analysis. If the diagnostic determination system becomes recognized all across Japan, then it will be possible to diagnose many more cases and also possibly detect unknown types of IEBAM. A complete lack of sterol C27-hydroxylase activity in mitochondria in the patients with CTX and β-oxidation side chain cleavage and amino acid conjugation abnormalities, however, cannot yet be identified by this system.

In addition, high-risk screening for cholestasis of unknown etiology will be promoted in Southeast Asian countries. Use of air delivery service for shipment of frozen urine samples from Southeast Asia to Japan is too costly to make this system useful for people there. To make this project possible, the possibility that physicians there could impregnate filter paper with a sufficient volume of patient urine, dry the paper and ship it by air delivery service is being investigated. If this 'dried urine spot paper' method can be used to screen for IEBAM, then the current status of IEBAM in Southeast Asia would thus be clarified. If a screening system with the dried urine dipstick method can be implemented in the future, then some pediatric patients who otherwise could not be identified or saved will have an opportunity to receive bile acid replacement therapy or a liver transplant and will hopefully be saved by new treatments that can be established in the future.

Conclusions

Gas chromatography–mass spectrometry of urinary bile acids was carried out for 10 years between July 1996 and June 2005 in Japan, targeting pediatric patients with cholestasis, liver cirrhosis and hepatic disorder of unknown etiology, who were suspected of having IEBAM. Requests for analysis of a total of 576 samples were made. Cholestasis was defined as serum D-Bil level ≥2.0 mg/dL and 10 patients with IEBAM (6.3%) were identified among 159 patients with cholestasis of unknown cause. This rate of detection was higher than those previously reported. Because these 10 patients had markedly high concentrations of abnormal bile acids, they could easily be distinguished based on analysis data. SLOS could also be diagnosed by this method. Of the remaining 149 patients, 91 (61.1%) were not able to be definitively diagnosed with this system, including patients in whom the concentrations of non-specific bile acids far exceeded reference values, those with intermediate concentrations of 3-oxo bile acids and those in whom unknown peaks were detected. Various aspects of this disorder have yet to be elucidated and much work remains to be done. Analysis of urinary bile acid data will continue and many more patients will be followed to improve diagnostic accuracy and identify new types of IEBAM.

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Original Article

Developmental pattern of urinary bile acid profile in preterm infants

Hiroshi Nishiura,[1] Akihiko Kimura,[1] Yasuhiko Yamato,[1] Kumiko Aoki,[2] Takahiro Inokuchi,[2] Takao Kurosawa[3] and Toyojiro Matsuishi[1]

[1]*Department of Pediatrics and Child Health and* [2]*Research Institute of Medical Mass Spectrometry, Kurume University School of Medicine, Kurume, Fukuoka and* [3]*Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido, Japan*

Abstract ***Background***: Bile acid metabolism in preterm infants is yet to be fully characterized. We compared the developmental pattern of urinary bile acid profiles in ten infants born at gestational ages from 25 to 33 weeks with previous data from full-term infants from birth to about 7 months of age.

Methods: Gas chromatography–mass spectrometry was performed on serial samples.

Results: Total urinary bile acid concentrations gradually increased until 1 to 2 months of age. After this peak of excretion (30 to 60 μmol/mmol creatinine), total urinary bile acid concentrations gradually decreased to less than 20 μmol/mmol creatinine. The percentage of usual bile acids (mainly cholic acid) relative to total urinary total bile acids gradually deceased from approximately 30% at birth to less than 15% at 7 months of age. On the other hand, 1β-hydroxylated bile acids (mainly 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid) relative to total urinary bile acids were increased gradually from 60% at birth to reach 70% to 80% at 1 month of age. The percentage of 1β-hydroxylated bile acids relative to total urinary bile acids then remained stable at a high percentage (70% to 90%) until the age of 7 months.

Conclusion: Physiological cholestasis in preterm infants persists longer than in full-term infants. Moreover, as large amounts of cholic and 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acids were detected in urine from preterm infants during this study, the 25-hydroxylation pathway may be particularly important for bile acid synthesis in early preterm infants.

Key words bile acid metabolism, developmental pattern, preterm infants.

Details of bile acid metabolism in preterm infants are not fully established. According to the few previous reports concerning fetal bile acid metabolism, the most likely main pathway of bile acid synthesis is the acidic pathway.[1] Bile collected from fetuses during early gestation (weeks 16 to 19) contains 4β-hydroxylated bile acids.[2] During the perinatal period 1β-, 6α-hydroxylated and/or 3β-hydroxy-Δ^5 bile acids, which can be detected in adults with liver dysfunction such as cholestasis, are commonly detected in the urine of neonates.[3,4] Finally, ketonic bile acids are abundant in amniotic fluid late in gestation.[5]

We previously reported bile acid profiles in meconium, feces, amniotic fluid, and urine from preterm and full-term infants,[6–12] focusing particularly on developmental patterns of urinary 1β-, 6α-, and 7β-hydroxylated and ketonic bile acids in full-term infants.[6,7,11] Preterm infants weighing about 1000 g at birth most often have a complication manifesting after birth, such as respiratory distress or congenital heart disease with cyanosis; they then develop prolonged jaundice. Accordingly, we need to understand the details of bile acid metabolism early in the life of preterm infants. Presently we compared the developmental pattern of the urinary bile acids profile between preterm and full-term infants, using gas chromatography–mass spectrometry (GC–MS) including monitoring of selected ions.

Methods

Study design

We investigated the urinary bile acid composition in ten preterm infants born at gestational ages between 25 and 33 weeks (Table 1), comparing the results with our previously reported data from full-term infants.[6,7,11] All subjects were characterized in terms of gender, gestational age, birth weight, APGAR score, mode of nutrition, age when milk intake began, serum total bilirubin concentration at 6 days of age, and diagnosis. No subject had a history or clinical signs of hepatobiliary or gastrointestinal disease. However, all preterm infants had physiological jaundice. They were fed milk via a nasogastric tube, supplemented with parenteral nutrition, during the first few

Correspondence: Akihiko Kimura, MD, 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

Abbreviations: GC–MS, gas chromatography–mass spectrometry; Me-TMS, methyl ester-trimethylsilyl ether; BSEP, bile salt exceed pump; OATP, organic anion transporting polypeptide; NTCP, Na^+ taurocholate cotransporting polypeptide; RDS, respiratory distress syndrome; PDA, patent ductus arteriosus; PS, pulmonary stenosis.

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Table 1 Characteristics of preterm infants studied

Patient	Gender	Gestational age (weeks)	Birth weight (g)	Apgar score (1 min/5 min)	Nutrition*	Milk intake initiation (days after birth)	Total bilirubin at 6 days (mg/dl)	Diagnosis at birth
1	F	25.0	714	5/7	Breast milk or formula	7	7.9	RDS, PDA
2	M	26.8	996	6/8	Breast milk	5	7.6	RDS, PDA
3	F	27.0	912	2/6	Breast milk	3	7.8	RDS, PDA
4	F	28.7	948	5/7	Breast milk or formula	4	4.9	Hypoglycemia with apnea
5	M	28.7	1026	7/8	Breast milk or formula	1	10.0	RDS, PDA
6	F	30.4	928	7/8	Breast milk or formula	2	6.1	Hypoglycemia with apnea, PS
7	F	31.5	1126	4/8	Breast milk or formula	1	6.1	RDS, PDA
8	F	31.5	1206	8/9	Breast milk or formula	1	5.5	RDS, PDA
9	M	31.2	1360	7/8	Breast milk or formula	9	8.3	RDS
10	M	33.0	1336	7/8	Breast milk or formula	2	7.6	Hypoglycemia with apnea

*By nasogastric tube.
PDA, patent ductus arteriosus; PS, pulmonary stenosis; RDS, respiratory distress syndrome.

weeks after birth. Informed consent for observations and analysis in this study was obtained from the parents of the infants.

