clinical symptoms or abnormal episodes during the neonatal or infantile period of CTLN2 patients.

In the last few years, our group has detected *SLC25A13* mutations in patients with neonatal intrahepatic cholestasis [8, 14], and we have uncovered the clinical features of neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; McKusick 605814) [9, 17]. In this study, we investigated nine infants with cholestatic jaundice of unknown origin, who were detected because of hypermethioninaemia, hyperphenylalaninaemia and/or hypergalactosaemia found by newborn screening, in order to determine the role of *SLC25A13* defects in children and to clarify the clinical features of NICCD.

Subjects and methods

Subjects

The nine patients who were retrospectively examined in this study had been referred to Tohoku University Hospital during the past 17 years. A newborn mass-screening programme for the early detection of phenylketonuria, maple syrup urine disease, homocystinuria and galactosaemia was started throughout Japan in 1977. The patients were detected by exhibiting positive results for any of these metabolic diseases (Table 1). The thorough metabolic examinations revealed that these nine infants had suffered neither from phenylketonuria, homocystinuria nor galactosaemia, but all had intrahepatic cholestatic liver disease in the neonatal period, lasting for more than 6 months. No known causes of neonatal cholestasis were found using appropriate diagnostic procedures [13] and these patients were given a diagnosis of idiopathic neonatal hepatitis. The case reports of patients 1, 3, 4, 5 and 6, and mutation analyses of patients 5, 6 and 8 have been described elsewhere [1, 8].

Methods

Blood phenylalanine (Phe) and methionine (Met) levels in mass screening were estimated by bacterial inhibition assays (Guthrie method) and galactose (Gal) was estimated by the Paigen method. Cut-off points for Phe, Met and Gal were 4, 1.5 and 8 mg/dl, respectively. The first blood specimen for newborn screening was obtained at 5–7 days of age. In the case of a positive result, a second measurement from a repeat dried blood specimen was obtained at 15–30 days of age. Serum amino acid analysis was performed using an L-8500 high speed amino acid analyser (Hitachi, Tokyo, Japan).

Table 1 Blood amino acid and galactose levels at mass-screening (mg/dl)

Patient no.	Gender	Initial testing[a]	Second testing[a]
1	F	Phe 8, Met 4	Met 3
2	M	Phe 4	Phe 8
3	F	Met 2	Met 3
4	F	Met 2	Met 4, Gal 16
5[b]	F	Phe 4, Met 1.2	Met 3
6[b]	M	Met 1.5, Gal 15	Met 3
7	M	Phe 4	Phe 4
8	F	Gal 10	Gal 20
9	F	Phe 9	Met 1.5, Gal 20

[a]Cutoff points: Phe 4 mg/dl, Met 1.5 mg/dl and Gal 8 mg/dl; only positive results are shown
[b]Siblings

Plasma Gal levels were measured by an enzymatic method using the Galactose Test (Boehringer Mannheim Co., Mannheim, Germany). The activities of vitamin K-dependent coagulation factors were measured by the Thrombotest (for patients 1 to 3) or the Hepaplastin test (Eisai, Tokyo, Japan; for patients 4 to 9).

Mutation analysis

The procedure for DNA analysis of *SLC25A13* mutations was previously reported in detail by Kobayashi et al. [5] and Ohura et al. [8]. Polymerase chain reaction (PCR) amplification was performed for the detection of the 851del4 mutation by using the primer sets IVS-8F2 (5'-GGTATATTTGTTGCTTGTGTTTG-3') and Ex-9B (5'-TCTTCCAGAGGAGCAATCCG-3'). PCR products were separated on a 4% Amplisize (BioRad, USA) gel and visualised by ethidium bromide staining. For the detection of the IVS11+1G>A mutation, we used the primer sets Ex-11F (5'-CAGCTTTGACTGTTTTAAGAAAGT-3') and IVS-11Bm2 (5'-AGGTATTGAGCATGTGGCACTG-3'). The underlined G is a mismatched base used to create a site for the Sau3AI restriction enzyme for mutant genes. PCR products were digested with Sau3AI and fragments were separated on a 3% Amplisize gel.

We performed a DNA diagnosis after written informed consent was obtained from parents. This study was approved by the Ethics Committee of the Tohoku University School of Medicine.

Results

Mutation analysis

Cases 5 and 6 were siblings and were homozygotes for the IVS11+1G>A mutation. Case 8 was a compound heterozygote for the 851del4 and IVS11+1G>A mutations [8]. Case 9 was a homozygote for the 851del4 mutation (data not shown). DNA samples from the other five patients (nos. 1, 2, 3, 4 and 7) were not available.

Clinical manifestations

The parents of the nine patients were not related. Mean birth weight was 2700 g (range 2382 to 2965 g). Four of the patients were small for gestational age infants (patients 1, 3, 6 and 9). Patient 8 was breast-fed and the other eight patients were mixed or bottle-fed. Jaundice and discoloured stools were observed in all patients except 8, while hepatomegaly was seen in six (patients 1,3, 5, 6, 7 and 8). Three patients showed poor weight gain (patients 1, 3 and 7). Patient 9, with hypergalactosaemia, had cataracts on admission.

Patients 1, 2, 4, 5, 8, and 9 shared common clinical features. They were referred to Tohoku University Hospital because of positive results from neonatal screening (Table 1), and subsequently revealed intrahepatic cholestasis. Patient 3 started vomiting at the age of 4 days. She was treated with an intravenous glucose infusion for 3 days and was discharged at the age of 10 days, but was again admitted to hospital because of feeding difficulties at the age of 16 days. She was later transferred to our clinic at the age of 29 days because hypermethioninaemia was detected by neonatal screen-

Table 2 Biochemical data of the nine patients. (*nd* not determined)

Patient no.	1	2	3	4	5	6	7	8	9	Reference range
Age at examination (days)	62	29	36	51	39	25	124	26	41	
Total bilirubin (mg/dl)	8.2	5.4	8.4	5.7	5.6	3.7	6.8	2.4	6.2	0.2–1.2
Conjugated bilirubin (mg/dl)	5.0	1.4	5.3	3.1	1.9	2.4	3.9	1.3	3.6	0–0.7
γ-GTP (IU/l)	177	272	168	113	186	253	100	220	394	1–49
ALT (IU/l)	93	63	22	31	41	43	79	42	46	3–28
Total protein (g/dl)	5.0	4.8	4.6	5.2	5.1	4.8	5.8	4.6	5.0	5.1–6.8
Ammonia (μg/dl)	53	76	132	72	34	nd	nd	84	133	<75
Total bile acids (μmol/l)	370	367	197	218	210	196	229	230	419	11–28[b]
Thrombotest/Hepaplastin test (%)[a]	46	18	50	32	31	(83)	(64)	82	35	>70

[a]Thrombotest used only in patients 4 to 9. Data in brackets obtained after vitamin K supplementation
[b]Normal values at 1 month of age

ing. Patient 6 was delivered by caesarean section because of fetal distress. The activities of vitamin K-dependent coagulation factors were less than 10% of normal controls at 3 days of age and he was treated with intravenous vitamin K injections. He was later referred to our clinic at 25 days because of hypermethioninaemia [8]. For patient 7, hyperphenylalaninaemia was detected by neonatal screening. Jaundice was observed during the follow-up examination, and the patient was admitted for further examination at the age of 63 days. After thorough investigations for cholestasis, including biliary atresia, he was given a diagnosis of idiopathic neonatal hepatitis and was referred to our hospital at the age of 110 days because his family had moved into our district.

Results of neonatal screening

The results of neonatal screening were unusual (Table 1). Patients 1 and 5 were positive for both hyperphenylalaninaemia and hypermethioninaemia in the first blood specimen, but only hypermethioninaemia was detected in the second. Patient 6 was positive for both hypermethioninaemia and hypergalactosaemia in the initial testing, but the Gal level had normalised by the second testing. There were some inconsistencies in the abnormal data between the initial and second testings. For example, in patient 9, hyperphenylalaninaemia was detected in the initial testing, whereas the blood Phe level had normalised and the blood levels of Met and Gal were elevated in the second specimen.

Biochemical data

Laboratory data obtained from the patients were comparable and suggested severe intrahepatic cholestasis (Table 2). Serum conjugated bilirubin ranged from 1.3 to 5.3 mg/dl. Serum γ-glutamyl transpeptidase (γ-GTP) activities were significantly elevated. Serum alanine aminotransferase (ALT) levels were only mildly elevated. Total protein levels of six patients decreased to below 5 g/dl. Blood ammonium levels were mildly elevated in three patients. The prominent feature was the elevation of serum total bile acid levels. These levels climbed above 190 μmol/l in all patients examined. The activities

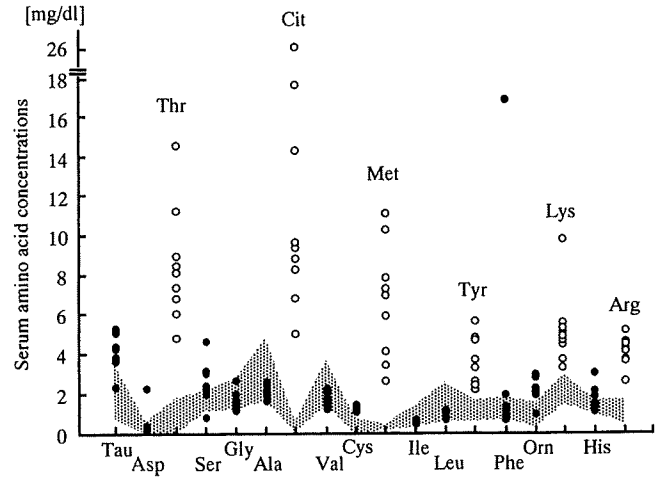

Fig. 1 Serum amino acid analysis of the nine patients on admission. Concentrations of threonine, citrulline, methionine, tyrosine, lysine and arginine are represented by *open circles*. The other amino acid levels are represented by *closed circles*, *dotted square*: Reference range

of vitamin K-dependent coagulation factors were low in seven of the patients. Blood Gal levels in patients 4, 8 and 9 were high at the time of newborn screening. On admission, the Gal levels of patients 8 and 9 were 16 and 43 mg/dl, respectively, but in patient 4 the Gal level was within the normal range (4 mg/dl).

