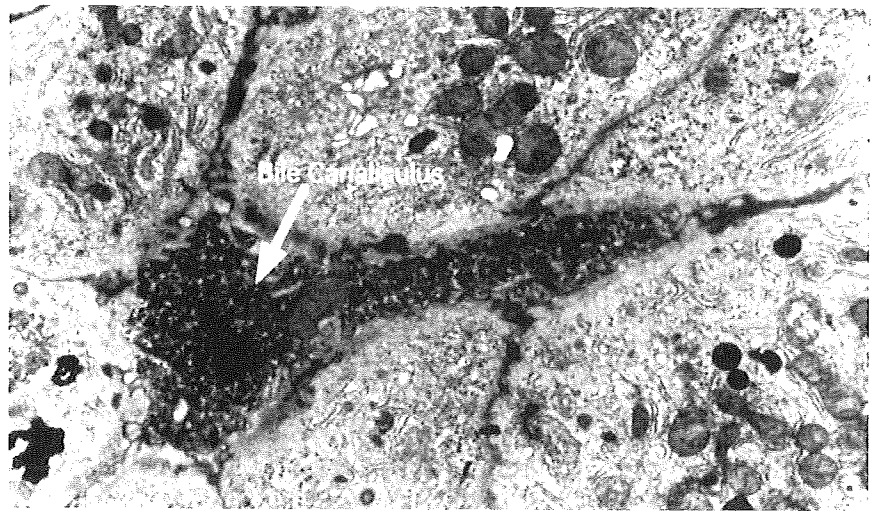

写真1　われわれが経験したPFIC type 1の毛細胆管内のbyler's bileを示す。

表2　胆汁酸代謝異常症とその特異的胆汁酸

3-Oxo-Δ^4-steroid 5β-reductase 欠損症：3-oxo-Δ^4 胆汁酸，allo 胆汁酸
3β-Hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase 欠損症：3β-dihydroxy-Δ^5 胆汁酸，3β-trihydroxy-Δ^5 胆汁酸
Oxysterol 7α-hydroxylase 欠損症：3β-monohydroxy-Δ^5 胆汁酸
Sterol 27-hydroxylase 欠損症：胆汁アルコール
Zellweger 症候群：THCA，DHCA

THCA, 3α-7α,12α-trihydroxy-5β-cholestanoic acid; DHCA, 3α-7α-dihydroxy-5β-cholestanoic acid.

遺伝子診断しかない。現在，遺伝子診断が出来る施設は国立成育医療センターで，遺伝診療科右田王介先生に相談されたい。

久留米大学病理学教室の鹿毛政義先生が病理組織からPFIC type 1とtype 2の鑑別が出来ないか症例の検討を始められている。是非，症例をお持ちの方はご連絡を(masakage@med.kurume-u.ac.jp)。

胆汁酸代謝異常症の分類（鑑別方法）

図1より胆汁酸代謝異常症が疑われた場合，まず，尿中胆汁酸分析を行い異常胆汁酸の検出の有無をみる。異常胆汁酸を検出したならば，次に血清の胆汁酸分析を勧める。血清胆汁酸分析で尿と同様に異常胆汁酸を検出したならば胆汁酸代謝異常症と診断して間違いないだろう。それぞれの胆汁酸代謝異常症で特異的な異常胆汁酸を検出する（表2）[17) 18)]。(尿中胆汁酸分析は，順伸クリニック入戸野博先生へ相談されるとよい。Tel：045-902-8818)

ただし，Zellweger症候群では，他の胆汁酸代謝異常症と異なりγ-GTPの上昇をみる。

ま と め

胆道閉鎖症を診断するための手立てについて述べた。類似疾患（肝内胆汁うっ滞症）の鑑別方法は進歩し可能になりつつあるものの胆道閉鎖症の診断は未だ困難である。乳児（特に60生日未満）の閉塞性黄疸，白色便をみたなら胆道閉鎖症を疑って欲しい。そして急いで診断すべきである。

文　献

1) Balistreri WF, Bezerra JA, Jansen P, Karpen SJ, Shneider BL, Suchy FJ. Intrahepatic cholestasis: summary of American Association for the Study of Liver Disease single-topic conference. Hepatology 2005 ; 42 : 222-235.
2) Balistreri WF, Bezerra JA. Whatever happened to "neonatal hepatitis" ? Clin Liver Dis 2006 ; 10 ; 27-53.
3) Sokol RJ, mack C, Narkewicz MR, Karrer FM. Pathogenesis and outcame of biliary atresia: current concepts. J Pediatr Gastroenterol Nutr 2003 ; 37 : 4-21.
4) Bezerra JA. The next challenge in pediatric cholestasis: deciphering the pathohenesis of biliary atresia. J Pediatr Gastroenterol Nutr 2006 ; 43 : S23-S29.
5) Harada K, Sato Y, Itatsu K, Isse K, Ikeda H, Yasoshima M, Zen Y,

matsui A, Nakanuma Y. Innate immunity to dsRNA in human biliary epithelial cell is associated with the pathogenesis of biliary atresia. Hepatology 2007 ; 46 : 1146-1154.
6) Matsui A, Dodoriki M. Screening for biliary atresia. Lancet 1995 ; 345 : 1181.
7) Chen S-M, Chang M-H, Du J-C, Lin C-C, Chen A-C, Lee H-C, Lau B-H, Yang Y-J, Wu T-C, Chu C-H, Lai M-W, Chen H-L, the Taiwan infant stool color card study group. Screening for biliary atresia by infant stool color card in Taiwan. Pediatrics 2006 ; 117 : 1147-1154.
8) 米沢俊一，佐々木美香，水野　大，島岡　理，佐々木純子．岩手県胆道閉鎖症（BA）マススクリーニング（MS）の4年間の結果報告：第1報．日児誌 2007 ; 111 : 605.
9) 秋山卓士．胆道閉鎖症．小児内科 2002 ; 34増刊号 : 546-550.
10) Deleuze JF, Jacquemin E, Dubuisson C, Cresteil D, Dumont M, Erlinger S, Bernard O, Hadchouel M. Defect of multidrug-resistance 3 gene expression in a subtype of progressive familial intrahepatic cholestasis. Hepatology 1996 ; 23 : 904-908.
11) de Vree JM, Jacquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, Deleuze JF, Desrochers M, Burdeiski M, Bernard O, Oude Elferink RP, Hadchouel M. Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. Proc Natl Acad Sci USA 1998 ; 95 : 282-287.
12) Jacquemin E, de Vree JM, Cresteil D, Sokal EM, Sturm E, Dumont M, Scheffer GL, Paul M, Burdeski M, Bosma PJ, Bernard O, Hadchouel M, Elferink RP. The wide spectrum of multidrug resistance 3 deficiency: from neonatal cholestasis to cirrhosis of adulthood. Gastroenterology 2001 ; 120 : 1448-1458.
13) Bull LN, Carlton VE, Stricker NL, baharloo S, DeYoung JA, Freimer NB, magid MS, Kahn E, markowitz J, DiCarlo FJ, McLoughlin L, Boyle JT, dahms BB, Faught PR, Fizgerald JF, Piccoli DA, witzleben CL, O'Connell NC, Setchell KD, Agostini RM Jr, Kocoshis SA, Reyes J, Knisely AS. Genetic and morphological findings in progressive familial intrahepatic cholestasis (Byler disease [PFIC-1] and Byler syndrome) : evidence for heterogeneity. Hepatology 1997 ; 26 : 155-164.
14) Bull LN, van Eijk MJ, Pawlikowska L, DeYoung JA, Juijn JA, Liao M, Klomp LW, Lomri N, Berger R, Scharschmidt BF, Knisely AS, Houwen RH, Freimer NB. A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. Nat Genet 1998 ; 18 : 219-224.
15) Jansen PL, Strautnieks SS, Jacquemin E, Hadchouel M, Sokal EM, Hooiveld GJ, Koning JH, De Jager Krikken A, Kuipers F, Stellaard F, Bijleveld CM, Gouw A, Van Goor H, Thompson RJ, Muller M. Hepatocanalicular bile salt export pump deficiency in patients with progressive intrahepatic cholestasis. Gastroenterology 1999 ; 117 : 1370-1379.
16) Wang L, Soroka CJ, Boyer JL. The role of gile salt export pump mutations in progressisve familial intrahepatic cholestasis type II. J Clin Invest 2002 ; 110 : 965-972.
17) Setchell KDR, Heubi JE. Defects in bile acid biosynthesis-diagnosis and treatment. J Pediatr Gastroenterol Nutr 2006 ; 43 : S17-S22.
18) Heubi JE, Setchell KDR, Bove KE. Inborn errors of bile acid metabolism. Semin Liver Dis 2007 ; 27 : 282-294.
19) 石川暢己，大浜和憲．当科における胆道閉鎖症症例の検討．日小外会誌 2007 ; 43 : 819.
20) Choi SO, Park WH, Lee HJ, Woo S-K. 'Triangular cord' : a sonographic finding applicable in the diagnosis of biliary atresia. J Pediatr Surg 1996 ; 31 : 363-366.
21) Kim M-J, Park YN, Han SJ, Yoon HS, Hwang EH, Chung KS,. Biliary atresia in neonates and infants: triangular area of high signal intensity in the port hapatis at T2-weighted MR cholangipancreatography with US and histopathologic correlation Radiology 2000 ; 215 : 395-401.
22) Jevon GP, Kimmick JE. Biliary atresia and cytomegalovirus infection: a DNA study. Pediatr Dev Pathol 1999 ; 2 : 11-14.
23) Morecki R, Glaser JH, Cho S, Balistreri WF, Horwitz MS. Biriary atresia and reovirus type 3 infection. N Engl J Med 1982 ; 307 : 481-484.
24) Riepenhoff-Talry M, Gouvea V, Evans MJ, Svensson L, Hoffenberg E, Sokol RJ, Uhnoo I, Greenberg SJ, Schakel K, Zhaori G, Fitzgerald J, Chong S, El-Yousef M, Nemeth A, brown M, Piccoli D, Hyams J, Ruffin D, Rossi T. Detection of group C rotavirus in infants with extrahepatic biliary atresia. J Infec Dis 1996 ; 174 : 8-15.
25) Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, Yasuda T, Ikeda S, Hirano R, Terazono H, Crackower MA, Kondo I, Tsui LC, Scherer SW, Saheki T. The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. Net Genet 1999 ; 22 : 159-163.
26) Tazawa Y, Kobayashi K, Ohura T, Abukawa D, Nishinomiya F, Hosoda Y, Yamashita M, nagata I, Kono Y, yasuda T, Yamaguchi N, Saheki T. Infantile cholestatis jaundice associated with adult-onset type II citrullinemia. J Pediatr 2001 ; 138 : 735-740.
27) Tomomasa T, Kibayashi K, Kaneko H, shimura H, Fukusato T, Tabata M, Inoue Y, Ohwada S, Kasahara M, Morishita Y, Kimura M, Saheki T, Morikawa A. Possible clinical and histologic manifestations of adult-oncet type II citrullinemia in early infancy. J Pediatr 2001 ; 138 : 741-743.
28) Tamamori A, Okano Y, Ozaki H, Fujimoto A, Kajiwara M, Fkuda K, Kobayashi K, Saheki T, Togami Y, Yamano T. Neaonatal intrahepatic cholestasis caused by citrin deficiency: swevere hepatic dysfunction in an infant requiring liver transplantation. Eur J Pediatr 2002 ; 161 : 609-613.
29) Lee WS, Sokol RJ. Liver disease in mitochondrial disorders. Semin Liver Dis 2007 ; 27 : 259-273.
30) Resmorduc O, Hermelin B, Boelle PY, Parc R, Taboury J, Poupon R. ABCB4 gene mutation-associated cholelithiasis in adults. Gastroenterology 2003 ; 125 : 452-459.
31) Jacquwmin E, Cresteil D, Manouvrier S, Boute O, Hadchouel M. Heterozygous non-sense mutation of the MDR3 gene in familial intrahepatic cholestasis of pregnancy. Lancet 1999 ; 353 ; 210-211.
32) Alagille D, Estrada A, hadchoued M, Gautier M, Odievre M, Dommergues JP. Syndromic paucity of interlobular bile ducts (Alagille syndrome or arteriohepatic dysplasia): review of 80 cases. J Pediatr 1987 ; 110 : 195-200.
33) Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS, Chandrasekharappa SC. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 1997 ; 16 : 234-242.
34) Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC, Qi M, Trask BJ, Kuo WL, Cochran J, Costa T, Pierpont MEM, Rand EB,

Piccoli DA, Hood L, Spinner NB. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 1997 ; 16 : 243-251.
35) Samejima H, Torii C, Kosaki R, Kurosawa K, Yoshihashi H, Muroya K, Okamoto N, Watanabe Y, Kosho T, Kubota M, Matsuda O, Goto M, Izumi K, Takahashi T, Kosaki K. Screenibg for Alagille syndrome mutations in the JAG1 and NOTCH2 genes using denaturing high-performance liquid chromatography. Genet Test 2007 ; 11 : 216-227.
36) Kahe E. Paucity of interlobular bile ducts: arteriohepatic dysplasia and nonsyndromic duct paucity. Perspect Pediatr Pathol 1991 ; 14 : 168-215.
37) Alagille D, Odievet M, Gautier M, Dommergues JP. Hepatic ductular hypoplasia associated with characteristic facies, vertebral malformations, retarded physical, mental, and sexual development, and cardiac murmur. J Pediatr 1975 ; 86 : 63-71.
38) Treem WR, Krzymowski GA, Cartun RW, Pedersen CA, hyams JS, Berman M. Cytokeratin immunohistochemical examination of liver biopsies in infants with Alagille syndrome and biliary atresia. J Pediatr Gastroenterol Nutr 1992 ; 15 : 73-80.
39) Snover DC, Horwitz CA. Liver disease in cytomegalovirus mononucleosis: a light microscopical and immunoperoxidase study of six cases. Hepatology 1984 ; 3 : 408-412.
40) Sacks SL, Freeman HJ. Cytomegalovirus hepatitis: evidence for direct hepatic viral infection using monoclonal antibodies. Gastroenterology 1984 ; 86 : 346-350.
41) Egawa H, Yorifuji T, Sumazaki R, Kimura A, Hashimoto M, Tanaka K. Intractable diarrhea after liver transplantation for Byler's disease: successful treatment with bile adsorptive resin. Liver Transpl 2002 ; 8 : 714-716.

