, involving 27-hydroxylation, until about 1 month of postnatal age.[24–26] However, our results reflected relatively low 27-hydroxylation. According to a previous report,[24] production of 27-hydroxycholesterol accounts for about 10% of bile acid synthesis in adults. Our results suggest that 25-hydroxylation may be an important pathway in the neonatal period, especially in preterm infants at approximately 30 weeks corrected age.

We determined the developmental pattern of each bile acid, such as 1β-hydroxylated bile acid and ketonic bile acid, and total bile acids (Figs 7,8). These determinations made clear that the developmental pattern of urinary bile acid profile in preterm infants differs from that of full-term infants,[11] although one should note that preterm infants in this study had complications. This suggests that bile acid metabolism is likely to differ between preterm and full-term infants.

In conclusion, we have identified metabolic differences likely to underlie prolonged physiological cholestasis in preterm infants. Specifically, we detected large amounts of cholic and 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acids in urine from preterm infants, leading us to suspect that the 25-hydroxylation pathway may be particularly important for bile acid synthesis in these infants.

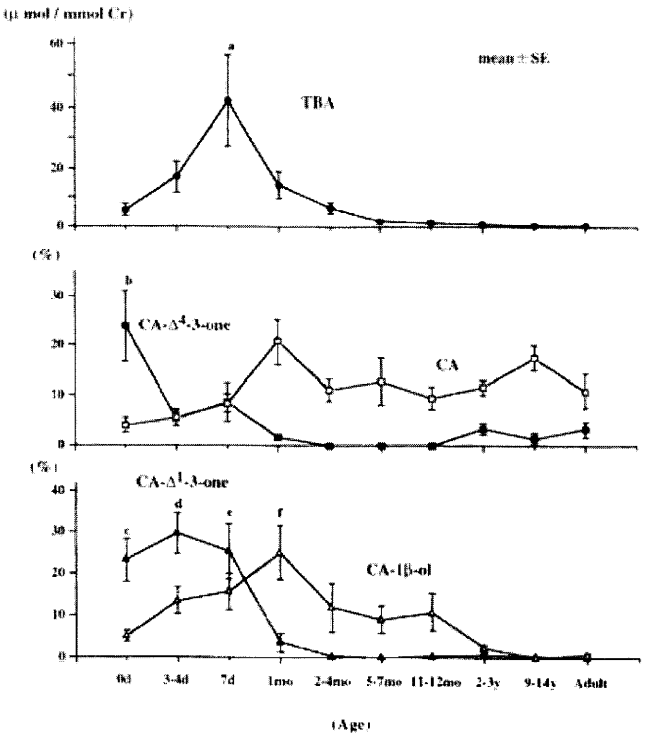


**Fig. 8** Developmental pattern of urinary excretion of bile acids in full-term infants. TBA, total bile acids (filled circle); CA, cholic acid (open squares); CA-$\Delta^4$-3-one, 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid (filled squares); CA-1β-ol, 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (open triangles); and CA-$\Delta^1$-3-one, 7α,12α-dihydroxy-3-oxo-5β-cholen-24-oic acid (filed squares). Percentages (%) refer to the fraction of each bile acid in relation to TBA. [a] $P <$ 0.05 vs 0 d, 2 to 4 months; $P < 0.01$ vs 5 to 7 months, 1 to12 months, 2 to 3 years, 9 to 14 years, and adults. [b] $P < 0.05$ vs 3 to 4 d; $P < 0.01$ vs 1 month, 2 to 4 months, 5 to 7 months, 11 to 12 months, 2 to 3 years, 9 to 14 years, and adults. [c] $P < 0.05$ vs 2 to 4 months, 5 to 7 months, 11 to12 months, 2 to 3 years, 9 to 14 years; $P < 0.01$ vs adults. [d] $P < 0.01$ vs 1 month; $P < 0.001$ vs 2 to 4 months, 5 to 7 months, 11 to12 months, 2 to 3 years, 9 to 14 years, adults. [e] $P < 0.05$ vs 1 month; $P < 0.01$ vs 2 to 4 months, 5 to 7 months, 11 to 12 months, 2 to 3 years, 9 to 14 years, adults. [f] $P < 0.05$ vs 2 to 3 years, 9 to 14 years, adults. Cr, creatinine.

## References


1 Zimniak P, Lester R. Bile acid metabolism in perinatal period. In: Lebenthal E (ed). *Human Gastrointestinal Development*. Raven Press, New York, 1989; 561–80.
2 Setchell KDR, Dumaswala R, Colombo C, Ronchi M. Hepatic bile acid metabolism during early development revealed from the analysis of human gallbladder bile. *J. Biol. Chem.* 1988; **263**: 16637–44.
3 Shoda J, Mahara R, Osuga T *et al.* Similarity of unusual bile acids in human umbilical cord and amniotic fluid from newborns and sera and urine from adult patients with cholestatic liver disease. *J. Lipid Res.* 1988; **29**: 847–59.
4 Shoda J, Osuga T, Matsuura K *et al.* Concurrent occurrence of 3β,12α-dihydroxy-5-cholenoic acid associated with 3β-hydroxy-5-cholenoic acid and their preferential urinary excretion in liver disease. *J. Lipid Res.* 1989; **30**: 1233–42.
5 Nakagawa M, Setchell KDR. Bile acid metabolism in early life: Studies of amniotic fluid. *J. Lipid Res.* 1990; **31**: 1089–98.
6 Kimura A, Yamakawa R, Ushijima K *et al.* Fetal bile acid metabolism during infancy: Analysis of 1β-hydroxylated bile acids in urine, meconium, and feces. *Hepatology* 1994; **20**: 819–24.
7 Inoue T, Kimura A, Aoki K, Tohma M, Kato H. Developmental pattern of 3-oxo-$\Delta^4$ bile acids in neonatal bile acid metabolism. *Arch. Dis. Child.* 1997; **77**: F52–6.
8 Yamato Y, Kimura A, Inoue T, Kurosawa T, Kato H. Fetal bile acid metabolism: Analysis of urinary 3β-monohydroxy-$\Delta^5$ bile acid on preterm infants. *Biol. Neonate* 2001; **80**: 19–25.
9 Ushijima K, Kimura A, Inokuchi T *et al.* Placental transport of bile acids: Analysis of bile acids in maternal serum and urine, umbilical cord blood, and amniotic fluid. *Kurume Med. J.* 2001; **48**: 87–91.
10 Maeda K, Kimura A, Yamato Y, Matsuishi T. Perinatal bile acid metabolism: Analysis of urinary unsaturated ketonic bile acids in preterm and full-term infants. *Acta Paediatr.* 2003; **92**: 216–20.
11 Kimura A, Mahar R, Inoue T *et al.* Profile of urinary bile acids in infants and children: Developmental pattern of excretion of unsaturated ketonic bile acids and 7β-hydroxylated bile acids. *Pediatr. Res.* 1999; **45**: 603–9.
12 Kumagai M, Kimura A, Takei H *et al.* Perinatal bile acid metabolism: Bile acid analysis of meconium of preterm and full-term infants. *J. Gastroenterol.* 2007; **42**: 904–10.
13 Tohma M, Mahara R, Takeshita H, Kurosawa T, Ikegawa S. Synthesis of the 1β-hydroxylated bile acids, unusual bile acids in human biological fluids. *Chem. Pharm. Bull.* 1986; **34**: 2890–9.
14 Tohma M, Mahara R, Takeshita H, Kurosawa T. A convenient synthesis of 3β,12α-, 3β,7α-, and 3β,7β-dihydroxy-5-cholen-24-oic acids: Unusual bile acids in human biological fluids. *Steroids* 1986; **48**: 331–8.
15 Leppik RA. Improved synthesis of 3-keto, 4-ene-3-keto, and 4,6-diene-3-keto bile acids. *Steroids* 1983; **41**: 475–84.
16 Chen H-L, Chen H-L, Liu Y-J *et al.* Developmental expression of canalicular transporter genes in human liver. *J. Hepatol.* 2005; **43**: 472–7.
17 Hardikar W, Ananthanarayanan M, Suchy FJ. Differential ontogenic regulation of basolateral and canalicular bile acid transport proteins in rat liver. *J. Biol. Chem.* 1995; **270**: 20841–6.
18 Zinchuk VS, Okada T, Akimura K, Seguchi H. Asynchronous expression and colocalization of Bsep and Mrp2 during development of rat liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2002; **282**: G540–8.
19 Gao B, St. Pierre M, Steiger B, Neier PJ. Differential expression of bile salt and organic anion transporters in developing rat liver. *J. Hepatol.* 2004; **41**: 201–8.
20 Lester R. Physiological cholestasis. *Gastroenterology* 1980; **78**: 864–5.
21 Suchy FJ, Balistreri WF, Heubi JE, Searcy JE, Levin RS. Physiological cholestasis: Elevation of the primary serum bile acid concentration in normal infants. *Gastroenterology* 1981; **80**: 1037–41.
22 Balistreri WF, Heubi JE, Suchy FJ. Immaturity of the enterohepatic circulation in early life: Factors predisposing to "physiologic" maldigestion and cholestasis. *J. Pediatr. Gastroenterol. Nutr.* 1983; **2**: 346–54.
23 Lee WS, Sokol RJ. Liver disease in mitochondrial disorders. *Semin. Liver Dis.* 2007; **27**: 259–73.
24 Setchell KDR, Schwarz M, O' Connell NC *et al.* Identification of a new inborn error in bile acid synthesis: Mutation of the oxysterol 7α-hydroxylase gene causes severe neonatal liver disease. *J. Clin. Invest.* 1998; **102**: 1690–703.
25 Schwarz M, Lund EG, Russell DW. Two 7α-hydroxylase enzymes in bile acid biosynthesis. *Curr. Opin. Lipidol.* 1998; **9**: 113–18.
26 Duane WC, Javitt NB. 27-hydroxycholesterol: Production rates on normal human subjects. *J. Lipid Res.* 1999; **40**: 1194–9.
27 Duane WC, Pooler PA, Hamilton JN. Bile acid synthesis in man: In vivo activity of the 25-hydroxylation pathway. *J. Clin. Invest.* 1988; **82**: 82–5.
28 Nakagawa M, Une M, Takenaka S, Kuramoto T, Abukawa D, Iinuma K. Urinary bile alcohol profile in infants with intrahepatic cholestasis: Identification of 5β-cholestane-3α,7α,24,25-tetrol. *Acta Paediatr.* 1999; **88**: 1078–82.
29 Axelson M, Ellis E, Mork B *et al.* Bile acid synthesis in cultured human hepatocytes: Support for an alternative biosynthetic pathway to cholic acid. *Hepatology* 2000; **31**: 1305–12.
30 Heubi JE, Setchell KDR, Bove KE. Inborn error of bile acid metabolism. *Semin. Liver Dis.* 2007; **27**: 282–94.
31 Nakagawa M, Une M, Takenaka S *et al.* Urinary bile alcohol in healthy and cholestatic children. *Clin. Chim. Acta* 2001; **314**: 101–6.
32 Clayton PT, Casteels M, Mieli-Vergani G, Lawson AM. Familial giant cell hepatitis with low bile acid concentrations and increased urinary excretion of specific bile alcohols: A new inborn error of bile acid synthesis? *Pediatr. Res.* 1995; **37**: 424–31.
33 Colombo C, Roda A, Roda R *et al.* Correlation between fetal and maternal serum bile acid concentration. *Pediatr. Res.* 1985; **19**: 227–31.