The most characteristic features were the abnormal amino acid patterns (Fig. 1). Serum amino acid analysis showed a significant elevation of citrulline and Met in all patients. The concentrations of threonine, tyrosine, lysine and arginine were also 2–4 times higher than the control levels. Other amino acid levels were within or near the reference ranges.

Histology

Percutaneous liver biopsies were performed on patients 1, 3, 4, 5 and 7. Typical findings are shown in Fig. 2. All five patients showed a uniform liver histology, namely cholestasis and fatty liver, with diffuse fatty changes in the hepatocytes, including micro- or macrovesicular fat droplets (Fig. 2A, patient no. 5). Mild infiltration with inflammatory cells, and portal fibrosis were also ob-

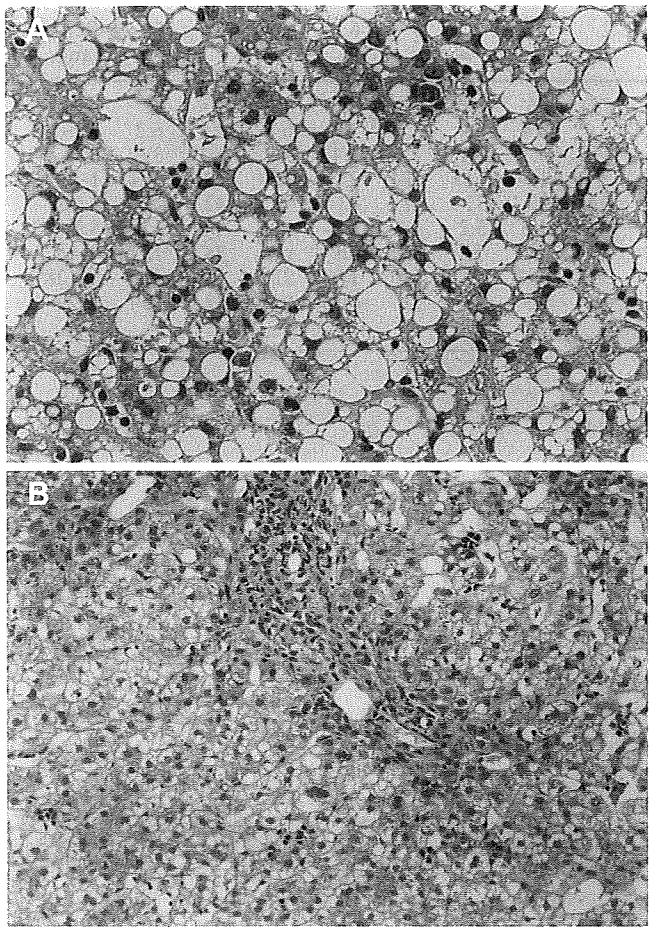

Fig. 2 Liver histology. **A** Patient 5, 55 days of age. **B** Patient 3, 43 days of age. Haematoxylin-eosin staining; original magnification A ×200; B ×100

served (Fig. 2B, patient no.3). Cholestasis was identified in the liver parenchyma, but there was no giant cell transformation of hepatocytes.

Treatment and outcome

The clinical courses of the nine patients were similar. All patients responded to nutritional management, including fat-soluble vitamins (A, D, K and E), formula containing medium-chain triglycerides, or lactose-free formula. Abnormal aminograms resolved at a mean age of 77 days (range 31 to 155 days). Concentrations of serum conjugated bilirubin and total bile acids had normalised at a mean age of 75 days (range 36 to 145 days) and 101 days (range 43 to 145 days), respectively. Serum γ-GTP and ALT activities had normalised at a mean age of 148 days (range 115 to 215 days) and 224 days (range 123 to 395), respectively. The clinical course of patient 5 shows a typical profile of the evolution of the biochemical data with age (Fig. 3). By the age of 1 year, all liver test results had normalised.

We have continued to follow-up four out of the nine patients. When the latest medical check-ups were performed, patients 5, 6, 8 and 9 were aged 10, 6, 3 and 1 year respectively and were mentally and physically normal. Unfortunately, the remaining five patients were lost to follow-up.

Discussion

The nine patients described in this paper were all referred to hospital because of positive results during neonatal screening. The clinical courses of these patients were similar. An abnormal aminogram persisted for a couple of months but then improved. The levels of serum conjugated bilirubin decreased first, then the levels of serum total bile acids gradually decreased, then all abnormal biochemical levels, including γ-GTP and ALT, improved. Liver biopsy revealed a fatty liver in all five patients examined. Since the results of specific tests for diseases associated with neonatal cholestasis remained negative, neonatal intrahepatic cholestasis of unknown origin was diagnosed in these patients [1]. The most characteristic features of the nine patients were transient multiple hyperaminoacidaemia and/or hypergalactosaemia. At first, we had speculated that these abnormalities might be secondary phenomena due to liver damage because hypermethioninaemia, hypertyrosinaemia and/or hypergalactosaemia are often associated with liver diseases [2, 6], but hypercitrullinaemia in these patients was unique and has not been reported in patients with liver diseases before. Thus, we speculated that these nine patients could be suffering from the same inborn error of metabolism disturbing the citrulline pathway. Hypercitrullinaemia with fatal hyperammonaemia is known to be detected in patients with CTLN1 or argininosuccinic aciduria during the neonatal period. In our series, CTLN1 and argininosuccinic aciduria were unlikely because the citrullinaemia was transient and serious hyperammonaemia was not detected.

The discovery of the gene responsible for CTLN2 prompted us to recall these patients and try to gather them for new DNA analyses. Finally, we were able to contact four out of the nine patients and could then perform DNA analysis for the *SLC25A13* mutation. Surprisingly, we detected *SLC25A13* mutations in all four patients examined. Although this analysis was not performed on five patients due to the unavailability of their DNA samples, the similarities of clinical manifestations and laboratory data, including transient hypercitrullinaemia, obtained from these nine patients suggests that all nine described in this paper suffered from *SLC25A13* mutations.

Establishment of a method for the DNA analysis of citrin deficiency revealed that the *SLC25A13* mutation is the cause of a particular type of neonatal intrahepatic cholestasis. Therefore, we designated these patients as NICCD [9, 17]. To date, *SLC25A13* mutations have been detected in 70 patients with NICCD [11]. Among them, about 40% of the NICCD patients were referred to hospital because of positive results from neonatal

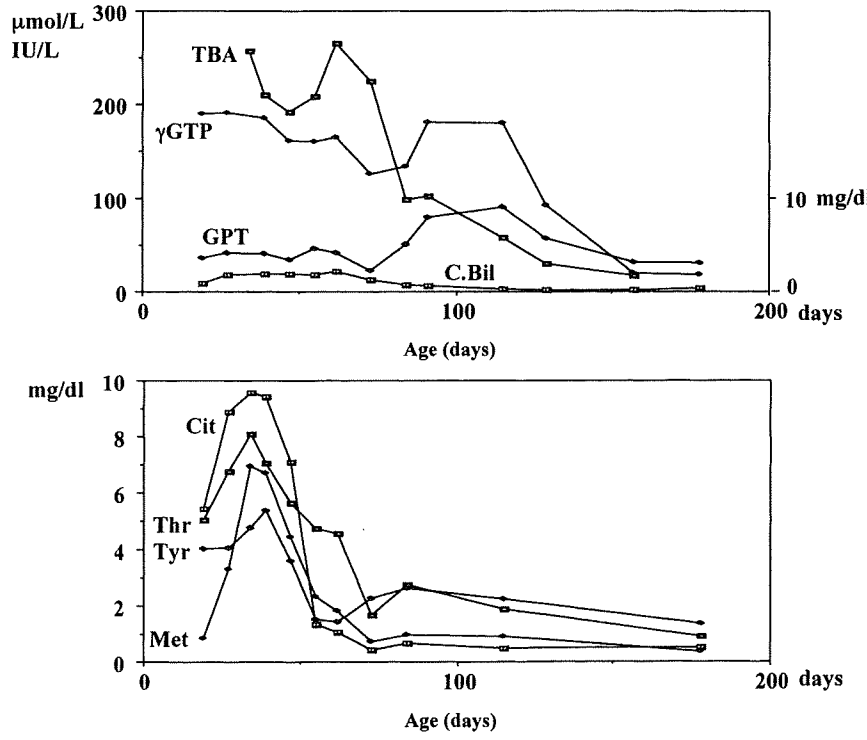

Fig. 3 Typical evolution of biochemical data with age in patient 5. (*C.Bil* conjugated bilirubin in mg/dl, *TBA* total bile acids in µmol/l)