Diagnostic approaches in neonatal cholestatic liver disease: early discovery of biliary atresia

Department of Pediatrics and Child Health, Kurume University School of Medicine

Akihiko KIMURA, Tatsuki MIZUOCHI, Yoshitaka SEKI and Hiroshi NISHIURA

There has been very little progress in diagnostic techniques and methods for biliary atresia in the last 20 years expected in the area of imaging technology. Therefore we often experience patients with biliary atresia who are not diagnosed until more than 60 days after birth. Incontrast, there has been steady improvement in the diagnosis of some cholestatic liver diseases, such as neonatal intrahepatic cholestasis caused by citrin deficiency, progressive familial intrahepatic cholestasis and inborn error of bile acid synthesis, as a result of improvements in the application of clinical and laboratory findings and development of molecular biological approaches.

Therefore, we discussed diagnostic approaches in neonatal cholestatic liver disease, especially those aimed at early discovery of biliary atresia. We hope that this report will be useful to pediatric hepatologists and young pediatricians.

Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2008.05669.x

HEPATOLOGY

SRD5B1 gene analysis needed for the accurate diagnosis of primary 3-oxo-Δ^4-steroid 5β-reductase deficiency

Isao Ueki,* Akihiko Kimura,* Huey-Ling Chen,† Tohru Yorifuji,‡ Jun Mori,§ Susumu Itoh,¶ Kenichi Maruyama,** Takashi Ishige,†† Hajime Takei,‡‡ Hiroshi Nittono,‡‡ Takao Kurosawa,§§ Masayoshi Kage¶¶ and Toyojiro Matsuishi*

*Department of Pediatrics and Child Health, ¶¶Department of Pathology, Kurume University School of Medicine, Kurume, Fukuoka, ‡Department of Pediatrics, Kyoto University, Graduate School of Medicine, Faculty of Medicine, §Department of Pediatrics, Kyoto Prefectural University of Medical Graduate School of Medical Sciences, Kyoto, ¶Department of Pediatrics, Faculty of Medicine, Kagawa University, Kida-gun, Kagawa, **Department of Neonatology, Gunma Children's Medical Center, Shibukawa, ††Department of Pediatrics, Gunma University, Graduate School of Medicine, Maebashi, Gunma, ‡‡Junshin Clinic, Yokohama, Kanagawa, §§Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido, Japan; and †Department of Pediatrics, National Taiwan University Hospital, Taipei, Taiwan

Key words
3-oxo-Δ^4 bile aciduria, inborn error of bile acid metabolism, mutation analysis, neonatal cholestasis, ursodeoxycholic acid therapy.

Accepted for publication 14 August 2008.

Correspondence
Dr Akihiko Kimura, Department of Pediatrics and Child Health, Kurume University School of Medicine, 67 Asahi-machi, Kurume-shi 830-0011, Japan. Email: hirof@med.kurume-u.ac.jp

Abstract

Background and Aim: We encounter hyper-3-oxo-Δ^4 bile aciduria in patients with severe cholestatic liver disease or fulminant liver failure during the neonatal period. However, simply by bile acid analysis, it is difficult to distinguish hyper-3-oxo-Δ^4 bile aciduria from primary 3-oxo-Δ^4-steroid 5β-reductase deficiency.

Methods: To determine whether 3-oxo-Δ^4-steroid 5β-reductase (*SRD5B1*) gene analysis is required for the accurate diagnosis of 3-oxo-Δ^4-steroid 5β-reductase deficiency, we evaluated the laboratory data, bile acid analysis and *SRD5B1* gene analysis from six patients with hyper-3-oxo-Δ^4 bile aciduria.

Results: Based upon the results, four patients who had developed neonatal liver failure were diagnosed as having neonatal hemochromatosis. Two patients with chronic cholestasis were diagnosed as having primary 3-oxo-Δ^4-steroid 5β-reductase deficiency by *SRD5B1* gene analysis. The *SRD5B1* gene in these two patients had a heterozygous mutation, G737A (Gly 223 Glu) in one patient and C217T (Arg 50 stop) in the other.

Conclusions: Based upon our limited data, we conclude that SDR5B1 gene analysis is required for the accurate diagnosis of 3-oxo-Δ^4-steroid 5β-reductase deficiency. Moreover, we think that it is important to elucidate whether there is a heterozygous or a compound heterozygous mutation of the *SRD5B1* gene in our two patients.

Introduction

We often encounter hyper-3-oxo-Δ^4 bile aciduria in children with severe cholestatic liver disease or fulminant liver failure during the neonatal period.[1–4] When greater than 75% of the total bile acids in the urine of patients with cholestasis consist of 3-oxo-Δ^4 bile acids, 3-oxo-Δ^4-steroid 5β-reductase deficiency should be suspected[5] (Fig. 1). However, using clinical symptoms, such as the absence of pruritus despite the presence of conjugated hyperbilirubinemia, or using laboratory data such as a normal value of γ-glutamyltransferase (GGT) activity, or total bile acid concentration by enzymatic assay using 3α-hydroxysteroid dehydrogenase, or by analysis of bile acids using gas chromatography-mass spectrometry (GC-MS), it is difficult to distinguish primary 3-oxo-Δ^4-steroid 5β-reductase deficiency from a variety of other cholestatic liver diseases causing hyper-3-oxo-Δ^4 bile aciduria. Therefore, we attempted to assess whether it is necessary to carry out gene analysis of 3-oxo-Δ^4-steroid 5β-reductase (SRD5B1 or AKR1D1) on chromosome 7q32-33[6] to establish the diagnosis.

To date, only five patients with primary 3-oxo-Δ^4-steroid 5β-reductase deficiency have been reported in the literature.[7,8] In this disease, if the diagnosis is delayed, primary bile acid treatment cannot be carried out[9,10] and patients suffer a poor clinical outcome as a result.

In the present study, we report the results of *SRD5B1* gene analysis in six patients with hyper-3-oxo-Δ^4 bile aciduria who were recently treated at Kurume, Kyoto, Kagawa, Gunma and Taiwan university hospitals. Moreover, we examined the clinical symptoms, laboratory data and liver histological findings between primary and secondary 3-oxo-Δ^4-steroid 5β-reductase deficiency.

Methods

We evaluated six patients who developed hyper-3-oxo-Δ^4 bile aciduria between August 2002 and July 2007. Their clinical

Journal of Gastroenterology and Hepatology **24** (2009) 776–785

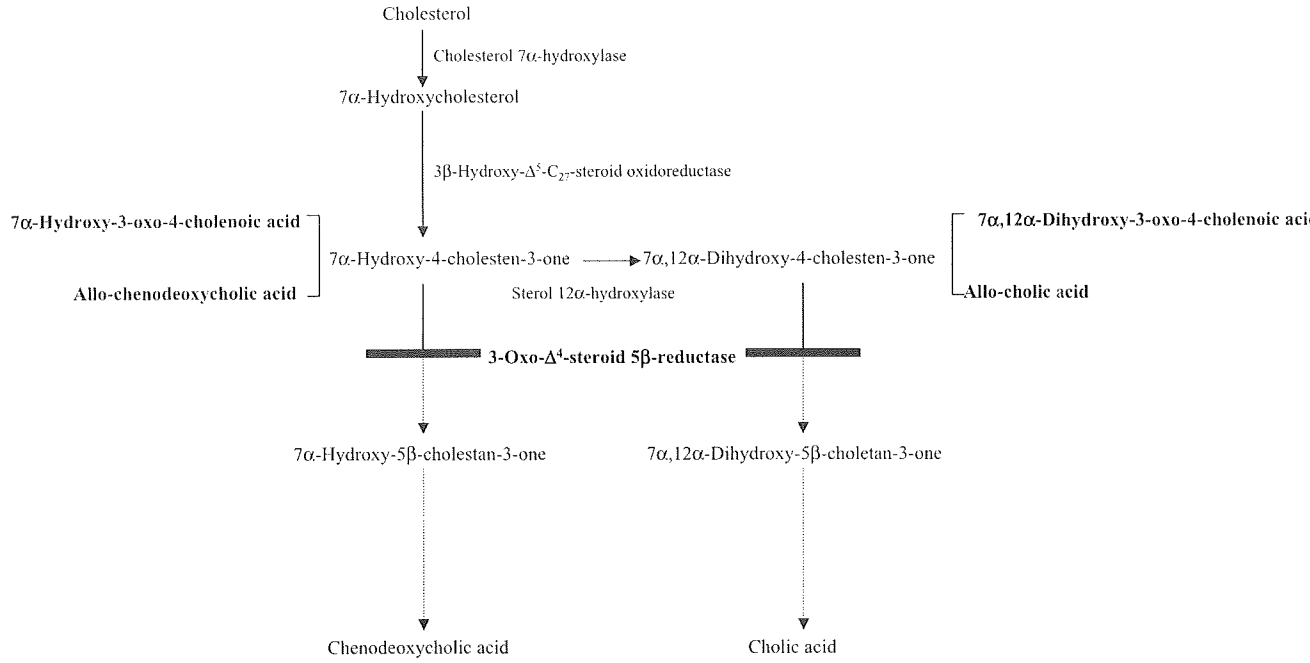

Figure 1 Effect of a defect of 3-oxo-Δ⁴-steroid 5β-reductase. Reduced synthesis of primary bile acids from cholesterol and increased synthesis of 3-oxo-Δ⁴ and allo-bile acids are shown in a flow chart of the classical pathway.

Table 1 Laboratory data of patients with hyper 3-oxo-Δ⁴ bile aciduria on admission

Patient (normal values)	1	2	3	4	5	6
Total bilirubin (0.3–1.5 mg/dL)	11.5	4.9	25.5	11.9	11.3	16.2
Direct bilirubin (<0.6 mg/dL)	6.9	2.0	4.3	4.1	8.3	8.9
Aspartate aminotransferase (13–33 U/L)	29	45	474	1352	934	1180
Alanine aminotransferase (8–42 U/L)	4	6	321	1079	679	878
γ-Glutamyltransferase (10–47 U/L)	36	78	19	17	76	52
Total bile acids (<10 μmol/L)	69.4	28.4	20.4	14.4	2.7	19.8
Serum ammonia (12–66 μg/dL)	270	241	176	187	116	n.d.
Ferritin (male: 23.0–183.0, female: 4.9–96.6 ng/mL)	n.d.	n.d.	18 070	3763	120	n.d.
Prothrombin activity (60–130%)	29	n.d.	7	20	43	n.d.

Prothrombin time in patient 6 was 19.8 s.
n.d., not done.

symptoms, laboratory data, bile acid analysis by GC-MS, and *SRD5B1* gene analysis were analyzed retro- and prospectively.

Case reports

Patient 1

At 35 weeks of gestational age, a Japanese boy was delivered by emergency cesarean section with complications and fetal distress. His birthweight was 1269 g. His mother had intrauterine growth retardation and oligohydramnios at 32 weeks of gestational age. On his first physical examination, general remarkable findings, such as periumbilical collateral vessel formation, also referred to as caput medusa, were noted. He had developed fulminant liver failure with hypoproteinemia (total protein 3.2 g/dL, albumin 1.9 g/dL), hypoglycemia (29 mg/dL), jaundice, respiratory distress and hypotension during his first day of life. His serum aminotransferase levels were normal although a severe coagulopathy and hyperammonemia were present (Table 1). At 29 days of age, his serum hyaluronic acid concentration was elevated (2020 ng/mL). Thereafter, he died at 44 days of age due to multiorgan failure. Liver pathological findings at autopsy revealed the appearance of ductular metaplasia and pericellular fibrosis (Fig. 2).

Patient 2

The sister of patient 1 was born 1 year later at 30 weeks of gestational age by emergency cesarean section after severe intrauterine growth retardation, oligohydramnios and fetal distress were noted. Her birthweight was 822 g. She developed severe hypoglycemia

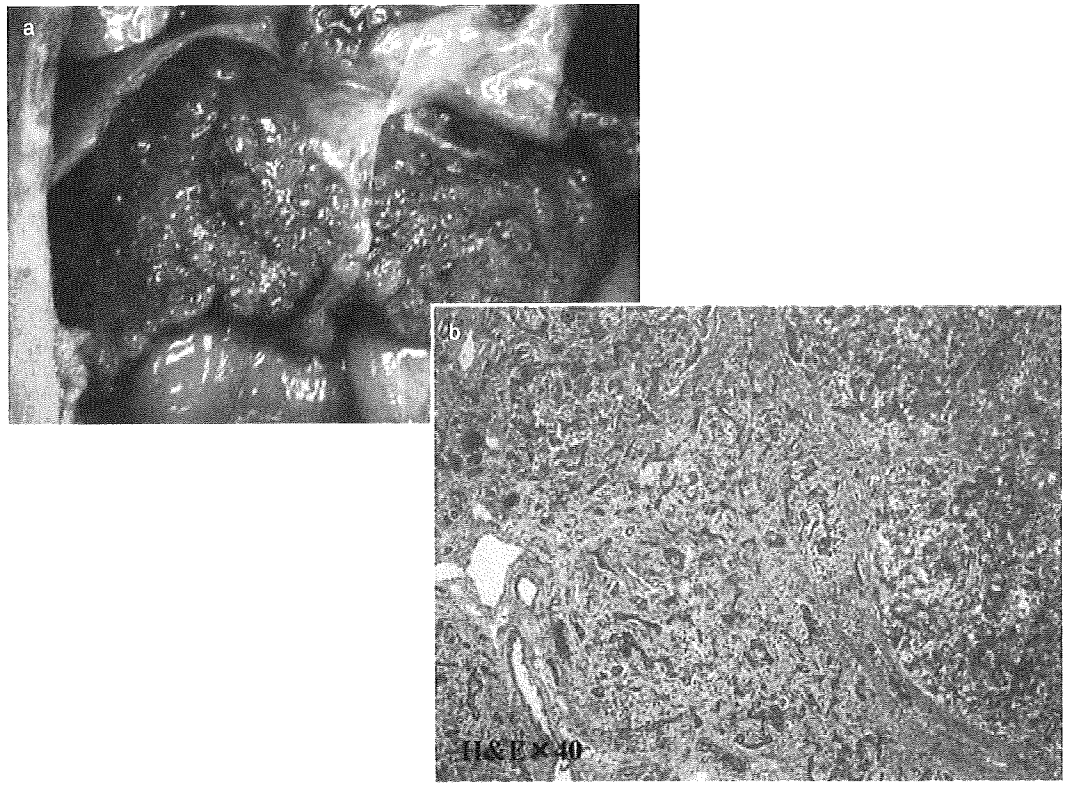

Figure 2 Liver pathological findings of patient 1. The liver was very atrophic and irregularly shaped on the surface at autopsy. Microscopically, the liver had extensive fibrosis, bile ductular proliferation of the surviving hepatocytes and extensive loss of hepatic parenchyma (hematoxylin and eosin staining; magnification ×40). The liver microscopic findings were very similar in patients 1 and 2.