0031-3998/10/6803-0258
PEDIATRIC RESEARCH

Vol. 68, No. 3, 2010
*Printed in U.S.A.*

# Molecular Genetic and Bile Acid Profiles in Two Japanese Patients With 3β-Hydroxy-$\Delta^5$-$C_{27}$-Steroid Dehydrogenase/ Isomerase Deficiency

TATSUKI MIZUOCHI, AKIHIKO KIMURA, ISAO UEKI, TOMOYUKI TAKAHASHI, TAKUJI HASHIMOTO, AKIRA TAKAO, YOSHITAKA SEKI, HAJIME TAKEI, HIROSHI NITTONO, TAKAO KUROSAWA, AND TOYOJIRO MATSUISHI

*Department of Pediatrics and Child Health [T.M., A.K., I.U., T.T., Y.S., T.M.], Division of Gene Therapy and Regenerative Medicine [T.T.], Kurume University School of Medicine, Kurume 830-0011, Japan; First Department of Pediatrics [T.H.], Toho University School of Medicine, Ohta-ku 143-8541, Japan; Division of Pediatrics [A.T.], Gifu Municipal Hospital, Gifu 500-8513, Japan; Institute of Bile Acid [H.T., H.N.], Junshin Clinic, Meguro-ku 152-0011, Japan; Faculty of Pharmaceutical Sciences [T.K.], Health Sciences University of Hokkaido, Ishikari-Tobetsu 061-0200, Japan*

**ABSTRACT:** We report definitive diagnosis and effective chenodeoxycholic acid (CDCA) treatment of two Japanese children with 3β-hydroxy-$\Delta^5$-$C_{27}$-steroid dehydrogenase/isomerase deficiency. Findings of cholestasis with normal serum γ-glutamyltransferase activity and total bile acid concentration indicated the need for definitive bile acid analysis. Large amounts of 3β-hydroxy-$\Delta^5$bile acids were detected by gas chromatography-mass spectrometry. *HSD3B7* gene analysis using peripheral lymphocyte genomic DNA from the patients and their parents identified four novel mutations of the *HSD3B7* gene in the patients. One had a homozygous mutation, 314delA; the other had compound heterozygous mutations: V132F, T149I, and 973_974insCCTGC. Interestingly, the second patient's mother had V132F and T149I mutations in one allele. Excessive 3β-hydroxy-$\Delta^5$-bile acids such as 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids were detected in the first patient's urine; the second patient's urine contained large amounts of 3β-hydroxy-5-cholenoic acid. Liver dysfunction in both patients decreased with ursodeoxycholic acid treatment, but unusual bile acids were still detected. Normalization of the patients' liver function and improvement of bile acid profiles occurred with CDCA treatment. Thus, we found mutations in the *HSD3B7* gene accounting for autosomal recessive neonatal cholestasis caused by 3β-hydroxy-$\Delta^5$-$C_{27}$-steroid dehydrogenase/isomerase deficiency. Early neonatal diagnosis permits initiation of CDCA treatment at this critical time, before the late cholestatic stage. **(*Pediatr Res* 68: 258–263, 2010)**

Deficiency of 3β-hydroxy-$\Delta^5$-$C_{27}$-steroid dehydrogenase/ isomerase (3β-HSD) was first described by Clayton *et al.* in 1987 (1). This inborn error of bile acid synthesis is very rare and shows autosomal recessive inheritance. The main findings in 3β-HSD deficiency are low or normal concentrations of total bile acid and normal activity of γ-glutamyltransferase (GGT) in serum, as well as absence of pruritus despite conjugated hyperbilirubinemia, elevated alanine aminotransferase (ALT), and fatty stools. In the synthesis of bile acids from cholesterol, 3β-HSD catalyzes the second of a series of reactions leading to excretion of 3β,7α-dihydroxy–5-cholenoic acid ($\Delta^5$-3β,7α-diol) and 3β,7α,12α-trihydroxy-5-cholenoic acid ($\Delta^5$-3β,7α,12α-triol) in the urine.

In the first reported patient, complete absence of 3β-HSD activity was found by Buchmann *et al.* in 1990 (2) based on the study of cultured fibroblasts. In 2000, Schwarz *et al.* (3) reported that the same patient had a homozygous mutation representing a 2-bp deletion in exon 6 of the 3β-HSD gene (*HSD3B7*) on chromosome 16p11.2-12. The human *HSD3B7* gene contains six coding exons and encodes 369 amino acids; so far, 13 distinct mutations causing 3β-HSD deficiency have been reported (4,5).

Here, we report genetic analyses of two Japanese patients with 3β-HSD deficiency: one previously reported patient (6,7) was diagnosed with 3β-HSD deficiency by bile acid analysis and the other newly reported patient showed different results in the bile acid analysis. Here, we describe definitive diagnosis by bile acid analysis using gas chromatography-mass spectrometry (GC-MS) and effective chenodeoxycholic acid (CDCA) treatment in two patients with 3β-HSD deficiency.

This study was approved by the Kurume University Review Board, and informed consent for the patient report including bile acid and gene analysis was obtained from the two patients and four parents.

## PATIENTS AND METHODS

***Patient 1.*** The family history and initial presentation of this patient have been described previously (6). This 2-mo-old male infant underwent medical therapy for cholestasis and liver dysfunction [total bilirubin (T Bil), 9.3 mg/dL; direct bilirubin (D Bil), 5.9 mg/dL; aspartate aminotransferase (AST), 587 IU/L; ALT, 596 IU/L; GGT, 23 IU/L; prothrombin time, 12.2 s] using ursodeoxycholic acid (UDCA, 12.5 mg/kg/d). Hepatic histologic findings indicated giant cell hepatitis with fibrosis. During treatment, cholestasis and

Received January 14, 2010; accepted May 17, 2010.
Correspondence: Akihiko Kimura, M.D., Ph.D., Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-Machi, Kurume-Shi 830-0011, Japan; e-mail: hirof@med.kurume-u.ac.jp

**Abbreviations: 3β-HSD,** 3β-hydroxy-$\Delta^5$-$C_{27}$-steroid dehydrogenase/ isomerase; **$\Delta^5$-3β-ol,** 3β-hydroxy-5-cholenoic acid; **$\Delta^5$-3β,7α-diol,** 3β,7α-dihydroxy–5-cholenoic acid; **$\Delta^5$-3β,7α,12α-triol,** 3β,7α,12α-trihydroxy-5-cholenoic acid; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **CA,** cholic acid; **CDCA,** chenodeoxycholic acid; **Cr,** creatinine; **D Bil,** direct bilirubin; **GC-MS,** gas chromatography-mass spectrometry; **GGT,** γ-glutamyltransferase; **T Bil,** total bilirubin; **UDCA,** ursodeoxycholic acid

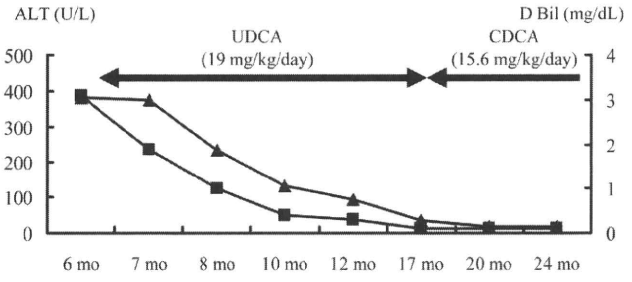


**Figure 1.** Clinical course of patient 2. Responses of the serum D Bil (■) and ALT (▲) to treatment with UDCA and CDCA are shown.

liver dysfunction improved. However, bile acid profiles in urine did not change, indicating that hepatotoxic 3β-hydroxy-$\Delta^5$-bile acids such as $\Delta^5$-3β,7α-diol and $\Delta^5$-3β,7α12α-triol accumulated in the patient's hepatocytes despite UDCA treatment. After treatment with CDCA (8.3 mg/kg/d), however, concentrations of 3β-hydroxy-$\Delta^5$-bile acids significantly decreased, suggesting that CDCA treatment of this disease would be useful in preventing cirrhosis (7). At present, the patient who is 12-yr-old is in good condition without liver dysfunction or excessive 3β-hydroxy-$\Delta^5$-bile acids in urine or serum on his current CDCA dose of 5.9 mg/kg/d. The patient did not have a follow-up liver biopsy performed.

***Patient 2.*** A male infant with a birth weight of 3310 g was delivered by spontaneous vaginal delivery without complications at a GA of 38 wk, after an uneventful pregnancy. He was noted to have jaundice at the age of 1 and 3 mo. The jaundice was mild when first noted and it gradually worsened. At the age of 6 mo, the patient was referred to Toho University Hospital because of hyperbilirubinemia and liver dysfunction (T Bil, 5.2 mg/dL; D Bil, 3.1 mg/dL; AST, 362 IU/L; ALT, 384 IU/L; GGT, 33 IU/L; total bile acids, 0.7 μM; and prothrombin activity, 21%). Initial physical examination on admission was nearly unremarkable, without hepatomegaly, obvious jaundice, or dark urine. Serial technetium-99m ($^{99m}$TC)-DISIDA cholescintigraphy visualized intestinal radioactivity. After the bile acid analysis, we started UDCA treatment (19 mg/kg/d), after which T Bil and ALT gradually decreased to the normal range, from 5.2 mg/dL and 384 IU/L to 0.3 mg/dL and 34 IU/L, respectively (Fig. 1). We chose not to perform a liver biopsy because the patient was in good health without liver dysfunction. After clinical diagnosis and UDCA treatment, we substituted CDCA treatment (15.6 mg/kg/d). Subsequently, liver function test results have remained within the normal range.

The initial bile acid analysis detected large amounts of 3β-hydroxy-5-cholenoic acid ($\Delta^5$-3β-ol), representing 84% of total urinary bile acids (Table 1). This led us to suspect oxysterol 7α-hydroxylase deficiency (8,9), but we could not detect a mutation of the *CYP7B1* gene. In the second urinary bile acid analysis during UDCA treatment, we detected large proportions of 3β-hydroxy-$\Delta^5$-bile acids such as $\Delta^5$-3β-ol and $\Delta^5$-3β,7α,12α-triol, when UDCA was excluded from calculations (Table 1). We ultimately diagnosed this patient with 3β-HSD deficiency.

After CDCA treatment, the concentration of 3β-hydroxy-$\Delta^5$-bile acids in urine decreased significantly during 4 mo, from 7.3 to 2.8 μmol/mmol creatinine (Cr) (Table 1).

The patients had neither steatorrhea nor pruritus. The parents of patients 1 and 2 were all in good health, without liver dysfunction.

***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 Cr concentration and expressed as micromoles per millimoles of Cr.

After synthesizing relevant unusual bile acids such as 3β-hydroxy-$\Delta^5$ (10), 3-oxo-$\Delta^4$ (11), and allo-bile acids (11), which occur in inborn errors of bile acid synthesis, analysis of the bile acids in urine and serum was undertaken by GC-MS using selected ion monitoring of characteristic fragments of methyl-ester-dimethylethylsilyl ether-methoxime derivatives of bile acids as described previously (11). Before GC-MS analysis, the samples were prepared by enzymatic hydrolysis (cholylglycine hydrolase, 30 U) and solvolysis (sulfatase, 150 U; Sigma Chemical Co. Chemical, St. Louis, MO). We did not use *N*-acetylglucodamine.

***Genetic analysis.*** With informed consent, *HSD3B7* gene analysis was performed using genomic DNA from peripheral lymphocytes from the two patients and four parents, as well as 100 healthy individuals using a QIAamp Mini Kit (Qiagen, Hilden, Germany). DNA fragments spanning the six coding regions of the *HSD3B7* gene were amplified by PCR using Gene *Taq* (Nippon Gene, Toyama, Japan) and five sets of primers to obtain DNA fragments of the optimal length for direct sequence analysis (Table 2). The PCR program included an initial denaturation step at 94°C for 3 min, followed by 30 cycles with denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min. A final extension step of 72°C for 10 min was performed using a T-Gradient Thermoblock (Biometra, Goettingen, Germany).

After enzyme processing with ExoSAP-IT (USB, Cleveland, OH), direct sequencing of the 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, Brea, CA).

After the four putative mutations were found in the patients, their parents and 100 healthy individuals were screened for these four mutations by direct sequence analysis.

**Table 1.** *Bile acid analysis of serum and urine using GC-MS in patient 2*

| | Initial | During UDCA treatment | During CDCA treatment |
|---|---|---|---|
| Serum (μmol/L) | | | |
| Cholic acid | n.d. | n.d. | n.d. |
| Chenodeoxycholic acid | n.d. | n.d. | 4.9 |
| Ursodeoxycholic acid | n.d. | 11.9 | n.d. |
| Deoxycholic acid | n.d. | n.d. | n.d. |
| Lithocholic acid | n.d. | n.d. | n.d. |
| Polyhydroxylated bile acids | n.d. | n.d. | n.d. |
| Allo bile acids | n.d. | n.d. | n.d. |
| Ketonic bile acids | n.d. | n.d. | n.d. |
| 3β-Hydroxy-5-cholenoic acid | 4.3 (64.7%) | n.d. | n.d. |
| 3β,7α-Dihydroxy-5-cholenoic acid | 1.6 (23.7%) | n.d. | n.d. |
| 3β,12α-Dihydroxy-5-cholenoic acid | n.d. | n.d. | n.d. |
| 3β,7α,12α-Trihydroxy-5-cholenoic acid | 0.8 (11.6) | n.d. | n.d. |
| Total bile acids | 6.7 | 11.9 | 4.9 |
| Urine (μmol/mmol Cr) | | | |
| Cholic acid | n.d. | n.d. | 0.1 |
| Chenodeoxycholic acid | n.d. | 0.4 | 0.2 |
| Ursodeoxycholic acid | n.d. | 41.5 (72.6%) | 0.1 |
| Deoxycholic acid | 1.1 | Trace | Trace |
| Lithocholic acid | n.d. | n.d. | n.d. |
| Polyhydroxylated bile acids | 0.2 | 6.7 | 0.1 |
| Allo bile acids | n.d. | n.d. | 0.1 |
| Ketonic bile acids | n.d. | 1.3 | 0.3 |
| 3β-Hydroxy-5-cholenoic acid | 15.6 (84.3%) | 2.9 (5.0%) [18.4%] | Trace |
| 3β,7α-Dihydroxy-5-cholenoic acid | 0.7 (3.9%) | 0.6 (1.0%) [3.7%] | 0.3 (8.1%) |
| 3β,12α-Dihydroxy-5-cholenoic acid | 0.2 (1.3%) | n.d. | Trace |
| 3β,7α,12α-Trihydroxy-5-cholenoic acid | 0.8 (4.1%) | 3.8 (6.7%) [24.5%] | 2.5 (67.6%) |
| Total bile acids | 18.6 | 57.1 | 3.7 |

n.d., not detected.
Excluding UDCA from the denominator is represented in [ ].

