Two neonatal cholestatic patients with mutation in *SRD5B1* (*AKR1D1*) gene: diagnosis and bile acid profiles during chenodeoxycholic acid treatment

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Abbreviations: 5β-rductase, 3-oxo-Δ^4-steroid 5β-rductase; TBA, total bile acids; GGT, γ-glutamyltranferase; ALT, alanine aminotransferase; GC-MS, gas chromatography-mass spectrometry; CDCA, chenodeoxycholic acid; AST, aspartate aminotransferase; UDCA, ursodeoxycholic acid; Cr, creatinine; CMV cytomegalovirus; CA, cholic acid.

Abstract

Background and Aim: In 2 Japanese neonatal cholestatic patients with 3-oxo-Δ^4-steroid 5β-reductase deficiency who had diagnosed as mutations of *SRD5B1* gene and detected unusual bile acids such as elevated 3-oxo-Δ^4 bile acids in serum and urine using gas chromatography-mass spectrometry, we discussed about effect of oral chenodeoxycholic acid treatment.

Patients and Methods: We evaluated the laboratory data, bile acid analysis, and *SRD5B1* gene analysis from 2 patients with neonatal cholestasis. *SRD5B1* gene and bile acid analysis are required using genomic DNA of peripheral lymphocyte and gas chromatography-mass spectrometry of serum and urine from 2 patients, respectively. Diagnosis and treatment of these 2 patients were retro- and prospectively investigated.

Results: Two patients were diagnosed as 3-oxo-Δ^4-steroid 5β-reductase deficiency by *SRD5B1* gene and bile acid analysis. One was novel heterozygote (R266Q) and the other was compound hetrozygote (G223E/R261C). Chenodeoxycholic acid treatment was effective to improve liver dysfunction and to reduce unusual bile acids such as 7α-hydroxy- and 7α,12α-dihydroxy-3oxochol-4en-24-oic acids in serum and urine.

Conclusion: Primary bile acid treatment using chenodeoxycholic acid is effective for these patients in the early neonatal period before the late stage of chronic cholestatic liver dysfunction.

Introduction

In 1988, Clayton *et al* reported that, in pediatric patients with severe liver disease such as cholestatic disease with cirrhosis and fumarylacetoacetase deficiency, the major unusual bile acids were 3-oxo-Δ^4 bile acids, such as 7α-hydroxy- and 7α,12α-dihydroxy-3oxochol-4en-24-oic acids (Clayton et al. 1988). At the same time, 3-oxo-Δ^4-steroid 5β-reductase (5β-reductase) deficiency was also described for the first time by Setchell *et al* (Setchell et al. 1988). This inborn error of bile acid synthesis is very rare disease and is autosomal recessive inheritance. The main findings of 5β-reductase deficiency were the normal or the slightly elevated concentration of total bile acids (TBA) and γ-glutamyltranferase (GGT) in serum, elevated conjugated hyper bilirubinemia and alanine aminotransferase (ALT), and fatty stools. During the course of synthesis of bile acids from cholesterol, named classical pathway, if activity of 5β-rductase enzymes decreased, reduced synthesis of primary bile acids and increased synthesis of 3-oxo-Δ^4 bile and allo-bile acids.

Lemonde *et al* and Gonzales *et al* reported *SRD5B1* (*AKR1D1*) gene analysis in 5β-rductase deficiency on chromosome 7q32-33 in 2003 and 2004, respectively (Lemonde et al. 2003,,Gonzales et al. 2004). According to these 2 reports, mutation in the *SRD5B1* gene were identified all 5 patients, 3 homozygous mutations and 2 compound heterozygous mutations (Lemonde et al. 2003,,Gonzales et al. 2004). Recently, we also reported 2 heterozygous mutations in patients with 5β-rductase deficiency that was diagnosed by bile acid analysis using gas chromatography-mass spectrometry (GC-MS) (Ueki et al. 2009).

We here reported to diagnose 2 infants, 6 months old Japanese boy, and 9 months old Japanese girl, with 5β-rductase deficiency resulting from one heterozygous and the other

compound heterozygous mutations in the *SRD5B1* gene. Moreover, we discuss about clinical effect of oral chenodeoxycholic acid (CDCA) treatment of these 2 patients with 5β-reductase deficiency.