(1 mg/dL), hypoproteinemia (total protein, 3.1 g/dL, albumin, 1.7 g/dL) and jaundice during her first day of life (her liver functional tests are listed in Table 1). On abdominal CT scan, ascites, an irregular liver edge and multiple low-density areas in the liver were observed. At 12 days of age, her hyaluronic acid concentration was elevated (4037 ng/mL). These findings suggested liver cirrhosis. This patient died as a result of liver and heart failure at 77 days of age. The liver pathological findings at autopsy were similar to those of patient 1.

Patient 3

A Japanese girl was born to a 36-year-old woman by spontaneous vaginal delivery without complication. Her birthweight was 3272 g. At 4 days of age, she was noticed to be deeply jaundiced (total bilirubin, 19.9 mg/dL); thereafter, she was admitted to Gunma Children's Medical Center due to liver dysfunction. Her admission laboratory studies are shown in Table 1. Her serum aminotransferase levels gradually decreased after admission; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) fell to 22 U/L and 16 U/L, respectively at 10 days of age, but she developed acute liver failure. Liver magnetic resonance imaging (MRI) findings revealed a very low signal, especially on the T_2-weighted images. We suspected neonatal hemochromatosis based upon the laboratory data and consulted her parents regarding a living donor liver transplantation. However, her parents refused. Despite medical treatment, she died as a result of liver and respiratory failure at 32 days of age. The liver and pancreas macroscopic pathological findings at autopsy revealed cirrhotic severe atrophy and their color had changed to a brown, rust-like hue. The liver microscopic findings showed massive hepatic necrosis and the presence of hemosiderin granules in the metaplastic ductular epithelium (Fig. 3a,b). Positive hemosiderin granules were present diffusely in myocardial (Fig. 3c) and pancreas cells (Fig. 3d).

Patient 4

A full-term Japanese boy was born to a 20-year-old woman by cesarean section with cephalopelvic disproportion. His birthweight was 3814 g. At 14 days of age, he had vomiting, hepatosplenomegaly and liver dysfunction (Table 1). His serum aminotransferase levels gradually decreased after admission; the AST and ALT fell to 17 U/L and 13 U/L, respectively, at 17 days of age. He developed fulminant liver failure. The results of abdominal ultrasonography at 4 weeks of life revealed a small liver with patent portal and hepatic veins and mild ascites. The liver and pancreas MRI findings revealed a very low signal, especially on the T_2-weighted images. He died as a result of liver failure due to sepsis at 87 days of age. The liver microscopic findings at autopsy resembled those of patient 3.

Journal of Gastroenterology and Hepatology **24** (2009) 776–785

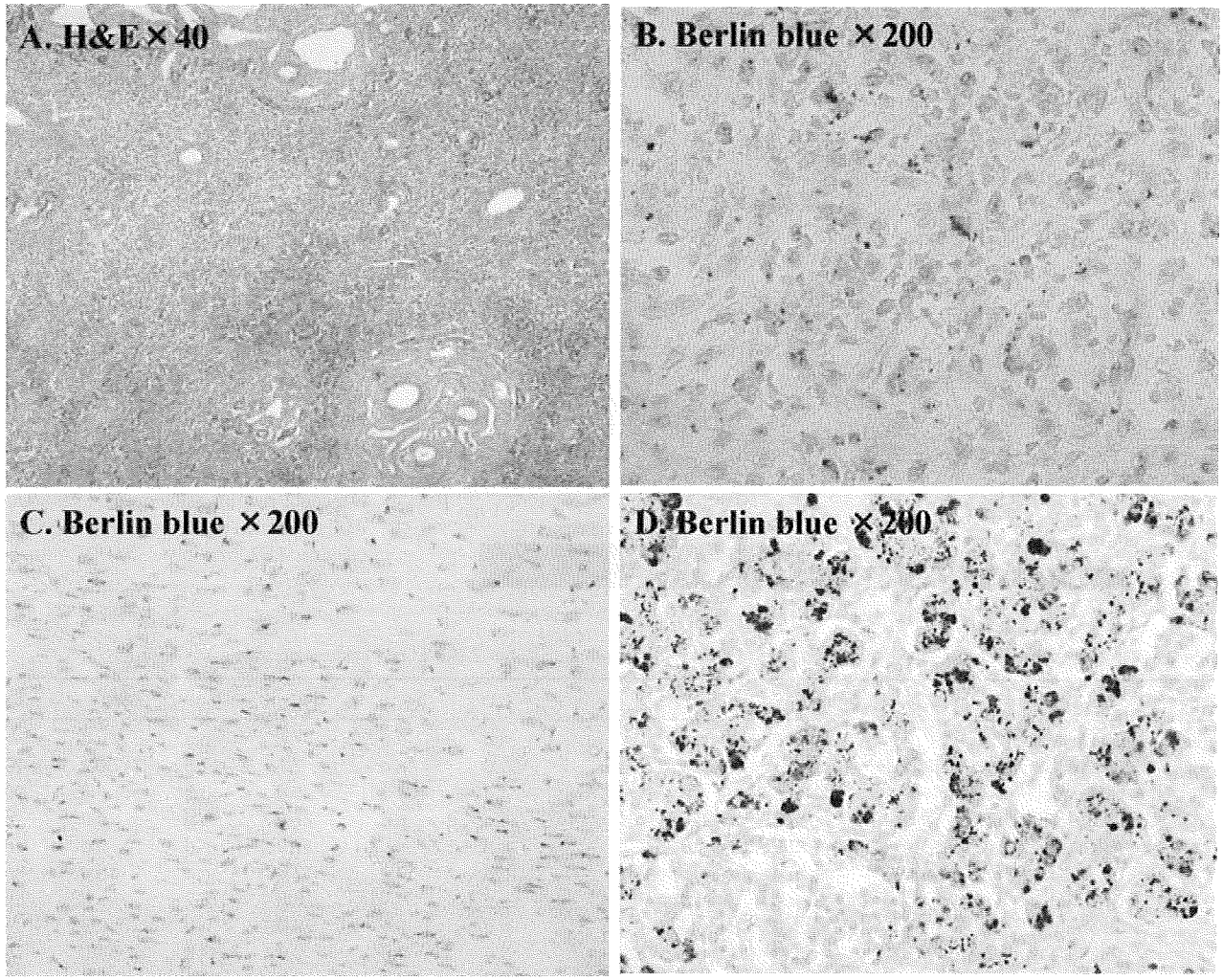

Figure 3 Liver pathological findings of patient 3. Microscopically, the liver specimen had extensive loss of hepatocytes with bile ductular proliferation and the portal areas approached one another (a, hematoxylin and eosin staining; magnification ×40). Hemosiderin was found predominantly in the metaplastic ductular epithelium (b, Berlin blue stain; magnification ×200). Positive hemosiderin granules are present diffusely in myocardial (c, Berlin blue stain; magnification ×200) and pancreas cells (d, Berlin blue stain; magnification ×200). The liver microscopic findings of patient 4 were similar to those of patient 3.

Patient 5

At 39 weeks of gestational age, a Japanese girl was born by spontaneous vaginal delivery without complication. Her birthweight was 2770 g. She was noticed to be jaundiced at 4 weeks of age; thereafter, her jaundice progressively worsened until 3 months of age. She became deeply icteric, with pale stools and dark urine. Her initial liver function tests on admission are shown in Table 1. Ursodeoxycholic acid (UDCA) (5 mg/kg per day) treatment was started immediately; thereafter, her serum bilirubin and aminotransferase levels gradually decreased (Fig. 4). However, we detected hyper-3-oxo-Δ^4 bile aciduria (Table 2). Accordingly, we recommended primary bile acid treatment for a suspected inborn error of bile acid synthesis. Her parents refused so we increased the dose of UDCA (to 10 mg/kg per day). At 11 months of age, her serum bilirubin and aminotransferase levels were within the normal range (Fig. 4). The serum concentration of the type IV collagen 7s domain also decreased to 4.0 ng/mL (normal range, <5.0 ng/mL) during UDCA treatment. However, we continued to detect hyper-3-oxo-Δ^4 bile aciduria after UDCA treatment. The liver biopsy microscopic findings at 8 months of age revealed giant cell transformation with bridging fibrosis and ductular proliferation (Fig. 5). We detected a heterozygous mutation in this patient (Fig. 6) by *SRD5B1* gene analysis and recommended *SRD5B1* gene analysis of the parents. However, her parents refused because she was in good health without liver dysfunction. At present, this patient remains in good health without any treatment.

Patient 6

A female Taiwanese infant with a birthweight of 3235 g was delivered at 38 weeks gestational age by spontaneous vaginal delivery

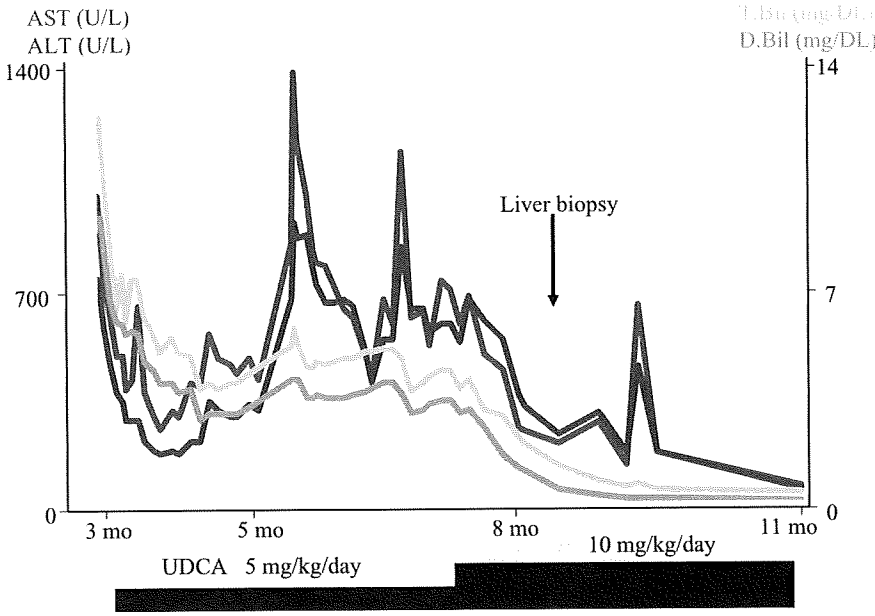

Figure 4 Clinical course of patient 5. Response of the serum bilirubin and aminotransferase levels to treatment with UCDA in patient 5. ALT, alanine aminotransferase; AST, aspartate aminotransferase; D.Bil, direct bilirubin; T.Bil, total bilirubin; UDCA, ursodeoxycholic acid.