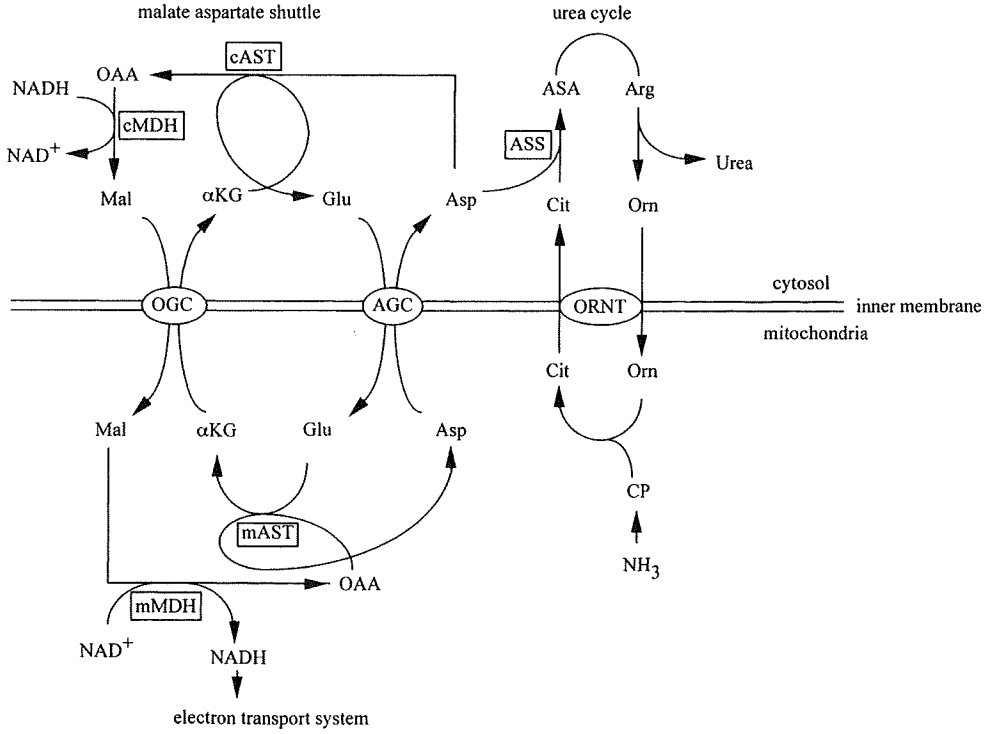

Fig. 4 Role of citrin as an aspartate glutamate carrier (AGC) in urea synthesis and in the malate aspartate shuttle. (αKG α-ketoglutarate, *Arg* arginine, *ASA* argininosuccinic acid, *Asp* aspartate, *cAST* cytosolic aspartate aminotransferase, *Cit* citrulline, *cMDH* cytosolic malate dehydrogenase, *CP* carbamoyl phosphate, *Glu* glutamate, *mal* malate, *mAST* mitochondrial aspartate aminotransferase, *mMDH* mitochondrial aspartate aminotransferase, *OAA* oxaloacetate, *OGC* oxoglutarate carrier, *Orn* ornithine, *ORNT* ornithine transporter)

screening. The rest of the NICCD patients were negative for newborn screening. These screen-negative patients manifested various symptoms such as prolonged jaundice, acholic stool and/or failure to thrive after 1 month of age, and were then referred to hospitals (unpublished data). We emphasise that neonatal screening provides an important opportunity for the diagnosis of NICCD [7, 8]. If a cholestatic infant exhibiting unusual results on mass screening, such as shown in Table 1, is encountered, the differential diagnosis should include NICCD. Hypercitrullinaemia could be detected directly through newborn screening if tandem mass specrometry were used.

Since the function of citrin has been found to be a mitochondrial aspartate glutamate carrier [10], the

various symptoms of NICCD may be understood as being caused by defective aspartate export from the mitochondria to the cytosol and by defects in the malate aspartate shuttle (Fig. 4). Defective aspartate export probably reduces aspartate availability for the ASS reaction in the cytosol, which leads to an accumulation of citrulline and a disturbance of urea cycle function. A deficiency of aspartate in the liver cytosol is also followed by an inhibition of protein synthesis, resulting in hypoproteinaemia in NICCD [11, 16]. The malate aspartate shuttle plays a role in the transport of the cytosolic reducing equivalent into the mitochondria. Therefore, citrin deficiency blocks the malate aspartate shuttle, thereby increasing the cytosolic NADH to oxidised nicotinamide adenine dinucleotide ratio ($NADH/NAD^+$). Since uridine diphosphate galactose 4-epimerase, requiring NAD as a cofactor, is strongly inhibited by NADH, galactosaemia in NICCD may represent a high NADH level in the cytosol of the liver [11], but the mechanism of transient multiple hyperaminoacidaemia is not clear. Patient 8, who was exclusively breast-fed, was positive only for galactosaemia on newborn screening, but after she was given a lactose-free formula, which contained a higher protein content compared to breast-milk, hyperaminoacidaemia became apparent. We speculate that both decreased protein synthesis and higher protein intake may give rise to multiple aminoacidaemia in NICCD [14]. The pathogeneses of transient and self-limited cholestasis in these infants with *SLC25A13* mutations remain unsolved. The improvement of NICCD symptoms within 1 year suggests some adaptation, compensation, or metabolic change during development [11].

The prognoses of the nine patients at the age of 1 year were fairly positive. Their symptoms resolved by 12 months without special treatment other than feeding programmes [8, 14], such as formulas containing medium-chain triglycerides or lactose-free formulas and supplementation with fat-soluble vitamins. There were three unrelated NICCD patients reported who developed progressive liver failure and were subsequently treated with liver transplantation before the age of 1 year [11, 15]. We must realise that the outcome of patients with NICCD is not always benign. We speculate that some NICCD patients may go on to develop CTLN2 with neuropsychiatric symptoms decades later [11, 16]. However, we do not know which NICCD patients, or how many, will go on to develop CTLN2. It is now important and urgent to identify the genetic or environmental factors that lead to the deterioration to CTLN2. We will continue to observe these patients regularly to see whether they develop symptoms of CTLN2.

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Clinical heterogeneity of neonatal intrahepatic cholestasis caused by citrin deficiency: case reports from 16 patients☆

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Abstract

A deficiency of citrin, which is encoded by the *SLC25A13* gene, causes both adult-onset type II citrullinemia (CTLN2) and neonatal intrahepatic cholestasis (NICCD). We analyzed 16 patients with NICCD to clarify the clinical features of the disease. Severe intrahepatic cholestasis with fatty liver was the most common symptom, but the accompanying clinical features were variable, namely; suspected cases of neonatal hepatitis or biliary atresia, positive results from newborn screening, tyrosinemia, failure to thrive, hemolytic anemia, bleeding tendencies and ketotic hypoglycemia. Laboratory data showed elevated serum bile acid levels, hypoproteinemia, low levels of vitamin K-dependent coagulation factors, and hypergalactosemia. Hypercitrullinemia was detected in 11 out of 15 patients examined. Most of the patients were given a lactose-free and/or medium chain triglycerides-enriched formula and lipid-soluble vitamins. The prognosis of the 16 patients is going fairy well at present, but we should observe these patients carefully to see if they manifest any symptom of CTLN2 in the future.

Keywords: Citrin deficiency; Citrullinemia; Neonatal intrahepatic cholestasis; Fatty liver; Newborn screening

Introduction

SLC25A13, the gene newly demonstrated to be responsible for adult-onset type II citrullinemia (CTLN2), encodes an aspartate glutamate carrier named citrin [1,2]. CTLN2 is characterized by a late onset (11–79 years), frequent attacks of unconsciousness with hyperammonemia, and ultimately death within a few

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years of its onset [1,3–7]. Until recently, very little was known about the clinical symptoms of CTLN2 in the neonatal/infantile period. However, we have detected *SLC25A13* mutations in six patients with neonatal hepatitis [8,9], and the clinical features of neonatal intrahepatic cholestasis caused by citrin deficiency (named NICCD [10]) have been uncovered [8,9,11–18]. It has become apparent that citrin deficiency causes two age-dependent phenotypes, namely, CTLN2 in adults and NICCD in infants. Most NICCD patients show symptoms, which ameliorate by 1 year of age, and several decades later, some patients develop CTLN2 [11,16,19,20]. So far, ten mutations have been reported in patients with a citrin deficiency [1,6,10,13], and 129 CTLN2 and 107 NICCD patients have been diagnosed genetically [21]. CTLN2 patients have been diagnosed on the basis of well-established criteria [1,3–7]. Whereas, as NICCD was discovered quite recently and shows various and transient symptoms, the criteria for clinical and biochemical diagnoses of NICCD are in the process of being established. In this study, we report 16 patients with NICCD in whom diagnoses were made by gene analyses, and examined the clinical heterogeneity of the disease.