## RESULTS

***Patient profile.*** Both patients were diagnosed with 3β-HSD deficiency by bile acid profiles and *HSD3B7* gene analysis. Liver dysfunction, such as T Bil, D Bil, AST, and ALT, improved in both patients with UDCA treatment (Ref. 7 and Fig. 1); however, the excess level of 3β-hydroxy-$\Delta^5$-bile acid

**Table 2.** *Oligonucleotides used for DNA amplification of the* HSD3B7 *gene*

| Product including | Sequence forward primer 5′→3′ | Sequence reverse primer 5′→3′ |
|---|---|---|
| Exon 1 | GCAGTAACAGGTGGTTGCAGC | AGCATCATCTGTTCCACTGCAG |
| Exon 2 | AGTGAGTCACATTGGGAACGTG | TCAATAGGACAACTTGTCCCAG |
| Exons 3 + 4 | ATGGGGAGGAGGAAGATGCAG | CTTGGGCTGGCAGGGTAAGG |
| Exon 5 | CCTTACCCTGCCAGCCCAAG | CTAGCCAGAGTCCACACTTCTC |
| Exon 6 | AGCAGCCTCGATGTGGTGTTG | TTCCCGTCCAGGGTGTGAGG |

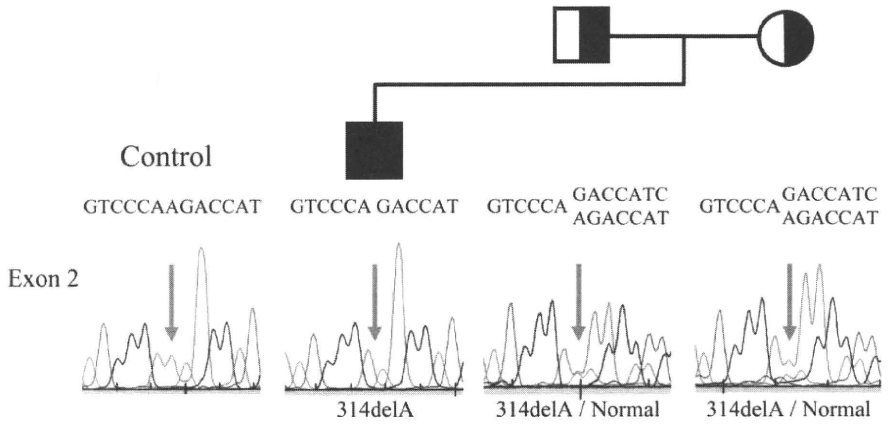


**Figure 2.** Pedigree for patient 1 shown with genomic DNA sequences in exon 2 of the *HSD3B7* gene in this patient, his parents, and a control. The *arrows* identify homozygous 314delA in the patient, heterozygous (314delA/normal) in his parents, and intact A in a control subject. The reverse strand sequence shows the same result. This represents a 314delA mutation causing a frameshift. Such a nucleotide deletion was not observed in 100 controls.

in urine did not change. After CDCA treatment, concentrations of 3$\beta$-hydroxy-$\Delta^5$-bile acids in urine and serum gradually decreased to the normal range. With CDCA treatment, these patients have maintained good condition without liver dysfunction, showing normal bile acid profiles with no 3$\beta$-hydroxy-$\Delta^5$-bile acids detected.

***Biochemical identification of the inborn error of bile acid synthesis.*** Results of urine and serum bile acid analysis for patient 2 are shown in Table 1. The serum bile acid concentration was normal in the initial analysis. We detected large amounts of $\Delta^5$-3$\beta$-ol in serum and urine (65% and 84% of total bile acids, respectively) as well as an evidence of oxysterol 7$\alpha$-hydroxylase deficiency in the initial bile acid analysis. In a second bile acid analysis during UDCA treatment, the main bile acid in serum was UDCA (100% of total bile acids). The main bile acid in urine was UDCA (73% of total bile acids), and we detected small amounts of 3$\beta$-hydroxy-$\Delta^5$-bile acids (13% of total bile acids), such as $\Delta^5$-3$\beta$-ol, $\Delta^5$-3$\beta$,7$\alpha$-diol and $\Delta^5$-3$\beta$,7$\alpha$,12$\alpha$-triol, in urine. When we excluded urinary UDCA from consideration at the second bile acid analysis, the main bile acids in urine were 3$\beta$-hydroxy-$\Delta^5$-bile acids (47% of total bile acids). After diagnosis, UDCA treatment was changed to CDCA treatment, after which the concentrations of 3$\beta$-hydroxy-$\Delta^5$-bile acids in urine gradually decreased.

Patient 1 showed large amounts of 3$\beta$-hydroxy-$\Delta^5$-bile acids (93% of total bile acids), such as $\Delta^5$-3$\beta$,7$\alpha$-diol and $\Delta^5$-3$\beta$,7$\alpha$,12$\alpha$-triol, in urine and serum (7) at the age of 18 mo.

***Identification of* HSD3B7 *gene defects.*** We identified four novel mutations in these two patients. In patient 1, a single homozygous mutation was found in exon 2, at nucleotide number 314, deletion A (314delA), resulting in a frameshift leading to formation of a stop codon at position 112. The mutation was detected in heterozygous form in the parents, whereas being absent in 100 healthy individuals (Fig. 2).

Patient 2 showed three heterozygous mutations. The first was in exon 3, at nucleotide 412, representing a G-to-T substitution, causing an amino acid change from valine to phenylalanine (V132F). The second mutation was in exon 4, at nucleotide number 464, representing a single C-to-T substitution, leading to an amino acid change from threonine to isoleucine (T149I). The third mutation in exon 6, between nucleotides 973 and 974, was a 5-bp insertion, CCTGC (973_974insCCTGC), causing a frameshift leading to formation of a stop codon at position 321. Interestingly, both V132F and T149I mutations in heterozygous form were detected in the patient's mother but were absent in the father and in 100 healthy individuals. A heterozygous 973_974insCCTGC mutation was detected in the father but was absent in the mother and in the 100 healthy individuals (Fig. 3).

Above nucleotide numbers indicating positions of individual mutations are based on those determined from human 3$\beta$-HSD cDNA (GenBank accession no. AF277719).

## DISCUSSION

In the clinical course of our two patients with 3$\beta$-HSD deficiency, UDCA treatment was very effective for treating conjugated hyperbilirubinemia and elevation of aminotransferase, such as ALT (Ref. 7, Fig. 1), in addition for treating idiopathic neonatal hepatitis. Therefore, the pediatricians may misdiagnose patients as having idiopathic neonatal hepatitis if UDCA restores apparent good health. We encountered these patients with normal values for GGT, total bile acids in serum, and suspected 3$\beta$-HSD deficiency. We also obtained prompt analysis of bile acids in serum and urine using GC-MS to detect any inborn errors of bile acid synthesis (12). Previous reports described two adult patients with 3$\beta$-HSD deficiency who were diagnosed in this manner (5,13).

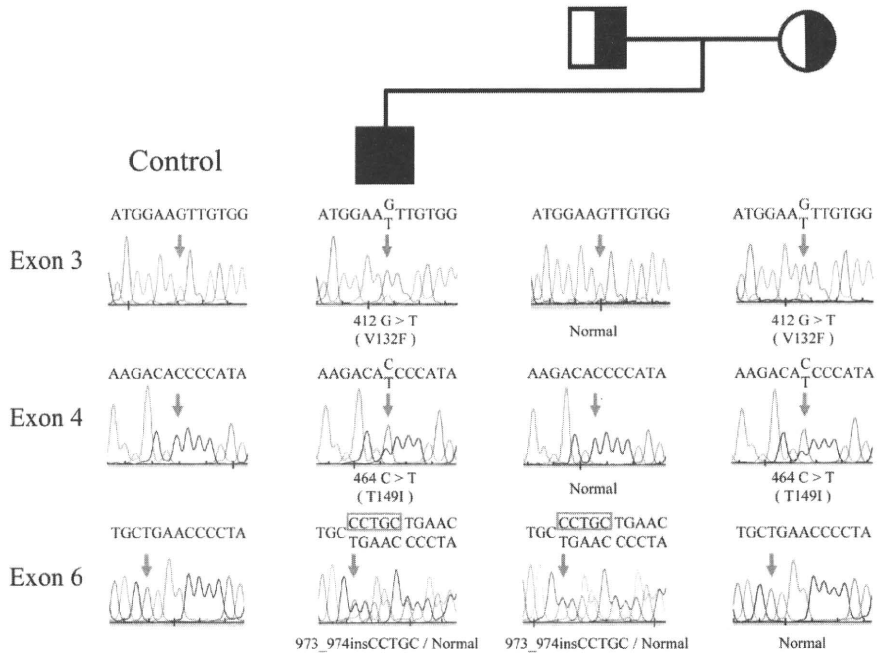


**Figure 3.** Pedigree for patient 2 shown with genomic DNA sequences in exons 3, 4, and 6 of the *HSD3B7* gene in this patient, his patients, and a control. The *arrow* in exon 3 identifies G/T in the patient and his mother, but G in his father and a control subject. The reverse strand sequence shows the same result. This represents a GTT-to-TTT mutation, affecting valine at position 132, where it is replaced by phenylalanine. The *arrow* in exon 4 identifies C/T in the patient and his mother, but C in his father and a control subject. The reverse strand sequence shows the same results. This represents an ACC-to-ATC mutation, affecting threonine at position 149, where it is replaced by isoleucine. Such nucleotide substitutions were not observed in 100 controls. The *arrows* and *squares* in exon 6 identify nucleotide number 974 (T/C) and heterozygous insertion of 5 bp, CCTGC, in the patient and his father; in his mother and a control subject, only T is present. The reverse strand sequence shows the same result. This represents a 973_974insCCTGC mutation causing a frameshift. Such a nucleotide insertion was not observed in 100 controls.

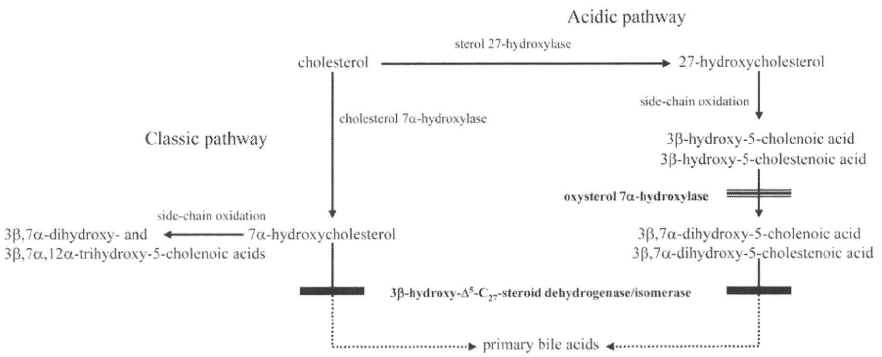


**Figure 4.** Effect of the defect of 3β-HSD. Reduced primary bile acid synthesis from cholesterol and increased synthesis of 3β-hydroxy-$\Delta^5$-bile acids are shown in the flow chart of the classic and acidic pathways.

Therefore, in a cholestatic patient, if the serum GGT activity is normal and the total serum bile acid concentration determined using 3α-hydroxysteroid dehydrogenase is normal or low, one needs to analyze bile acids in serum and urine using methods such as GC-MS.