We divided 119 urine samples into six groups: those from infants less than 30 weeks old by corrected age ($n = 13$); 31 to 35 weeks corrected age ($n = 39$); 36 to 40 weeks corrected age ($n = 42$); 41 to 45 weeks corrected age ($n = 15$); 46 to 50 weeks corrected age ($n = 5$); and more than 51 weeks corrected age ($n = 5$). We compared urinary concentrations of each bile acid, such as 1β-hydroxylated bile acids or ketonic bile acids, between the corrected-age groups, and also determined changes in the concentration of each bile acid over time.

Sample collection

Urine samples were collected at various times from each preterm infant and stored at −25°C until assay. Urine samples were kept for no more than 2 years. We performed bile acid analysis on a total of 119 urine samples obtained from the ten preterm infants. Concentrations of individual bile acids in the urine from each subject were corrected for creatinine (Cr) concentration and expressed as μmol/mmol of Cr. We analyzed each sample only once. However, when we made calibration curves for determination of bile acid, we analyzed each sample 4 times.

Materials and reagents

The following bile acids were synthesized as described previously[13–15]: 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 1β,3α,7α-trihydroxy-5β-cholan-24-oic acid; 3β,7α,12α-trihydroxy-5-cholen-24-oic acid; 3β,7α-dihydroxy-5-cholen-24-oic acid; 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid; and 7α-hydroxy-3-oxo-4-cholen-24-oic acid. Other bile acids were obtained from Sigma Chemical (St. Louis, MO).

Gas chromatography–mass spectrometry (GC–MS)

GC–MS was performed with a Hewlett-Packard 5972A instrument (Hewlett-Packard Japan, Tokyo) using an HP-5MS gas chromatographic column (30 m × 0.25 mm inside diameter; film thickness, 0.25 μm; and a fused silica capillary column bound with methylsilicon (J & W Scientific, Folsom, CA). Figure 1 shows a chromatogram obtained by selected ion monitoring of the characteristic fragments of the methyl ester-trimethylsilyl ether (Me-TMS) derivatives of a mixture of reference bile acids.

Analysis of bile acids in urine

In the standard procedure, samples of human biological fluids were prepared routinely for GC–MS analysis as described in our previous reports.[8–12] Calibration curves for determination of bile acids were obtained by plotting the peak area ratio that corresponded to the monitored ion for each bile acid and the corresponding internal standard versus the amount of each bile acid. A linear relationship ($r > 0.976$) was obtained over a range of 1.5 to 10 ng for each bile acid.

Identification and quantitation of individual bile acids

GC–MS data for individual bile acids are summarized in Table 2, including their characteristic fragment ions and relative abundance.

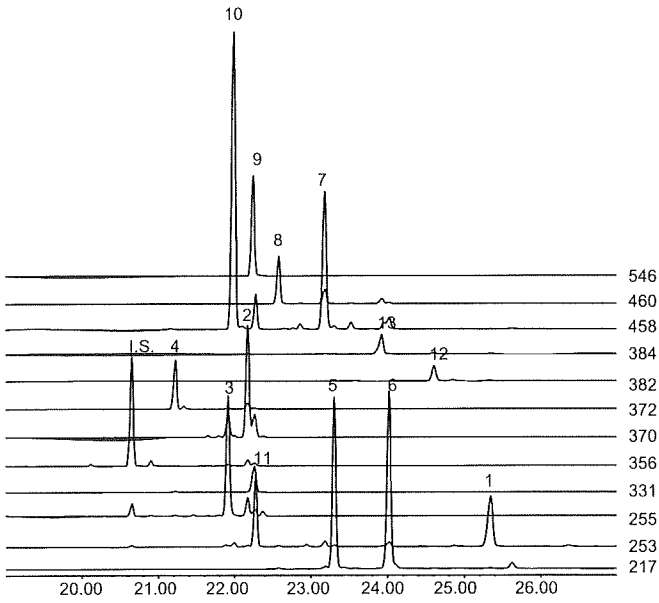

Fig. 1 Selected ion gas chromatography–mass spectrometry (GC–MS) chromatogram of methyl ester-trimethylsilyl ether (Me-TMS) derivatives of reference bile acids. Peak numbers and compounds are the same as in Table 2.

Statistical analysis

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

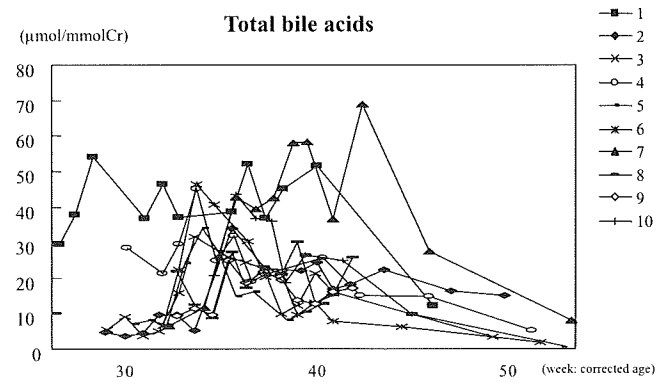

Fig. 2 Developmental pattern of urinary excretion of total bile acids (μmol/mmol Cr) in 10 preterm infants. Numbers correspond to patient numbers in Table 1.

Results

Clinical diagnoses in ten preterm infants (Table 1)

The main diagnosis in seven preterm infants (patients 1, 2, 3, 5, 7, 8, and 9) was respiratory distress syndrome (RDS), while the other three (patients 4, 6, and 10) had severe hypoglycemia with apnea after delivery. Additionally, seven of the preterm infants (patients 1, 2, 3, 5, 6, 7, and 8) had congenital heart disease such as patent ductus arteriosus (PDA) or pulmonary stenosis (PS).

Total bile acids (Fig. 2, Table 3)

Total urinary bile acid concentrations showed low excretion during 2 or 3 weeks after birth. Concentrations of total urinary bile acids then gradually increased over 1 or 2 months, peaking at 30 to 60 μmol/mmol Cr and ultimately decreasing gradually to less than 20 μmol/mmol Cr.

Table 2 Gas chromatography-mass spectrometric data for methyl ester-trimethylsilyl ether derivatives of bile acids

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

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

Table 3 Urinary excretion of bile acids in preterm infants

Groups	1	2	3	4	5	6
Corrected age (weeks)	<30	31–35	36–40	41–45	46–50	51<
Number	13	39	42	15	5	5
Total bile acids (μmol/mmol Cr)	17.9 ± 17.1[a]	23.3 ± 13.1[b]	25.2 ± 13.3[c]	21.1 ± 15.2[d]	14.8 ± 8.7	6.1 ± 5.8
Usual bile acids (%)	21.6 ± 13.5[e]	15.0 ± 9.0[f]	11.1 ± 6.2[g]	10.0 ± 6.6[h]	4.5 ± 1.8	7.0 ± 4.1
1β-Hydroxylated bile acids (%)	65.1 ± 13.8[i]	78.2 ± 9.5[j]	83.3 ± 7.7	82.3 ± 6.9	86.1 ± 6.6[k]	76.3 ± 7.6
Unsaturated ketonic bile acids (%)	7.3 ± 5.8[l]	2.9 ± 3.6[m]	0.9 ± 0.5[n]	1.5 ± 0.9[o]	1.6 ± 1.0[p]	4.4 ± 3.4

Usual bile acids, cholic, chenodeoxycholic, deoxycholic, and lithocholic acids; 1β-hydroxylated bile acids, 1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic and 1β,3α,7α-Trihydroxy-5β-cholan-24-oic acids; Unsaturated ketonic bile acids, 7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic and 7α-Hydroxy-3-oxo-4-cholen-24-oic acids; [a]$P < 0.05$ vs 36 to 40 weeks; [b]$P < 0.05$ vs >51 weeks; [c]$P < 0.01$ vs >51 weeks; [d]$P < 0.01$ vs >51 weeks; [e]$P < 0.01$ vs 36 to 40 weeks, 41 to 45 weeks, 46 to 50 weeks; $P < 0.05$ vs 51< weeks; [f]$P < 0.001$ vs 46 to 50 weeks; $P < 0.05$ vs <51 weeks; [g]$P < 0.05$ vs 46 to 50 weeks; [h]$P < 0.05$ vs 46 to 50 weeks; [i]$P < 0.01$ vs 31 to 35 weeks; $P < 0.0001$ vs 36 to 40 weeks; $P < 0.001$ vs 41 to 45 weeks; $P < 0.01$ vs 51< weeks; [j]$P < 0.05$ vs 36 to 40 weeks, 46 to 50 weeks; [k]$P < 0.05$ vs 51< weeks; [l]$P < 0.05$ vs 31 to 35 weeks, $P < 0.001$ vs 36 to 40 weeks, $P < 0.01$ vs 41 to 45 weeks; [m]$P < 0.05$ vs 36 to 40 weeks; [n]$P < 0.05$ vs 41 to 45 weeks, $P < 0.05$ vs 46 to 50 weeks, $P < 0.001$ vs >51 weeks; [o]$P < 0.05$ vs >51 weeks; [p]$P < 0.05$ vs >51 weeks. Cr, creatinine.