Materials and methods

Patients

We performed a DNA analysis of *SLC25A13* in 17 cholestatic infants to whom we had given a diagnosis of neonatal hepatitis with fatty liver of unknown origin over the past 28 years. Among them, 14 patients were proven to be homozygotes or compound heterozygotes for *SLC25A13* mutations, and were consequently enrolled in this study (patients 1–14, 5–28 years of age). *SLC25A13* mutations were not detected in the three remaining patients. After the publication of NICCD cases [8,9,11], we have newly diagnosed two more patients as having NICCD and added them to the present study (patients 15 and 16, 2 and 1 years of age, respectively).

Seven of our 16 patients (patients 9–15) were previously reported [8,9,17]. The parents of patient 1 were first cousins, and patients 12 and 13 were siblings.

Genetic analyses for the *SLC25A13* mutations were performed after informed consent was obtained. The Ethics Committees of the Tohoku University School of Medicine and the Kagoshima University Graduate School of Medical and Dental Sciences approved this study.

DNA diagnosis of 11 mutations of the SLC25A13 gene

The procedure for the DNA diagnosis of nine *SLC25A13* mutations has previously been reported in detail [1,6,10]. In the present study, we used the following primers and conditions: mutation I (851del4), the primer sets IVS8F2 (5′-GGTATATTTGTTGCTTGTG TTTG-3′) and Ex9B (5′-TCTTCCAGAGGAGCAA TCCG-3′), direct electrophoresis on a 4% Amplisize (Bio-Rad) agarose gel; mutation II (IVS11 + 1G→A), Ex11F2 (5′-GAAAGTGCTACGCTATGAAGG-3′) or Ex11F (5′-CAGCTTTGACTGTTTTAAGAAAGT-3′) and IVS11Bm2 (5′-AGGTATTGAGCATGTGGCAC TG-3′, the underlined G is a mismatched base), *Sau*3AI digestion, electrophoresis on a 3.5 or 3% Amplisize gel; mutation III (1638ins23), IVS15F2 (5′-TGTTGTGTC TCTCCTGCAGG-3′) and Ex16B (5′-GCAGTCTAT CACTCCGCTGT-3′), direct electrophoresis on a 3% Amplisize gel; mutation IV (S225X), IVS6F (5′-GGAG AGTACAAGTTCTGGTC-3′) and IVS7B (5′-ACTAG TTGCCTTCTTCACCC-3′), *Alu*I digestion, electrophoresis on a 2.5% Amplisize gel; mutation V (IVS13 + 1G→A), Ex13F (5′-GTGAACGATTTTGTGAGGGA TA-3′) and IVS13Bm (5′-GAAGAGAGCTTCAAAA GGTACTTC-3′), *Pst*I digestion, electrophoresis on a 3% Amplisize gel; mutation VI (1800ins1), IVS16F (5′-CTAATTATATCTGTGATTTCTCCA-3′) and IVS17B (5′-GGAGTTGATACATTCTCATCAG-3′), *Tru*1I digestion, electrophoresis on a 3.5% Amplisize gel; and mutation VIII (E601X), IVS16F (5′-CTAATTATATC TGTGATTTCTCCA-3′) and Ex17NBm6 (5′-AATGT AGAACCATCGCTGTAGG-3′), *Eco*RI digestion, electrophoresis on a 3% Amplisize gel. Mutations I to VIII were confirmed by the GeneScan and SNaPshot methods [10]. Two novel mutations, X (IVS6 + 5G→A) and XIX (IVS16ins3kb), will be published elsewhere.

Results

Genotype analyses

Table 1 shows the DNA diagnoses of the 15 families. The 851del4 and the IVS11 + 1G > A mutations comprised 70% (21/30) of the total alleles, as recently described [10,20,21]. Homozygotes of the IVS11 + 1G > A mutation were frequent, as was previously reported [10].

Case reports

Clinical manifestations of the 16 patients (6 boys and 10 girls) are listed in Table 1. Birth weights ranged from 1946 to 3140 g and mean birth weights for male and female patients were 2834 and 2523 g, respectively. The average standard deviation for birth weight was -1.1 ± 0.6. Seven patients (Nos. 1, 7, 8, 9, 10, 13, and 16) were small-for-gestational age babies. Seven infants were breast-fed, five were formula-fed, and the rest were mixed-fed, when they visited one of our hospitals. All patients were referred to hospitals prior to 6 months of age (18–167 days old).

Table 1
Genotype and clinical characterizations of 16 patients with NICCD

Patient Nos.	Genotype[a]	Sex	Gestational age (weeks)	Birth weight (g)	Feeding modality	Age[b] (days)	Clinical features
1	II/II	M	40	2700	BM	63	OJ, FTT, hemolytic anemia
2	II/II	F	38	2674	BM	45	OJ, FTT, cataracts
3	II/V	F	37	2730	FM	52	Vitamin K deficiency, OJ
4	V/XIX	M	40	3140	BM	46	OJ, hemolytic anemia
5	II/III	M	41	2940	MM	118	OJ, FTT, cataracts
6	II/V	M	41	3010	MM	70	OJ
7	II/II	F	36	1946	BM	150	OJ, FTT
8	VIII/X	F	41	2650	FM	167	OJ, FTT
9[c]	I/II	F	38	2360	FM	147	OJ, FTT
10[c]	IV/VI	F	37	2050	BM	132	MAS, FTT, subcutaneous bleeding
11[c]	I/II	M	37	2700	MM	39	NBS (Gal), OJ
12[d,e]	II/II	F	38	2958	FM	19	NBS (Met), OJ
13[d,e]	II/II	M	40	2514	FM	25	fetal distress, NBS (Met), OJ
14[d,e]	I/II	F	40	2840	BM	19	NBS (Gal)
15[e]	I/I	F	37	2460	MM	38	NBS (Gal & Met), OJ, cataracts
16	II/II	F	39	2560	BM	18	NBS (Phe), OJ, cataracts

M, male; F, female; BM, breast-fed; MM, mixed-fed; FM, formula-fed; OJ, obstructive jaundice; FTT, failure to thrive; MAS, meconium aspiration syndrome; NBS, newborn screening positive; Gal, hypergalactosemia; Met, hypermethioninemia; Phe, hyperphenylalaninemia. Patients 12 and 13 were siblings.

[a] I: 851del4, II: IVS11 + 1G > A, III: 1638ins23, IV: S225X, V: IVS13 + 1G > A, VI: 1800ins1, VIII: E601X, X: IVS6 + 5G > A, XIX: IVS16ins3kb

[b] Age at first visit to one of our hospitals.

[c] Ref. [9].

[d] Ref. [8].

[e] Ref. [17].

The chief complaints of seven patients (patients 2, 4–9) were similar, i.e., jaundice and/or discolored stools, and they were referred to one of our hospitals as suspected cases of neonatal hepatitis or biliary atresia. Among them, five patients were revealed to exhibit a failure to thrive (patients 2, 5, and 7–9).

Patient 1 was admitted to a local hospital at the age of 1 month because of hepatosplenomegaly. He was suspected of having hemolytic anemia and was transferred to a University hospital at the age of 2 months for further evaluation. Laboratory data revealed obstructive jaundice and liver damage, and a diagnosis of neonatal hepatitis was made. Patient 3 was referred to the hospital because of a low activity (28%) of vitamin K-dependent coagulation factors at the age of 1 month, at that point obstructive jaundice and hyperamino acidemia were noticed. Patient 4 manifested not only cholestatic jaundice but also anemia. His laboratory data obtained at the age of 1 month showed normocytic anemia; RBC $255 \times 10^6/mm^3$, hemoglobin 7.8 g/dL, reticulocytes 4.6%, Fe 75 μg/dL, ferritin 447 ng/mL (normal range: 15–220), haptoglobin <1 mg/dL (normal range: 19–170). He was suspected of having hemolytic anemia in addition to liver injury, but results of Coombs' test were negative. Patient 10 had been admitted at the neonatal intensive care unit for 2 months, as she was a low birth weight infant and suffered from meconium aspiration syndrome. She was referred to the hospital at 4 months of age because subcutaneous bleeding was noticed about her neck. Laboratory analysis revealed cholestatic jaundice and a low activity (<8%) of vitamin K-dependent coagulation factors. Patient 13 was delivered by caesarean section because of fetal hypoxia. He was transferred to a local hospital at the age of 3 days because of liver dysfunction and a low activity (<10%) of vitamin K-dependent coagulation factors. He was treated with vitamin K injections and discharged on day 21, but was again referred to the hospital because of a positive result from newborn screening [8,17].

Newborn screening

Six infants (Nos. 11–16) out of the 16 (6/16 = 38%) were referred to hospitals because of positive results from newborn screenings. Hypermethioninemia was detected in two (patients 12 and 13), hypergalactosemia in two (patients 11 and 14), hyperphenylalaninemia in patient 16, and both hypergalactosemia and hypermethioninemia were detected in patient 15. Thorough metabolic examinations revealed that these six patients had suffered from neither phenylketonuria, homocystinuria nor hereditary galactosemia, but had intrahepatic cholestasis [8,9,17].