In the initial bile acid analysis, we detected large amounts of 3β-hydroxy-$\Delta^5$-bile acids such as $\Delta^5$-3β-ol in serum and urine from patient 2, a finding also reported in oxysterol 7α-hydroxylase deficiency (8,9). We speculate that the main pathway of bile acid synthesis was the acidic pathway, based on the results of initial bile acid analysis in serum and urine. Low or absent activity of cholesterol 7α-hydroxylase enzyme in this patient reflected previous observations that cholesterol 7α-hydroxylase enzyme activity physiologically is low or absent in fetal and neonatal life (8,14), and that low or absent activity of oxysterol 7α-hydroxylase enzyme in this patient could be physiologic (as in knockout mice lacking a functional cholesterol 7α-hydroxylase enzyme). Cholestasis precedes up-regulation of oxysterol 7α-hydroxylase activity in the acidic pathway (15,16). Oxysterol 7α-hydroxylase activity was first detected in 3- to 4-wk-old mice and remained detectable in the livers of older mice (16). We detected increased 3β-hydroxy-$\Delta^5$-bile acids such as $\Delta^5$-3β,7α-diol and $\Delta^5$-3β,7α,12α-triol in our patient's second urinary bile acid analysis (Table 1, Fig. 4).

CDCA treatment was recommended for patients with 3β-HSD deficiency by Ichimiya *et al.* (17,18), who reported that treatment with CDCA was very effective in improving the clinical status and liver function, reflecting reduced cholesterol catabolism because of inhibition of cholesterol 7α-hydroxylase. As a result, hepatotoxic 3β-hydroxy $\Delta^5$ bile acids were decreased. The treatment should also improve absorption of cholesterol from the gut. Together, these two mechanisms might contribute to an increased serum cholesterol concentration. The importance of bile secretion stimulation for avoiding hepatotoxicity could be evaluated by treatment with UDCA, which does not inhibit cholesterol 7α-hydroxylase and therefore does not prevent further synthesis of hepatotoxic 3β-hydroxy $\Delta^5$ bile acids (18,19). Even with short-term UDCA treatment available, primary bile acid treatment should be the first choice upon definitive diagnosis. Actually, our patients showed less liver dysfunction with short-term UDCA treatment (Ref. 7, Fig. 1). After diagnosis was made using GC-MS bile acid analysis,

both patients, especially the first, maintained good condition without liver dysfunction. Unusual bile acids such as 3$\beta$-hydroxy-$\Delta^5$-bile acids gradually decreased on long-term CDCA treatment. Subramaniam *et al.* (20) support cholic acid (CA) and CDCA treatment in 3$\beta$-HSD deficiency, but CDCA cannot be used when patients with 3$\beta$-HSD deficiency present late with chronic liver disease, at which point CDCA can be hepatotoxic. Alternatively, Jacquemin *et al.* (21) and Gonzales *et al.* (22) found oral CA treatment to be safe and effective in treating most common inborn errors of bile acid synthesis, including 3$\beta$-HSD deficiency. Therefore, in 3$\beta$-HSD deficiency, CA treatment may be better because CA activates negative feedback regulation of bile acid synthesis to inhibit production of hepatotoxic metabolites and is not itself hepatotoxic. Unfortunately, however, CA is not available for clinical use in Japan. We think that CDCA treatment may be effective for 3$\beta$-HSD deficiency because of the high potency of CDCA in suppressing bile acid synthesis relative to CA (23–25).

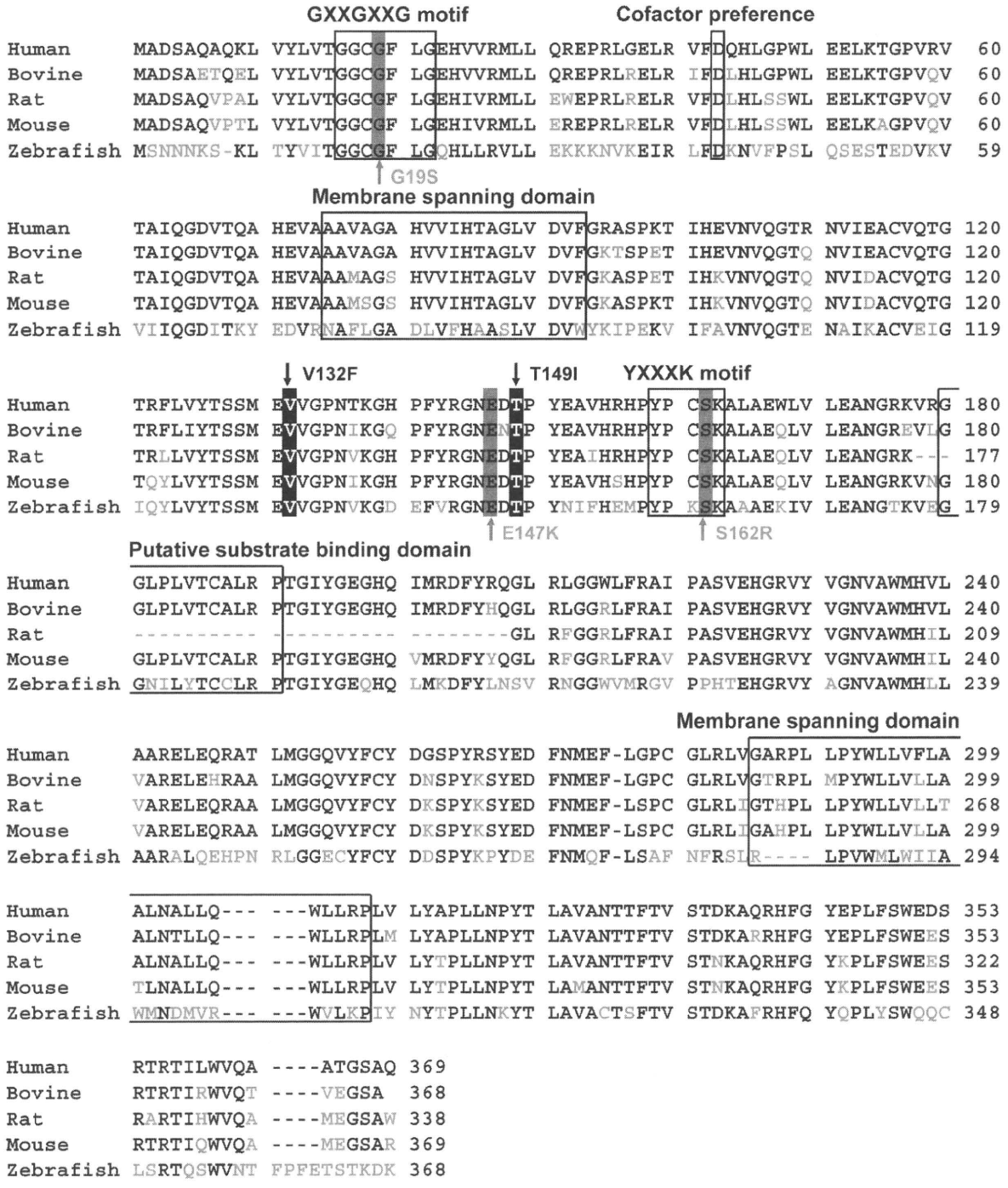


**Figure 5.** Aligned amino acid sequences for *HSD3B7*, comparing human with bovine, rat, mouse, and zebrafish sequences. Amino acids identical with those in humans are in *black* as opposed to *gray* letters. Amino acids are numbered at the right. *Black arrows* identify the two novel missense mutations, V132F and T149I, described in this study. *Gray arrows* identify previously reported missense mutations, G19S, E147K, and S162R. GenBank accession numbers for human, cattle, rats, mice, and zebrafish are NM_025193, BC105259, NM_139329, BC132605, and NM_199809, respectively.

Previous reports of mutations in the *HSD3B7* gene in 17 patients identified 13 distinct mutations causing 3β-HSD deficiency (4,5). This enzyme deficiency has been characterized as showing autosomal recessive transmission. Here, we describe genetic analysis of the *HSD3B7* gene in two patients with 3β-HSD deficiency. Patient 1 had a homozygous 314delA mutation. The protein encoded by the 314delA mutation is composed of 98 amino acids from the normal protein fused to a 13-residue extension. Patient 2 had three heterozygous mutations: V132F, T149I, and 973_974insCCTGC. The protein encoded by the 973_974insCCTGC mutation is composed of 318 amino acids from the normal protein fused to a 2-residue extension. We screened for the two potentially informative substitution mutations, V132F and T149I, in 100 healthy individuals by direct sequence analysis; neither was found. As for interspecies comparisons corresponding protein, the valine 132 and threonine 149 residues are conserved among humans, cattle, rats, mice, and zebrafish as well as in the previously reported missense mutations G19S, E147 K, and S162R (4,5). Moreover, amino acids adjoining valine 132 and threonine 149 are conserved across species (Fig. 5), which suggests an important catalytic or structural role in the dehydrogenase/isomerase. Accordingly, we concluded that either or both of the V132F and T149I mutations could have contributed to loss of 3β-HSD enzyme function in the proband, considering that patient 2 received one allele with V132F and T149I mutations from the mother, whereas the other allele from his father contained 973_974insCCTGC, also affecting the *HSD3B7* gene. His mother was asymptomatic despite having the two missense mutations because both occurred together on only one allele, representing heterozygosity. In the two patients, in this study, we identified a total of four novel mutations.

Patient 1 was a homozygote and patient 2 was a compound heterozygote. Both parents of each patient were the heterozygous for a mutation, strongly suggesting that the patient inherited one or more mutated genes from each parent.

Finally, the results of *HSD3B7* gene analysis led us to identify four novel mutations in the *HSD3B7* gene that can underlie 3β-HSD deficiency, an autosomal recessive form of neonatal cholestasis. Because the liver function in this disease is improved by UDCA treatment, patients may be misdiagnosed with idiopathic neonatal hepatitis. However, a low or normal serum concentration of total bile acid and a normal serum GGT concentration in a neonate with cholestasis should lead us to suspect inborn errors of bile acid synthesis such as 3β-HSD deficiency. After diagnosis of 3β-HSD deficiency, we recommend prompt initiation of primary bile acid treatment using CDCA (and/or CA, where available) in the early neonatal period, before the late stage of chronic cholestatic liver dysfunction.