Table 2 Bile acid analysis of the serum and urine using GC-MS in patients with hyper 3-oxo-Δ⁴ bile aciduria on admission

Patient		1	2	3	4	5-a	5-b	6-a	6-b	6-c
Serum	Cholic acid (%)	n.d.	n.d.		n.d.	n.d.	1.6	n.d.	n.d.	n.d.
	Chenodeoxycholic acid (%)	90.1	81.3		100.0	n.d.	7.1	17.5	99.2	79.1
	Ursodeoxycholic acid (%)	n.d.	n.d.		n.d.	34.3	n.d.	8.8	n.d.	20.9
	Allo-cholic acid (%)	n.d.	n.d.		n.d.	n.d.	27.0	n.d.	n.d.	n.d.
	Allo-chenodeoxycholic acid (%)	9.9	n.d.		n.d.	n.d.	17.9	n.d.	n.d.	n.d.
	CA-Δ⁴-3-one (%)	n.d.	n.d.		n.d.	27.2	17.6	n.d.	n.d.	n.d.
	CDCA-Δ⁴-3-one (%)	n.d.	n.d.		n.d.	38.4	6.8	73.7	n.d.	n.d.
	Others (%)	n.d.	18.7		n.d.	0.1	22.0	n.d.	0.8	n.d.
	Total bile acids (μmol/L)	30.3	0.5		8.4	8.5	9.8	9.2	54.9	6.1
Urine	Cholic acid (%)	4.4	5.5	n.d.	8.8	0.4	2.1	0.1	0.2	3.6
	Chenodeoxycholic acid (%)	2.4	n.d.	6.3	7.7	0.4	0.1	1.1	43.5	22.2
	Ursodeoxycholic acid (%)	0.1	2.9	0.4	n.d.	17.3	n.d.	8.2	0.2	24.7
	Allo-cholic acid (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Allo-chenodeoxycholic acid (%)	0.7	n.d.	14.7	2.5	n.d.	0.3	n.d.	n.d.	n.d.
	CA-Δ⁴-3-one (%)	5.1	48.3	n.d.	n.d.	64.7	87.6	51.0	18.8	7.6
	CDCA-Δ⁴-3-one (%)	86.2	40.9	63.3	73.1	14.3	6.5	35.8	27.7	15.8
	Others (%)	1.1	2.4	15.3	7.9	2.9	3.4	3.8	9.6	26.1
	Total bile acids (μmol/mmol Cr)	57.9	4.6	5.1	6.2	169.1	114.1	53.1	174.7	0.8

5-b, after the end of ursodeoxycholic acid (UDCA) treatment in patient 5 at 12 months of age; 6-a, before chenodeoxycholic acid treatment in patient 6 at 3 months of age; 6-b, 7 days after starting chenodeoxycholic acid treatment in patient 6 at 3 months of age; 6-c, mother of patient 6; we did not carry out a serum bile acid analysis in patient 3; CA-Δ⁴-3-one, 7α,12α-dihydroxy-3-oxo-4-cholenoic acid; CDCA-Δ⁴-3-one, 7α-hydroxy-3-oxo-4-cholenoic acid; n.d., not detected; 5-a, during UDCA treatment in patient 5 at 8 months of age.

after an uneventful pregnancy. At 2 months of age, the patient was referred to Taiwan University Hospital with a chief complaint of progressive jaundice (16.8 mg/dL). The stool color was light yellow. The jaundice present since birth was initially mild. Her initial physical examination on admission was nearly unremarkable without hepatosplenomegaly, jaundice or dark urine. Her liver functional tests at admission are shown in Table 1. Serial technetium-99*m* (^{99m}Tc)-DISIDA cholescintigraphy revealed visualization of intestinal radioactivity at 4.5 h postinjection. We started primary bile acid treatment with chenodeoxycholic acid (CDCA) (12 mg/kg per day) after analyzing her bile acids using GC-MS and her *SRD5B1* gene because we detected hyper 3-oxo-Δ⁴ bile aciduria (Table 2). A heterozygous mutation of the *SRD5B1* gene was found (Fig. 7). One month following the onset of CDCA administration, her total bilirubin and ALT levels decreased from 17.9 mg/dL and 342 U/L to 9.7 mg/dL and 235 U/L, respectively. CDCA treatment has continued until the present time. Her liver microscopic biopsy findings at 5 months of

Journal of Gastroenterology and Hepatology **24** (2009) 776–785

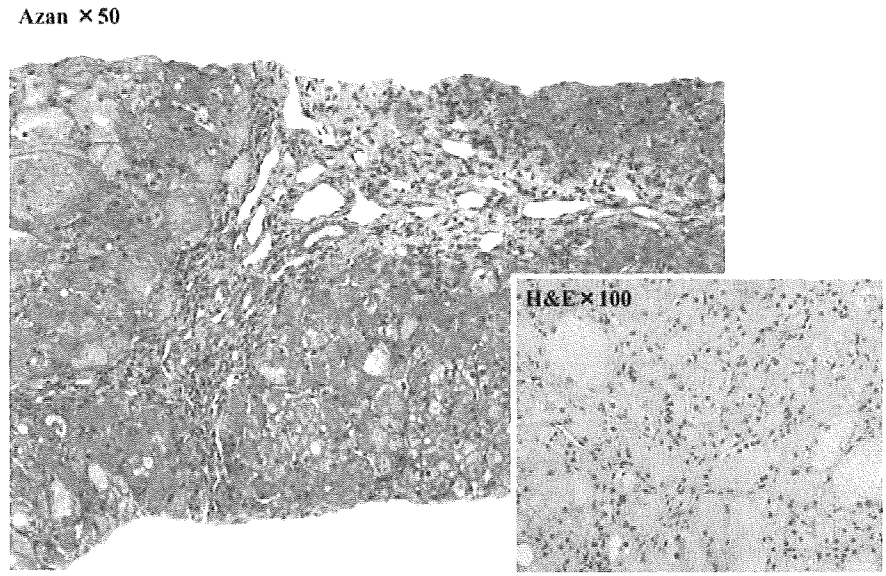

Figure 5 Liver pathological findings of patient 5. Liver biopsy specimen from patient 5 at 8 months shows lobular disarray resulting from extensive giant cell transformation (hematoxylin and eosin staining; magnification ×100) and consistent with cirrhosis showing wide fibrotic bands at the portal areas (Azan stain; magnification ×50). Bile ductular proliferation was noted. The liver microscopic findings of patient 6 were very similar to those of patient 5.

age revealed giant cell hepatitis with fibrosis similar to that of patient 5. Unfortunately, her liver function subsequently deteriorated and she was admitted to Taiwan University Hospital to be prepared for liver transplantation.

Patients 1–4 in the present study were treated with supportive care, such as blood exchange, administration of fresh frozen plasma, vitamin K and antibiotics. In all patients, the serum antibody titers showed no evidence of infection with hepatitis B or C virus, herpes simplex virus, Epstein–Barr virus or cytomegalovirus.

The parents of patients 1–6 were also in good health without liver dysfunction. However, in the mother of patient 6, we detected a high percentage of 3-oxo-Δ^4 bile acids relative to total bile acids (23.4%) in her urine (Table 2). Her liver functional tests were normal, including AST (17 U/L), ALT (7 U/L), total bilirubin (0.4 mg/dL) and GGT (7 U/L). Her GGT value was very low.

Qualitative and quantitative bile acid analysis

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

After we synthesized some specific unusual bile acids, such as 3β-hydroxy-Δ^5,[11] 3-oxo-$\Delta^{4,12}$ and allo-bile acids,[13] as seen in inborn errors of bile acid synthesis, we routinely analyzed the bile acids in the urine and serum by GC-MS using selected ion monitoring of the characteristic fragments of the methyl ester-dimethylethylsilyl ether-methoxime derivatives of the bile acids as described previously,[14] after enzymatic hydrolysis (choloylglycine hydrolase 30 units) and solvolysis (sulfatase 150 units; Sigma Chemical Co., St Louis, MO, USA).

All the patients in this study had the bile acids in their serum and urine analyzed using GC-MS on admission.

Genetic analysis

With informed consent, liver tissue (patients 1 and 2), blood (patients 3, 5 and 6) and nails (patient 4) were collected from the patients and parents of patient 6, as well as 103 healthy Asian individuals. The genomic DNA was extracted from liver tissues, peripheral leukocytes and nails using a QIAamp Mini Kit (Qiagen, Hilden, Germany).

The DNA fragments spanning the nine coding regions of the *SRD5B1* genes were amplified by polymerase chain reaction (PCR) using Gene Taq (Nippon Gene, Toyama, Japan) and nine sets of primers (F1: 5′-CTTTCTTTGATGGAATAGGC-3′ and B1: 5′-AGTAAGTCAATGAGATCTGC-3′, F2: 5′-TGTACATGCAA AATGTCCTG-3′ and B2: 5′-ATGAGTGCAATTACACACAC-3′, F3: 5′-TTACAAAGAAAAAGGGGCTG-3′ and B3: 5′-CTTC ATGCACATAGCTATTG-3′, F4: 5′-GCTCACAATTATGAAGA CTG-3′ and B4: 5′-TCATTGAAAGTAAAGGGTGC-3′, F5: 5′-TGCTTATTAACATACCCAGG-3′ and B5: 5′-ATTTAGGTGG AGCAATCATG-3′, F6: 5′-AATTGCATTCAACAACGTGG-3′ and B6: 5′-AACCAAAAGGCATTCCAATC-3′, F7: 5′-GAGG AGGATGGTTTATTAAC-3′ and B7: 5′-GGTTTCCTATTAAG CTGAAC-3′, F8: 5′-TTCATACATCTTTGGAAGGC-3′ and B8: 5′-TCAGGCATGTTAACATTCAG-3′, F9: 5′-AACAGCAGAGG AATGAATAG-3′ and B9: 5′-AACCCTCTCTCTTCATTTTC-3′) to obtain the optimal length of DNA fragments suitable for direct sequence analysis.[15] The temperature program included an initial denaturation step of 94°C for 2 min followed by 30 cycles of a denaturation step of 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min. A final extension step of 72°C for 10 min was used using a T3 thermocycler (Whatman, Kent UK).

After enzyme processing with ExoSAP-IT (USB Co., Cleveland, OH, USA), direct sequencing of the amplified PCR products was carried out with the DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's protocol,

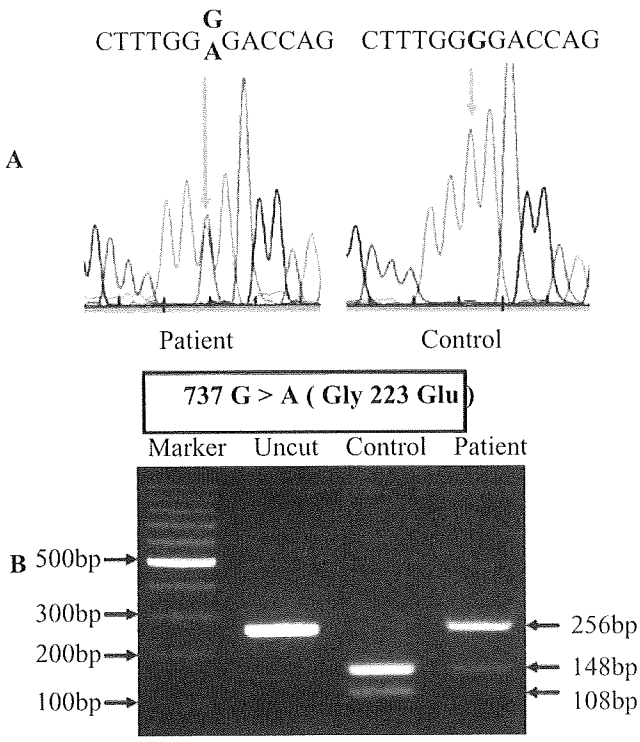

Figure 6 DNA analysis in patient 5 and a healthy control. (a) Genomic DNA sequence of the 3-oxo-Δ⁴-steroid 5β-reductase gene. The position of the nucleotide sequence in the mutation is shown with arrows. The arrow identifies G/A in the patient and G in a control subject. The reverse-strand sequence showed the same result. This represents a CGA-to-TGA mutation, affecting glycine at position 223, where it is replaced by glutamate. Such a nucleotide substitution was not observed in the 103 controls (the wild-type nucleotide 'G' is seen in the control example). (b) Digestion of amplified SDR5B1 exon 6 polymerase chain reaction (PCR) fragment with *Ava*II. To screen for the novel G-to-A mutation at nucleotide 737, we amplified the PCR products of *SRD5B1* gene exon 6. The PCR products from the healthy control allele were digested by the *Ava*II enzyme (at G/GWCC, W. A or T) into two fragments, whereas the mutant form of the allele was not digested. The patient was confirmed to be heterozygous for G-to-A substitution, as he showed digested and non-digested fragments.

using the same primers as for PCR amplification. The sequencing reaction product was analyzed electrophoretically, using a SEQ2000XL analyzer (Beckman Coulter).

Once the two putative mutations were found, six patients, the parents of patient 6 and 103 healthy Asian individuals were screened for these two mutations by digesting the appropriate PCR fragment with a restriction enzyme. *Ava*II (Takara, Shiga, Japan) was used to recognize the G/GW (A or T) CC sequence in the exon 6 PCR fragment for the mutation found in patient 6, while *Kpn*I (Takara, Shiga, Japan) was used to recognize the GGTAC/C sequence in the exon 2 PCR fragment for the mutation found in patient 5. The restricted DNA fragments were separated by electrophoresis on a 1.5% agarose gel and then stained with ethidium bromide.

Results

Patient profile and liver pathology

Patient profile and liver pathology are shown in Table 1 and Figures 2, 3 and 5. Four patients, patients 1–4, developed neonatal liver failure, while the other patients, patients 5 and 6, showed chronic cholestasis. Patients 1 and 2 immediately developed fulminant liver failure after emergency cesarean sections due to fetal distress at late gestational age. Moreover, these patients had complications evident during pregnancy, such as intrauterine growth retardation and oligohydramnios, and did not have an elevated AST or ALT after birth. Patients 3 and 4 developed liver failure 1 or 2 weeks after delivery, respectively, with elevated ferritin concentrations. All patients had severe jaundice and abnormal prothrombin times except for one patient. The total bile acid and GGT values were low in all but two patients, patients 1 and 2 and patients 2 and 5, respectively. In addition, the liver pathological findings of the patients are shown in Figures 2, 3 and 5.

Biochemical identification of an inborn error of bile acid synthesis

Biochemical identification of an inborn error of bile acid synthesis is shown in Table 2. In the serum, the main bile acid in the patients with liver failure, patients 1, 2 and 4, was CDCA. We detected large amounts of 3-oxo-Δ⁴ bile acids in the serum of patients with chronic cholestasis, patients 5 and 6, prior to treatment. Patient 5 had a decreased percentage of 3-oxo-Δ⁴ bile acids relative to the total bile acids in the serum after the end of the UDCA treatment. We did not detect 3-oxo-Δ⁴ bile acids in the serum of patient 6 during the CDCA treatment.

We detected large amounts of 3-oxo-Δ⁴ bile acids in the urine of all the patients. In the patients with liver failure, the main 3-oxo-Δ⁴ bile acid in the urine was 7α-hydroxy-3-oxo-4-cholenoic acid. Patients 5 and 6 had increased excretion of urinary bile acids during bile acid treatment compared to that without treatment, and a decreased percentage of 3-oxo-Δ⁴ bile acids relative to the urinary total bile acids during the bile acid treatment.