Laboratory findings

Laboratory data (Table 2) obtained from the 16 patients were comparable and suggested severe intrahepatic cholestasis. Serum levels of total bile acids and conjugated bilirubin were elevated in all patients. Activities of gamma glutamyltranspeptidase and alkaline phosphatase were also significantly elevated, but

Table 2
Laboratory findings in 16 patients with NICCD

Patient Nos.	TB and CB (mg/dL)	Total bile acids (μmol/L)	AST and ALT (IU/L)	γGTP and ALP (IU/L)	Total protein and albumin (g/dL)	Vitamin K-dependent coagulation factors (%)	Galactose (mg/dL)	Ammonia (μg/dL)
1	9.6/6.8	205	85/44	NE/865	5.7/3.8	66	NE	NE
2	7.5/2.6	215	81/20	183/1628	4.2/2.7	95	99[a]	NE
3	13/8.5	172	133/45	78/1570	3.8/2.6	24	NE	NE
4	9.0/3.4	249	96/38	206/2411	4.9/3.2	75	<4.0	31
5	8.2/4.2	327	88/33	154/2555	5.3/3.9	49	83	165
6	9.9/5.4	331	109/50	408/3728	5.1/4.0	37	NE	112
7	5.2/2.6	210	210/84	164/2397	6.1/4.2	100	NE	NE
8	5.4/3.5	252	208/127	132/2280	5.9/4.0	55	NE	NE
9	6.2/3.7	213	302/167	65/1582	6.1/4.3	50	NE	NE
10	6.2/2.4	126	83/24	90/405	4.6/2.9	<4	NE	NE
11	12.6/2.6	120	31/20	142/2230	3.9/2.6	26	25	NE
12	5.6/1.9	210	62/41	186/773	5.1/3.5	30	4.0	94
13	3.7/2.4	196	75/43	253/1306	4.9/3.1	<10[b]	<4.0	NE
14	2.9/1.4	230	57/33	202/2140	4.6/3.4	82	17	84
15	6.2/3.3	419	126/46	394/6745	5.1/3.8	35	43	133
16	9.2/5.6	248	103/59	136/2056	4.8/3.2	47	14	60
Normal value	<1.2/<0.4	<36	<30/<35	<57/<450	>5.0/>3.3	>60	<4.0	<60

TB, total bilirubin; CB, conjugated bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, gamma-glutamyltranspeptidase; ALP, alkaline phosphatase; NE, not examined. [a,b]Measured at the age of 72 and 3 days, respectively, and all other biochemical data were evaluated around the time of their first visit to one of our hospitals.

alanine aminotransferase activities increased only mildly. Total protein levels of eight patients decreased to less than 5.0 g/dL. The blood ammonium concentration was measured in seven infants and mild hyperammonemia (>60 μg/dL) was detected in five.

Other characteristic features included bleeding tendencies and cataracts. Levels of vitamin K-dependent coagulation factors were low in 11 patients. Among them, patients 10 and 13 manifested a critical decrease of coagulation factors, which was probably not only due to a malabsorption of vitamin K, but also to severe liver damage. Hypergalactosemia was detected in six out of nine patients examined. Among them, four infants were revealed to have cataracts.

Amino acid levels

Plasma amino acid analyses showed a significant elevation of citrulline and/or methionine in 11 out of 15 patients examined (Table 3). The concentrations of

Table 3
Amino acid levels in 15 patients with NICCD (μmol/L)

Patients	Threonine	Citrulline	Methionine	Tyrosine	Lysine	Arginine
1	180	31	44	189	NE	120
2	455	223	44	98	317	154
3	666	581	854	559	575	354
4	175	43	26	34	133	45
5	669	839	417	191	427	283
6	372	124	97	131	277	172
7	115	42	40	70	NE	NE
8	516	119	468	214	493	249
9	479	496	426	287	439	140
10	NE	NE	NE	NE	NE	NE
11	153	33	44	352	NE	NE
12	681	547	465	264	347	262
13	619	286	229	143	220	148
14[a]	415	485	240	185	384	315
15	1217	1518	300	237	722	305
16	683	1210	108	424	648	318
Reference range	66–153	5–37	13–32	34–94	102–203	28–99

NE: not evaluated.
Amino acid levels were measured around the time of their first visit to one of our hospitals (except for patient 14).
[a] Measured at the age of 26 days.

threonine, tyrosine, lysine, and arginine were also 2–8 times higher than the control levels. Other amino acid concentrations were within or near normal ranges (data not shown). Tyrosinemia without citrullinemia was noticed in two patients (Nos. 1 and 11).

Histology

Among the 16 patients, a liver needle biopsy was performed on 12 (Nos. 1–12). All 12 patients showed uniform liver histological findings, i.e., cholestasis and fatty liver (data not shown) [9,17]. The liver histology obtained from patient 8 at 5 months old showed not only a fatty change, but also a giant cell transformation (Fig. 1).

Clinical management

We retrospectively reviewed the medical records of 14 patients (patients 1–14), who had been given a diagnosis of neonatal hepatitis with fatty liver of unknown origin before the DNA diagnosis of *SLC25A13* was performed. Special milk formulas were used on all except patient 10. A medium chain triglycerides (MCT)-enriched formula was given to five (patients 1, 3, 8, 9, and 12) and lactose-free milk was prescribed to four (patients 2, 11, 14, and 15) patients. Six infants (patients 4–7, 13, and 16) were fed with formula milk, which contained MCT and no lactose. Patient 3 was fed with a phenylalanine–tyrosine-free formula because the patient was diagnosed as having hypertyrosinemia.

All our infants received lipid-soluble vitamins according to protocol while cholestasis persisted. Vitamin K was administered intravenously to patients 10 and 13 because of a critical vitamin K deficiency. Ursodeoxycholic acid was prescribed to five patients (Nos. 3, 5, 8–10), phenobarbital to four patients (Nos. 3–6) and cholestyramine to one patient (No. 3). Patient 4 received a blood transfusion because of anemia at 1 month old. Fresh frozen plasma was administered to patient 10 in order to supply coagulation factors.

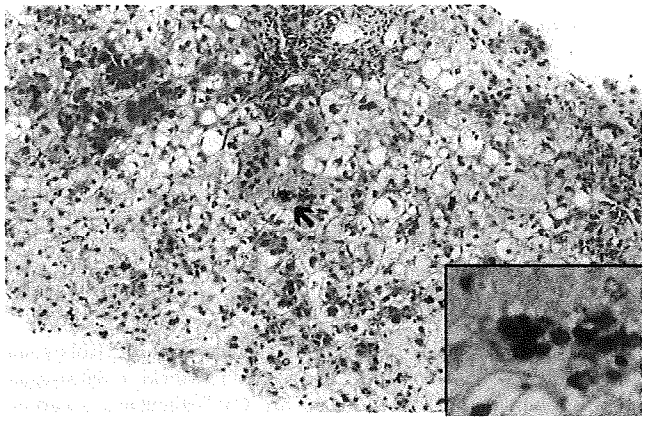

Fig. 1. Focal macrovesicular fatty liver in the periportal area and a giant cell transformation in the parenchyma, pointed out by the arrow, are seen in the liver specimens obtained from patient 8. The inset shows a giant cell present in the area.

Discussion

We have pointed out that neonatal screening provides an important opportunity for the diagnosis of NICCD [8,17]. Shigematsu et al. [22] reported that they found three patients with NICCD in 102,200 newborns by detecting hypercitrullinemia using electrospray tandem mass spectrometry. Their data suggested that the incidence of NICCD be calculated at 1 in 34,000. However, the frequency of homozygotes with mutated *SLC25A13* is higher, and has been estimated to be 1 in 19,000 [20,21]. It is suggested that NICCD patients could be detected through newborn screening only if their symptoms are manifested in the neonatal period. Screening-negative patients most likely developed their symptoms at a later date. It is possible that some of the NICCD patients might have apparently been healthy during their entire infantile period.

Hypergalactosemia is a common finding of NICCD. In patients 2 and 5, hypergalactosemia was not detected through newborn screening, but later their serum galactose levels were revealed to be extremely high (Table 2). Tamamori et al. [23] reported that the galactose level was increased at one month in comparison with day 5. It is important that a galactose assay be done in all cases in which NICCD is suspected, even if the result from newborn screening was negative.

Among the 11 patients with hyperamino acidemia, six patients were being formula-fed, three mixed-fed, and two breast-fed at the time of the evaluation. Hypercitrullinemia was not detected in four patients (Nos. 1, 4, 7, and 11), of whom three were exclusively breast-fed and one was mixed-fed. All six of the patients (Nos. 3, 8, 9, and 12–14) who were exclusively formula-fed presented with hyperamino acidemia. Patient 14 was originally breast-fed (Table 1) and was referred to the hospital because of a positive result from newborn screening at the age of 19 days. At that time she was positive only for galactose, with levels of methionine and phenylalanine being within normal ranges. She was started on a lactose-free formula, and the hyperamino acidemia became apparent at the age of 26 days (Table 3). Infants fed with formula milk were prone to having hyperamino acidemia, but this was also detected in two infants, who were exclusively breast-fed (patient Nos. 2 and 16). These data suggest that plasma amino acid levels may be influenced by multiple factors, such as protein intake, endogenous protein synthesis and additional unknown factors. We, however, did not check the daily intake of milk.