## REFERENCES


1. Clayton PT, Leonard JV, Lawson AM, Setchell KD, Andersson S, Egestad B, Sjövall J 1987 Familial giant cell hepatitis associated with synthesis of 3β,7α-dihydroxy- and 3β,7α,12α-trihydroxy-5-cholenoic acids. J Clin Invest 79:1031–1038
2. Buchmann MS, Kvittingen EA, Nazer H, Gunasekaran T, Clayton PT, Sjövall J, Björkhen I 1990 Lack of 3β-hydroxyΔ5-C27-steroid dehydrogenase/isomerase in fibroblasts from a child with urinary excretion of 3β-hydroxy-Δ5-bile acids. J Clin Invest 86:2034–2037
3. Schwarz M, Wright AC, Davis DL, Nazer H, Björkhem I, Russell DW 2000 The bile acid synthesis gene 3β-hydroxyΔ5-C27-steroid oxidoreductase is mutated in progressive intrahepatic cholestasis. J Clin Invest 106:1175–1184
4. Cheng JB, Jacquemin E, Gerhardt M, Nazer H, Cresteil D, Heubi JE, Setchell KD, Russell DW 2003 Molecular genetics of 3β-hydroxyΔ5-C27-steroid oxidoreductase deficiency in 16 patients with loss of bile acid synthesis and liver disease. J Clin Endocrinol Metab 88:1833–1841
5. Fischler B, Bodin K, Stjernman H, Olin M, Hansson M, Sjövall J, Björkhen I 2007 Cholestatic liver disease in adults may be due to an inherited defect in bile acid biosynthesis. J Intern Med 262:254–262
6. Terazawa S, Kimura A, Inoue T, Murai T, Kurosawa T, Takao A 1998 An infant with 3β-hydroxyΔ5-C27-steroid dehydrogenase/isomerase deficiency presenting with typical neonatal hepatitis syndrome: the first Japanese case. Acta Paediatr Jpn 40:638–640
7. Yamato Y, Kimura A, Murai T, Yoshimura T, Kurosawa T, Terazawa S, Takao A, Maeda K, Nakashima E, Yamashita Y, Kato H 2001 3β-HydroxyΔ5-C27-steroid dehydrogenase deficiency: diagnosis and treatment. J Paediatr Child Health 37:516–519
8. Setchell KD, Schwarz M, O'Connell NC, Lund EG, Davis DL, Lathe R, Thompson HR, Weslie Tyson R, Sokol RJ, Russell DW 1998 Identification of a new inborn error in bile acid synthesis: mutation of the oxysterol 7alpha-hydroxylase gene causes severe neonatal liver disease. J Clin Invest 102:1690–1703
9. Ueki I, Kimura A, Nishiyori A, Chen H-L, Takei H, Nittono H, Kurosawa T 2008 Neonatal cholestatic liver disease in an Asian patient with a homozygous mutation in the oxysterol 7α-hydroxylase gene. J Pediatr Gastroenterol Nutr 46:465–469
10. Tohma M, Mahara R, Takeshita H, Kurosawa T 1986 A convenient synthesis of 3β,12α-, 3β,7α-, and 3β,7β-dihydroxy-5-cholen-24-oic acids: unusual bile acids in human biological fluids. Steroids 48:331–338
11. Kimura A, Mahara R, Inoue T, Nomura Y, Murai T, Kurosawa T, Tohma M, Noguchi K, Hoshiyama A, Fujisawa T, Kato H 1999 Profile of urinary bile acids in infants and children: developmental pattern of excretion of unsaturated ketonic bile acids and 7β-hydroxylated bile acids. Pediatr Res 45:603–609
12. Sundaram SS, Bove KE, Lovell MA, Schol RJ 2008 Mechanisms of disease: inborn errors of bile acid synthesis. Nat Clin Pract Gastroenterol Hepatol 5:456–468
13. Kobayashi M, Koike M, Sakiyama M, Okuda S, Okuda M, Tanaka T, Unno A, Nittono H, Takei H, Murai T, Yoshimura T, Kurosawa T 2000 3β-Hydroxy-Δ5-C27 dehydrogenase/isomerase deficiency in a 23-year-old woman. Pediatr Int 42:685–688
14. Gustafsson J 1986 Bile acid biosynthesis during development: hydroxylation of C27-sterols in human fetal liver. J Lipid Res 27:801–806
15. Ishibashi S, Schwarz M, Frykman PK, Herz J, Russell DW 1996 Disruption of cholesterol 7α-hydroxylase gene in mice. I. Postnatal lethality reversed by bile acid and vitamin supplementation. J Biol Chem 271:18017–18023
16. Schwarz M, Lund EG, Setchell KD, Kayden HJ, Zerwehk JE, Björkhem I, Herz J, Russell DW 1996 Disruption of cholesterol 7α-hydroxylase gene in mice. II. Bile acid deficiency is overcome by induction of oxysterol 7α-hydroxylase. J Biol Chem 271:18024–18031
17. Ichimiya H, Nazer H, Gunasekaran T, Glayton P, Sjövall J 1990 Treatment of chronic liver disease caused by 3β-hydroxy-Δ5-C27 dehydrogenase deficiency with chenodeoxycholic acid. Arch Dis Child 65:1121–1124
18. Ichimiya H, Egestad B, Nazer H, Baginski ES, Clyton PT, Sjövall J 1991 Bile acids and bile alcohols in a child with hepatic 3β-hydroxy-Δ5-C27 dehydrogenase deficiency: effect of chenodeoxycholic acid treatment. J Lipid Res 32:829–841
19. Jacquemin E, Setchell KD, O'Connell NC, Estrada A, Maggiore G, Schmitz J, Hadchoael M, Bernard O 1994 A new cause of progressive intrahepatic cholestasis: 3β-hydroxy-C27 dehydrogenase/isomerase deficiency. J Pediatr 125:379–384
20. Subramaniam P, Clayton PT, Portmann BC, Mieli-Vergani G, Hadžić N 2010 Variable clinical spectrum of the most common inborn error of bile acid metabolism-3β-hydroxy-Δ5-C27-steroid dehydrogenase deficiency. J Pediatr Gastroenterol Nutr 50:61–66
21. Jacquemin E, Gerhardt M, Cresteil D, Fabre M, Taburet AM, Hadchouel M, Trivin F, Setchell KD, Bernard O 2001 Long-term effects of bile acid therapy in children with defects of primary bile acid synthesis: 3β-hydroxy-C27-steroid-dehydrogenase/isomerase and Δ4-3-oxosteroid 5β-reductase deficiencies. In: von Berge Henegouwen GP, Keppler D, Leuschner U, Paumgartner G, Stiehl A (eds) Biology of Bile Acids in Health and Disease. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 278–282
22. Gonzales E, Gerhardt MF, Fabre M, Setchell KD, Davit-Spraul A, Vincent I, Heubi JE, Bernard O, Jacquemin E 2009 Oral cholic acid for hereditary defects of primary bile acid synthesis: a safe and effective long-term therapy. Gastroenterology 137:1310–1320
23. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned MR, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B 1999 Identification of a nuclear receptor for bile acid. Science 284:1362–1365
24. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consier TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM 1999 Bile acids: natural ligands for an orphan nuclear receptor. Science 284:1365–1368
25. Ellis E, Axelson M, Abrahamsson A, Eggertsen G, Thörne A, Nowak G, Ericzon B-G, Björkhem I, Einarsson C 2003 Feedback regulation of bile acid synthesis in primary human hepatocytes: evidence that CDCA is the strongest inhibitor. Hepatology 38:930–938

Biochimica et Biophysica Acta 1800 (2010) 313–315

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

# Pyruvate therapy for Leigh syndrome due to cytochrome *c* oxidase deficiency

Hirofumi Komaki [a,b], Yutaka Nishigaki [c], Noriyuki Fuku [c], Hiroko Hosoya [c], Kei Murayama [d], Akira Ohtake [e], Yu-ichi Goto [a,b], Hiroyuki Wakamoto [f], Yasutoshi Koga [g], Masashi Tanaka [c,*]

[a] *Department of Pediatric Neurology, National Center Hospital of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan*
[b] *Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Kodaira, Tokyo 187-8502, Japan*
[c] *Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi, Tokyo 173-0015, Japan*
[d] *Department of Metabolism, Chiba Children's Hospital, Chiba 266-0007, Japan*
[e] *Department of Pediatrics, Saitama Medical University, Moroyama, Saitama 350-0495, Japan*
[f] *Ehime Rehabilitation Center for Children, Toon, Ehime 791-0212, Japan*
[g] *Department of Pediatrics, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, Japan*

ARTICLE INFO

*Article history:*
Received 15 May 2009
Accepted 8 July 2009
Available online 17 July 2009

*Keywords:*
Pyruvate therapy
Leigh syndrome
Cytochrome *c* oxidase deficiency
Exercise intolerance
Lactate-to-pyruvate ratio

ABSTRACT

*Background:* Recently we proposed the therapeutic potential of pyruvate therapy for mitochondrial diseases. Leigh syndrome is a progressive neurodegenerative disorder ascribed to either mitochondrial or nuclear DNA mutations.
*Methods:* In an attempt to circumvent the mitochondrial dysfunction, we orally applied sodium pyruvate and analyzed its effect on an 11-year-old female with Leigh syndrome due to cytochrome *c* oxidase deficiency accompanied by cardiomyopathy. The patient was administered sodium pyruvate at a maintenance dose of 0.5 g/kg/day and followed up for 1 year.
*Results:* The exercise intolerance was remarkably improved so that she became capable of running. Echocardiography indicated improvements both in the left ventricle ejection fraction and in the fractional shortening. Electrocardiography demonstrated amelioration of the inverted T waves. When the pyruvate administration was interrupted because of a gastrointestinal infection, the serum lactate level became elevated and the serum pyruvate level, decreased, suggesting that the pyruvate administration was effective in decreasing the lactate-to-pyruvate ratio.
*Conclusions:* These data indicate that pyruvate therapy was effective in improving exercise intolerance at least in a patient with cytochrome *c* oxidase deficiency.
*General significance:* Administration of sodium pyruvate may prove effective for other patients with cytochrome *c* oxidase deficiency due to mitochondrial or nuclear DNA mutations.


## 1. Introduction

Mitochondrial diseases are intractable disorders, including encephalomyopathy, cardiomyopathy, hearing or visual loss, and diabetes; and they are caused by either mitochondrial or nuclear DNA mutations. In spite of the research efforts for gene therapy aiming at removal of a specific mitochondrial DNA mutation by use of restriction enzymes, *e.g.*, SmaI or XmaI for the m.8993T>G mutation [1], definite therapies have not been established for mitochondrial diseases. The supplementation of vitamins and cofactors are not satisfactory except for a limited number of patients, such as those with thiamine-responsive pyruvate dehydrogenase complex deficiency [2] or those with defects in the biosynthetic pathway of coenzyme Q [3, 4]. Earlier we proposed that pyruvate has a therapeutic potential for mitochondrial diseases, because: (a) pyruvate can stimulate the glycolytic pathway by reducing the NADH/NAD ratio in the cytoplasm, (b) pyruvate can activate the pyruvate dehydrogenase complex (PDHC) by inhibiting pyruvate dehydrogenase kinase, and (c) pyruvate can scavenge hydrogen peroxide by a non-enzymatic reaction [5].

Leigh syndrome (LS) is an early-onset progressive neurodegenerative disorder characterized by developmental delay or regression, lactic acidosis, and bilateral symmetrical lesions in the basal ganglia, thalamus, and brainstem [6, 7]. The disease is caused by mutations in both nuclear and mitochondrial genes involved in energy metabolism; however, the underlying gene defects remain unidentified in nearly half of the patients [8, 9]. Because of the clinical and genetic heterogeneity of the disorder, there is no established treatment for patients with LS.

Our recent trial showed that sodium pyruvate produced a slightly favorable change in the plasma lactate and pyruvate levels for the treatment of mitochondrial disease [5]. This preliminary result prompted us to apply sodium pyruvate to a patient with LS due to cytochrome *c* oxidase. In the present report, we describe our clinical experience with pyruvate therapy in an adolescent with cytochrome *c* oxidase deficiency.

* Corresponding author. Tel.: +81 3 3964 3241x3095; fax: +81 3 3579 4776.
*E-mail address:* mtanaka@tmig.or.jp (M. Tanaka).

0304-4165/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2009.07.008

## 2. Administration of pyruvate to a patient with cytochrome *c* oxidase deficiency

An 11-year-old female complained of frequent falls during walking and slowness in running. This patient was born with a weight of 3590 g after a normal pregnancy. At the age of 6 years, she complained of double and blurred vision. Neurological examinations revealed gaze nystagmus and bilateral paresis of the abducens nerve. Cranial magnetic resonance imaging (MRI) demonstrated bilateral lesions in the putamen (Fig. 1). The lactate level was elevated in the cerebrospinal fluid (31 mg/dL, normal 10–20 mg/dL). Histopathological study of the skeletal muscle revealed the presence of diffuse cytochrome *c* oxidase-negative fibers (Fig. 2) without ragged-red fibers (RRF) or strongly succinate dehydrogenase-reactive blood vessels (SSV). Biochemical analysis of the mitochondria isolated from the skeletal muscle indicated a marked deficiency of cytochrome *c* oxidase activity (17% of the normal control value). The sequencing of the entire mtDNA identified no pathogenic mutations either in the protein-coding regions or in the ribosomal and transfer RNA genes. From these findings she was diagnosed as having LS due to cytochrome *c* oxidase deficiency. From the age of 8 years oral administration of coenzyme Q was started, but her motor dysfunction became gradually aggravated and her easy fatigability, enhanced. Neurological examination revealed dystonia and an ataxic gait. She sometimes needed assistance in walking, and her speech became gradually slurred. At the age of 10 years, echocardiography revealed mild cardiac dysfunction: her left ventricular ejection fraction was 52% (normal 55%–80%), and the fractional shortening was 26% (normal>28%). An electrocardiogram revealed inverted T waves in leads V3 and V4, suggesting cardiac muscle involvement. The blood lactate and pyruvate levels were 20.5 mg/dL and 1.13 mg/dL, respectively, with a lactate-to-pyruvate ratio of 18.1. The levels of lactate and pyruvate in the cerebrospinal fluid were 32.4 mg/dL and 1.21 mg/dL, respectively, giving a lactate-to-pyruvate ratio of 26.8.

At the age of 11 years, administration of sodium pyruvate (0.5 g/kg/day) was started. After the administration both the blood lactate and pyruvate levels decreased to 10.3 mg/dL and 0.88 mg/dL, respectively, with a reduction in the lactate-to-pyruvate ratio to 11.7.