We detected urinary 3-oxo-Δ⁴ bile acids in the mother of patient 6; however, we could not detect 3-oxo-Δ⁴ bile acids in the serum or urine in the father of patient 6.

Identification of SDR5B1 gene defects

We did not detect a mutation in the *SRD5B1* gene of patients 1–4. We have identified two single-nucleotide changes in two patients. A single substitution of G to A at nucleotide position 737 was confirmed in exon 6 of the *SRD5B1* gene, which causes an amino acid change from glycine (GGG) to glutamate (GAG) at amino acid position 223 in patient 5. This novel mutation was identified as a heterozygous mutation (Fig. 6).

In patient 6, a single substitution of C to T at nucleotide position 217 was confirmed in exon 2 of the SDR5B1 gene, which causes an amino acid change from arginine (CGA) to stop (TGA) at amino acid position 50. This novel mutation was identified as a heterozygous mutation in the patient and her mother (Fig. 7).

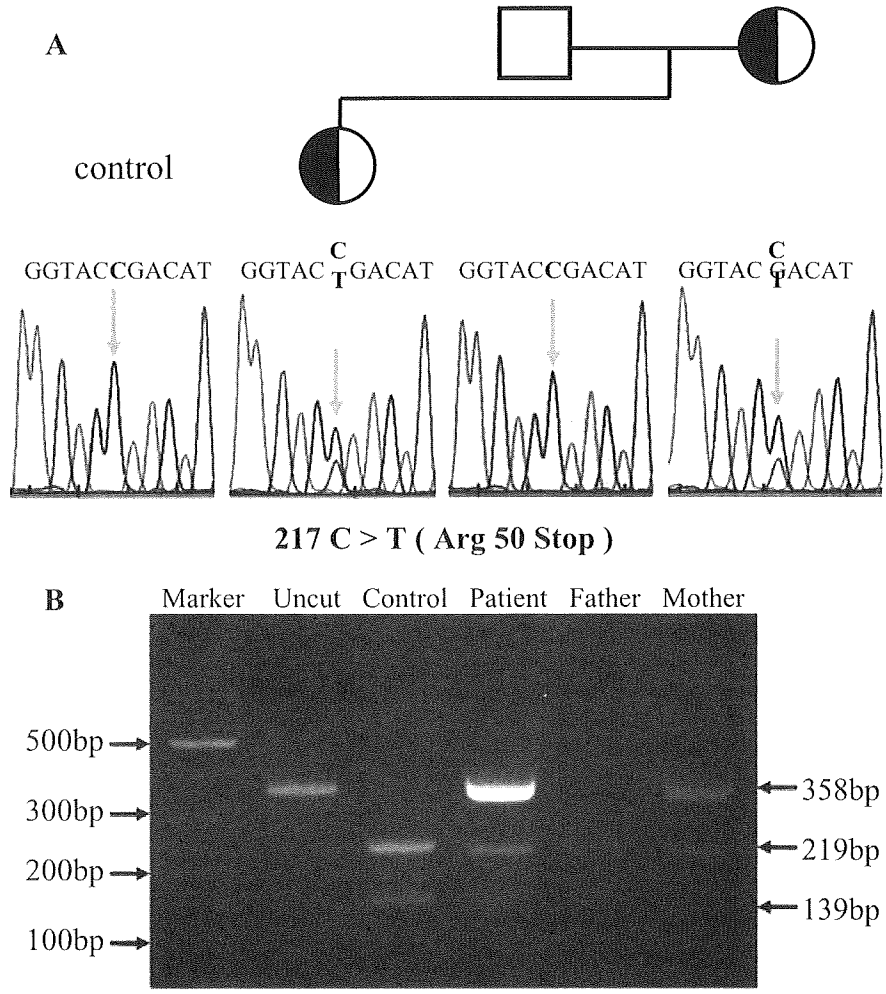

Figure 7 DNA studies of patient 6, her parents and a control. (a) Pedigree and DNA sequence of the 3-oxo-Δ⁴-steroid 5β-reductase gene in genomic DNA samples. The position of the mutant nucleotide sequence is shown with arrows, indicating C/T in the patient and her mother, and C in her father and a control subject. The reverse strand sequence showed the same result. This represents a CGA-to-TGA mutation, affecting arginine at position 50, which is replaced by a stop codon. Such a nucleotide substitution was not observed in the 103 controls (the wild-type nucleotide 'C' appears in the selected control). (b) Digestion of amplified SRD5B1 exon 2 PCR fragment with *Kpn*I. To screen for the novel C-to-T mutation at nucleotide 217, we amplified the PCR products of *SRD5B1* gene exon 2. The PCR products from the patient's father and a control example were digested by the *Kpn*I enzyme (GGTAC/C) into two fragments. The patient and her mother showed both digested and non-digested fragments, with the latter representing the mutation; this indicates that they were heterozygous for the mutation.

Neither of these mutations was found in 103 healthy Asian individuals.

Discussion

We identified two patients with heterozygous mutations of primary 3-oxo-Δ⁴-steroid 5β-reductase deficiency by *SRD5B1* gene analysis. According to previous reports[7,8] of the mutations in the *SRD5B1* gene of the five reported patients, three were homozygous and two were compound heterozygous. Furthermore, this enzyme deficiency was reported as displaying an autosomal recessive transmission pattern. Although our two patients appear to have an autosomal recessive transmission pattern, we suspect that the father of patient 6 may have a heterozygous mutation outside the coding region, such as in the promoter region or in an intron. We do not know about patient 5 because we could not carry out *SRD5B1* gene analysis in the parents of patient 5. However, we suspect that one of the parents of patient 5 may have heterozygous mutations outside the coding region. Therefore, we suggest that our two patients with chronic cholestasis may be compound heterozygotes of the *SRD5B1* gene.

Although both patients 5 and 6 have a heterozygous mutation in the *SRD5B1* gene, these patients may have cholestasis with liver dysfunction only during infancy; patient 5 actually had a favorable course without bile acid replacement therapy. Thereafter, the cholestasis with liver dysfunction in this disease may gradually

improve, as the mother of patient 6 does not have liver dysfunction. Because neonatal bile acid metabolism is different from that of adults, 3-oxo-Δ^4-steroid 5β-reductase may be essential during the newborn period. In healthy full-term infants, we detect a high percentage of urinary 3-oxo-Δ^4 bile acids in the total bile acids during the early neonatal period. This reflects the normal development of bile acid metabolism, including the initial immaturity of 3-oxo-Δ^4-steroid 5β-reductase.[14,16] In the newborn period, patients with a heterozygous mutation in the *SRD5B1* gene may develop cholestasis with liver dysfunction that can resolve later. However, the same patient may develop a severe stage of disease similar to that of patient 6.

Moreover, we would still suspect that patients with a heterozygous mutation of the *SRD5B1* gene may have mutations in another gene. We have completed coding region analysis of the bile salt export pump gene (BSEP) in patient 6. However, we could not find mutations in BSEP, except for a heterozygous Val 444 Ala polymorphism.

In this study, a primary 3-oxo-Δ^4-steroid 5β-reductase deficiency could not be diagnosed solely by low or normal levels of GGT activity and the total bile acid concentration by an enzymatic technique using 3α-hydroxysteroid dehydrogenase, or the presence of hyper 3-oxo-Δ^4 bile aciduria. Patients with fulminant hepatic failure, patients 1–4, did not have detectable 3-oxo-Δ^4 bile acids in their serum, whereas it was detected in the serum of patients 5 and 6. This may be a very important observation. Lemonds and Clayton also suggested that the detection of 3-oxo-Δ^4 bile acids and trace amounts of CDCA in the serum are important findings in the differential diagnosis.[7] However, Sumazaki had reported a patient with an extremely reduced activity of 3-oxo-Δ^4-steroid 5β-reductase without any genetically defined defects and who had large amounts of 3-oxo-Δ^4 bile acids in the serum and urine.[17] Therefore, we suggest that the pediatric physician must analyze the bile acids in the serum and urine using GC-MS when one suspects a primary 3-oxo-Δ^4-steroid 5β-reductase deficiency based upon the values of the GGT activity and the total bile acid concentration. If high concentrations of 3-oxo-Δ^4 bile acids are detected in the serum and urine, one should analyze the *SRD5B1* gene.[7]

In our patient 5, the liver function tests, including type IV collagen 7s domain, gradually improved with UDCA treatment (Fig. 4). However, we detected 3-oxo-Δ^4 bile acids in the serum and urine after UDCA treatment (or when not on any current treatment) (Table 2).

Of the patients with fulminant hepatic failure, patients 1–4 were diagnosed as having neonatal hemochromatosis by their clinical symptoms, such as intrauterine growth retardation, oligohydramnios, fetal distress and hypoglycemia, their laboratory data, such as elevated ferritin concentration, the liver pathological findings, and the results of *SRD5B1* gene analysis. We suggest that patients 1 and 2 had fetal hemochromatosis whereas patients 3 and 4 were diagnosed as having infantile (neonatal) hemochromatosis.[18,19] The reason for the reduced activity of hepatic 3-oxo-Δ^4-steroid 5β-reductase was a partial enzyme deficiency predisposing to severe hepatocyte damage.[16] Therefore, we suggest that these patients did not have a primary defect in the *SRD5B1* gene. We believe that pediatric hepatologists should not stop searching for a cause of any hepatocyte damage after the discovery of hyper 3-oxo-Δ^4 bile aciduria with neonatal liver failure;[20] that is, pediatric hepatologists should carry out *SRD5B1* gene analysis to distinguish between a primary 3-oxo-Δ^4-steroid 5β-reductase deficiency from a secondary defect in the presence of hyper-3-oxo-Δ^4 bile aciduria. Our experience indicates that until a specific diagnostic test is devised, *SRD5B1* gene analysis will remain essential for research and for accurate diagnosis of primary 3-oxo-Δ^4-steroid 5β-reductase deficiency, especially when the deficiency occurs sporadically.

In conclusion, we suggest that patients with fulminant hepatic failure who have hyper-3-oxo-Δ^4 bile aciduria during the neonatal period may have secondary 3-oxo-Δ^4-steroid 5β-reductase deficiency based upon our results of *SRD5B1* gene analysis. However, patients with chronic cholestasis who have hyper-3-oxo-Δ^4 bile aciduria are more likely to have primary 3-oxo-Δ^4-steroid 5β-reductase deficiency based upon the detected heterozygous mutations in the *SRD5B1* gene. Therefore, SDR5B1 gene analysis is necessary for the accurate diagnosis of 3-oxo-Δ^4-steroid 5β-reductase deficiency. Moreover, from the results of the *SRD5B1* gene analysis, we think that it is important to elucidate whether there is a heterozygous or compound heterozygous mutation in our two patients.

References

1 Shneider BL, Setchell KDR, Whitington PF, Neilson KA, Suchy FJ. Δ^4-3-Oxosteroid 5β-reductase deficiency causing neonatal liver failure and hemochromatosis. *J. Pediatr.* 1994; **124**: 234–8.

2 Siafakas CG, Jonas MM, Perez-Atayde AR. Abnormal bile acid metabolism and neonatal hemochromatosis: a subset with poor prognosis. *J. Pediatr. Gastroenterol. Nutr.* 1997; **25**: 321–6.

3 Kimura A, Suzuki M, Murai T *et al.* Urinary 7α-hydroxy-3-oxochol-4-en-24-oic and 3-oxochola-4,6-dien-24-oic acids in infants with cholestasis. *J. Hepatol.* 1998; **28**: 270–9.

4 Kimura A, Suzuki M, Tohma M *et al.* Increased urinary excretion of 3-oxo-Δ^4 bile acids in Japanese patients with idiopathic neonatal cholestasis. *J. Pediatr. Gastroenterol. Nutr.* 1998; **27**: 606–9.

5 Setchell KDR, Suchy FJ, Welsh MB, Zimmer-Nechemias L, Heubi J, Balistreri WF. Δ^4-3-Oxosteroid 5β-reductase deficiency described in identical twins with neonatal hepatitis. *J. Clin. Invest.* 1988; **82**: 2148–57.

6 Kondo K-H, Kai M-H, Setoguchi Y *et al.* Cloning and expression of cDNA of human Δ^4-3-oxosteroid 5β-reductase and substrate specificity of the expressed enzyme. *Eur. J. Biochem.* 1994; **219**: 357–63.

7 Lemonde HA, Custard EJ, Bouquet J *et al.* Mutations in SRD5B1 (AKR1D1), the gene encoding Δ^4-3-oxosteroid 5β-reductase, in hepatitis and liver failure in infancy. *Gut* 2003; **52**: 1494–9.

8 Gonzales E, Cresteil D, Baussan C, Dabadie A, Gerhardt M-F, Jacquemin E. SRD5B1 (AKR1D1) gene analysis in Δ^4-3-oxosteroid 5β-reductase deficiency: evidence for primary genetic defect [letter]. *J. Hepatol.* 2004; **40**: 716–18.

9 Daugherty C, Setchell KDR, Heubi J, Balistreri WF. Resolution of liver biopsy alterations in three siblings with bile acid treatment of an inborn error of bile acid metabolism (Δ^4-3-Oxosteroid 5β-reductase deficiency). *Hepatology* 1993; **18**: 1096–101.

10 Clayton PT, Molls KA, Johnson AW, Barabino A, Marazzi MG. Δ^4-3-Oxosteroid 5β-reductase deficiency: failure of ursodeoxycholic acid treatment and response to chenodeoxycholic acid plus cholic acid. *Gut* 1996; **38**: 623–8.