Patients and Methods

Patient report

Patient 1: A male Japanese infant was delivered at 37 weeks gestational age an uneventful pregnancy. The parents were not consanguineous and were healthy, without liver disease. At birth, he was noticed abdominal distension and metabolic acidosis. We detected abdominal free air by abdominal X-ray and CT scanning. Therefore, we performed operation the suspected gastrointestinal perforation at second days after birth. After operation at Saitama Medical University Hospital, he was diagnosed meconium peritonitis due to perforation of jejunum with neonatal intussuception.

Thereafter, he persistence jaundice until at 6 months of age. We performed the serial technetium-99m (^{99m}Tc)-DISIDA cholescintigraphy and liver biopsy at 2 and 3 months of age, respectively. No cholescintigraphy revealed visualization of intestinal radioactivity. His liver microscopic findings revealed giant cell transformation and consistent with fibrosis showing wide fibrotic bands at portal areas.

His laboratory studies disclosed an aspartate aminotransferase (AST) concentration in serum of 344 U/L (normal range <37); ALT, 441 I/L (<31); and total/direct bilirubin, 4.4/3.4 mg/dL (<1.2/0.4). GGT concentration in serum was 46 U/L (<52); and we did not examine serum TBA at admission. We suspected the inborn errors of bile acid synthesis from above data,

and analyzed serum and urine bile acids using GC-MS, as results of bile acid analysis showed Table 1. From results of bile acid analysis, we suspected the 5β-reductase deficiency. At that time he was received ursodeoxycholic acid (UDCA) treatment.

Patient 2: A female Japanese infant with a birth weight of 2832 g was delivered by spontaneous vaginal delivery without complications at a gestational age of 38 weeks, after an uneventful pregnancy, her mother's first. The parents were not consanguineous and were healthy, without liver disease.

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

On physical examination, growth and development were within normal range. No dysmorphic features were present. Hepatomegaly and jaundice were noted. Neurologic findings were normal. Stools were gray. Initial laboratory results included serum concentration of AST, 315 U/L; ALT, 229 U/L; alkaline phosphatase, 2717 U/L (115 to 359); total/direct bilirubin, 6.3/3.6 mg/dL; albumin, 4.8 g/dL (4.0 to 5.0); total cholesterol, 216 mg/dL (128 to 219); prothrombin time, 14.8 seconds (10.0 to 13.5); and blood ammonia, 66 μg/dL (<66). Serum GGT was 61 U/L and serum TBA, 5.2 μmol/L (<10). Other causes of liver disease such as autoimmune hepatitis, chronic viral hepatitis, and other metabolic conditions were excluinded by appropriate investigations. Abdominal ultrasonography showed a visible gallbladder and hepatomegaly; no choledochal cyst, bile duct dilation, or ascites was demonstrated. Serial technetium-99m (^{99m}Tc)-DISIDA cholescintigraphy indicated that tracer entered the intestine. We suspected the inborn errors of bile acid synthesis from above data, and analyzed serum and urine bile acids using GC-MS at 9 months of age, as results of bile acid analysis showed Table 1.

From results of bile acid analysis, we suspected the 5β- reductase deficiency.

Qualitative and quantitative bile acid analysis

The serum and urine samples were collected and stored at -25℃ 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 (Tohma et al. 1986), 3-oxo-Δ^4 (Leppik 1983) and allo-bile acids (Iida et al. 1993), 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 (Kimura et al. 1999), after enzymatic hydrolysis (choloylglycine hydrolase 30 units) and solvolysis (sulfatase 150 units; Sigma Chemical Co., St Louis, MO, USA).

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

Genetic analysis

With informed consent, *SRD5B1* gene analysis was performed using genomic DNA from peripheral lymphocytes from 2 patients, their parents, as well as 50 healthy individuals. DNA fragments spanning the 9 coding regions and exon-intron junctions of the *SRD5B1* gene were amplified by polymerase chain reaction (PCR) using Gene Taq (Nippon Gene, Toyama, Japan) and 9 sets of primers to obtain DNA fragments of the optimal length for direct sequence analysis

(Ueki et al. 2009).

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, CA).

After the 3 putative mutations were found in the patients, their parents and 50 healthy individuals were screened for these 3 mutations by direct sequence analysis.