We have reported the common features of liver histologies obtained from patients with NICCD, i.e., a diffuse

fatty change in hepatocytes with micro- and macrovesicular fat droplets, dilated canaliculi containing bile, and mild to moderate fibrosis with minimal lymphocyte infiltration in the portal area [9,17]. But, an interesting histological finding, a focal fatty liver in the background of the parenchymal giant cell transformation, was seen in patient 8. The giant cell transformation of hepatic cells, which has frequently been detected in patients with neonatal hepatitis, has not been reported in patients with NICCD before. It has been suggested that the parenchymal giant cell transformation represents a non-specific reaction of an infant's hepatocytes to various types of injuries [24]. Patient 8 was the oldest (167 days old), at the time when she first visited the hospital, of the 16 NICCD patients. We have speculated that she had been suffering from prolonged cholestasis and liver injury, which might have resulted in the giant cell transformation.

The clinical management for NICCD is directed at treating the consequences of hypergalactosemia and cholestasis. Naito et al. [12] reported that a lactose challenge test given to a patient at the age of 56 days worsened the liver function test and she again became hypergalactosemic, but re-challenging with lactose at 152 days did not worsen these findings. We propose that lactose may be toxic to patients with NICCD and should be avoided while cholestasis persists. We must prevent complications of prolonged cholestasis, such as malnutrition and a lipid-soluble vitamin deficiency. In patients with a failure to gain weight adequately, their diets should be supplemented with MCT, which can be absorbed without interluminal bile salts. The decrease of vitamin K-dependent coagulation factors due to a vitamin K deficiency is an immediate and constant risk. While cholestatic jaundice persists, the prothrombin time should be checked repeatedly, and vitamin K should be given if prolonged. But in the case of severe liver damage, such as in case 10, fresh frozen plasma is needed to increase coagulation factors.

Clinical courses of the 16 patients were similar to those of previously reported cases [8,9,12,17]. Laboratory data normalized within 12 months of life, and all except one (patient 8) have been well without any neuropsychological signs. Patient 8 had suffered an afebrile convulsion at the age of 16 months although her EEG was normal, and a mild delay in psychomotor development was noticed. But a re-examination at 4 years and 6 months old showed that her mental and physical development had caught up to within the normal range (DQ 93). Tanaka et al. [16] reported on a male patient who developed CTLN2 in his teens and had shown mental retardation and growth failure from early childhood. Further investigations of the natural history of patients with NICCD should be undertaken to establish a long term prognosis of the disease, especially regarding psychomotor development. Patient 7 has developed frequent attacks of ketotic hypoglycemia in the mornings at the age of one and a half years. Tamamori et al. [15] reported two patients who also developed hypoglycemia. They speculated that the hypoglycemia was caused by the disturbance of gluconeogenesis, because the aspartate glutamate carrier (citrin) functions to provide substrates for gluconeogenesis as a part of the pathway for the conversion of amino acids to glucose [15,20]. Hypoglycemia may be a common feature in patients with citrin deficiency.

We had speculated the prognosis of patients with NICCD as being fairly good, because all of the patients we described had recovered spontaneously before the age of 1 year [8,9,17]. But Tamamori et al. [15] reported a patient with NICCD who had been suspected of having tyrosinemia type 1, but no succinylacetone had been detected in her urine. She developed progressive liver failure and subsequently underwent a liver transplantation at the age of 11 months. Tomomasa et al. also reported on a patient with NICCD who developed CTLN2 at 16 years of age and underwent living-related liver transplantation therapy [11,19]. We must realize that patients with NICCD are not always benign, although all 16 patients in this paper have apparently remained healthy to this day.

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A possible mechanism of neonatal intrahepatic cholestasis caused by citrin deficiency

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Abstract

For this study, we investigated why cholestasis develops into neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), and concluded that primary mitochondrial impairment associated with the delayed maturity of bile acid metabolism may contribute to the occurrence of NICCD.

Keywords: Neonatal cholestasis; Citirn deficiency

1. Introduction

Citrullinemia caused by an argininosuccinate synthetase deficiency (ASS) has been classified into two groups; classical citrullinemia types I and III having a generalized defect in ASS activity, and adult-onset citrullinemia type II (CTLN2) which has a reduction of ASS protein specifically in the liver [1]. Recently, the responsible gene for CTLN2 was uncovered as SLC25A13, located on chromosome 7q21.3 [2], 14 mutations having been reported [2–5]. The product of the SLC25A13 gene, designated citrin, has been disclosed to be a Ca^{2+}-stimulated aspartate/glutamate carrier (AGC) in mitochondria [6], a special shuttle systems carrying reducing equivalents from cytosolic NADH into the mitochondria. The most active NADH shuttle is called the malate-aspartate shuttle [7,8]. The transporter, AGC, catalyzes an irreversible step in this aspartate-malate NADH shuttle [7].

Hyperammonemia and fatty liver, which develop around the 20th year of life, are clinical core features of CTLN2 [1]. However, advances in molecular biology have discovered neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) [9–12]. In adult-onset CTLN2, intrahepatic cholestasis is rare [13], but NICCD patients have transient neonatal intrahepatic cholestasis [9–12]. In this study, we preliminary investigated why cholestasis develops in NICCD.

2. Materials and methods

Fourteen patients, from 19 to 167 (mean ± S.D., 80 ± 52) days of life, who were proven to be homozygotes or com-

Abbreviations: AGC, aspartate/glutamate carrier; ASS, argininosuccinate synthetase deficiency; BC, biliary cirrhosis; CA, cholic acid; CDC, chenodeoxycholic acid; CTLN2, adult-onset citrullinemia type II; EBA, extrahepatic biliary atresia; INH, idiopathic neonatal hepatitis; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency

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Table 1
Comparison of results of liver function tests in patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), idiopathic neonatal hepatitis (INH) and extrahepatic biliary atresia (EBA)

	NICCD, $n=14$	INH, $n=14$	EBA, $n=14$	Significance* vs. NICCD	
				INH (*P*-value)	EBA (*P*-value)
Total bilirubin (mg/100 ml)	7.0 (2.7)	8.3 (2.7)	9.3 (2.5)	NS	.0158
Direct bilirubin (mg/100 ml)	3.4 (1.4)	5.4 (2.2)	6.2 (2.1)	.0054	.0009
Total bile acids (μmol/l)	229 (59)	150 (50)	121 (38)	.0022	.0002
Total bile acids/direct bilirubin	77 (37)	32 (17)	21 (10)	.0002	<.0001
ALP (IU/l)	1805 (171)	824 (209)	1054 (121)	.0003	.0013
GGTP (IU/l)	181 (26)	73** (28)	256** (46)	.0002	NS
AST (IU/l)	114 (73)	318 (253)	236 (280)	.0179	NS
ALT (IU/l)	56 (42)	286 (259)	146 (148)	.0011	.0131
AST/ALT	2.1 (0.6)	1.2*** (0.2)	1.5*** (0.3)	<.0001	.0067
Total cholesterol (mg/100 ml)	172 (35)	158 (42)	155 (61)	NS	NS

Values are mean (S.D.). NS: not significant. Normal upper range: total bilirubin, <1.0 mg/100 ml; direct bilirubin, <1.0 mg/100 ml; total bile acids, <36 μmol/l (<10 μmol/l in adult); ALP, <450 IU/l; GGTP, <74 IU/l; AST, <63 IU/l; ALT, <35 IU/l; total cholesterol, <197 mg/100 ml.
* Mann–Whitney *U*-test.
** $P<.0001$ (EBA vs. INH).
*** $P=.0047$ (EBA vs. INH).

pound heterozygotes for SLC25A13 gene mutations, were recruited for this study. Gene analysis [5] were performed with the informed consent of their parents. Six of the 14 patients had been previously reported [9,10]. Twelve of the 14 patients examined had diffuse fatty liver, and parenchymal cellular infiltration associated with hepatic fibrosis. Another 14 patients with idiopathic neonatal hepatitis (INH), between 22 and 165 (76 ± 47) days of life, or with extrahepatic biliary atresia (EBA), between 19 and 101 (55 ± 22) days of life, were also recruited in this study, as infantile cholestatic disease controls. All liver biopsy specimens obtained from the 28 patients with INH or EBA showed no fatty liver. In all INH patients, known causative diseases of neonatal hepatitis were eliminated: infectious hepatitis (hepatitis A, B, and C viruses, cytomegalovirus, Epstein–Barr virus, and sepsis), metabolic and inherited diseases (α_1-antitrypsin deficiency, cystic fibrosis, Niemann-Pick disease, and progressive familial intrahepatic cholestasis), chemical hepatic injury, and biliary tract diseases. Results of an urinary organic acid analysis were negative in all 14 NICCD patients. Statistical analyses was performed by the Mann–Whitney's *U*-test.

In addition, urinary bile acid analyses [14] before treatment were performed in three NICCD infants (Nos. 1–3), three INH infants (Nos. 4–6), and three biliary cirrhosis (BC) infants (Nos. 7–9) for a comparative study. The three BC patients had suffered from INH, EBA and hereditary tyrosinemia type I (acute form), respectively.

3. Results

In comparison with INH and EBA patients, NICCD patients had a lower level of serum direct bilirubin, or serum ALT, and had a higher level of serum total bile acids, total bile acids/direct bilirubin ratio, serum ALP, and AST/ALT ratio. Furthermore, these patients had a higher level of serum γ-GTP, and a lower level of serum AST activity than those of INH (Table 1).