Usual bile acids (Fig. 3, Table 3)

Relative to total urinary bile acids, usual bile acids (cholic, chenodeoxycholic, deoxycholic, and lithocholic acids) gradually decreased after birth from a high percentage (20% to 40%), to become stable at a low percentage (less than 15%), by about 40 weeks corrected age. Among the usual bile acids, cholic acid was most abundant (approximately 90% of usual bile acids; data not shown). Trace amounts of secondary bile acids (deoxycholic and lithocholic acids) were detected.

1β-Hydroxylated bile acids (Fig. 4, Table 3)

This study determined that the percentage of 1β-hydroxylated bile acids (1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic and 1β,3α,7α-trihydroxy-5β-cholan-24-oic acids) relative to total urinary bile acids gradually increased from birth to 3 or 4 weeks of age (from about 60% to 70% or 90%). This high percentage was then maintained until sampling ended at about 7 months of age. The principal 1β-hydroxylated bile acid was 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (approximately 80% of 1β-hydroxylated bile acids; data not shown).

Unsaturated ketonic bile acids (Fig. 5, Table 3)

Relative to total urinary bile acids, the percentage of unsaturated ketonic bile acids (7α,12α-dihydroxy-3-oxo-4-cholen-24-oic and 7α-hydroxy-3-oxo-4-cholen-24-oic acids) decreased rapidly

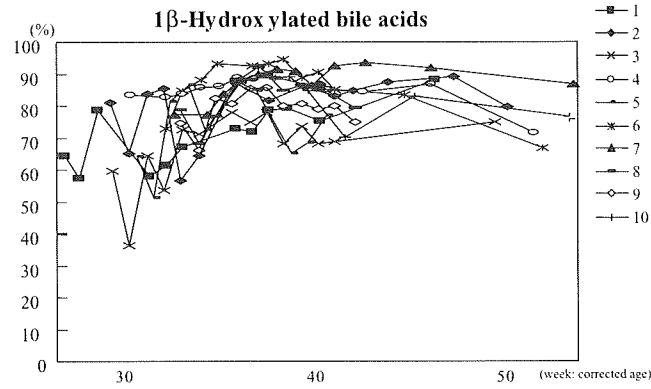

Fig. 4 Developmental pattern of the percentage of 1β-hydroxylated bile acids in 10 preterm infants, as a percentage relative to total bile acids.

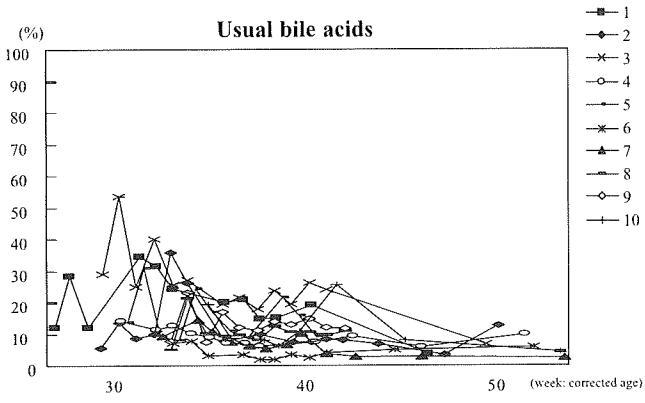

Fig. 3 Developmental pattern of that percentage of usual bile acids in 10 preterm infants, as a percentage in relative to total bile acids.

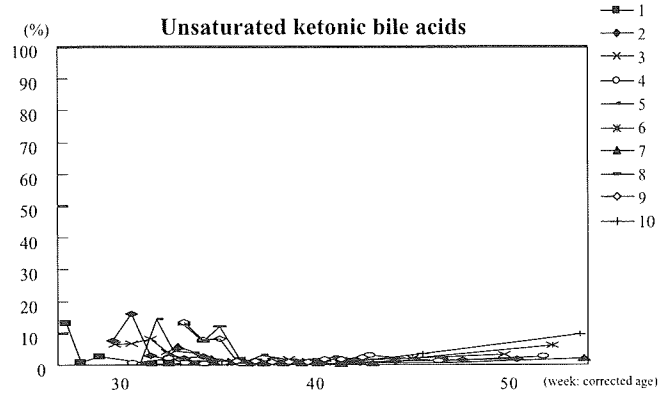

Fig. 5 Developmental pattern of the percentage of unsaturated ketonic bile acids in 10 preterm infants, as a percentage relative to total bile acids.

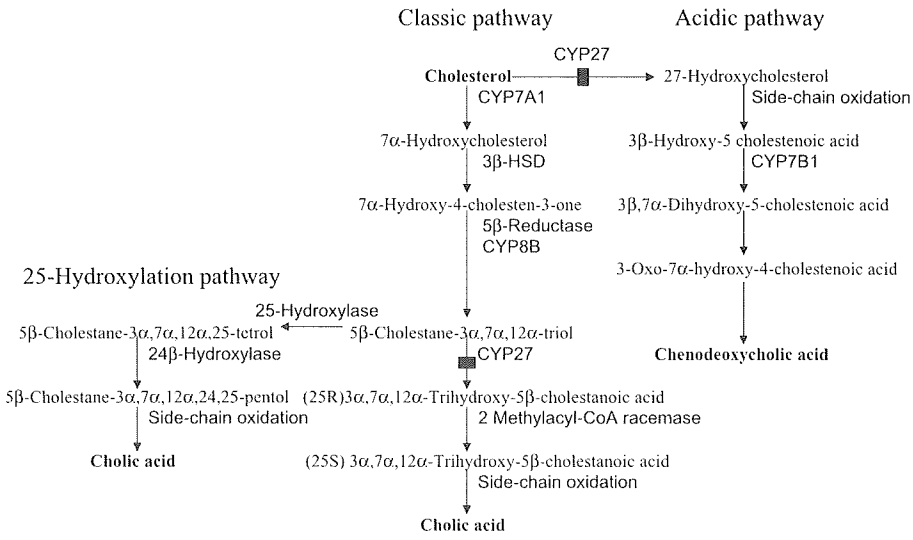

Fig. 6 Metabolic pathways for the synthesis of primary bile acids including the classic pathway, acidic pathway, and 25-hydroxylation pathway. CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP27, cholesterol 27-hydroxylase; CYP8B, cholesterol 12α-hydroxylase; 3β-HSD, 3β-hydroxy-Δ^5-C_{27}-steroid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

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

Other bile acids

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

Discussion

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

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

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

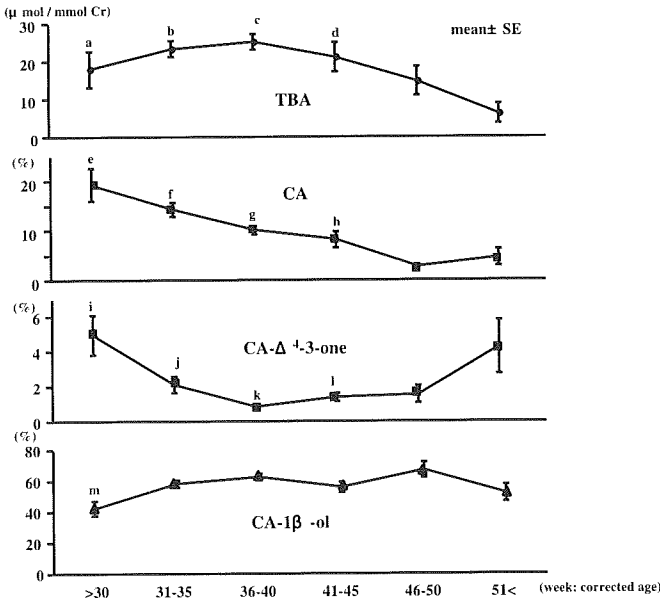

Fig. 7 Developmental pattern of urinary excretion of bile acids in preterm infants. TBA, total bile acids (filled circle); CA, cholic acid (open squares); CA-Δ^4-3-one, 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid (filled squares); and CA-1β-ol, 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (open triangles).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