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

Results

Biochemical identification of an inborn error of bile acid synthesis

Patient 1: He had jaundice since early neonatal period, especially after operation. So far, condition of this patient was stable due to UDCA (7.5 mg/kg/day) treatment. UDCA treatment was effective for liver dysfunction, such as elevated ALT and total bilirubin. After diagnosis, we started combined UDCA and CDCA (5 mg/kg/day) treatment. Liver dysfunction was improved by combined UDCA and CDCA treatment completely (Fig 1-A). Serum value of total bilirubin was decreased to normal range before decreased that of ALT. While, concentrations of 3-oxo-Δ^4 bile acids in serum and urine were not reduced by UDCA treatment. After combined UDCA and CDCA treatment, serum 3-oxo-Δ^4 bile acids gradually decreased to not detectable, less than 1 μmol/L. While urinary 3-oxo-Δ^4 bile acids decreased after fluctuations. Since UDCA treatment

stopped at 14 months of age, liver function and bile acid profiles in serum and urine were stable by CDCA mono-treatment.

Patient 2: She was received UDCA (7 mg/kg/day) treatment at 8 months of age but no improvement of clinical symptoms and liver dysfunction. After diagnosis, we started combined UDCA (10 mg/kg/day) and CDCA (10 mg/kg/day) treatments at 9 months of age. Liver dysfunction, ALT and serum TBA, increased but 3-oxo-Δ^4 bile acids in serum decreased. All most serum TBA is CDCA. While concentrations of TBA and 3-oxo-Δ^4 bile acids in urine were increased respectively (Fig. 1-B). Then we reversed dose of CDCA from 10 to 5 mg/kg/day, thereafter, combined UDCA (10 mg/kg/day) and CDCA (5 mg/kg/day) treatment was effective for liver dysfunction. Serum value of total bilerubin was also decreased to normal range before decreased that of ALT as well as patient 1 and concentrations of TBA and 3-oxo-Δ^4 bile acids in serum and urine were gradually decreased. Clinical course of liver dysfunction and bile acid profiles in serum and urine were gradually decreased by CDCA mono-treatment at 15 months old of age. At elevated ALT and TBA in serum and urine, we detected elevated cytomegalovirus (CMV) antibodies. EIA units of serum IgM antibodies against CMV (< 0.8) were under 0.34 at 6 months of age and elevated to 1.27 at 11 months of age. The serum IgG antibodies (< 0.2) at 6 months of age were under 2.0 and elevated to 28.0 at 11 months of age.

Identification of *SRD5B1* gene defects

We identified 1 novel mutation and 2 reported mutations in these 2 patients.

Patient 1: A single heterozygous mutation, novel, was found in exon 7, at nucleotide number 866, representing a G-to-A substitution, causing an amino acid change from arginine to

glutamine (R266Q). The mutation was detected in heterozygous form in the mother, but absent in the father and controls (Fig. 2).

Patient 2: Two heterozygous mutations, previously reported, were found. One was detected in exon 6, at nucleotide number 737, representing a G-to-A substitution, causing an amino acid change from glycine to glutamic acid (G223E). G223E was detected in heterozygous form in the father, but absent in the mother and 50 healthy individuals. The other was detected in exon 7, at nucleotide number 850, representing a C-to-T substitution, causing an amino acid change from arginine to cysteine (R261C). R261C was detected in heterozygous form in the mother, but absent in the father and controls (Fig. 3).

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

Discussion

In our 2 patients with 5β-reductase deficiency who had *SRD5B1* mutation, CDCA treatment was effective because of improved liver dysfunction and reduced unsaturated ketonic bile acids in serum and urine. Oral bile acid treatment such as cholic acid (CA) was safe and effective intreating most common inborn errors of bile acid synthesis, including 5β-reductase deficiency (Gonzales et al. 2009). CA treatment may be better because activates negative feedback regulation of bile acid synthesis to inhibit production of hepatotoxic metabolites and is not itself hepatotpxic better than CDCA. However CA is not available for clinical use in Japan. According to previous report (Clayton et al. 1996), combined CA and CDCA treatment for 5β-reductase deficiency was also used. We suggested that oral bile acid treatment using CDCA

mono-treatment is effective for patient with 5β-reductase deficiency.