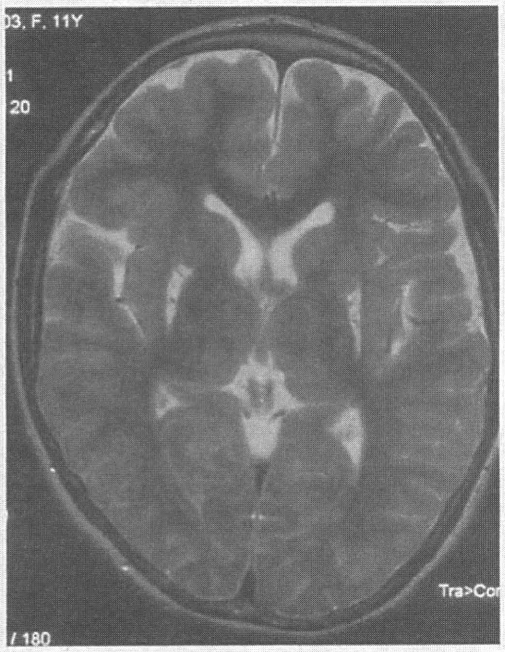


**Fig. 1.** T2-weighed magnetic resonance imaging (MRI) of the brain of the patient at 11 years of age.

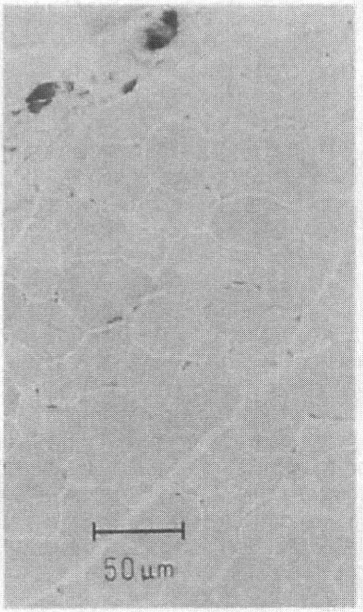


**Fig. 2.** Histochemical staining for cytochrome c oxidase in the biopsied skeletal muscle of the patient. Diffuse deficiency of cytochrome *c* oxidase is to be noted.

Interestingly, the exercise tolerance of the patient improved after the start of pyruvate administration; and she became capable of participating in athletic games in school. One year after the start of pyruvate administration, although none of the neurological symptoms or signs had significantly improved, her cardiac function returned to within the normal ranges: left ventricular ejection fraction of 58% and fractional shortening of 30%. Inverted T waves in leads V3 and V4 of the electrocardiogram were diminished. These findings suggest that pyruvate administration might have beneficial effects on mitochondrial cardiomyopathy.

When pyruvate administration was interrupted because of a gastrointestinal infection, the serum lactate level of the patient increased from 11.3 mg/dL to 14.3 mg/dL; and her serum pyruvate level decreased from 0.96 mg/dL to 0.94 mg/dL, suggesting that the pyruvate administration was effective in decreasing the lactate-to-pyruvate ratio.

The present observations suggest that oral administration of sodium pyruvate at a dose of 0.5 g/kg/day had no harmful effects, although diarrhea was sometimes observed when the pyruvate was administered at a high concentration. We therefore recommend administering sodium pyruvate at 16.5 g/L (150 mM) diluted in either water, milk or fruit juice.

## 3. Discussion

In the present study, we reported a patient with LS who responded to pyruvate administration. The histochemical finding of diffuse cytochrome *c* oxidase deficiency indicated that this condition was distinct from the benign infantile mitochondrial myopathy due to reversible cytochrome *c* oxidase deficiency [10]. The sustained levels of blood lactate and pyruvate suggested that the enzyme defect itself was persisting in the present patient.

We also administered sodium pyruvate to several patients with mitochondrial encephalomyopathies in advanced stages. In such patients having respiratory disturbance necessitating artificial ventilation, dysphagia requiring tube feeding or a gastric fistula, severe psychomotor developmental delay, and/or multiple organ failure, we were unable to assess the efficacy of pyruvate administration. Considering the progressive nature of LS, and given that the pyruvate

administration is efficacious in preventing neurodegeneration, therapeutic intervention should be started in the early stage of disease progression.

There are some limitations in the present study. First, despite vigorous analysis of mitochondrial DNA mutations, we were unable to identify the causative etiology of LS in the present patient. Further survey for nuclear DNA mutations is needed. Second, this was a clinical study on one patient, in which the results must be interpreted with caution. For validation of our findings, multi-institutional research including the present case should be conducted.

In a patient with LS associated with cardiomyopathy examined previous to the present one (Wakamoto et al., unpublished observation), MRI conducted 1 year after the start of pyruvate therapy demonstrated remarkable improvement with distinct decreases in the size and intensity of the lesions located in the basal ganglia. Echocardiography also demonstrated marked improvements in the values of left ventricular end-diastolic diameter, left ventricular end-systolic diameter, fractional shortening, and left ventricular ejection fraction; although the degree of hypertrophy of the heart muscle was not influenced by the pyruvate administration. These observations indicated improved cardiac function after the treatment of this LS patient.

In another patient with LS, a marked improvement in the electroencephalographic findings was noticed after administration of sodium pyruvate (Koga et al., unpublished). Because LS is caused by a wide variety of the molecular and genetic defects, we need to identify the specific subtypes that are responsive to pyruvate therapy. For this purpose, we have started constructing a rapid and comprehensive detection system for pathogenic mutations of mitochondrial DNA by use of the Luminex suspension array technology (Nishigaki et al., in preparation). Efficient and systematic screening for nuclear DNA mutations should be also established.

Hermann et al. investigated the effect of intracoronary pyruvate in 8 patients with congestive heart failure, and concluded that pyruvate had a favorable inotropic effect [11]. Pyruvate affects energy metabolism by its input into the tricarboxylic-acid (TCA) cycle in 2 ways. First, pyruvate enters the TCA cycle as acetyl-CoA after decarboxylation via pyruvate dehydrogenase. Second, pyruvate enriches the TCA cycle after carboxylation to oxaloacetate and/or malate via pyruvate carboxylase and/or malic enzyme. Actually the $^{13}C$ NMR spectroscopic study by Weiss et al. demonstrated that the addition of 0.8 mM pyruvate significantly increased in the levels of citrate in the rat heart perfused with 5 mM [2-$^{13}C$]acetate [12]. This anaplerotic effect of pyruvate would increase the flux through the TCA cycle, supplementing oxidative phosphorylation. The exact mechanisms by which pyruvate improved the exercise intolerance in the present patient with cytochrome *c* oxidase deficiency should be further investigated.

We previously demonstrated that pyruvate infusion lowered the lactate-to-pyruvate ratio and corrected the deficit in ureogenesis in the liver of citrin-knockout ($Ctrn^{-/-}$) mice, a model of adult-onset type II citrullinaemia [13]. Recently, Mutoh et al. reported the use of arginine and sodium pyruvate for the treatment of a citrin-deficient patient at the early stage of adult-onset type II citrullinaemia [14]. Oral administration of arginine and sodium pyruvate for over 3 years improved the clinical symptoms and almost completely normalized the laboratory findings of the patient. The authors concluded that the administration of arginine and sodium pyruvate with low-carbohydrate meals may be an effective therapy for patients with citrin deficiency in order either to prolong metabolic normalcy or to provide a safer and more affordable alternative to liver transplantation [14]. Thus, extended studies are needed to confirm the therapeutic potential of pyruvate for both citrin deficiency and mitochondrial respiratory defects.

In conclusion, although the pathogenic mutation causing the mitochondrial dysfunction was not determined, our results suggest that exercise intolerance, mild cardiac dysfunction, and lactic acidosis were ameliorated by the pyruvate administration. Administration of sodium pyruvate may prove effective for other patients with cytochrome *c* oxidase deficiency due to mitochondrial or nuclear DNA mutations. Validation of our findings will require their replication with additional patients having different mitochondrial abnormalities confirmed by genetic or biochemical analysis.

## Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research [C-18590317 to Y.N., and C2-10832009 and A (2)-15200051 to M.T.], a Grant-in-Aid for Young Scientists (B-18700541 to N.F.), and a Grant-in-Aid for Exploratory Research (20650113 to N.F.) from the Ministry of Education, Culture, Sports, Science and Technology; by a grant from the Third-Term Comprehensive 10-year Strategy for Cancer Control (to M.T.); by grant 20B-13 from the program Research Grants for Nervous and Mental Disorders of the Ministry of Health, Labour, and Welfare (to M.T.); and by grants for scientific research from the Kao Research Council for the Study of Healthcare Science (to Y.N.), the Takeda Science Foundation (to Y.N. and to M.T.), and the Sankyo Foundation of Life Science (to Y.N.).

## References

[1] M. Tanaka, H.J. Borgeld, J. Zhang, S. Muramatsu, J.S. Gong, M. Yoneda, W. Maruyama, M. Naoi, T. Ibi, K. Sahashi, M. Shamoto, N. Fuku, M. Kurata, Y. Yamada, K. Nishizawa, Y. Akao, N. Ohishi, S. Miyabayashi, H. Umemoto, T. Muramatsu, K. Furukawa, A. Kikuchi, I. Nakano, K. Ozawa, K. Yagi, Gene therapy for mitochondrial disease by delivering restriction endonuclease SmaI into mitochondria, J. Biomed. Sci. 9 (2002) 534–541.

[2] E. Naito, M. Ito, I. Yokota, T. Saijo, Y. Ogawa, Y. Kuroda, Diagnosis and molecular analysis of three male patients with thiamine-responsive pyruvate dehydrogenase complex deficiency, J. Neurol. Sci. 201 (2002) 33–37.

[3] L.C. Lopez, M. Schuelke, C.M. Quinzii, T. Kanki, R.J. Rodenburg, A. Naini, S. Dimauro, M. Hirano, Leigh syndrome with nephropathy and CoQ10 deficiency due to decaprenyl diphosphate synthase subunit 2 (PDSS2) mutations, Am. J. Hum. Genet. 79 (2006) 1125–1129.

[4] C. Quinzii, A. Naini, L. Salviati, E. Trevisson, P. Navas, S. Dimauro, M. Hirano, A mutation in para-hydroxybenzoate-polyprenyl transferase (COQ2) causes primary coenzyme Q10 deficiency, Am. J. Hum. Genet. 78 (2006) 345–349.

[5] M. Tanaka, Y. Nishigaki, N. Fuku, T. Ibi, K. Sahashi, Y. Koga, Therapeutic potential of pyruvate therapy for mitochondrial diseases, Mitochondrion 7 (2007) 399–401.

[6] D. Leigh, Subacute necrotizing encephalomyelopathy in an infant, J. Neurol. Neurosurg. Psychiatry 14 (1951) 216–221.

[7] J. Finsterer, Leigh and Leigh-like syndrome in children and adults, Pediatr. Neurol. 39 (2008) 223–235.

[8] A.A. Morris, J.V. Leonard, G.K. Brown, S.K. Bidouki, L.A. Bindoff, C.E. Woodward, A.E. Harding, B.D. Lake, B.N. Harding, M.A. Farrell, J.E. Bell, M. Mirakhur, D.M. Turnbull, Deficiency of respiratory chain complex I is a common cause of Leigh disease, Ann. Neurol. 40 (1996) 25–30.

[9] S. Rahman, R.B. Blok, H.H. Dahl, D.M. Danks, D.M. Kirby, C.W. Chow, J. Christodoulou, D.R. Thorburn, Leigh syndrome: clinical features and biochemical and DNA abnormalities, Ann. Neurol. 39 (1996) 343–351.

[10] S. DiMauro, J.F. Nicholson, A.P. Hays, A.B. Eastwood, A. Papadimitriou, R. Koenigsberger, D.C. DeVivo, Benign infantile mitochondrial myopathy due to reversible cytochrome *c* oxidase deficiency, Ann. Neurol. 14 (1983) 226–234.

[11] H.P. Hermann, B. Pieske, E. Schwarzmuller, J. Keul, H. Just, G. Hasenfuss, Haemodynamic effects of intracoronary pyruvate in patients with congestive heart failure: an open study, Lancet 353 (1999) 1321–1323.

[12] R.G. Weiss, S.T. Gloth, R. Kalil-Filho, V.P. Chacko, M.D. Stern, G. Gerstenblith, Indexing tricarboxylic acid cycle flux in intact hearts by carbon-13 nuclear magnetic resonance, Circ. Res. 70 (1992) 392–408.

[13] M. Moriyama, M.X. Li, K. Kobayashi, D.S. Sinasac, Y. Kannan, M. Iijima, M. Horiuchi, L.C. Tsui, M. Tanaka, Y. Nakamura, T. Saheki, Pyruvate ameliorates the defect in ureogenesis from ammonia in citrin-deficient mice, J. Hepatol. 44 (2006) 930–938.

[14] K. Mutoh, K. Kurokawa, K. Kobayashi, T. Saheki, Treatment of a citrin-deficient patient at the early stage of adult-onset type II citrullinaemia with arginine and sodium pyruvate, J. Inherit. Metab. Dis. (2008) electronic publication pubmed id: 18958581.