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

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

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

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

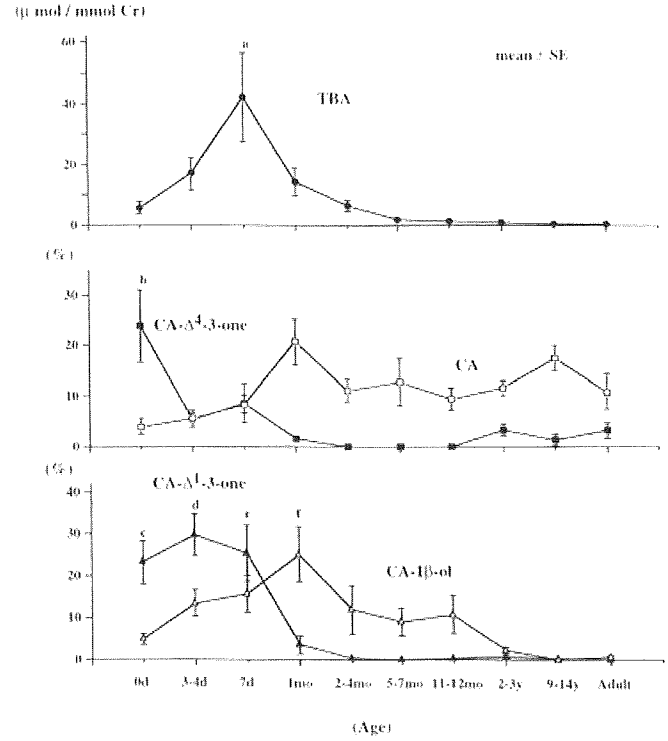

Fig. 8 Developmental pattern of urinary excretion of bile acids in full-term infants. TBA, total bile acids (filled circle); CA, cholic acid (open squares); CA-Δ^4-3-one, 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid (filled squares); CA-1β-ol, 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid (open triangles);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

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Original Article

Histological findings in the livers of patients with neonatal intrahepatic cholestasis caused by citrin deficiency

Akihiko Kimura,[1] Masayoshi Kage,[2] Ikuo Nagata,[3] Sotaro Mushiake,[4] Toshihiro Ohura,[5] Yusaku Tazawa,[6] Shunichi Maisawa,[7] Takeshi Tomomasa,[8] Daiki Abukawa,[5] Yoshiyuki Okano,[9] Ryo Sumazaki,[10] Masaki Takayanagi,[11] Akiko Tamamori,[12] Tohru Yorifuji,[13] Yasuhiko Yamato,[1] Kohji Maeda,[1] Masami Matsushita,[1] Toyojiro Matsuishi,[1] Ken Tanikawa,[2] Keiko Kobayashi[14] and Takeyori Saheki[14]

[1]*Department of Pediatrics and Child Health,* [2]*Department of Pathology, Kurume University School of Medicine, Kurume,* [3]*Department of Pediatrics, Faculty of Medicine, Tottori University, Yonago,* [4]*Department of Pediatrics, Faculty of Medicine, Osaka University, Osaka,* [5]*Department of Pediatrics, Tohoku University School of Medicine, Sendai,* [6]*Department of Pediatrics, South Miyagi Medical Center, Shibata,* [7]*Department of Pediatrics, Morioka Children's Hospital, Morioka,* [8]*Department of Pediatrics, Gunma University School of Medicine, Maebashi,* [9]*Department of Pediatrics, Osaka City University Graduate School of Medicine, Osaka,* [10]*Department of Pediatrics, Institute of Clinical Medicine, University of Tsukuba, Tsukuba,* [11]*Department of Pediatrics, Chiba Children's Hospital, Chiba,* [12]*Department of Pediatrics, Fujiidera City Hospital, Fujiidera,* [13]*Department of Pediatrics, Kyoto University, Kyoto, and* [14]*Department of Molecular Metabolism and Biochemical Genetics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan*

Aim: To characterize the histological features of the livers of patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), we studied specimens from 30 patients diagnosed with NICCD by genetically analyzing the *SLC25A13* gene.

Methods: Liver biopsy specimens were subjected to hematoxylin–eosin, Azan, and Berlin-blue staining.

Results: Most specimens showed varying degrees of fibrosis. The degree of inflammation varied among the specimens, with half showing moderate or severe inflammatory changes. Fat deposition in hepatocytes was observed in almost all of the specimens, and severe fatty liver was noted in 20 (67%) of them. There was a mixture of two types of hepatocytes with macrovesicular or microvesicular fat droplets, and cholestasis was observed at a rate of 77%. Hemosiderin deposition, mostly mild and localized in periportal hepatocytes and macrophages in portal areas, was observed in 57% of the specimens.

Conclusion: A combination of mixed macrovesicular and microvesicular fatty hepatocytes and the above-described findings, such as fatty liver, cholestasis, necroinflammatory reaction and iron deposition, are almost never observed in other liver diseases in infants and adults. We believe that NICCD is a disease with characteristic hepatopathological features.

Key words: citrin, citrullinemia, fatty liver, fibrosis, neonatal intrahepatic cholestasis caused by citrin deficiency, *SLC25A13*.

INTRODUCTION

SAHEKI *ET AL.* reported that the enzyme abnormalities of citrullinemia can be classified as qualitative, type I and type III, or quantitative, type II.[1,2] The first, the classical form (CTLN1), is found in most patients with neonatal/infantile-onset citrullinemia, and was first described by McMurray *et al.*[3] In CTLN1, the enzyme defect is found in all tissues in which argininosuccinate synthetase (ASS) is expressed.[1,2,4] The second form, type II citrullinemia (CTLN2) is an adult- or late childhood-onset liver disease characterized by a liver-specific defect in ASS, and most of these patients have a fatty liver.[5] This enzyme abnormality is caused by a deficiency in citrin, a calcium-binding

Correspondence: Professor Masayoshi Kage, Department of Pathology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. Email: masakage@med.kurume-u.ac.jp
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mitochondrial solute carrier protein which is encoded by the *SLC25A13* gene.[6]

Recently, several cases of *SLC25A13* mutations have been reported in early infancy with cholestatic liver disease.[7–13] Yamaguchi *et al.*[14] designated these findings as neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). Citrin deficiency causes two age-dependent phenotypes, CTLN2 in adults and NICCD in infants.[15] Most NICCD patients showed hypoproteinemia, galactosemia, multiple aminoacidemia including citrullinemia, methionemia and tyrosinemia, cholestasis, and have a fatty liver.[7–13] Only a few papers have described the pathology of the NICCD[8,9,11,13] or CTLN2[5] liver.

Therefore, the present study was designed to clarify the histological findings of the NICCD liver.

METHODS

Patients

WE STUDIED THE liver histological findings of 30 patients aged 2.9 ± 1.7 months with a range of 1–7 months consisting of 17 men and 13 women who had been diagnosed with NICCD with *SLC25A13* mutations by genetic analysis including five patients who were documented in previous reports.[7–11] Moreover, mutations in *SLC25A13* were detected in both alleles of 29 patients and in a single allele of one patient. Mutation detection and DNA diagnosis of the *SLC25A13* gene were performed as previously described ([6,14,16] and T. Saheki et al., 2006, unpublished data). Moreover, we examined biochemical data within 1 week before or after liver biopsy for 30 patients with NICCD.

Methods

Liver biopsy specimens from 30 patients diagnosed with NICCD were subjected to hematoxylin–eosin, Azan, and Berlin-blue staining. The grading of fibrosis and inflammation was based on Ludwig's Classification with slight modifications (Table 1).[17] The other histopathological features were graded as none, mild, moderate and severe, and scored as 0, 1, 2 and 3, respectively.