11 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. *Steroid* 1986; **48**: 331–8.
12 Leppik RA. Improved synthesis of 3-keto, 4-ene-3keto-, and 4,6-diene-3-keto bile acids. *Steroid* 1983; **41**: 475–84.
13 Iida T, Nishida S, Chang FC, Niwa T, Goto J, Nambara T. Potential bile acid metabolism. XXI. A new synthesis of allochenodeoxycholic and allocholic acids. *Chem. Pharm. Bull.* 1993; **41**: 763–5.
14 Kimura A, Mahara 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.
15 Charbouneau A, Luu The V. Genomic organization of a human 5β-reductase and its pseudogene and substrate selectivity of the expressed enzyme. *Biochem. Biophys. Acta* 2001; **1517**: 228–35.
16 Inoue T, Kimura A, Aoki K, Tohma M, Kato H. Developmental pattern of 3-oxo-Δ^4 bile acids in neonatal bile acid metabolism. *Arch. Dis. Child* 1997; **77**: F52–6.
17 Sumazaki R, Nakamura N, Shoda J, Kurosawa T, Tohma M. Gene analysis in Δ^4-3-oxosteroid 5β-reductase deficiency. *Lancet* 1997; **349**: 329.
18 Whitington PF, Kelly S, Ekong UD. Neonatal hemochromatosis: fetal liver disease leading to liver failure in the fetus and newborn. *Pediatr. Transplant.* 2005; **9**: 640–5.
19 Whitington PF. Fetal and infantile hemochromatosis. *Hepatology* 2006; **43**: 654–60.
20 Clayton PT. Δ^4-3-oxosteroid 5β-reductase deficiency and neonatal hemochromatosis [letter]. *J. Pediatr.* 1994; **125**: 845–6.

Pediatrics International (2009) **51**, 535–543 doi: 10.1111/j.1442-200X.2008.02799.x

Original Article

Diagnostic determination system for high-risk screening for inborn errors of bile acid metabolism based on an analysis of urinary bile acids using gas chromatography–mass spectrometry: Results for 10 years in Japan

Hiroshi Nittono,[1,2] Hajime Takei,[1,2] Atsushi Unno,[1,2] Akihiko Kimura,[3] Toshiaki Shimizu,[2] Takao Kurosawa,[4] Sadahiko Tohma[4] and Mizuho Une[5]

[1]*Junshin Clinic, Bile Acid Institute,* [2]*Department of Pediatrics, Juntendo University School of Medicine, Tokyo,* [3]*Department of Pediatrics and Child Health, Kurume University School of Medicine, Fukuoka,* [4]*Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Hokkaido,* [5]*School of Pharmacy, Hiroshima International University, Hiroshima, Japan*

Abstract ***Background***: Some patients with cholestasis of unknown cause may have inborn errors of bile acid metabolism (IEBAM) thus causing abnormalities of bile acid biosynthesis. Although seven types of bile acid synthetic defects have thus far been reported for this disorder, no detailed information on its incidence and so on in Japan is yet available. In order to elucidate the current status of IEBAM in Japan, in July 1996 a diagnostic determination system was established for high-risk screening for IEBAM.

Methods: Urinary bile acids were analyzed on gas chromatography–mass spectrometry (GC-MS) and quantitative analysis was done using selected ion monitoring (SIM).

Results and conclusions: In a total of 576 samples analyzed over the 10 year period prior to June 2005, 159 patients were found with cholestasis of unknown etiology. Of these patients, 10 (6.3%) were found to have IEBAM by this system, while 91 (61.1%) had cholestasis without a definitive diagnosis. This diagnostic determination system with GC-MS of urinary bile acids is therefore considered useful for detecting IEBAM.

Key words cholestasis, GC-MS, inborn errors of bile acid metabolism, screening, urinary bile acid.

In 1972 Eyssen *et al.* reported cases of Zellweger syndrome complicated by inborn errors of bile acid metabolism (IEBAM).[1] In 1980 Oftebro *et al.* demonstrated a complete lack of sterol C-27 hydroxylase activity in mitochondria in patients with cerebrotendinous xanthomatosis (CTX).[2] In 1987 Clayton *et al.* reported 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase HSD3B7 deficiency.[3] In 1988 Setchell *et al.* reported Δ^4-3-oxo-steroid 5β-reductase (AKR1D1) deficiency.[4] In 1994 abnormal bile acid excretion was reported in patients with Smith-Lemli-Opitz syndrome (SLOS) caused by abnormalities in 3β-hydroxysteroid Δ^7-reductase, an enzyme involved in the final step of cholesterol biosynthesis.[5] More recently Clayton *et al.* reported abnormalities in the 25-hydroxylation pathway in 1995,[6] and Setchell *et al.* reported oxysterol 7α-hydroxylase (SYP7B1) deficiency in 1998.[7] β-Oxidation abnormalities[8,9] associated with side-chain cleavage and amino acid conjugation abnormalities[10,11] have also been reported.

Correspondence: Hiroshi Nittono, MD, Junshin Clinic (Nittono Clinic) Bile Acid Institute, 2-1-24, Haramachi, Meguro-ku, Tokyo 152-0011, Japan. Email: junsin@h4.dion.ne.jp

Received 11 April 2008; revised 7 September 2008; accepted 10 November 2008.

In 1996, when these new types of IEBAM were reported in various parts of the world, a urinary bile acid analysis system was developed for screening for IEBAM to elucidate the current status of IEBAM in Japan and detect new types of this disorder.[12] There have been several reports concerning changes in bile acid metabolism over time from the prenatal to postnatal period, infancy and early childhood.[13–17] The reference range for urinary bile acid in healthy children, which was reported by Kimura *et al.* using the method of Suzuki *et al.* for analysis,[15,18] was used for high-risk screening to detect IEBAM in patients with cholestasis of unknown etiology. Urinary bile acid levels are expressed in mmol/mol·creatinine (or mmol/mol·cre).

Methods

The target patients included children with hepatic disorder, the cause of which could not be identified on conventional liver function testing or hepatitis virus testing, patients with liver cirrhosis of unknown etiology, sibling cases (of liver cirrhosis) and patients with cholestasis who exhibited serum levels of total bile acid, γ-glutamyltranspeptidase (γ-GTP) and total cholesterol within the normal range. The age of patients for whom analysis of urinary bile acids was requested ranged between 1 month after birth and 22 years. A total of 576 samples was analyzed during

the 10 years between July 1996 and June 2005. Cholestasis was diagnosed if the serum level of direct bilirubin (D-Bil) was ≥2.0 mg/dL and a total of 159 patients met this criterion.

Bile acid analysis system

Physicians who treat patients with cholestasis or hepatic dysfunction of unknown etiology and who wish to request full-set gas chromatography–mass spectrometry (GC-MS) of urinary bile acids should apply to the Bile Acid Institute for analysis by phone or fax. Next, they should fill in the designated Bile Acids Analysis Request Form (Fig. 1) and send it with a urine sample (spot urine stored frozen below −20°C immediately after collection) to the Bile Acid Institute via refrigerated delivery service. The Bile Acid Institute will perform a full-set analysis of urinary bile acid on GC-MS and send the analysis data thus obtained and the Analysis Request Form (in which the patient's clinical signs and symptoms are documented) to the Department of Pediatrics and Child Health, Kurume University School of Medicine. The Bile Acid Institute will notify the requesting physicians of the results within 1 month after the request, with comments by Dr Kimura of the Department of Pediatrics and Child Health, Kurume University School of Medicine, as well as the data and interpretation on the results of bile acid analysis. If the Bile Acid Institute finds it difficult to analyze and interpret data for some patients, the Institute will then send the samples to the Faculty of Pharmaceutical Sciences, Health Sciences University of

Bile Acids Analysis Request Form

Analysis request day : day / month / year / /

patient's name : M・F birth day : / / age : y m d

(parent's name) : conceptual age : w d weight at birth : g

present complaint :

clinical progress :

Samples

urine collected day : / /

serum collected day : / /

bile collected day : / /

medications in collected samples
UDCA, phenobal, steroids, taurine, glycyrrhizin
others :

Family history

consanguineous marriage : Yes・No・Unknown

no history of death due to liver desease in family : Y・N・U

others :

Past history

low weight birth : Y・N・U | apparent death : Y・N・U | IVH(TPN) : Y・N・U | gathrie reacall : Y・N・U

others :

Clinical examination

T-Bil		mg/dL	γ-GTP		IU/L	s-TBA		μmol/L
D-Bil		mg/dL	T-Cho		mg/dL	citrulline		μmol/L
AST		IU/L	NH_3		μg/dL	tyrosine		μmol/L
ALT		IU/L	HPT		%	galactose		μmol/L

Liver biopsy finding, liver, biliary system echo and scintigraphy findings, others :

Check lists

hypocoagulability	Y・N・U	consciousness disorder	Y・N・U	cardiac murmur	Y・N・U
jaundice (from birth)	Y・N・U	muscle hypotonia	Y・N・U	retinal pigment degeneration	Y・N・U
continuous jaundice	～	vitamin E defect	Y・N・U	mental retardation	Y・N・U
chronic diarrhea	Y・N・U	cutaneous pruritus	Y・N・U	anterior fontanel distension	Y・N・U
contaract	Y・N・U	specific fajes	Y・N・U	head CT・MRI abnormality	Y・N・U
xanthomatosis	Y・N・U	grayish stool	Y・N・U	hepatomegaly cm	splenomegaly cm

Client

doctor's name : e-mail :

hospital name and faculty :

address :

phone : facsimile :

note :

* Please send us this completed request form together with the samples via refrigerated delivery service.

Nittono Clinic Bile Acids Research Institute e-mail : bile-res@b01.itscom.net facsimile : +81-3-5704-5820
1-24 Haramachi-2-chome Meguro-ku Tokyo, Japan. 152-0011

Fig. 1 Bile Acids Analysis Request Form.

Hokkaido for a detailed analysis. If abnormalities in the metabolism of bile alcohols, such as CTX, are suspected, the Institute will request the School of Pharmacy, Hiroshima International University, to perform the analysis (Fig. 2).

Prior simplified classification using the bile acid analysis request forms

The Analysis Request Form provides not only space for the patient's age, sex and clinical progress but also a checklist for the determination of the type of IEBAM and approximate discrimination from other cholestatic disorders with similar clinical manifestations. In the column for clinical examination, (abnormal) data on the direct and indirect bilirubin and transaminases (alanine aminotransferase and aspartate aminotransferase [AST]) can be compared, at a glance, with data on γ-GTP, serum total bile acids (s-TBA), total cholesterol (T-Cho) and so on, the latter of which tend to be normal in many types of IEBAM. In addition, the Analysis Request Form provides spaces for citrullin and tyrosine levels to discriminate this disorder from neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD).[19] In the check lists the presence or absence of the following clinical symptoms can be checked: jaundice, hypocoagulability, steatorrhea, cutaneous pruritus, vitamin E deficiency and chronic diarrhea, as well as muscle hypotonia and large fontanels (both of which are typical findings of Zellweger syndrome), xanthomatosis and cataract (both characteristic of CTX), cardiac murmur (characteristic of Alagille syndrome) and mental retardation (frequently observed in patients with serious disease due to abnormality in cholesterol biosynthesis). The Analysis Request Form also provides free-text space for comments on findings of a liver biopsy, liver/biliary-system echo (hepatobiliary ultrasound) and hepatobiliary scintigraphy.

Results

Between July 1996 and June 2005, a total of 576 samples (serum, bile and stool samples were analyzed for bile acids, as requested. These requests came from 36 prefectures across Japan as well as Taiwan (16 samples). Of these patients, 159 were diagnosed with cholestasis, who met the predetermined criterion: D-Bil ≥2.0 mg/dL. Of these 159 patients, 10 were differentially diagnosed with IEBAM and an associated metabolic disorder on GC-MS of urinary bile acids, which included: 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase (HSD3B7) deficiency in one, Δ^4-3-oxo-steroid 5β-reductase (AKR1D1) deficiency in five, Zellweger syndrome in three and oxysterol 7α-hydroxylase (CYP7B1) deficiency in one. For two patients who had already been clinically diagnosed with SLOS, bile acids were analyzed and the excretion of abnormal bile acids was confirmed. Although markedly tall, unknown peaks were detected in samples obtained from a few other patients, no new bile acids could be identified (thus, these peaks are still unknown) and analysis is continuing for their identification. Table 1 lists the results of the bile acid analysis, that is, data on specific bile acid levels by the disorder diagnosed.

The profile of each disorder diagnosed on bile acid analysis is described in the following sections.

3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase deficiency

This subject was a female patient who was 22 years of age at the time of diagnosis. Since the age of 5 years she had exhibited transient direct-type hyperbilirubinemia, hepatic dysfunction and a bleeding tendency due to a vitamin K deficiency. Although she had obstructive jaundice, the levels of s-TBA and γ-GTP were within the normal range and she had no cutaneous pruritus. She was treated as a patient with liver cirrhosis of unknown cause and a request for urinary bile acid analysis came from the treating physician. GC-MS of bile acids indicated a high s-TBA level (56.0 mmol/mol·cre) and a high proportion (94.6%) of unusual bile acids to total bile acids (3β,7α,12α-trihydroxy-5-cholen-24-oic acid; Δ^5-3β, 7α, 12α-triol, 71.9%; 3β,7α-dihydroxy-5-cholen-24-oic acid; Δ^5-3β, 7α-diol, 22.2%; and 3β-hydroxy-5-cholen-24-oic acid; Δ^5-3β-ol, 0.6%).[21] Figure 3 shows the analysis chart for this patient.