The three NICCD patients who underwent urinary bile acid analysis had no significant urinary excretion of 3-oxo-Δ^4 bile acids, or 3β-hydroxy-5-cholenoic acid. However, these patients had a lower excretion of 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid (cholic acid, CA), and a lower CA to 3α, 7α-dihydroxy-5β-cholan-24-oic acid (chenodeoxycholic acid, CDCA) ratio (C/CDC), ≤ 0.51. The three INH patients had a high ratio of C/CDC, ≥ 4.17, and the three BC patients had a low ratio of C/CDC, ≤ 1.29, which was associated with a large amount of urinary 3-keto-Δ^4 bile acids, $\geq 31\%$ of the total urinary bile acids (Table 2).

4. Discussion

Among CTLN2, intrahepatic cholestasis is rare [13], but NICCD patients have transient intrahepatic cholestasis. In NICCD, mitochondrial morphological abnormalities have been reported [9]. However, we do know whether mitochondrial diseases are often associated with intrahepatic cholestasis [15–18]. Citrin is a protein localized in the inner membranes of human mitochondria [6,19]. Citrin dysfunction leads to NADH paucity and ATP depletion in mitochondria. On the other hand, biliary excretions of bile acids and organic anions are mediated by canalicular ATP-dependent transporters; the bile salt export pump (Bsep) and multidrug resistance protein 2 (Mrp2) [20,21]. In this study, we showed that NICCD patients have mild direct hyperbilirubinemia but severe intrahepatic cholestasis. In the cholestatic liver, possible pro-oxidant compounds, such as copper and hydrophobic bile acids, are retained in the hepatocytes. These mitochondrial toxins further cause the inhibition of normal respiration and electron flow through the respiratory chain [22]. Accordingly, we speculate that neonatal citrin deficiency, in the

Table 2
Urinary total bile acids of NICCD and non-NICCD infantile cholestatic patients

	Diagnosis								
	NICCD			INH			BC/INH	BC/HT	BC/EBA
	No. 1/1[a]	No. 2/4[a]	No. 3/5[a]	No. 4/1[a]	No. 5/3[a]	No. 6/4[a]	No. 7/5[a]	No. 8/5[a]	No. 9/6[a]
Total bile acids (μmol/mmol Creatinine)	39.9	11.3	45.1	94.8	40.4	8.4	55.7	14.2	39.2
Cholic acid (%)	5.4	21.6	10.1	65.5	48.4	35.1	20.8	5.4	8.0
Chenodeoxycholic acid (%)	48.8	42.2	53.1	2.8	11.5	8.4	16.1	51.9	17.3
C/CDC	0.11	0.51	0.19	23.30	4.20	4.17	1.29	0.10	0.46
1β, 3α, 7α, 12α-Tetrahydroxy-5β-cholan-24-oic acid (%)	9.7	2.4	13.3	7.9	2.0	26.8	2.0	0.0	0.0
1β, 3α, 7α-Trihydroxy-5β-cholan-24-oic acid (%)	7.6	7.3	6.4	0.9	3.2	6.0	1.6	2.0	0.0
Hyocholic acid (%)	17.1	13.1	11.1	1.6	8.8	9.9	2.4	6.3	0.1
Ursodeoxycholic acid (%)	0.4	0.6	0.3	0.0	1.6	5.4	4.5	0.0	5.7
Deoxycholic acid (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
Lithocholic acid (%)	0.0	0.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0
3β-Hydroxy-5-en-24-oic acid (%)	3.4	5.9	0.6	0.4	0.5	1.3	1.4	2.4	0.1
Total of 3-oxo-Δ^4 bile acids (%)	7.1	6.1	3.6	20.6	23.6	6.7	50.5	68.3	31.8

INH: idiopathic neonatal hepatitis; HT: hereditary tyrosinemia type I (acute form); BC: biliary cirrhosis; EBA: extrahepatic biliary atresia (postoperative); C/CDC: cholic acid/chenodeoxycholic acid ratio; total of 3-oxo-Δ^4 bile acids, include CA-Δ^4-3-one, CA-$\Delta^{4,6}$-3-one, CDCA-Δ^4-3-one, CDCA-$\Delta^{4,6}$-3-one; all-cholic acid, allo-chenodeoxycholic acid 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid, and 3α, 7α-dihydroxy-5β-cholestanoic acid, were not detected in all patients.

[a] Patient/age (months of life).

background of physiological cholestasis [23], might cause intrahepatic cholestasis.

The present study further showed that the AST/ALT ratio of NICCD patients was significantly greater than those of age-matched cholestatic controls. The AST/ALT ratio of patients with adult-onset CTLN2 associated with fatty liver is generally <1.00 [24–26], as well as in non-alcoholic steatohepatitis in adults [27] or idiopathic steatohepatitis in children [28,29]. A low AST/ALT ratio is explained as being due to a larger degree of ALT leakage from the hepatocyte cytoplasm in which AST is located at a lower concentration than ALT. On the other hand, AST is predominantly located in the mitochondria [27]. A high serum AST/ALT ratio is seen in alcoholic steatohepatitis with mitochondrial damage [30]. However, electron microscopy showed normal mitochondrial appearance in a patient with NICCD [9]. Further studies to solve the mechanism of a high AST/ALT ratio seen in NICCD are needed.

In neonatal cholestatic diseases, CA is predominantly excreted into the urine [31], with a small amount of 3-keto-Δ^4 bile acids, from 6% to 23% of the total bile acids [32]. In an infant with liver cirrhosis, urinary 3-keto-Δ^4 bile acids increase as the disease progresses, reaching 64% of the total bile acids [32]. The present study showed that the INH patients examined in this study had a high C/CDC ratio in their urinary specimens, ≥4.17, and a small amount of urinary 3-keto-Δ^4 bile acids, from 6% to 23% of the total bile acids, and that infants with BC had a low C/CDC ratio in their urinary specimens, ≤1.29, and a large amount of urinary 3-keto-Δ^4 bile acids, from 31% to 68% of the total bile acids. This suggests that a low C/CDC ratio of urinary specimens or a large amount of urinary 3-keto-Δ^4 bile acids indicates the presence of advanced liver disease. On the other hand, our NICCD patients had a low C/CDC ratio of urinary specimens and a small amount of 3-keto-Δ^4 bile acids. This suggests the absence of advanced liver disease, and a low activity of 12α-hydroxylase, as seen in fetal bile acid metabolism [33], in NICCD.

In summary, the combination of a primary mitochondrial defect and a delayed maturity of bile acid metabolism in citrin deficiency, in the background of physiological cholestasis, may form a vicious circle of intrahepatic cholestasis in NICCD, and cause transient intrahepatic cholestasis.

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Clinical pictures of 75 patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD)

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Summary We clarified the clinical features of NICCD (neonatal intrahepatic cholestasis caused by citrin deficiency) by retrospective review of symptoms, management and long-term outcome of 75 patients. The data were generated from questionnaires to paediatricians in charge of the patients. Thirty of the patients were referred to hospitals before 1 month of age because of positive results in newborn screening (hypergalactosaemia, hypermethioninaemia, and hyperphenylalaninaemia). The other 45, the screen-negative patients, were referred to hospitals with suspected neonatal hepatitis or biliary atresia because of jaundice or discoloured stool. Most of the screen-negative patients presented before 4 months of age, and 11 had failure to thrive. Laboratory data showed elevated serum bile acid concentrations, hypoproteinaemia, low levels of vitamin K-dependent coagulation factors and hypergalactosaemia. Hypoglycaemia was detected in 18 patients. Serum amino acid analyses showed significant elevation of citrulline and methionine concentrations. Most of the patients were given a lactose-free and/or medium-chain triglyceride-enriched formula and fat-soluble vitamins. Symptoms resolved in all but two of the patients by 12 months of age. The two patients with unresolved symptoms suffered from progressive liver failure and underwent liver transplantation before their first birthday. Another patient developed citrullinaemia type II (CTLN2) at age 16 years. It is important to recognize that NICCD is not always a benign condition.

Abbreviations

CTLN2	citrullinaemia type 2
NICCD	neonatal intrahepatic cholestasis caused by citrin deficiency

Introduction

SLC25A13, the gene newly implicated as the cause of adult-onset type II citrullinaemia (CTLN2, OMIM #603471), encodes an aspartate–glutamate carrier called citrin (Kobayashi et al 1999; Palmieri et al 2001). CTLN2 is characterized by late onset (11–79 years), frequent loss of consciousness with hyperammonaemia, and ultimately death within a few years of onset (Imamura et al 2003; Kobayashi et al 1993, 1997, 1999; Saheki et al 1987; Yasuda et al 2000). Until recently, there was little information about the manifestations of CTLN2 in the neonatal/infantile period. However, *SLC25A13* mutations have been detected in patients with neonatal hepatitis syndrome (Ohura et al 2001; Tazawa et al 2001; Tomomasa et al 2001) and the clinical features of neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM #605814) have been revealed (Ben-Shalom et al 2002; Hachisu et al 2005; Lee et al 2002; Naito et al 2002; Ohura et al 2001, 2003; Tamamori et al 2002; Tanaka et al 2002; Tazawa et al 2001, 2004; Tomomasa et al 2001; Yamaguchi et al 2002). It is now apparent that citrin

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References to electronic databases: CTLN2, OMIM #603471; NICCD, OMIM #605814

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deficiency causes two age-dependent phenotypes, namely CTLN2 in adults and NICCD in infants. While CTLN2 patients have been diagnosed on the basis of well-established criteria (Imamura et al 2003; Kobayashi et al 1993, 1997, 1999; Saheki et al 1987; Yasuda et al 2000), NICCD is less clearly defined. Patients manifest various and transient symptoms, and the criteria for clinical and biochemical diagnosis are still being established. In this study we review clinical data on 75 patients who have been diagnosed with NICCD by DNA analysis.