Grading was independently performed by three pathologists, and the grade for each specimen was determined by consensus between two or three of them.

Relationship between age and histological findings

To clarify the relationship between age and the histological findings, the cases were divided into three groups according to their ages: group A, less than 2 months old; group B, 3–4 months old; and group C, more than 5 months old. The average of the grading score of the histological findings for each group was then obtained.

Table 1 Histological classification of liver biopsy

Stage of fibrosis	
Stage 0	No portal fibrosis
Stage 1	Mild to moderate fibrous expansion of portal tract
Stage 2	Bridging fibrosis between portal tracts without lobular distortion
Stage 3	Bridging fibrosis between portal tracts with lobular distortion
Stage 4	Liver cirrhosis
Grade of inflammation	
Grade 0	None (0)
Grade 1	Mild (1–3)
Grade 2	Moderate (4–6)
Grade 3	Severe (≥7)

Parentheses indicate scores derived by Ludwig's scoring system.

Statistical analysis

The data regarding the relationship between age and histological findings were analyzed using the Mantel–Haenszel linear trend test. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Patients

THE PROGNOSIS OF almost NICCD patients at 1 year of age was fairly well. However, some NICCD patients had developed progressive liver failure by then. For example, two patients developed liver failure by 6 months (patient 28) and 7 months (patient 30)[10] of age and one patient (patient 9) developed behavioral aberrations, which included shouting and episodes of violence, by 16 years of age.[9,18] Two patients, one with liver failure[10] and one with mental derangement,[9,18] received a living-related liver transplant. Therefore, the outcomes of the NICCD patients were not always favorable. We obtained four sets of follow-up liver biopsy specimens from patients 8, 9, 13 and 18 (data not shown).

From the clinical laboratory data, serum levels of citrulline, α-fetoprotein, ferritin and pancreatic secretory trypsin inhibitor (PSTI) were noted to have generally increased (Table 2). We also detected high serum levels of total and direct bilirubin, aspartate (AST) and/or alanine aminotransferase (ALT), total bile acids and

Table 2 Biochemical data on liver biopsy in the 30 patients with neonatal intrahepatic cholestasis caused by citrin deficiency

Patient No.	1	2	3	4	5	6	7	8	9	10	11
Age (months)/sex	1/M	1/M	1/M	1/M	1/F	1/F	2/M	2/M	2/M	2/M	2/M
Total/direct bilirubin (mg/dL)	9.0/3.4	12.6/2.6	3.3/2.2	10.4/5.8	5.6/1.9	3.3/0.7	6.2/3.8	9.9/5.4	7.6/3.3	6.6/2.6	3.6/1.6
AST/ALT (IU/L)	96/38	31/20	115/61	121/24	62/41	43/21	112/28	109/50	41/20	100/30	190/53
Total bile acids (μM)	250	120	513	298	210	52	323	331	n.d.	240	212
γ-GTP (IU/L)	206	142	131	251	186	148	142	408	130	n.d.	125
Total cholesterol (mg/dL)	212	195	n.d.	181	161	158	175	206	133	n.d.	196
Total protein/albumin (g/dL)	4.9/3.2	3.9/2.6	5.3/4.0	4.5/3.0	5.1/3.5	4.4/3.3	4.7/2.6	–/–	3.6/1.9	–/–	4.7/2.8
Citrulline (nmol/mL)	4.3	n.d.	85.0	n.d.	40.5	149.0	74.3	12.6	n.d.	117.0	211.0
α-Fetoprotein (ng/mL)	n.d.	n.d.	n.d.	200 700	n.d.	n.d.	n.d.	n.d.	29 600	n.d.	n.d.
PSTI (ng/mL)	n.d.	n.d.	n.d.	91.0	n.d.	40.0	24.0	n.d.	n.d.	n.d.	110.0
Ferritin (ng/mL)	447	n.d.	n.d.	2656	n.d.	117	502	1830	n.d.	n.d.	n.d.
Prothrombin activity (%)	75	26	93	55	37	88	70	37	9	n.d.	76
Mutation type	V/XIX	I/II	I/I	I/II	II/II	I/V	II/V	II/V	II/II	I/V	I/V

12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
2/M	2/F	2/F	2/F	2/F	3/M	3/M	3/M	3/F	3/F	4/M	4/M	4/F	4/F	5/M
10.2/3.9	11.1/3.6	13.0/8.5	6.9/2.7	5.3/2.4	6.1/3.5	6.0/3.8	9.6/2.7	8.8/3.2	12.0/2.6	5.1/2.5	6.7/3.7	5.4/3.5	6.2/2.4	15.0/10.1
106/22	86/23	133/45	78/25	74/44	98/36	232/48	85/44	95/39	75/19	95/90	295/105	208/100	83/24	146/66
240	320	172	290	143	302	269	205	389	157	283	172	253	127	355
213	132	78	209	160	124	249	n.d.	149	198	145	270	132	90	129
n.d.	n.d.	204	232	n.d.	194	n.d.	140	223	256	128	169	n.d.	n.d.	n.d.
4.9/3.7	4.0/3.5	3.8/2.6	4.1/2.7	n.d.	5.3/3.9	n.d.	5.7/3.8	5.1/3.1	4.8/3.0	4.2/3.5	4.8/3.1	5.5/3.5	n.d.	4.0/2.8
242.0	478.0	581.0	n.d.	291.7	839.1	208.0	n.d.	32.2	392.0	675.0	524.0	27.5	28.4	5.8
n.d.	n.d.	87 000	n.d.	91 940	n.d.	n.d.	n.d.	n.d.	n.d.	75 300	n.d.	n.d.	n.d.	10 578
n.d.	24.0	n.d.	n.d.	57.0	n.d.	n.d.	n.d.	62.0	12.9	12.5	n.d.	n.d.	n.d.	188.0
743	n.d.	775	n.d.	1651	n.d.	n.d.	n.d.	n.d.	200	n.d.	n.d.	503	n.d.	n.d.
87	n.d.	n.d.	25	51	43	n.d.	66	50	75	29	39	69	15	15
IV/IV	II/II	II/V	II/II	II/II	II/III	I/I	II/II	VI/VI	I/II	I/–	I/II	VIII/X	IV/VI	II/V

27	28	29	30	Mean ± SD	Range	Normal range
5/F	6/M	6/F	7/F			
5.8/3.4	5.5/3.9	6.2/2.0	5.9/2.9	7.6 ± 3.0/3.6 ± 2.0 (n = 30)	3.3–15.0/0.7–10.1	0.2–1.1/0.0–0.4
260/169	123/87	127/38	191/67	120.3 ± 63.7/49.2 ± 33.3 (n = 30)	31–295/20–169	6–40/5–40
213	n.d.	150	168	241.3 ± 96.1 (n = 28)	52–513	5–25
67	149	65	292	168.6 ± 75.0 (n = 28)	65–408	5–32
n.d.	148	n.d.	168	183.1 ± 34.8 (n = 19)	133–256	130–220
6.4/4.7	4.5/3.0	4.6/2.7	6.0/3.2	4.7 ± 0.7/3.2 ± 0.6 (n = 25)	3.6–6.4/1.9–4.7	6.5–8.3/3.7–5.2
48.2	11.0	41.3	86.8	179.1 ± 199.2 (n = 25)	4.3–291.7	17–43
n.d.	11 000	329 000	207 000	115 790.9 ± 108 111.0 (n = 9)	11 000–329 000	<10 000
n.d.	n.d.	21.9	n.d.	58.5 ± 53.6 (n = 11)	12.5–188.0	22–46
n.d.	n.d.	n.d.	197	874.6 ± 816.3 (n = 11)	117–2656	12–80
88	9	41	29	51.3 ± 26.0 (n = 25)	9–93	70–140
I/II	I/II	I/II	I/II			

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; PSTI, pancreatic secretory trypsin inhibiter; M, male, F, female; n.d., not done; I, 851del4; II, IVS11 + 1G > A; III, 1638ins23; IV, S225X; V, IVS13 + 1G > A; VI, 1800ins1; VIII, E601X; X, IVS6 + 5G > A; XIXIVS16ins3kb; –, unknown; SD, standard deviation.