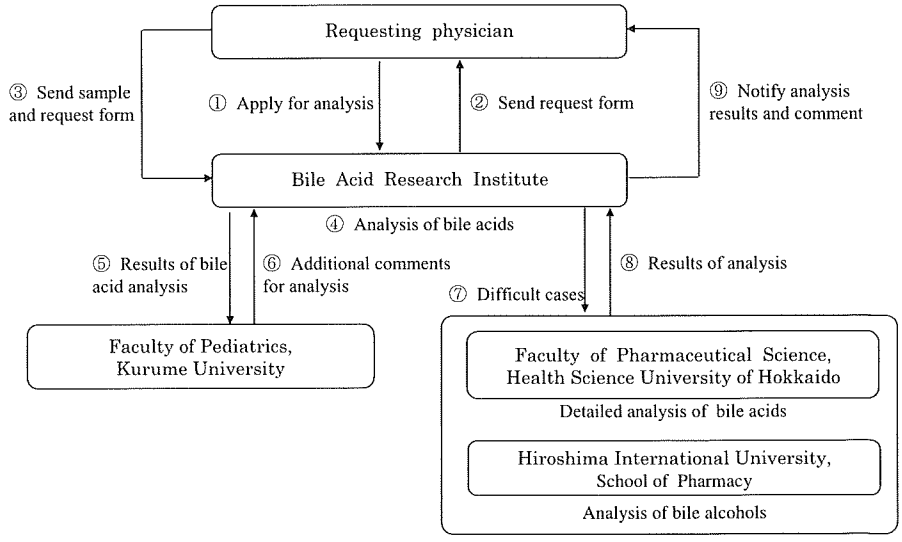

Fig. 2 Diagnostic system for inborn errors of bile acid metabolism.

Table 1 Diagnostic system for IEBAM and urinary bile acid levels vs disease

IEBAM	No. patients	Total bile acids (mmol/mol·cre)	Ratio of bile acids (%)					
			Normal	Added hydroxy-	5-cholen	CA-Δ^4	CDCA-Δ^4	Long chain
3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase	1	56.0	1.6	1.7	94.6	0.2	1.9	0.0
Δ^4-3-oxo-steroid 5β-reductase (primary)	2	114.1	2.3	0.1	3.1	87.6	6.5	0.0
		57.9	6.8	0.2	0.9	5.1	86.2	0.0
Δ^4-3-oxo-steroid 5β-reductase (secondary)	3	9.3	20.4	0.5	2.3	14.3	60.6	0.0
		13.7	5.9	0.4	6.4	2.4	78.5	0.0
		44.5	17.6	3.7	2.4	27.8	47.6	0.0
Zellweger	3	126.8	4.0	24.1	0.2	0.0	0.0	71.3
		11.2	26.5	5.7	1.9	0.0	0.0	58.1
		41.8	2.5	0.8	1.1	0.0	0.0	89.6
Oxysterol-7α-hydroxylase	1	293.0	29.4	2.1	67.6	0.1	0.7	0.0
SLOS	2	33.0	39.7	12.4	40.3	6.8	0.0	0.0
		1.5	52.2	5.2	30.5	0.0	0.0	0.0

Normal: total amounts of cholic acid (CA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), deoxycholic acid (DCA) and lithocholic acid (LCA); added hydroxy-: total amounts of 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (CA-1β-ol), 2β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (CA-2β-ol), 3α,4β,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (CA-4β-ol), 3α,6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (CA-6α-ol), 1β,3α,7α-trihydroxy-5β-cholan-24-oic acid (CDCA-1β-ol), 2β,3α,7α-trihydroxy-5β-cholan-24-oic acid (CDCA-2β-ol), 3α,4β,7α-trihydroxy-5β-cholan-24-oic acid (CDCA-4β-ol) and 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid (CDCA-6α-ol); 5-cholen: total amounts of 3β-hydroxy-5-cholen-24-oic acid (Δ^5-3β-ol), 3β,7α-dihydroxy-5-cholen-24-oic acid (Δ^5-3β,7α-triol), 3β,7β-dihydroxy-5-cholen-24-oic acid (Δ^5-3β,7β-triol), 3β,12α-dihydroxy-5-cholen-24-oic acid (Δ^5-3β,12α-ol), 3β,7α,12α-trihydroxy-5-cholen-24-oic acid (Δ^5-3β,7α-diol) and 3β,7β,12α-trihydroxy-5-cholen-24-oic acid (Δ^5-3β,7β,12α-triol); CA-Δ^4: total amounts of 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid (CA-Δ^4-3-one) 7α,12α-dihydroxy-3-oxo-chol-4,6-dien-24-oic acid (CA-$\Delta^{4,6}$-3-one); CDCA-Δ^4: total amounts of 7α-hydroxy-3-oxo-4-cholen-24-oic acid (CDCA-Δ^4-3-one) and 7α-hydroxy-3-oxo-chol-4,6-dien-24-oic acid (CDCA-$\Delta^{4,6}$-3-one); long chain: total amounts of 3β,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) and 3β,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA).
IEBAM, inborn errors of bile acid metabolism; SLOS, Smith-Lemli-Opitz syndrome.

Δ^4-3-oxo-steroid 5β-reductase deficiency

In principle this disorder is diagnosed as a primary disorder if a genetic mutation is identified in the responsible enzyme and as a secondary disorder if the enzymatic activity is secondarily reduced without a mutation. Among the patients suspected of having a primary disorder, however, no genetic mutation can be detected in many cases and the detailed characteristics of this disorder have yet to be elucidated. Based on the results of urinary

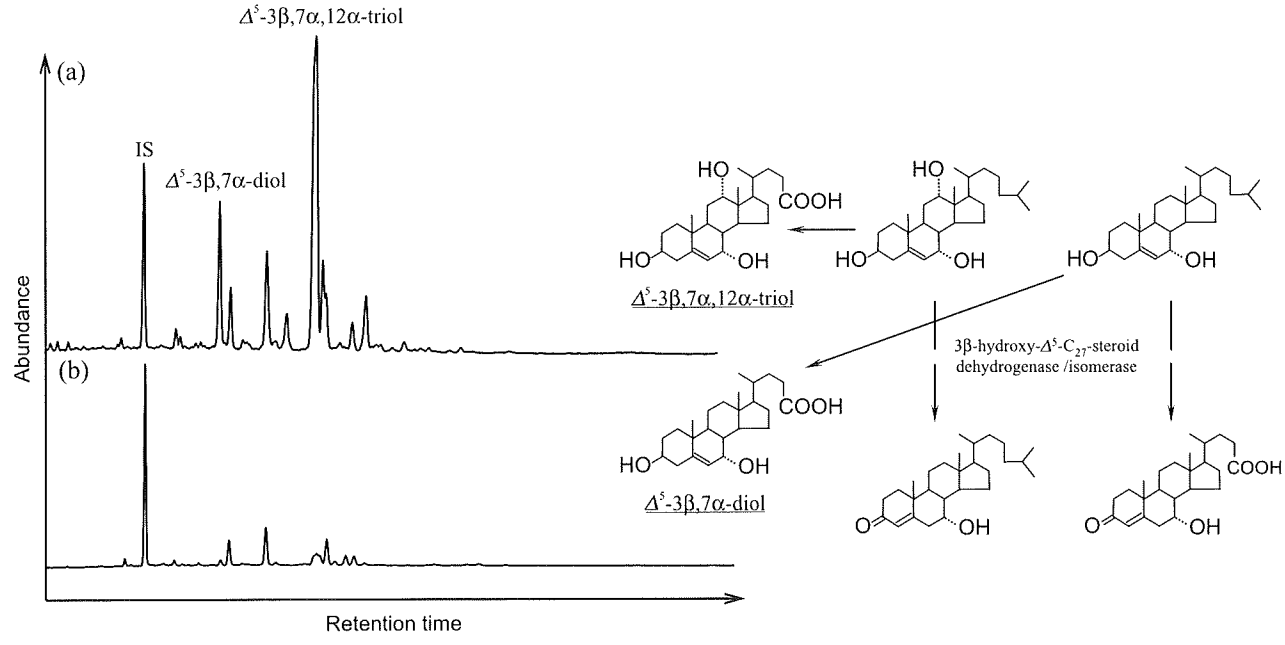

Fig. 3 Total ion chromatogram of urinary bile acids in (a) female 21-year-old patient with 3b-hydroxy-*D*5-C27-steroid dehydrogenase/isomerase deficiency and (b) female 26-year-old control. IS, internal standard.

bile acid analysis, cases were arbitrarily classified as primary disorders if 3-oxo bile acids accounted for >90% of the total bile acids and as secondary disorders if the proportion of 3-oxo bile acids ranged from 70% to 90%.

Primary Δ^4-3-oxo-steroid 5β-reductase deficiency was diagnosed in two patients. In one of them the diagnosis was confirmed by a gene analysis performed by Ueki *et al.*[21] This patient was a female infant aged 6 months at the time of the request for analysis. The urinary level of total bile acids was 114.1 mmol/mol·cre and the proportions of CA-Δ^4-3-oxo bile acid (7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid) and CDCA-Δ^4-3-oxo bile acids (7α-hydroxy-3-oxo-4-cholen-24-oic acid) were 87.6% and 6.5%, respectively. She is now 3 years old and the proportion of CA-Δ^4-3-oxo bile acids is still high, around 90% in the abnormal range, but she has no cholestatic symptoms and her liver function test values and other laboratory values are all within the normal ranges. The other patient was a male infant aged 1 month at the time of the request for analysis. The level of total bile acids was 57.9 mmol/mol·cre and the proportions of CA-Δ^4-3-oxo bile acids and CDCA-Δ^4-3-oxo bile acids were 5.1% and 86.2%, respectively. He died of liver failure at the age of 2 months.

A secondary Δ^4-3-oxo-steroid 5β-reductase deficiency was diagnosed in three patients. A female infant, who was 3 months old at the time of the request, had moderate jaundice and steatorrhea (grayish stool). Abdominal ultrasound indicated marked atrophy of the liver without ascites. The laboratory values were: D-Bil 6.3 mg/dL, AST 144 IU/L, γ-GTP 28 U/L and s-TBA 118 μmol/L. GC-MS indicated that the urinary total bile acid level was 9.3 mmol/mol·cre and that the proportion of 3-oxo bile acids was 74.9% (the proportions of CA-Δ^4- and CDCA-Δ^4-3-oxo bile acids were 14.3% and 60.6%, respectively). Another patient was a male infant aged 2 weeks at the time of the request. He had hyperammonemia and liver failure with D-Bil 2.1 mg/dL (T-Bil 12.6 mg/dL), AST 37 IU/L and s-TBA 18 μmol/L. GC-MS showed that the urinary total bile acid level was 13.7 mmol/mol·cre and that the proportion of 3-oxo bile acids was 80.9% (the proportions of CA-Δ^4- and CDCA-Δ^4-3-oxo bile acids were 2.4% and 78.5%, respectively). The last patient was also a male infant aged 2 weeks at the time of the request, with D-Bil 19.3 mg/dL, AST 207 IU/L, γ-GTP 36 U/L and s-TBA 232 μmol/L. Biliary scintigraphy indicated an accumulation of tracer in the liver alone, without excretion of it into the bile ducts. GC-MS indicated that urinary total bile acid level was 44.5 mmol/mol·cre and that the proportion of 3-oxo bile acids was 75.4% (the proportions of CA-Δ^4-3-oxo bile acids and CDCA-Δ^4-3-oxo bile acids were 27.8% and 47.6%, respectively). No information on the clinical outcome of these three patients is available. Figure 4 shows the analysis chart for primary Δ^4-3-oxo-steroid 5β-reductase deficiency.

Zellweger syndrome

In the process of bile acid biosynthesis, enzymes in the peroxisomes play roles in the oxidation and cleavage of the cholesterol side chain. In patients with Zellweger syndrome, all enzymes in the peroxisomes are damaged and the levels of bile acid precursors 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) and 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA) intermediates before side-chain cleavage are elevated. THCA and DHCA were detected in the samples obtained from three patients, who were diagnosed with Zellweger syndrome. As observed in patients with Zellweger syndrome, the levels of THCA and DHCA are also elevated in patients with peroxisomal thiolase deficiency (deficiency of an enzyme involved in bile acid metabolism; a single peroxisomal enzyme deficiency), chondrodysplasia punctata rhizomelic type and pseudo-infantile Refsum disease

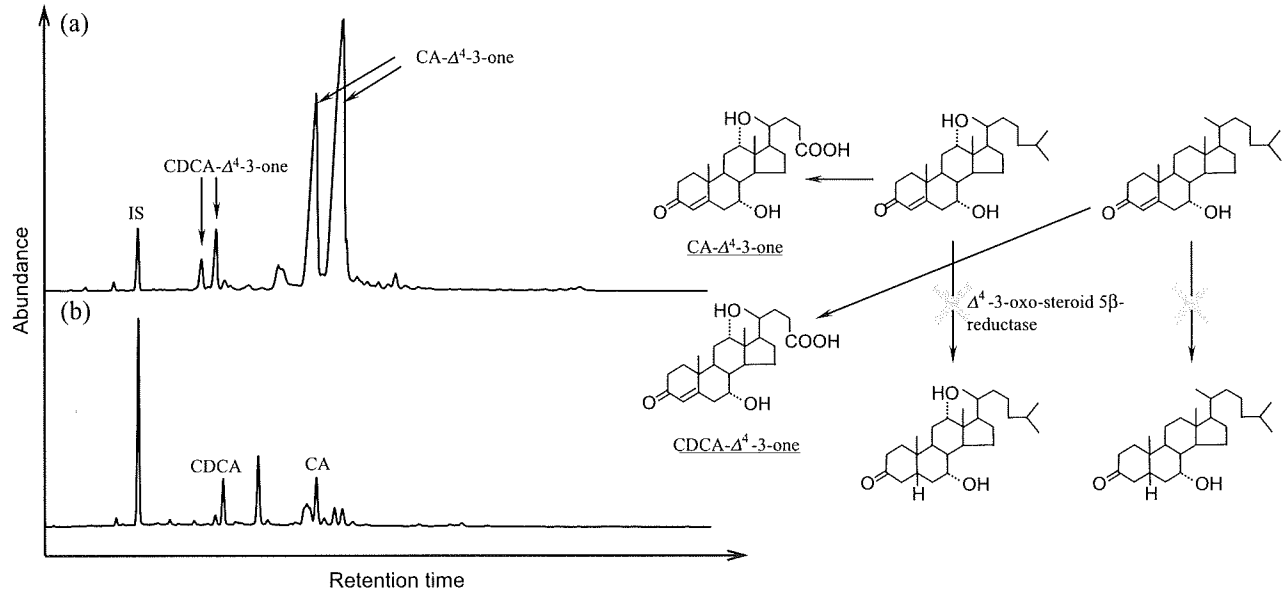

Fig. 4 Total ion chromatogram of urinary bile acids in (a) female 6-month-old patient with *D*4-3-oxo-steroid 5b-reductase deficiency and the (b) female 6-month-old control. CA, cholic acid; CDCA, chenodeoxycholic acid; IS, internal standard.