In bile acid analysis during combined UDCA and CDCA treatment, external UDCA was detected in urine more than that in serum, while external CDCA was detected in serum more than that in urine (data not shown). We think that hydrophilic UDCA may be prone to excrete from kidney to urine by multidrug resistance-related protein 4 after transport from hepatocytes to blood by multidrug resistance-related protein 4 in the basolateral membrane (Wagner et al. 2005, Marschall et al. 2005, Stapelbroek et al. 2010). Unsaturated ketonic bile acids, 3-oxo-Δ^4 bile acids, were detected in urine more than in serum. 3-Oxo-Δ^4 bile acids were across the basolateral membrane via multidrug resistance-related protein 3 from hepatocytes to blood (Tamaguchi et al. 2010). Thereafter, 3-oxo-Δ^4 bile acids excrete to urine immediately by some bile acid transporter. As the results, we may be detected large amounts of urinary 3-oxo-Δ^4 bile acids in this disease.

In spite of normal liver function, we detected 3-oxo-Δ^4 bile acids in serum and urine, especially urine. We speculate that negative feedback via 5mg/kg/day of CDCA may be incomplete. However if CDCA dose increase, liver function may be aggravated by some possibility. CDCA dose such as 10 mg/kg/day may be very effective negative feedback to cholesterol 7α-hydroxylase via the farnesoid X receptor (Gonzales et al. 2009). Therefore, we should be very carefully following these patients. Best following liver functional test may be analysis of bile acid in serum and urine, marker of fibrosis such as type IV collagen 7s domain, and especially pathologic findings by liver biopsy. However, fluctuations of urinary 3-oxo-Δ^4 bile acids may be not always showed stage of disease. Because since we analyze urinary bile acid using spot sample of serum and urine, we do not deny the relation of the diet. Moreover,

some liver disease reduced activity of 5β-reductase, such as metabolic, viral (Clayton et al. 1988, Kimura et al. 1998), and drug induced hepatitis. Therefore, it is very carefully to affect the viral infection such as cytomegaro virus infection in neonatal period or to be used medicine. Actually we speculated that 3-oxo-Δ^4 bile acids in serum and urine of our patient 2 were increased by itself hepatotoxic of CDCA (Fig. 1-B) or effect of CMV hepatitis..

Interestingly, duration until become to normalizing laboratory data during oral bile acid treatment using combined UDCA and CDCA treatment or CDCA mono-treatment, of patient with 5β-reductase deficiency spent long-term period more than that of patient with 3β-hydroxy-Δ^5-C_{27}-steroid dehydrogenase/isomerase deficiency from our experience (Yamato et al. 2001, Mizuochi et al. 2010). From these results, we recommended that treatment of patient with 5β-reductase deficiency should be start the oral bile acid treatment in the early neonatal period before the late stage of chronic cholestatic liver dysfunction because of 5β-reductase deficiency will be development to cholestatic liver failure until less than about 10 months of age (Gonzales et al. 2004, Ueki et al. 2009).

The human *SRD5B1* gene contains 9 coding exons corresponding to 326 amino acids; so far, 7 distinct mutations causing 5β-reductase deficiency have been reported (Lemonde et al. 2003,,Gonzales et al. 2004, Ueki et al. 2009). This enzyme deficiency has been characterized as showing autosomal recessive transmission. Here we describe genetic analysis of the *SRD5B1* gene in 2 patients with 5β-reductase deficiency, and identified 1 novel mutation (R266Q) and 2 reported mutations (G223E and R261C) (Gonzales et al. 2004, Ueki et al. 2009, Drury et al. 2010). Screenibg for the potentially informative mutation R266Q was undertaken for 50 healthy individuals, but this mutation was absent in all of them. Moreover, the R266Q mutation was

predicted to probably have an adverse effect (score, 0.995) with Polymorphism Phenotyping version 2 (Adzhubei et al. 2010). Accordingly, we believe that the R266Q mutation may have contributed to a loss of function of 5β-reductase in our patients. Patient 1 has only a single heterozygous mutation (R266Q) in one allele from his mother. We would still suspect that patient 1 with a heterozygous mutation of the *SRD5B1* gene may have a mutation in another allele and he is compound heterozygote. Patient 2 has two heterozygous mutations (G223E/R261C). The patient 2 received 1 allele with G223E mutation from the father, and the other allele with R261C from the mother, making her a compound heterozygote for the *SRD5B1* gene.

In conclusion, we diagnosed 2 patients with 5β-reductase deficiency, one had heterozygous and the other had compound heterozygous mutations in *SRD5B1* gene. Primary bile acid treatment using CDCA (5 mg/kg/day) is effective for these patients with 5β-reductase deficiency in the early neonatal period before the late stage of chronic cholestatic liver dysfunction.

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Figure legends

Figure 1. Clinical course of patient 1 (A) and patient 2 (B).