γ-glutamyl transpeptidase. Prothrombin activity, total protein and albumin were decreased. The mutation types were 851del4/IVS11 + 1G > A throughout most of late infancy, being more than 5 months of age in patients 27, 28, 29 and 30.

Histological findings

Histological findings of the 30 patients are shown in Table 3. The results of the fibrosis staging and inflammation grading are shown in Figure 1.

Fibrosis

Most specimens showed varying degrees of fibrosis; 35% of all specimens were classified as stage 0, while stages 1 and 2 together accounted for 50%. However, there was a wide spectrum of fibrosis: more advanced liver lesions with distorted lobular architecture (stage 3) (Fig. 2) and cirrhosis were observed in four and one specimens, respectively. One patient with cirrhosis developed hepatic failure. Therefore, this patient underwent a living-related liver transplant. One patient with cirrhosis developed at 10 months of age.[10]

Inflammatory reaction

The degree of inflammation varied with the specimens, where half showed moderate or severe inflammatory changes. Inflammatory cell infiltration in the portal tracts and piecemeal necrosis were observed (Fig. 3). Inflammatory cells present in the portal tracts were predominantly lymphocytes. Focal necrosis and acidophilic bodies in the parenchyma were seen in 23 (77%) and 12 (40%) specimens, respectively. The sinusoids showed the proliferation of mononuclear cells with scarce neutrophils and the activation of Kupffer cells.

Fat deposition in hepatocytes

Fat deposition in hepatocytes was observed in all specimens except one and severe fatty liver was noted for 20 (67%) specimens (Fig. 4a). Fat droplets deposited in the cytoplasm of hepatocytes varied in size, and fat-laden hepatocytes were classified as those with macrovesicular fat droplets, those with foamy, microvesicular fat droplets, and those with mixed macrovesicular and microvesicular fat droplets. Hepatocytes with microvesicular fat droplets had a centrally located nucleus. In 80% of 29 specimens with fat deposition including all 20 specimens which showed severe fatty livers, there was a mixture of macro- and microvesicular fat droplets (Fig. 4b,c). Macrovesicular and microvesicular fatty liver alone accounted for three (10%) and one (4%) specimens, respectively. A moderate and severe fatty liver with an inflammatory reaction and lipogranuloma were diagnosed as steatohepatitis, which accounted for 60% of the patients. The histopathological findings in this disease were different from those in non-alcoholic steatohepatitis. The clinical features of one patient who had no fat deposition in hepatocytes did not differ from that of other patients with such fat deposition.

Cholestasis

Cholestasis was observed in 77% of the specimens and was severe in 57%. The acinar arrangement of hepatocytes was prominent in specimens with severe cholestasis (Fig. 5) and multinucleated giant cell transformation was found in one case (Fig. 6).

Hemosiderin deposition

Hemosiderin deposition, mostly mild and localized in periportal hepatocytes and macrophages in portal areas (Fig. 4b), was observed in 57% of the specimens.

A combination of all five features, fatty liver, inflammatory cell infiltration, fibrosis, cholestasis and hemosiderin deposition was observed in the same liver biopsy specimen in 12 (40%) of the total specimens.

Relationship between the age and the histological findings

The mean score of each histological finding in each of groups A, B and C are summarized in Table 4. The degree of fibrosis, necroinflammatory reaction such as focal necrosis and acidophilic bodies, acinar arrangement of hepatocytes, cholestasis and steatohepatitis of infants more than 3 months old (groups B and C) were more accentuated than those of the early infants of group A. Conversely, hemosiderosis and extramedullary hematopoiesis in groups B and C were less pronounced than in group A. The staging score of fibrosis, grade of inflammation and steatohepatitis were significantly higher in the older than in the younger group in order of group A, B and C.

Histological findings of follow-up biopsy

Follow-up biopsies were conducted for patients 8, 9, 13 and 18, and the findings were as follows: patients 8, 9 and 13 showed histological deterioration of cholestasis and fatty change. Of note, patient 9 underwent a liver transplant at the age of 16 years because of hepatic failure. The findings for the explant liver were

Table 3 Histological findings of liver biopsy in the 30 patients with neonatal intrahepatic cholestasis caused by citrin deficiency

Patient no.	1	2	3	4	5	6	7	8	9	10
Stage of fibrosis	0	0	1	0	0	0	0	0	3	2
Grade of inflammation	1	2	2	1	1	1	2	1	1	1
Focal necrosis[a]	1	1	2	0	0	0	1	0	0	1
Acidophilic body[b]	0	1	0	2	0	1	0	1	0	0
Acinar arrangement[c]	0	1	3	3	0	1	0	1	2	1
Cholestasis[d]	0	3	3	3	1	0	1	2	3	1
Degree of fat deposit[e]	1	3	3	3	3	3	2	3	3	3
Type of fat deposit[f]	1	3	0	3	3	3	1	3	0	0
Steatohepatitis[g]	0	1	1	1	0	1	1	1	0	2
Hemosiderosis[h]	0	2	1	2	0	0	1	2	0	2
Extramedullary hematopoiesis[i]	0	2	0	3	2	1	0	2	0	0
Patient no.	11	12	13	14	15	16	17	18	19	20
Stage of fibrosis	0	2	2	1	0	0	3	2	1	1
Grade of inflammation	1	1	1	2	1	2	2	2	3	1
Focal necrosis	1	0	1	1	1	2	1	1	3	0
Acidophilic body	1	0	0	1	0	0	1	0	0	0
Acinar arrangement	2	0	0	2	2	1	1	1	2	1
Cholestasis	3	0	0	3	3	2	2	2	3	3
Degree of fat deposit	3	0	2	2	3	2	3	3	2	3
Type of fat deposit	3	0	2	3	3	3	3	3	3	3
Steatohepatitis	2	0	0	1	1	1	1	1	2	1
Hemosiderosis	2	0	1	0	2	1	1	0	2	1
Extramedullary hematopoiesis	0	0	0	3	2	0	1	0	0	0
Patient no.	21	22	23	24	25	26	27	28	29	30
Stage of fibrosis	2	2	0	2	2	3	1	3	3	4
Grade of inflammation	3	2	1	2	3	2	1	2	3	3
Focal necrosis	1	2	1	1	3	1	1	1	2	1
Acidophilic body	1	2	0	1	1	1	0	0	0	2
Acinar arrangement	3	2	0	2	2	1	2	1	3	2
Cholestasis	3	3	0	3	0	3	3	3	3	3
Degree of fat deposit	3	3	3	3	1	3	2	3	3	3
Type of fat deposit	3	3	3	3	1	3	3	3	3	3
Steatohepatitis	0	3	2	1	0	2	1	3	3	3
Hemosiderosis	3	1	1	1	0	1	1	0	0	0
Extramedullary hematopoiesis	1	0	1	1	2	0	0	0	1	0

[a]Focal necrosis was graded from 0–3 based on the number of counts per 10 fields at a magnification of ×40. A score of denotes 0 is none, 1 denotes 1–2; 2 denotes up to 4, and 3 denotes >4.
[b]Acidophilic bodies were counted and graded from 0–3, as similar to that for focal necrosis.
[c]The acinar arrangements of the hepatocytes were graded 0–3. A rating of 0 denotes none, 1 denotes involvement up to 30% of the hepatocytes, 2 denotes 30–60%, and 3 denotes >60%.
[d]Cholestasis was graded from 0–3. A score of 0 denotes none, 1 denotes cholestasis without a bile plug, 2 denotes scattered bile plugs, and 3 denotes frequent bile plugs.
[e]The degree of fat deposition in hepatocytes was graded from 0–3 based on the percentage of hepatocytes in the biopsy involved. A rating of 0 denotes none; 1 denotes up to 30%, 2 denotes 30–60%, and 3 denote >60%.
[f]The type of fat deposit was classified as being between 0–3. A score of 0 denotes no fatty change, 1 denotes predominantly macrovesicular fat droplets, 2 denotes predominantly microvesicular fat droplets, and 3 denotes mixed microvesicular and macrovesicular fat droplets.
[g]Steatohepatitis was graded from 0–3, where 0 denotes none, 1 denotes steatosis involving up to 60% and intra-acinar inflammation with no or mild portal inflammation, 2 denotes steatosis (>66%) with both acinar and portal inflammation, and 3 denotes panacinar steatosis with intra-acinar inflammation and piecemeal necrosis.
[h]Hepatocellular iron was graded between 0–3, where 0 denotes none, 1 denotes localized deposition in the portal and /or periportal area; 2 denotes iron deposition involving up to 60% of the parenchyma, and 3 denotes >60%.
[i]Extramedullary hematopoiesis was graded between 0–3, similar to that for focal necrosis.