(both of which are multiple enzyme deficiencies). These disorders, however, cannot yet be identified on this system. Figure 5 shows the analysis chart for Zellweger syndrome. Bile acid replacement therapy was performed for two of these three patients, but all of them died.[22]

Oxysterol-7α-hydroxylase deficiency

In patients with cholesterol-7α-hydroxylase deficiency[23] and those with an oxysterol-7α-hydroxylase deficiency,[7] high concentrations of 3β-hydroxy-5-cholen-24-oic acid (bile acid unhydroxylated at position 7) are detected in both the serum and urine. Because the analysis data could not distinguish these two disorders, genetic testing was performed for a definitive diagnosis. This patient was an infant aged 5 months at the time of the request for analysis, who exhibited persistent jaundice. Giant cell formation was observed in the liver and the following laboratory values were notable: D-Bil 4.3 mg/dL, AST 1080 IU/L, γ-GTP 45 U/L and s-TBA 4 μmol/L. After a genetic analysis, Ueki *et al.* recently made a definitive diagnosis of oxysterol-7α-hydroxylase deficiency in this infant.[24] There are two pathways of (cholesterol) 7α-hydroxylation. One is a neutral pathway in which cholesterol is hydroxylated by cholesterol-7α-hydroxylase and the other is an acidic pathway in which cholesterol is hydroxylated and oxidized at position 27 and then hydroxylated by oxysterol-7α-hydroxylase. In the neutral pathway, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized, while only CDCA is synthesized in the acidic pathway. Applying simple logic to this classification, it can be presumed that both CA and CDCA are synthesized in patients with oxysterol-7α-hydroxylase deficiency, while very little CA is synthesized in patients with cholesterol-7α-hydroxylase deficiency. This patient, however, had a Δ^5-3β-ol level of 197.6 mmol/mol·cre, a CA level of 17.1 mmol/mol·cre and a CDCA level of 2.6 mmol/mol·cre. The increase in urinary CA level was suggestive of an oxysterol-7α-hydroxylase deficiency. Figure 6 shows the analysis chart for this patient.

Smith-Lemli-Opitz syndrome

Prior to bile acid analysis by this system, two patients had already been clinically diagnosed with SLOS. In SLOS patients the synthesis of cholesterol is insufficient due to a decrease in the activity of 3β-hydroxysteroid Δ^7-reductase, an enzyme involved in the final step of cholesterol biosynthesis, and the synthesis of steroids and bile acids (both synthesized from cholesterol) is thereby affected. The urinary total bile acid levels were 33.0 and 1.5 mmol/mol·cre in these two patients. The levels of Δ^5-3β-ol were 2.1 and 0.30 mmol/mol·cre and those of 3β,7β-dihydroxy 5-cholen-24-oic acid (Δ^5-3β,7β-diol) were 9.3 and 0.15 mmol/mol·cre. The proportions of 5-cholenic acids to total bile acids were high, at 40.3% and 30.5%. Figure 7 shows the analysis chart for these patients.

Discussion

Among the patients with cholestasis of unknown etiology, the prevalence of IEBAM is reported to be approximately 1–2.5%.[25] The prevalence, however, is 6.3%. This difference was considered to be due to selection of patients for bile acid analysis by high-risk screening.

In making a diagnosis of IEBAM, urinalysis is generally performed, because the concentration of unusual bile acids is higher in excreted urine than in serum. Urinalysis findings suggestive of IEBAM have already been reported. In the patients with IEBAM, 3α-bile acids cannot be synthesized in the liver. Therefore, the s-TBA level on the enzymatic method in IEBAM was low. Furthermore, the low concentration of bile acid in the bile duct may show normal γ-GTP.[26] In fact, the urinalysis findings were

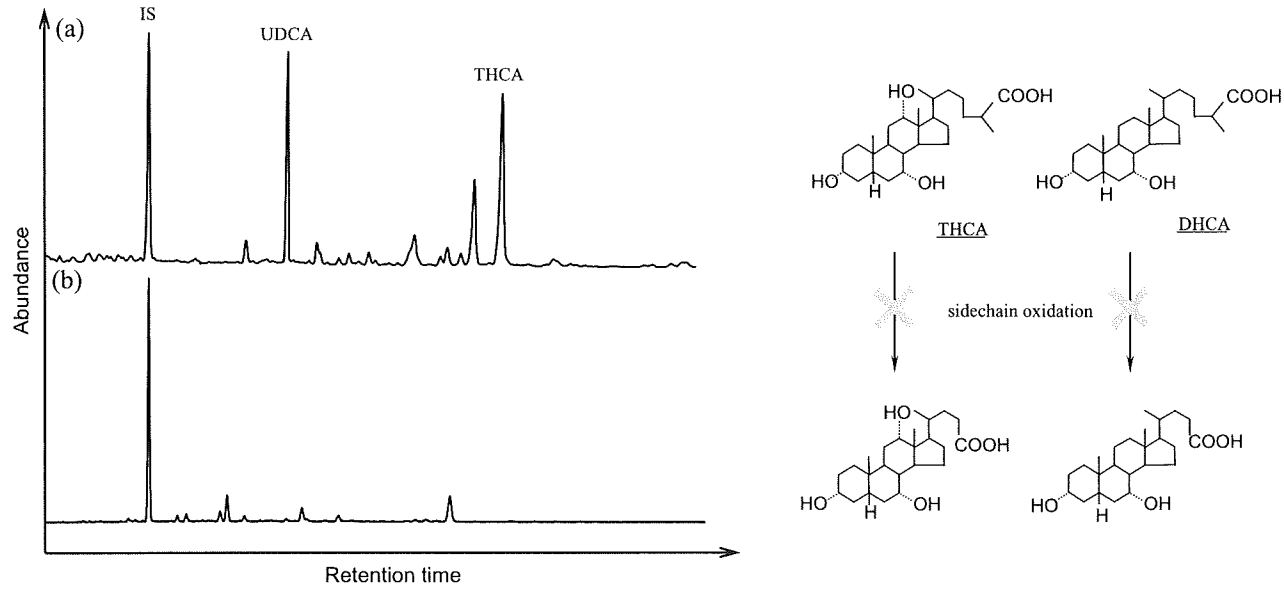

Fig. 5 Total ion chromatogram of urinary bile acids in (a) male 2-month-old patient with Zellweger syndrome and (b) male 2-month-old control. IS, internal standard; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; UDCA, ursodeoxycholic acid.

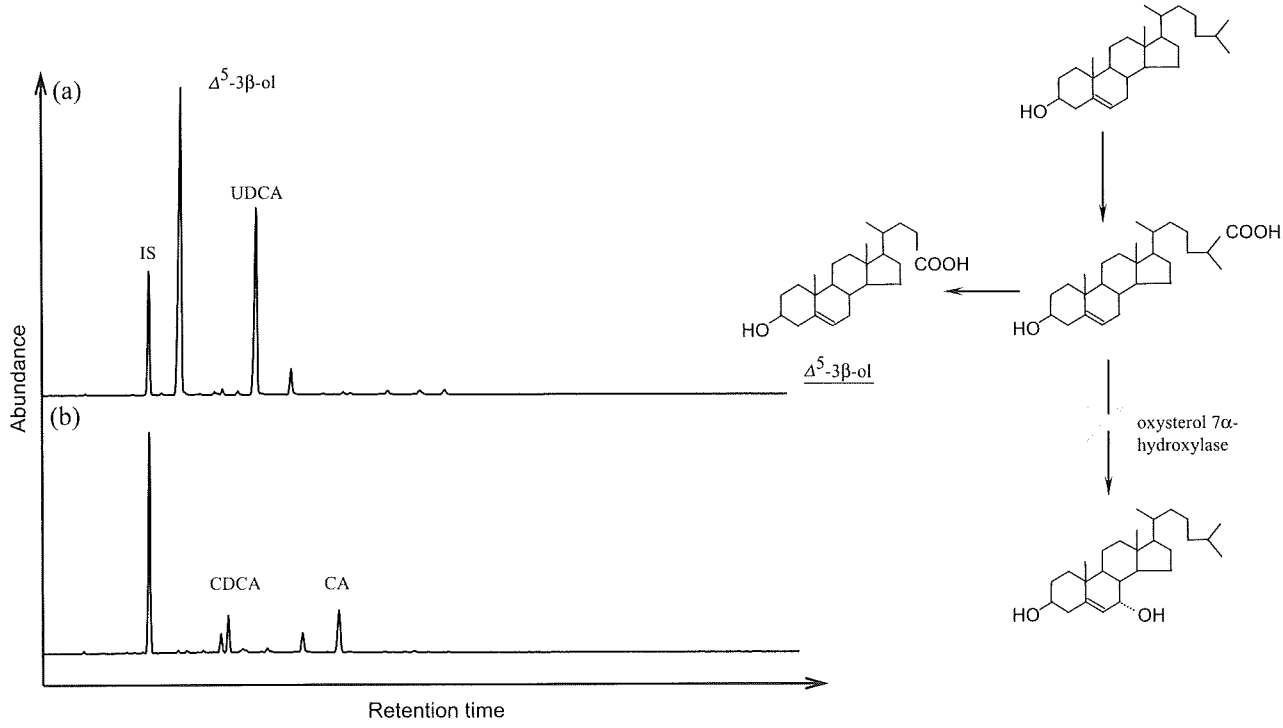

Fig. 6 Total ion chromatogram of urinary bile acids in (a) male, 5-month-old patient with oxysterol-7a-hydroxylase and (b) male, 5-month-old control. CA, cholic acid; CDCA, chenodeoxycholic acid; IS, internal standard; UDCA, ursodeoxycholic acid.

useful in making the diagnosis in patients who exhibited no increase in serum TBA or γ-GTP despite the presence of obstructive jaundice.

In the 10 years that the urinary bile acid analysis system has been operating, many requests have come from institutions with physicians specializing in pediatric liver disease (who are located in certain limited regions of Japan). Requests for a sample analysis have been received from approximately 80% of the prefectures of Japan. Awareness of IEBAM needs to be increased in regions from which we received no requests for bile acid

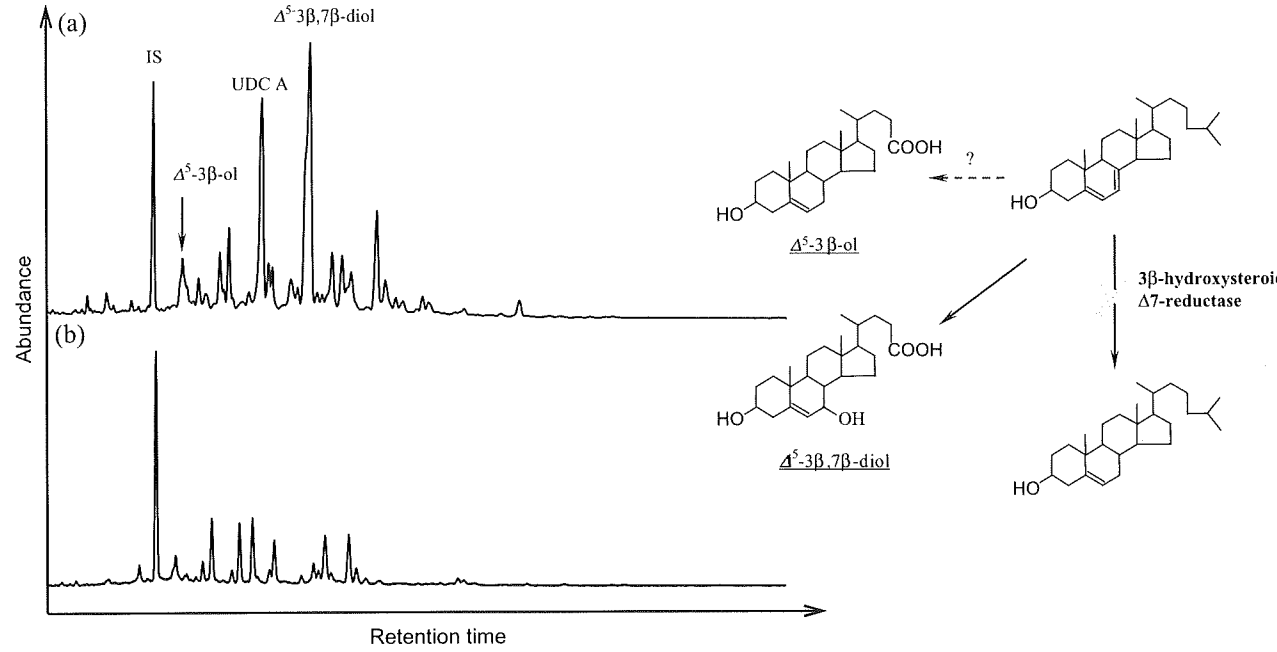

Fig. 7 Total ion chromatogram of urinary bile acids in (a) male, 5-year-old patient with Smith-Lemli-Opitz syndrome and (b) male, 5-year-old control. IS, internal standard; UDCA, ursodeoxycholic acid.