Responses of the serum alanine aminotransferase (ALT; closed circle), total bilirubin (T Bil; open circle), serum total bile acids (s-TBA; closed square), serum 3-oxo-Δ^4 bile acids (s-3-oxo-Δ^4; open square), urine total bile acids (u-TBA; closed triangle), and urine 3-oxo-Δ^4 bile acids (u-3-oxo-Δ^4; open circle) to treatment with combined ursodeoxycholoc acid (UDCA) and chenodeoxycholic acid (CDCA) treatment or CDCA mono-treatment are shown.

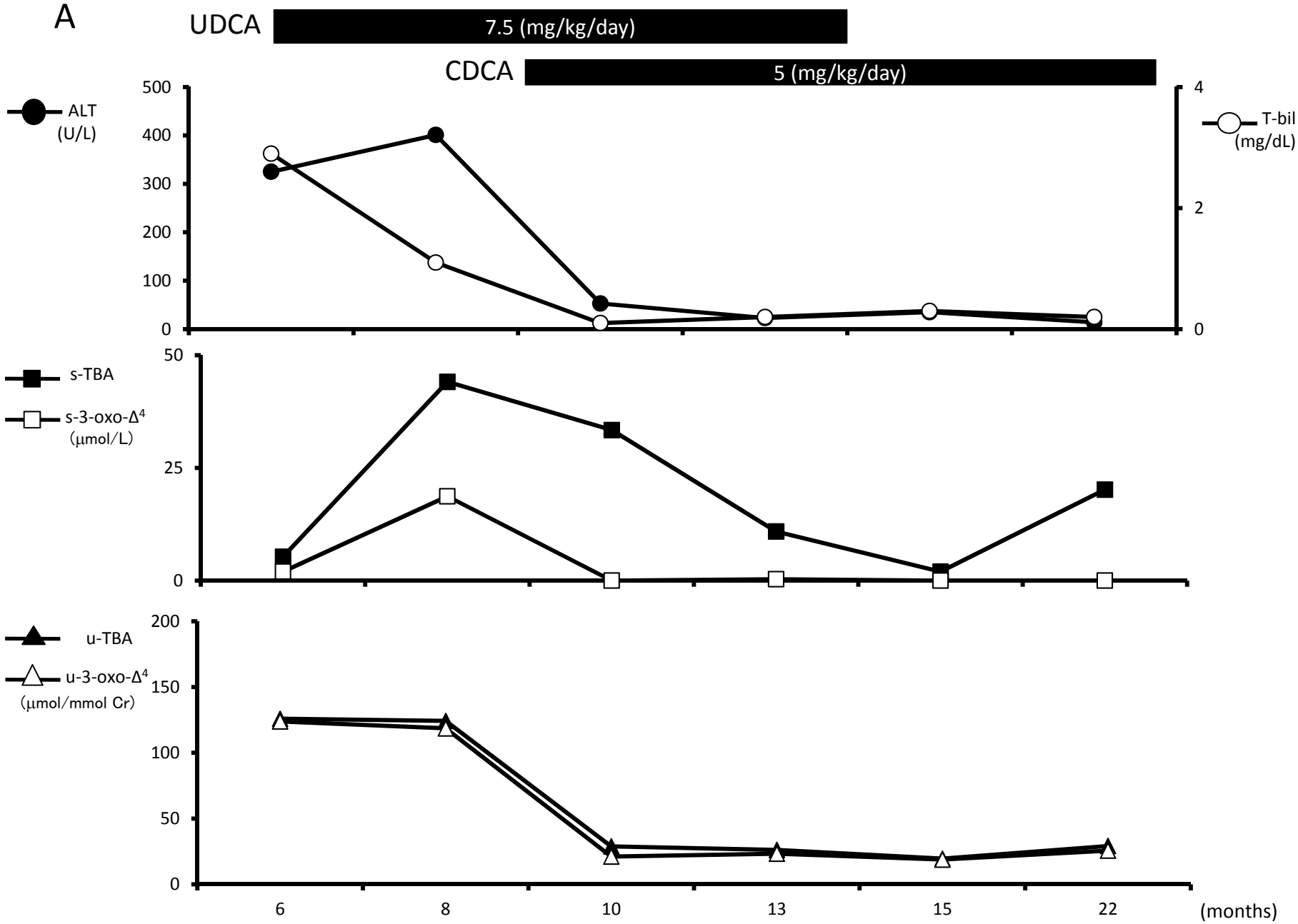
Figure 2. Pedigree for patient 1 shown with genomic DNA sequences in exon 7 of the *SRD5B1* gene in this patient, his parents, and a control.