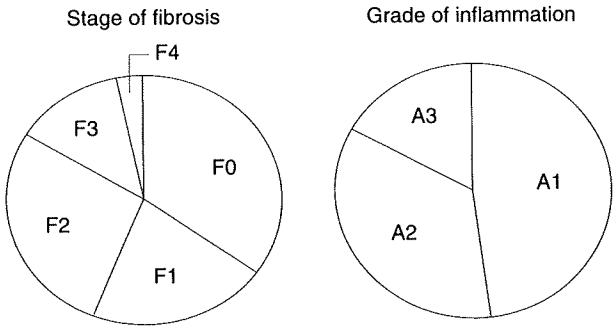

Figure 1 Results of fibrosis and the grade of necroinflammation.

more pronounced than those of the biopsy. Patient 8 showed progression of fibrosis from stage 1–3 and more pronounced portal inflammation. In contrast, patient 18 showed marked improvement of every

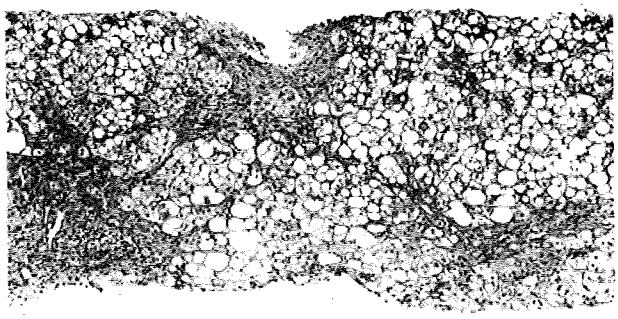

Figure 2 Severe fatty liver with distorted lobular architecture due to extensive fibrosis in stage 3 with portal inflammation (hematoxylin–eosin, original magnification ×50).

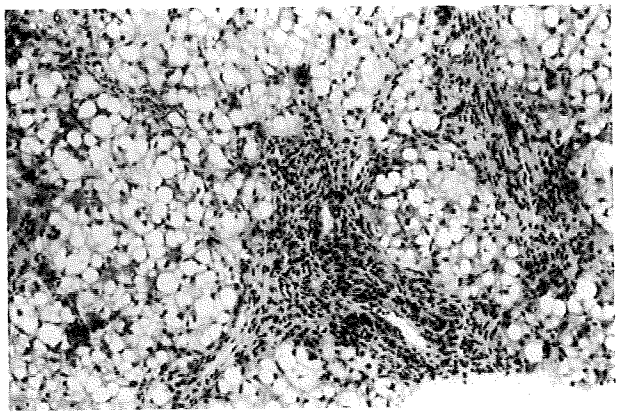

Figure 3 Fatty liver with moderate inflammatory cell infiltration in the portal tract and parenchyma, which is indicative of steatohepatitis (hematoxylin–eosin, original magnification ×100).

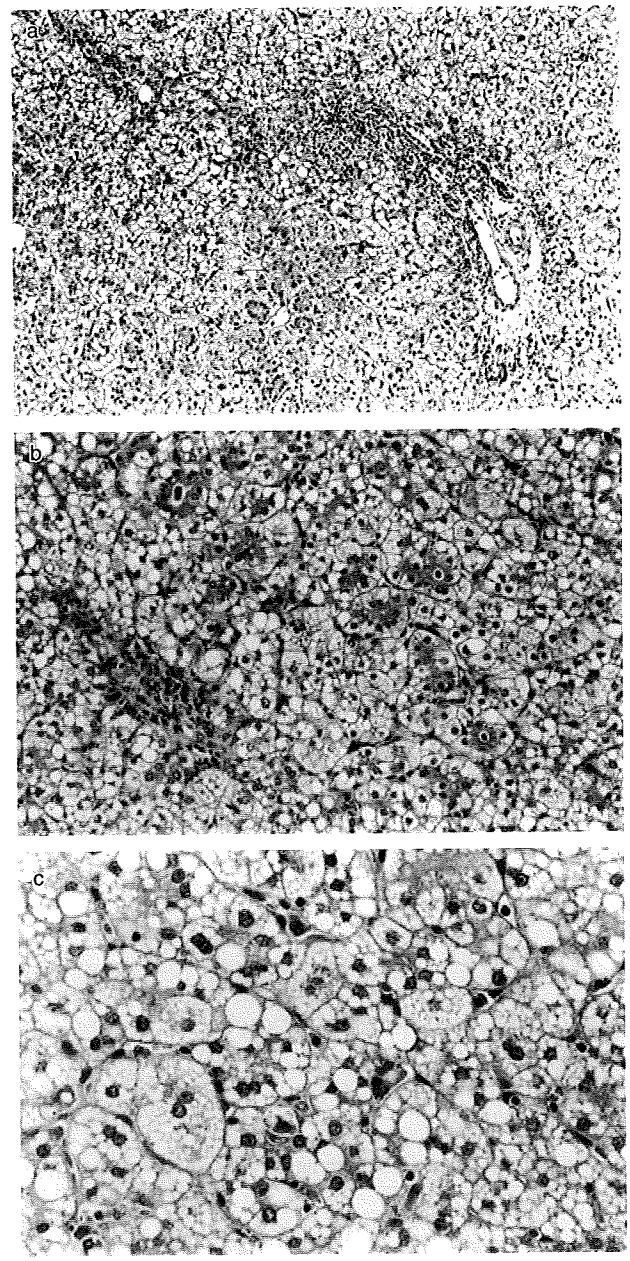

Figure 4 (a) Severe fatty liver with cholestasis. The portal tracts show mild inflammatory cell infiltration (hematoxylin–eosin [HE], original magnification ×50). (b) Pseudo-acinor transformation with bile plugs is observed. Hemosiderin-laden macrophages are present in a portal tract (HE, original magnification ×100). (c) Macro- and microvesicular-type fatty droplets. Some of the swollen hepatocytes have a foamy appearance and their cytoplasm packed with micro-fat droplets. Kupffer cells are activated (HE, original magnification ×200).

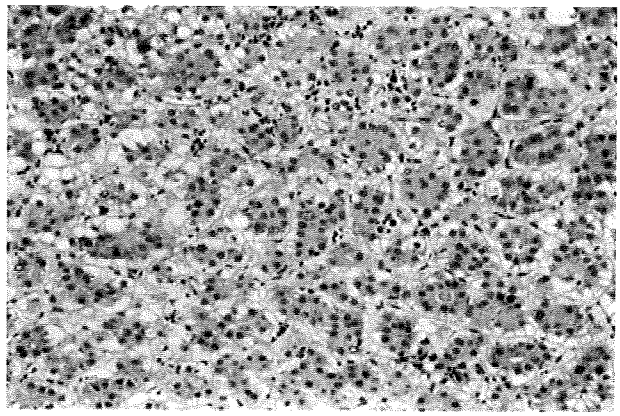

Figure 5 Striking pseudo-acinor transformation of the hepatic cords containing bile plugs. Small fatty droplets are present at the periphery of hepatocytes arranged in an acinar fashion (hematoxylin–eosin, original magnification ×100).