# 2．ミトコンドリア病（ミトコンドリア呼吸鎖異常症）

## ―最も頻度の高い先天代謝異常症―

村山　圭[*1]，森　雅人[*2]，大竹　明[*3]

KEY WORDS
小児期ミトコンドリア病
呼吸鎖酵素解析
組織特異性
遺伝子診断
mtDNA 枯渇症候群（Mitochondrial DNA Depletion Syndrome；MDS）

Kei Murayama

### はじめに―MELAS や Leigh 脳症だけではない―

ミトコンドリア病（ミトコンドリア呼吸鎖複合体異常症）は呼吸鎖（電子伝達系）または酸化的リン酸化の障害であり，多彩な臓器症状を呈する先天代謝異常症である。その有病率は少なくとも5,000人に１人と言われている[1]。しかし，多くの小児科医にとってその実感はおそらくない。その理由として，①MELAS や Leigh 脳症に代表されるような神経疾患だけであると思っている（実際には小児ミトコンドリア病全体の１/３程度である），②原因として最も多いと思われる新生児期のミトコンドリア病（致死性・非致死性乳児ミトコンドリア障害）に触れる機会がない，③急性脳症，原因不明の心筋症，肝症において本症の検索がなされていない，④本症の診断は時に難しい（後述）などが挙げられる。本稿をお読みいただいた読者が，実際の臨床の場において，ミトコンドリア病はありふれた代謝疾患であると感じていただきたいと切に願う次第であり，そこから治療へ向けた様々な発展が起こってくるものと確信している。本稿では日頃のミトコンドリア病の診断を進めていく過程でよく受ける質問等を大いに考慮しながら，本症の診断や治療について述べていきたい。

＊1：千葉県こども病院　代謝科（〒266-0007　千葉県千葉市緑区辺田町579-1）E-mail：kmuraya@mri. biglobe. ne. jp
＊2：自治医科大学　小児科
＊3：埼玉医科大学　小児科

〔大基準〕

Ⅰ．臨床症状
以下の3項目（①～③）をすべて満たす臨床的に確定診断されたミトコンドリア脳筋症
またはミトコンドリアサイトパチー[※1]
①他の病因では説明できない多臓器にまたがる症状が存在する：少なくとも以下の3系統以上の臓器にまたがること
　1）神経系，2）筋肉，3）心臓，4）腎臓，5）消化器系，6）肝臓，7）内分泌，8）造血器，
　9）耳科，10）眼科，11）皮膚科，12）奇形症候群
②発作性進行性経過：しばしば感染を機に増悪する
　または
　母系遺伝を思わせる家族歴：母方の親戚の1人以上にミトコンドリア呼吸鎖
　異常症の疑い例または可能性例が存在する
③代謝性あるいは非代謝性の除外診断を確実に行うこと

Ⅱ．病理組織像
骨格筋　　2％以上の ragged red fiber（赤色ぼろ線維）

Ⅲ．酵素活性
①抗体染色：COX（－）fiber　　50歳以下の場合2％以上
　　50歳以上の場合5％以上
②In vitro 呼吸鎖酵素活性[※2]
1つの臓器で20％以下または2つ以上の臓器にまたがって30％以下
1つの培養細胞で30％以下

Ⅳ．機能解析
線維芽細胞の ATP 合成能：平均マイナス3SD 以下

Ⅴ．分子生物学
核またはミトコンドリアの明らかな病原遺伝子異常が見つかること

〔小基準〕

Ⅰ．臨床症状
1つでもミトコンドリア脳筋症に合致した症状[※3]があること

Ⅱ．病理組織像
骨格筋：ragged red fiber　30～50歳　1～2％
　　30歳以下　少しでもあればよい
　筋線維膜下のミトコンドリアの異常集積　16歳以下で2％以上
臓器は問わず：ミトコンドリアの電顕的異常

Ⅲ．酵素活性
①抗体染色による呼吸鎖酵素欠損の証明
②In vitro 呼吸鎖酵素活性[※2]
1つの臓器で20～30％または2つ以上の臓器にまたがって30～40％
1つの培養細胞で30～40％

Ⅳ．機能解析
①線維芽細胞の ATP 合成能：平均マイナス2～3SD
②Galactose medium 中で成育できない線維芽細胞

診断基準（文献5，6より改変）

V．分子生物学
核またはミトコンドリアの可能性のある遺伝子異常がみつかること

VI．呼吸鎖異常を示唆する1つ以上の検査所見
①血中，髄液中乳酸・ピルビン酸・アラニン高値
②髄液中タンパクの増加（KSS 疑いの時）
③$^{31}$P-MRSまたは PET の異常所見（筋肉 or 脳）
④エルゴメター異常所見（$VO_2max$，$AVO_2D$，乳酸閾値の低下）

Definite：確定例
　大基準2つ　または，大基準1つプラス小基準2つ
Probable：疑い例
　大基準1つプラス小基準1つ　または，小基準3つ
Possible：可能性例
　大基準1つ　または，
　小基準のI（臨床症状）プラス他の小基準もう1つ

下線は千葉県こども病院・埼玉医大小児科で解析できるものを表す。
（Blue Native 電気泳動は，この診断基準には含まれていない。）

〔補足〕

※1 ミトコンドリア脳筋症またはミトコンドリアサイトパチー
①Leigh disease ②Alpers症候群 ③LIMD 致死型乳児ミトコンドリア病 ④Pearson症候群 ⑤KSS（Kearns-Sayre症候群） ⑥MELAS（mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes） ⑦MERRF（myoclonic epilepsy with ragged-red fibers） ⑧NARP（neuropathy, ataxia, and retinitis pigmentosa） ⑨MNGIE（mitochondrial neuro-gastro-intestinal encephalomyopathy） ⑩LHON（Leber's hereditary optic neuropathy）

※2 呼吸鎖酵素活性は，クエン酸合成酵素（CS）または，complex IIとの比を正常対照平均に対する％で表す。

※3 ミトコンドリア脳筋症に一般的な症状：
①筋肉
　1）眼筋症：外眼筋麻痺，眼けん下垂
　2）運動不耐症，筋力低下，脱力感
　3）心筋伝導障害
　4）肥大型　or　拡張型（まれ）心筋症
　5）筋痛症，横紋筋融解症
②神経
　1）失調症
　2）感音性難聴
　3）痴呆症　or　精神遅滞
　4）網膜色素変性症
　5）視神経萎縮
　6）癲癇／ミオクローヌス
　7）卒中様発作
　8）末梢性神経症
③その他
　1）成長障害
　2）De Toni-Fanconi-Debre 症候群
　3）糖尿病
　4）肝障害，肝不全
　5）消化管運動障害 and/or 吸収不良
　6）多発性対称性脂肪腫症
　7）汎血球減少症

これらに小児科的には以下の症状を加える
　①胎動が乏しく流産に至った既往
　②新生児死亡
　③動きの乏しい児
　④重症体重増加不良
　⑤新生児筋緊張の低下
　⑥新生児筋緊張の亢進
（成人基準では筋または神経症状が必須であるが，小児基準には必ずしも適切ではない）

## Ⅰ．ミトコンドリア病の診断―診断に最も有効な検査は酵素解析である―

ミトコンドリアの最も重要な仕事は，糖質，蛋白，脂質を燃料としてATPというエネルギーを作り出すことであり，その最大の場がミトコンドリア呼吸鎖である。呼吸鎖はミトコンドリアDNA（mtDNA）と核DNAの共同作業によりATPを産生するが，多くを核に依存しているため，特に小児期発症のミトコンドリア病は核DNAの異常に基づくほうが遙かに多い（90～95％）[2][3]。

ミトコンドリア病は，いかなる症状，いかなる臓器・組織，いかなる年齢にでも発症する。そして，母系遺伝も含めあらゆる遺伝形式をとりうる[4]。本症の診断基準において最も有用なものはBernierらの基準[5]である（表1）。一見複雑そうであるが，多くのデータに基づきつくられたよくできた診断基準である。診断ツールは，①臨床症状，②生化学検査，③病理組織，④酵素活性，⑤機能解析，⑥DNA解析の6つからなる（図1）。このうち①②は臨床の現場で行えるが，他は特殊検査となる。⑥はMELASであればmtDNAの検索も有効であるが，その他の疾患はmtDNAの検索では分らないことがほとんどである。現実的には生検を行ったうえで行う③ないし④に絞られる。もしフレッシュな組織（採取後速やかにそのまま－80°Cに保存してある組織）があるのであれば，酵素活性に勝るものはない。

もう一つ大切なことは，他の代謝疾患をしっかりrule outできているかどうかである。代謝疾患のrule outについては，正書を参考にしていただきたいが，少し乱暴に言えば，アミノ酸分析，尿中有機酸分析，アシルカルニチン分析は行っておきたい検査である。尿素サイクル異常症，有機酸代謝異常症，脂肪酸代謝異常症はミトコンドリアの二次的障害を伴うことが非常に多い[7]。こうした疾患のrule outは診断基準の中にもしっかり記載されている。

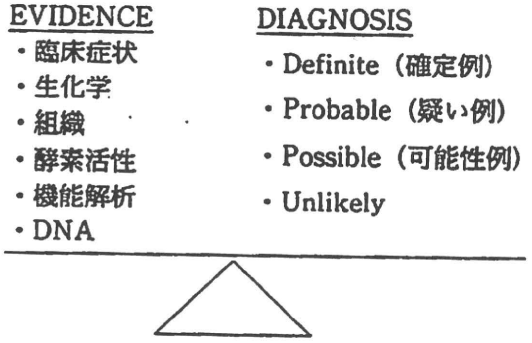


図1　呼吸鎖異常症の診断

本症を考えるとき，「何でもあり」と思っていただけると診断に近づけるものと考える。例えば，単一臓器の障害だけでは説明のできない病態，複数臓器にまたがる病態は，本症を疑う重要な鍵となる。私たちが経験した新生児発症の難治性分泌性下痢症の症例は，まさに「何でもあり」を如実に表しているのでぜひ一読していただきたい[8]。

高乳酸血症は本症を疑う重要な手がかりとなる。特にピルビン酸との比（L/P比）が20を超える場合は，呼吸鎖の障害が示唆される。また，ケトン体比（3-OHB/AcAc）が3以上の時も呼吸鎖の障害が考えられる。乳酸はピルビン酸とセットで提出しておきたい。しかし，ミトコンドリア病の約20％は乳酸値正常である[9]。したがって，乳酸値が低いから本症を否定したことにはならないし，乳酸値だけにこだわることも賢明ではない[8]。あくまで参考にとどめるべきである。

病理に関して一言加えたい。RRF（ragged red fiber）は小児では一般的ではないが，ミトコンドリア異常症を示す非常に有用な所見であり，Bernierの大基準に記載されている。その時には，フレッシュな組織であれば必ず酵素活性も低下を認める。逆にRRFがないからといって「ミトコンドリア

病でない」ことにはならない。酵素活性の測定も併せて行うべきである。

## II. 酵素解析について

酵素解析のメインは呼吸鎖の酵素活性である。筆者の施設では，分光光度計を用いて，Complex IからIVおよびミトコンドリアマーカーとしてTCA回路の構成酵素であるクエン酸合成酵素（CS；Citrate Synthase）を測定している。Bernierらの基準[5]にあるとおり，活性の絶対値だけでなく，CS比，Complex II比で表すことが重要である。

測定検体は，組織は筋肉，肝臓，心筋，腎臓，培養細胞では皮膚由来の線維芽細胞である。組織に関しては−80℃にて凍結した生検体を用いている。肝の針生検ではバードモノプティを使うと1本が5〜10mg程度に相当する。1本で何とかできるが3本程度あることが望ましい。筋肉も40mg（3〜4㎜立方体くらい）程度あると問題なく行える。心筋は非常にミトコンドリアが豊富にあるため，比較的少量でも可能である。

また，病理解剖後の酵素活性の測定は，死亡後12時間以内に採取し，すぐに−80℃に保存しておくことが望ましい。酵素活性は比較的不安定なComplex IIから低下を始めてしまうため，その評価が難しくなる。可能であるならば，すぐに針による肝臓Autopsyや皮膚の採取（死亡後すぐであれば培養が可能）を行うとよい。−80℃に保存されれば，少なくとも10年以上にわたって酵素活性は保たれる。

Blue-Native PAGEを用いたイムノブロット解析は，近年ミトコンドリア病の強力な診断法として広く行われている。酵素活性の残り検体でアプライできるため，酵素活性を補完する意味は勿論，分子量の違いによりアセンブリー異常を推定することも可能である[10]。

## III. 組織特異性について

ミトコンドリア病の大きな特徴のひとつは，組織特異性（Tissue Specificity）である。この組織特異性はmtDNAの異常でも核DNAの異常でも起こってくる。ミトコンドリア蛋白の50%は組織特異性（tissue specificity）があるといわれている[11]。例えば，筋肉と肝臓での呼吸鎖の酵素活性が異なってくることは，実際によく見られることである。Thorburnらは，肝臓において明らかな酵素欠損があっても，骨格筋で同様の酵素欠損が見つかる率は25%にも満たないと述べている[12]。自験例でも同様であり，肝機能異常を認めたミトコンドリア病26例のうち，22例が肝臓を用いて診断できている。特に肝不全を呈してくる肝型mtDNA枯渇症候群は12例全例が肝臓での診断であり，線維芽細胞などは正常活性であった。また，骨格筋や肝臓で酵素欠損があった場合，線維芽細胞でも同様の結果が出るのは50%にも満たない。