The arrow in exon 7 identifies G/A 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 CGA-to-CAA mutation, affecting arginine at position 266, where it is replaced by glutamine.

Figure 3. Pedigree for patient 2 shown with genomic DNA sequences in exons 6 and 7 of the *SRD5B1* gene in this patient, his parents, and a control.

The arrow in exon 6 identifies G/A in the patient and her father, but G in her mother and a control subject. The reverse strand sequence shows the same result. This represents a GGG-to-GAG replacing, affecting glycine at position 223, where it is replaced by glutamic acid. The arrow in exon 7 identifies C/T in the patient and her mother, but C in her father and a control subject. The reverse strand sequence shows the same result. This represents a

CGT-to-TGT mutation affecting arginine at position 261, where it is replaced by cysteine.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7.5 (mg/kg/day)
CDCA
5 (mg/kg/day)
ALT
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T-bil
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2
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s-TBA
s-3-oxo-Δ4
(μmol/L)
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25
0
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u-3-oxo-Δ4
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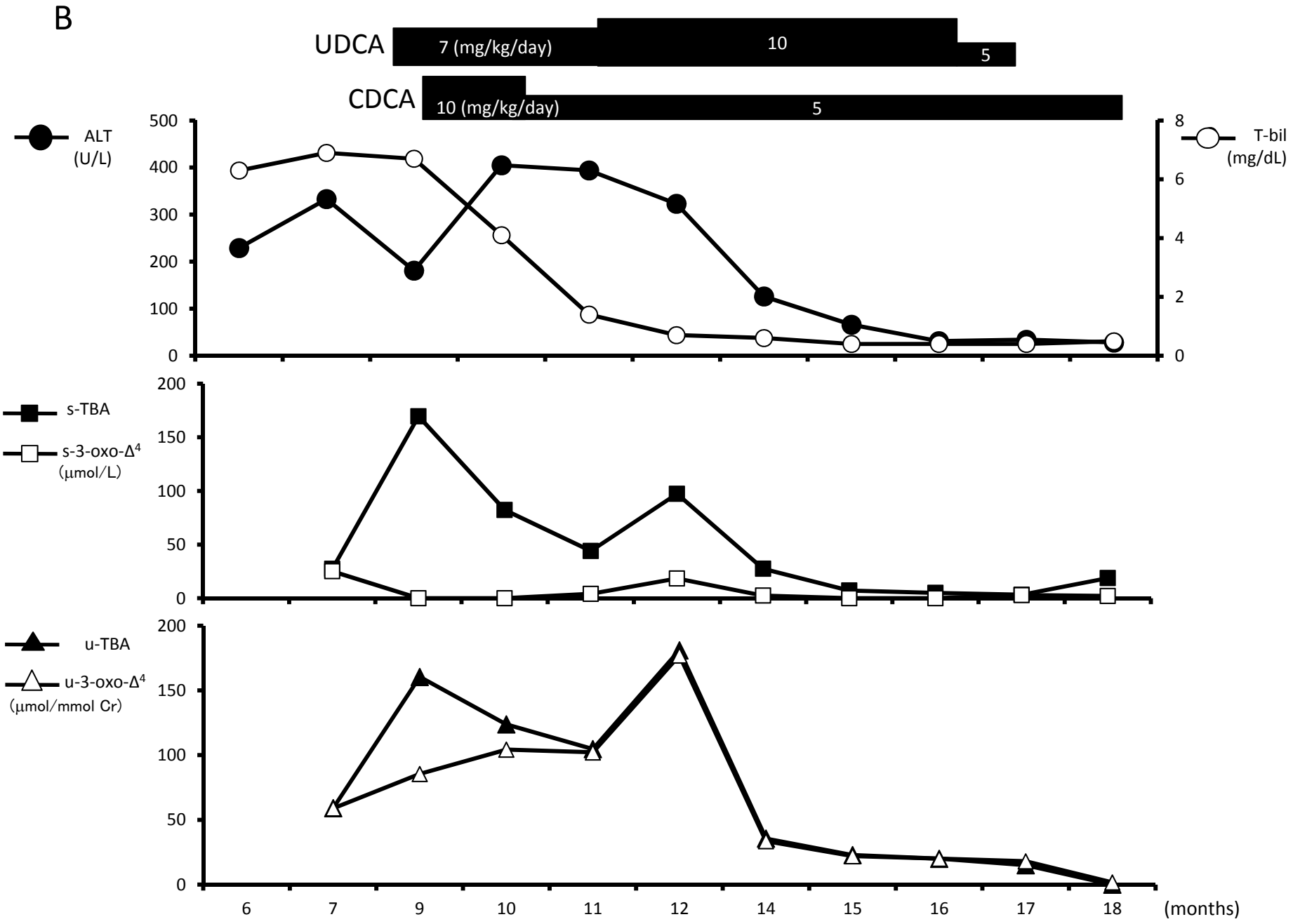
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7 (mg/kg/day)
10
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CDCA
10 (mg/kg/day)
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ALT
(U/L)
T-bil
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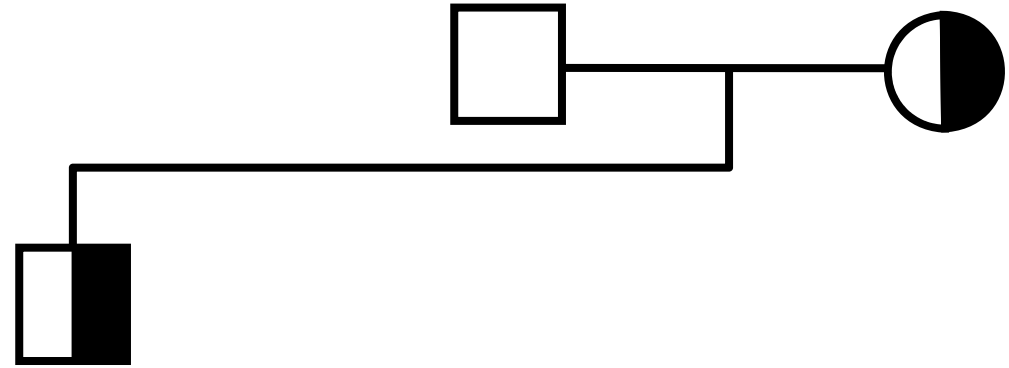