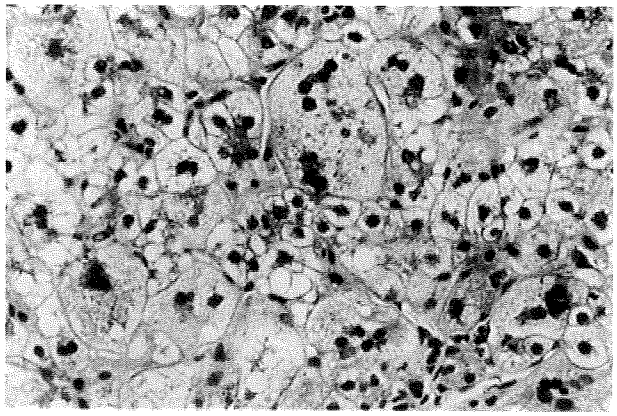

Figure 6 Giant cell hepatitis and cholestasis. Multinucleate giant cells contain several nuclei (hematoxylin–eosin, original magnification ×200).

histological finding, including decreased portal fibrosis and inflammation.

DISCUSSION

THE CAUSE OF liver dysfunctions such as fatty liver, hypoglycemia and galactosemia in this disease is as follows.[15] Citrin deficiency blocks the malate aspartate shuttle, which may increase the ratio of cytosolic nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide ($NADH/NAD^+$), which in turn is associated with the inhibition of glycolysis and makes reduced alcohol metabolism. This may be why CTLN2 patients dislike carbohydrates and cannot drink alcohol, and why alcohol consumption often results in psychiatric symptoms. An increased $NADH/NAD^+$ ratio is also characteristic of the inhibition of gluconeogenesis involving reduced substrates.[19] This, together with the reduction in alanine metabolization to urea and glucose due to citrin deficiency may cause hypoglycemia in NICCD patients. Although NICCD patients suffer from galactosemia, which sometimes even leads to the development of cataracts, no abnormalities in the enzymes involved in galactose metabolism have been found.[20] Because uridine diphosphate-glucose epimerase which requires NAD as a cofactor is strongly inhibited by NADH,[21] galactosemia in NICCD may represent a high NADH level in the cytosol of the liver.

From the biochemical data of this study, 50% of the high level of total bilirubin was associated with direct bilirubin, but it was not always dominant. The levels of AST were increased to more than twice the levels of ALT. Low levels of total protein, albumin and prothrombin

Table 4 Relationship between age and histological changes

Pathological findings	Group A ($n = 16$) <2 months	Group B ($n = 9$) 3–4 months	Group C ($n = 5$) >5 months	*P*-value
Stage of fibrosis	0.69 ± 1.01	1.67 ± 0.87	2.80 ± 1.10	$P = 0.001$
Grade of inflammation	1.31 ± 0.48	2.11 ± 0.78	2.20 ± 0.84	$P = 0.004$
Focal necrosis	0.75 ± 0.68	1.44 ± 1.01	1.20 ± 0.45	$P = 0.063$
Acidophilic body	0.44 ± 0.63	0.67 ± 0.71	0.60 ± 0.89	$P = 0.523$
Acinar arrangement	1.19 ± 1.05	1.56 ± 0.88	1.80 ± 0.84	$P = 0.172$
Cholestasis	1.75 ± 1.29	2.11 ± 1.27	3.00 ± 0.00	$P = 0.059$
Degree of fat deposit	2.44 ± 0.89	2.67 ± 0.71	2.80 ± 0.45	$P = 0.333$
Steatohepatitis	0.81 ± 0.66	1.22 ± 0.97	2.40 ± 0.89	$P = 0.008$
Hemosiderosis	1.00 ± 0.89	1.11 ± 0.93	0.40 ± 0.55	$P = 0.356$
Extramedullary hematopoiesis	0.94 ± 1.18	0.67 ± 0.71	0.20 ± 0.45	$P = 0.297$

The data are expressed as means ± standard deviation.
P-values are by the Mantel–Haenszel linear trend test.

activity and high levels of citrulline, α-fetoprotein, ferritin and PSTI were observed as previously described in NICCD patients.[6–13] However, 11 patients showed high levels of ferritin, which were not observed in previous reports on NICCD patients. Therefore, the pediatric hepatologist should suspect NICCD when a neonatal cholestatic patient has high levels of AST of more than twice the ALT value, citrulline, α-fetoprotein and ferritin, and low levels of total protein and prothrombin activity.

The histological findings in this study such as a fatty liver, cholestasis, necroinflammatory reaction and iron deposition are not pathognomonic findings because they occur in various liver diseases.[22] However, the combination of mixed macrovesicular and microvesicular fatty hepatocytes and these histological findings are almost never observed in other liver diseases in infants and adults. Microvesicular fatty deposition was found in NICCD, this type of fatty change is a characteristic feature of Reye syndrome[23] and other rare conditions.[22] However, the histogenesis of the microvesicular fatty deposition in NICCD is unclear. It might reflect the acute impairment of β-oxidation of fatty acid due to mitochondrial dysfunction as in Reye syndrome.

Although our series of NICCD patients shared common liver histological findings as described above, there seemed a tendency that late infants of group C had more advanced fibrosis and more accentuated inflammation than those of early infants of group A. The duration of illness may be an aggravating factor of the progression of the disease in some cases. There was no difference between the liver histological findings and mutation type. Interestingly, however, the mutation type of patients with severe fibrosis who were 6 and 7 months of age was 851del4/IVS11 + 1G > A. Because evidence for this relationship between the mutation type and the progression of fibrosis is not clear, further investigation is needed. Moreover, in the follow-up liver biopsy patients, we observed improvements in their liver histological findings as the liver dysfunction was ameliorated. Therefore, we speculate that the correlations between the stage of the liver histological findings and the biochemical test data exist.

This study found that NICCD is a disease with characteristic hepatopathological features. If NICCD is suspected in the presence of cholestasis during infancy, a liver biopsy should be performed to screen for liver diseases. We believe that a liver biopsy is of high diagnostic value for NICCD, and is useful for accurately assessing inflammation and the degree of the progression of fibrosis.

Although we were not able to elucidate the natural history of the disease, we previously found that despite a benign course in the majority of the patients, it leads to the development of liver cirrhosis in some patients with CTLN2.[5,10] This suggests that it involves the risk of progressive fibrosis and eventually leads to the development of cirrhosis. This possibility is suggested by the above histopathological findings characteristic of NICCD in the patients who progressed to stage 3 chronic hepatitis and cirrhosis. Although the process responsible for the progression of liver lesions is not clear, some patients with steatohepatitis including non-alcoholic steatohepatitis (NASH) progress to cirrhosis.[24] In this study, steatohepatitis was found in 60% of the specimens. It is likely that, in NICCD, steatohepatitis repeatedly deteriorates, persists and progresses.

In conclusion, if NICCD is suspected in the presence of cholestasis during infancy, a liver biopsy should be performed to screen for other liver diseases. NICCD is a disease with characteristic hepatopathological features, such as the combination of mixed macrovesicular and microvesicular fatty hepatocytes, cholestasis, necroinflammatory reaction and iron deposition. Therefore, it is possible to diagnose NICCD based on histological liver findings in most cases. However, when cirrhosis with fat deposition is detected in hepatocytes in liver specimens, the patient should be carefully observed, because the prognosis of NICCD patients is not always fair, with some developing progressive liver failure by 1 year of age. Finally, we could not infer the development of CTLN2 from the histological findings of the patients with NICCD who were examined in this study.

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はじめとして

小児期に胆汁うっ滞性の病態を示す疾患は多様である。胆道閉鎖、新生児肝炎、硬化性胆管炎などが主要な疾患であるが、シトリン欠損症を代表とする代謝性疾患のほか、Alagille 症候群などの遺伝性胆汁うっ滞性疾患があり、その診断や鑑別診断は困難である場合が多い。

Alagile 症候群や代謝性あるいは遺伝性疾患は、比較的稀であり、またその臨床像の多様性から。一般的レベルでは診断が困難であり、適切な診断システムが必須である。現在、究極の治療法として「肝臓移植」が選択される場合があるが、正確な診断は「移植肝」をセーブし、患者さんや家族の負担を軽減すると共に、社会的医療資源の温存にも繋がる点で、重要なプロセスであると考えられる。

この論文集は、Alagille 症候群や代謝性あるいは遺伝性疾患の診断にかかわるこれまでの主要な業績を集約した。今後の実態調査等で活用され、研究成果に寄与することを願っている。

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仙台医療センター小児科
田澤雄作

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