以上から帰結されることは，ミトコンドリア病の診断は，できるだけ障害臓器を用いた酵素解析を行うということである。診断だけを考えれば障害臓器の検索が最も有用である一方で，侵襲度の低さを考えれば皮膚生検→線維芽細胞の培養による検索であり，実際の患児の状態や家族の希望をもとに，総合的に判断し診断を進めて行くことが大切だと考える。なお，線維芽細胞の培養には数カ月要することも付け加えておく。悩んだ場合は遠慮なく相談していただければ幸いである。

## IV. 遺伝子診断について

先述したとおり，一般に小児期早期におけるミトコンドリア病はほとんど核遺伝子の変異によって起こるため，mtDNAの検索は必ずしも適応とはならない[2]。mtDNAの検索は，臨床症状が特定の症候群に当てはまる症

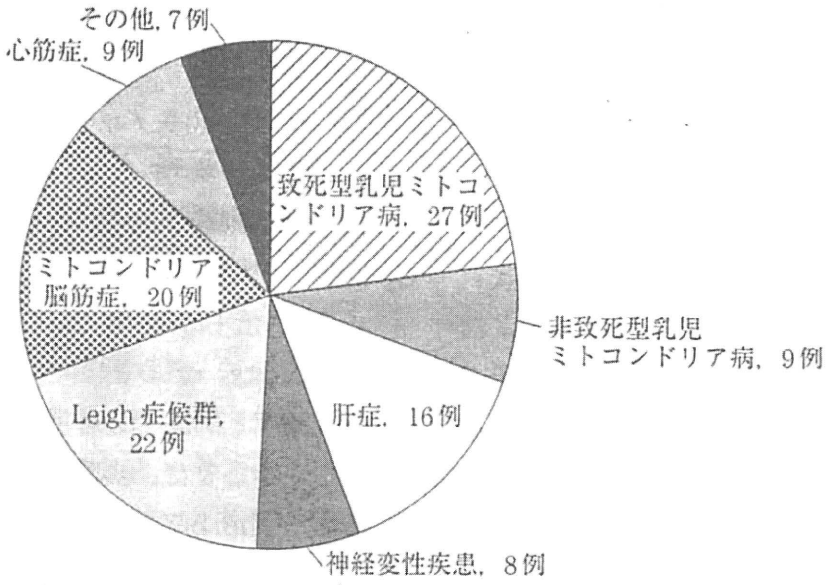


**図2　ミトコンドリア病と診断された患者の臨床診断（症例数）**（2010年7月現在，千葉県こども病院・埼玉医科大学小児科）

例に有用である（Pearson，NARP，MELAS，MERRF など）。しかし，時に mtDNA の変異はミトコンドリアの一部にしか存在しないことがある（heteroplasmy）。病因となる変異は必ずしも血液に見いだされるとは限らず，障害臓器に限局されることもある。

核遺伝子の検索は非常に難しい。それは次の理由による。①候補遺伝子が多すぎる，②ほとんどが家系特異的変異（private mutation）である，③Common mutation がわずかである（*POLG*，*SURF1*，*LRPPRC* など），④臨床型と遺伝子型の関連（Genotype/phenotype correlation）がしばしば乏しい（例えば Leigh 脳症はすでに30以上の責任遺伝子が分っている）。

したがって大規模研究として行うか，個々のchallengeに賭けるかになってしまうところが核遺伝子の診断の難しいところである。このことからも遺伝子診断に繋げるためにも，酵素診断の重要性がわかっていただけるのではないだろうか。

## Ⅴ．本邦におけるミトコンドリア病

筆者らが2010年7月までに診断した118例の臨床診断を図2に示す。臨床診断が多岐にわたっていることに注目していただきたい。小児神経科医だけでなく，新生児科医，小児肝臓や循環器の専門医も十分認識しておくべき疾患であるといえる。なかでも乳児期のミトコンドリア障害（いわゆる先天性高乳酸血症）が最も多いことが分かる。新生児期発症のミトコンドリア病は本邦でも報告されているが[13]，筆者らのまとめた新生児発症のミトコンドリア病は，小児ミトコンドリア病全体の約半数（93例中44例）を占めた[14]。また，44例中27例が死亡しており，特に新生児期発症のミトコンドリア病は予後が悪いといえる。新生児期の症状に関して，Bernier ら[5]の診断基準の最後に補足・記載されている。流産の既往，新生児期の死亡，動きの乏しい児，重度の体重増加不良，新生児期の筋緊張低下・亢進は，本症を疑わせる所見である。

また，小児期発症では急性脳症や臨床的 Reye 症候群を示す症例も数例経験しているが，診断されていないものが相当数あるように思われる。

臨床診断されたものを，酵素診断で分けたものを図3に示す。酵素診断で最も多いのは Complex Ⅰ欠損症であり，全体の約4割を

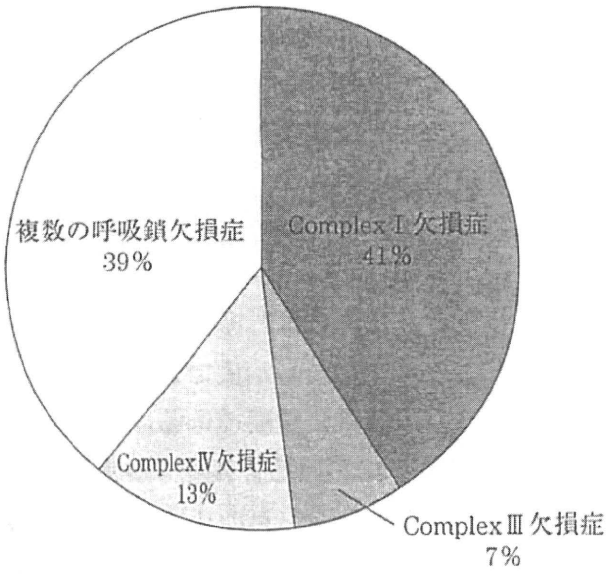


**図3　ミトコンドリア病と診断された患者の酵素診断の内訳**（2010年7月現在，千葉県こども病院・埼玉医科大学小児科）

占める。次いで多いものは，Ⅰ＋Ⅳ，Ⅰ＋Ⅲ，Ⅰ＋Ⅲ＋Ⅳといったような複数にわたる酵素欠損であり，Complex Ⅰ同様に4割近くを占める。Complex Ⅱ欠損症は世界的にも報告は少ない。次いで多いのは Complex Ⅳ欠損症であるが，Complex Ⅳ欠損症は cytochrome c oxidase（COX）とも呼ばれる。Leigh脳症に報告が多いが，それ以外の病態（ミオパチーや乳児ミトコンドリア障害など）でも起こる。

余談になるが，筆者らは酵素活性の検体量が極めて少ない場合には，欠損頻度の多い順に検索すべくCS→Complex Ⅰ→Ⅱ→Ⅳ→Ⅲの順にできるところまで行うことにしている。また，118例中，mtDNA の異常を認めたものは20例であり，多くは核の異常によると推定される。

## Ⅵ. mtDNA 枯渇症候群（Mitochondrial DNA Depletion Syndrome；MDS）

MDS は mtDNA の複製や核酸合成に関わる蛋白の異常によって，mtDNA の量が低下した結果，Complex Ⅱ以外の呼吸鎖Ⅰ，Ⅲ，Ⅳ，Ⅴの活性の低下がみられてくるミトコンドリア病の疾患群である（Complex Ⅱは核DNA のみにコードされている）。脳肝型，脳筋型，筋型に分類される（肝型，筋型と簡便に呼ぶことが多い）。いずれも核遺伝（今のところ常染色体劣性のみ）である。mtDNA が減少するだけでミトコンドリアの数が減少するわけではない。ミトコンドリアはむしろ増加し，クエン酸合成酵素の活性が異常高値になる。

酵素欠損は，Complex Ⅰ＋Ⅲ＋Ⅳ欠損，Complex Ⅰ＋Ⅲ欠損，Complex Ⅰ欠損のパターンをとりうる。臨床症状と酵素診断から推定し，mtDNAの比較定量（qPCR）を行えば診断は容易である[15]。これまで筆者らは12例の肝型 MDS，6例の筋型 MDS を診断してきたが，いずれも非常に重篤な症例ばかりである。ミトコンドリア病全体としても1～2割程度は占めるものと思われる。

## Ⅶ. ミトコンドリア病の治療

ミトコンドリア病は様々な臓器障害を起こすため，各臓器障害に応じた治療は非常に重要である。これらは各専門医に相談しながら対応していく必要がある。

薬物療法に関しては，今のところ決め手となる治療はなく，症状を緩和する治療が主体である。栄養に関しては，小児科医の腕の見せ所であると考える。酵素診断や遺伝子診断がしっかりなされることにより，今後様々な治療が生まれてくるはずである。臨床医がまずできることは，しっかりと診断をし，症例を積み重ねていくことに尽きる。以下に現在の主な治療についてあげていきたい。

### 1．ミトコンドリア機能をサポートするビタミンや補酵素等の投与

基本的にはいずれの薬物も有用であるという十分なエビデンスはない。しかし，ミトコンドリア障害が考えられるとき，原発性であ

表2　ミトコンドリアレスキュー薬
（千葉県こども病院モデル）

| | |
|---|---|
| アリナミンF（$VitB_1$） | 100mg |
| シナール（VitC） | 1g |
| ビオチン（VitH） | 5mg |
| ユベラ（VitE） | 100mg |
| ノイキノン（CoQ） | 50mg |
| カルニチン | 300mg |
| 分2～3 | |

各種脳症，metabolic crisis（代謝性アシドーシスを伴う意識障害）の急性期ほか，最近は尿素サイクル異常症などにも使っています．1歳用（10kg）につくってありますので，適宜調整してください．
商品名は千葉県こども病院採用のものです．

っても2次性であっても各種ビタミン剤や補酵素などを投与することは悪いことではない。各種代謝性疾患はミトコンドリアの2次的障害を伴うことが多く[7]，筆者らは表2に示すミトコンドリアレスキュー薬を急性脳症，各種急性代謝異常症，尿素サイクル異常症などに使用している。ミトコンドリア肝症についてはトランスアミナーゼの改善がよく経験される[16]。一方で，神経学的所見が強い症例や心筋症などは効果に乏しい傾向にある。

ビタミンC+Kの併用は，Complex IIIをバイパスさせる治療として報告されている[18]。Complex IV欠損症に対するピルビン酸療法も近年取り上げられており，症例の蓄積が期待される[19]。

2．栄養

脂肪酸代謝異常症が否定されているのであれば，糖質を抑え，脂質を多めに与えるとよい。Kajiらは，*MPV17*遺伝子異常に基づく肝型mtDNA枯渇症候群において，コハク酸を含むComplex IIをtargetにした薬物療法とケトン乳+MCTミルクを投与することにより，トランスアミナーゼは正常化し，さらに感染時の肝機能の悪化を抑えたことを報告している[16]。また，低血糖を頻回とする肝型MDSの場合，糖質を頻回（3時間ごと）投与することにより低血糖を抑え肝機能も改善されたとの報告もある[19]。呼吸鎖障害時の糖負荷はNADHを過剰にさせる方向に動くため，低血糖にならない程度の糖質は与えた方が良い。これらはしっかりと酵素解析を行ってこそ考えられる治療であるため，障害臓器の検索は非常に重要である。

3．MELASにおけるアルギニン療法

アルギニン—NO—シトルリンサイクルの基質として投与することにより，NOの産生を促し，脳血管の拡張を引き起こしstrokeを改善させることを意図する。医師主導型治験として本邦で行われており[20]，2009年の国際先天代謝異常学会（San Diego）でも取り上げられ注目されている。また，経口投与（アルギU顆粒）によりstrokeの予防にも効果があると思われる。このことはMELAS患者の体内においてアミノ酸のimbalanceがあることを示唆している。

4．ミトコンドリア肝症に対する肝移植

主に肝不全を呈する肝型MDSが対象となる。これまでも肝移植は多く行われてきた。本邦でも酵素診断が確立される以前は，特に肝不全を改善させる緊急避難的に肝移植は行われてきた。肝移植の適応の是非についての記載は散見されるが[21]，現在のところ不定である。肝外症状を改善させるわけではなく，神経症状が強い場合や心筋症など手術侵襲に耐え難い症状がある場合は，適応外とせざるを得ない。組織特異性を認識したうえで，個々の症例について慎重に検討すべき事項であろう。

## おわりに

2009年にSan Diegoで開かれた国際先天代謝異常学会において，最も演題が多かったのがライソゾーム病であり，次いでミトコン