control

Exon 7

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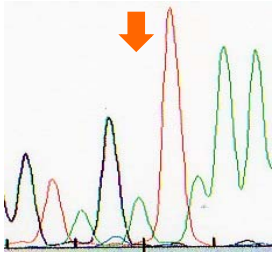

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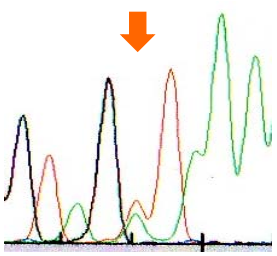
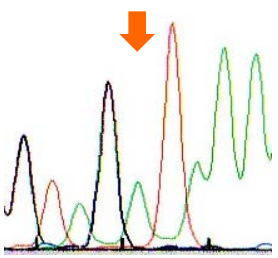


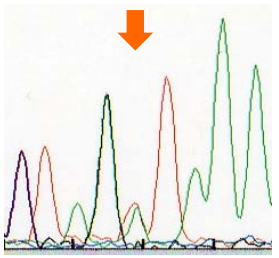
866 G > A
(R266Q)

CAGCGAGGG

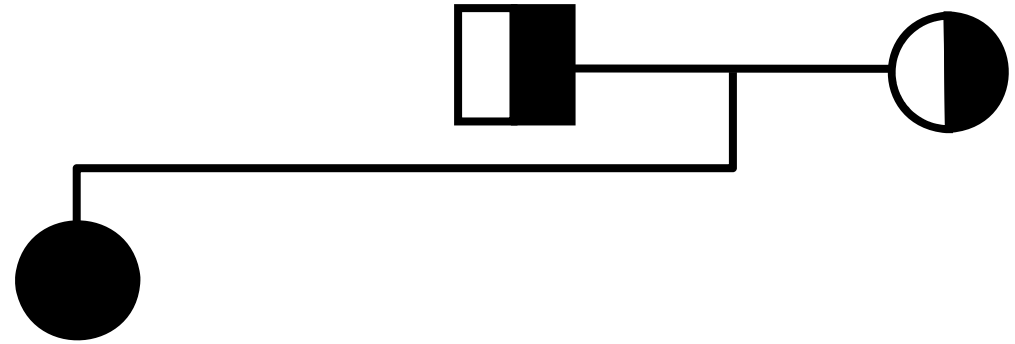


Wild type

CAGC G/A AGGG



866 G > A
(R266Q)



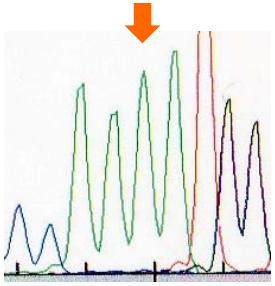
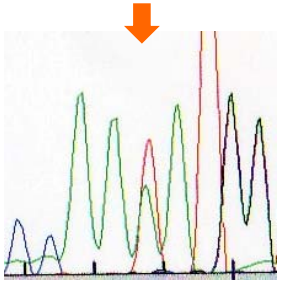
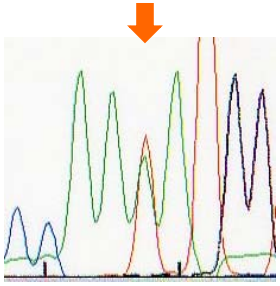
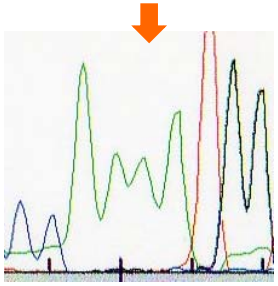
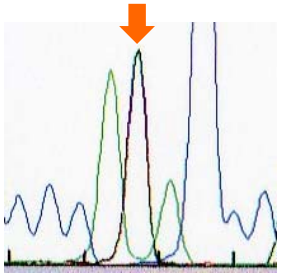
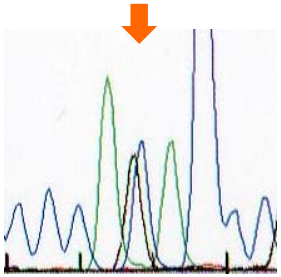
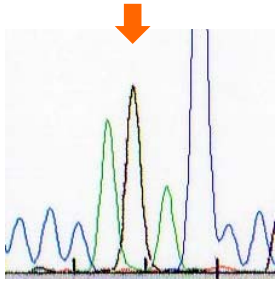
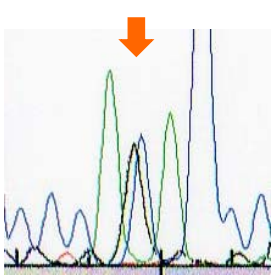
	control			
Exon 6	TTGGGGACC	TTGG$^{G}_{A}$GACC	TTGG$^{G}_{A}$GACC	TTGGGGACC
				
		737 G > A （G223E)	737 G > A （G223E)	Wild type
Exon 7	TTTGCGTTT	TTTG$^{C}_{T}$GTTT	TTTGCGTTT	TTTG$^{C}_{T}$GTTT
				
		850 C > T (R261C)	Wild type	850 C > T (R261C)

Table 1. Bile acid analysis using GC-MS in 2 patients with 3-oxo-Δ^4-steroid 5β-reductase deficiency

	Patient 1 (6 months of age)	Patient 2 (9 months of age)
Serum (μmol/L)		
Cholic acid	n.d.	n.d.
Chenodeoxycholic acid	0.1	n.d.
Deoxycholic acid	0.2	n.d.
Lithocholic acid	n.d.	n.d.
Ursodeoxycholic acid	1.5	n.d.
Allo-cholic acid	n.d.	n.d.
Allo-chenodeoxycholic acid	0.9	n.d.
7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid	0.3	14.4
7α-Hydroxy-3-oxo-4-cholen-24-oic acid	1.7	10.7
Others	0.6	2.3
Total bile acids	5.3	27.4
Urine (μmol/mmol Cr)		
Cholic acid	0.2	n.d.

Chenodeoxycholic acid	n.d.	n.d.
Deoxycholic acid	0.1	n.d.
Lithocholic acid	n.d.	n.d.
Ursodeoxycholic acid	0.9	n.d.
Allo-cholic acid	0.8	n.d.
Allo-chenodeoxycholic acid	n.d.	n.d.
7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid	121.1	37.5
7α-Hydroxy-3-oxo-4-cholen-24-oic acid	2.8	21.4
Others	n.d.	0.2
Total bile acids	125.9	59.1

n.d., not detected.