Patients and methods

Patients

We collected data on the clinical and biochemical features in 75 patients with NICCD by sending out a questionnaire to their paediatricians. All patients had undergone newborn screening for phenylketonuria, homocystinuria, maple syrup urine disease, galactosaemia, hypothyroidism and congenital adrenal hyperplasia at the age of 4–6 days using standard methods. Symptoms, laboratory data, management and long-term outcome of these patients were reviewed retrospectively.

DNA diagnosis of mutations of the *SLC25A13* gene

Genetic analyses for the *SLC25A13* mutations were performed with the consent of the patients' families. The Ethics Committees of the Tohoku University School of Medicine and the Kagoshima University Graduate School of Medical and Dental Sciences approved the study. The procedure for the DNA diagnosis of *SLC25A13* mutations has been reported in detail previously (Kobayashi et al 1999; Tomomasa et al 2001; Yasuda et al 2000).

Results

Patients and mutation types

The 75 patients were classified into two groups, screen-positive and screen-negative (Table 1). Five of the patients were siblings. The female-to-male ratios of the screen-positive and screen-negative patients were 2:1 (30 patients) and 1:1 (42 patients), respectively. The sex of 3 of the screen-negative patients was undetermined. There was no significant difference in the gestational age or birth weight between the two groups, but the birth weight of the NICCD patients overall was lower than the standard birth weight in Japan (3050 g). All patients were confirmed to have citrin deficiency by genetic analysis. The two most frequently detected mutations were 851del4 and IVS11+1G>A (99/140, or 71% of the total alleles). These are the same mutations as those reported most frequently in earlier reviews (Kobayashi et al 2003; Saheki and Kobayashi, 2002; Yamaguchi et al 2002).

Table 1 Perinatal history and *SLC25A13* mutations in 75 patients

	Newborn screening	
	Positive	Negative
Number	30[a]	45[b]
Sex (female: male)	20:10	21:21[c]
Gestational age (wk)	38.5 ± 1.6	39.1 ± 1.4
Birth weight (g)	2533 ± 301	2598 ± 317
Range of birth weight	(1930–3235)	(1988–3202)
Mutation	No. of mutated alleles	
851del4	19	25
IVS11+1G>A	22	33
1638ins23	2	3
S225X	2	1
IVS13+1G>A	3	8
1800ins1	1	4
R605X	0	1
E601X	1	3
Other mutations	1	3
Not determined	3	5

[a]including three siblings; [b]including two siblings; [c]three were gender undetermined

Results of newborn screening

Thirty of the 75 patients were detected through newborn screening (Table 2). All of the screen-positive patients were referred to hospital for further evaluation before the age of 1 month. Thorough metabolic evaluation revealed intrahepatic cholestatic liver disease. Hypergalactosaemia was detected in 8 of the 30 screen-positive patients, hypermethioninaemia was detected in 4, and hyperphenylalaninaemia in 5. Surprisingly, 11 of the screen-positive patients were positive for both methionine and galactose, 1 was positive for both methionine and phenylalanine, and 1 was positive for all three.

Table 2 Results of newborn screening from 30 patients

Positive tests	No. of patients
Gal	8
Met	4
Phe	5
Met & Gal	11
Met & Phe	1
Met, Gal & Phe	1

Gal, galactose; Met, methionine; Phe, phenylalanine

Table 3 Age of the screen-negative patients at the initial visit

Age (mo)	<1	1	2	3	4	5
No. of patients	1	11	16	8	7	2

Table 4 Chief complaints of the 45 screen-negative patients at the initial visit

	No. of patients
Jaundice and/or acholic stools	39
Failure to thrive	11
Increased prothrombin time	2
Hepatomegaly	2
Other symptoms (one patient each):	
Subcutaneous bleeding, Hemolytic anemia, Ascites, Hypoglycemic convulsion, Watery diarrhea, Lethargy	

Some patients had more than one manifestation.

Ages and chief complaints of the screen-negative patients at their first visit

Table 3 shows the age at which the screen-negative patients were first admitted to hospital. All were seen before age 5 months, and more than half were first seen before 2 months.

The chief complaints of the 45 screen-negative patients are presented in Table 4. Presenting features included prolonged jaundice, acholic stool and poor weight gain. Most were referred to hospital as suspected cases of neonatal hepatitis or biliary atresia. Failure to thrive was reported in 11 of these 45 patients, increased prothrombin time in 2, and hepatomegaly in 2. Other manifestations included subcutaneous bleeding, haemolytic anaemia, ascites due to hypoproteinaemia, hypoglycaemic convulsion, watery diarrhoea, and lethargy.

Laboratory data

Laboratory data in the two groups showed similar evidence of liver damage and severe intrahepatic cholestasis (Table 5). Serum total bile acids and conjugated bilirubin were elevated in most patients. Serum transaminase concentrations were mildly elevated, with AST levels usually higher than ALT levels. Total serum protein was measured in 64 of the patients and in 39 it was less than 50 g/L. Levels of vitamin K-dependent coagulation factors were low (<50% of the normal range) in 34 of 49 patients screened. Serum galactose concentration was greater than 1.1 mmol/L in 20 patients out of the 33 in whom it was measured. Three of 54 patients tested for blood ammonia were confirmed to have hyperammonaemia (>110 μmol/L) but none was symptomatic.

Table 5 Laboratory data (mean ± SD)

	NBS-pos. (*n*)	NBS-neg. (*n*)	Reference range
T. Bil (mg/dl)	6.26 ± 2.7 (30)	7.72 ± 3.2 (43)	0.2–1.2
C. Bil (mg/dl)	3.15 ± 1.6 (26)	3.81 ± 1.7 (43)	0–0.7
γ-GTP (IU/L)	301 ± 164 (26)	183 ± 88 (40)	8–57
AST (IU/L)	90.0 ± 88 (29)	147 ± 81 (43)	12–30
ALT (IU/L)	45.9 ± 34 (30)	63.2 ± 40 (43)	8–35
TBA (μmol/L)	254 ± 111 (26)	246 ± 67 (38)	11–28
TP (g/L)	46.5 ± 5.1 (25)	50.5 ± 8 (39)	51–68
PT (%)	37.5 ± 18.1 (19)	43.1 ± 20 (30)	>70
Gal (mmol/L)	2.29 ± 1.76 (22)	2.31 ± 2.54 (11)	<0.2
NH_3 (μmol/L)	69 ± 24 (24)	67 ± 37 (30)	<44

NBS, newborn Screening; pos, positive; neg, negative; *n*, number of patients examined; T. Bil, total bilirubin; C. Bil, conjugated bilirubin; γ-GTP, γ-glutamyl transpeptidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TP, total protein; PT, prothrombin time; Gal, galactose; NH_3, ammonia.

Amino acid levels

The most characteristic feature of the patients was an abnormal amino acid pattern, with significant elevation of citrulline and methionine concentrations (Table 6). Threonine, tyrosine, lysine and arginine were also 2–4 times higher than the control range. Other amino acids were within or near their normal ranges. Hypercitrullinaemia was detected in all of the screen-positive patients. However, the citrulline concentration was normal in 6 of the patients who were negative on newborn screening.

Treatments

Treatments are shown in Table 7. Neonatal hepatitis syndrome or hypergalactosaemia was suspected before the DNA diagnosis of *SLC25A13* in most of the patients. As a result, many received lactose-free or medium-chain triglyceride-enriched formula. Phenylalanine–tyrosine-free formula, protein-free formula and low-methionine formula were used for patients with suspected tyrosinaemia,

Table 6 Amino acid analysis (μmol/L, mean ± SD)

	NBS-pos. (*n*)	NBS-neg. (*n*)	Reference range
Threonine	794 ± 294 (25)	463 ± 235 (37)	65–153
Citrulline	582 ± 360 (27)	314 ± 257 (38)	5.14–37.1
Methionine	346 ± 261 (27)	268 ± 349 (37)	13.4–32.2
Tyrosine	266 ± 143 (26)	203 ± 171 (36)	34.2–93.8
Phenylalanine	114 ± 133 (24)	61.7 ± 33.3 (37)	41.8–112
Lysine	429 ± 150 (24)	360 ± 178 (35)	102–203
Arginine	263 ± 115 (26)	208 ± 121 (37)	28.1–98.7

NBS, Newborn Screening; pos, positive; neg, negative
n, number of patients examined.

Table 7 Treatments

	No. of patients	
	NBS-pos. ($n = 30$)	NBS-neg. ($n = 45$)
Special milk formula		
Lactose-free	19	9
MCT-enriched	7	19
Other formulas	8	15
None	2	16
Medicine		
Fat-soluble vitamins	14	37
Ursodeoxycholic acid	4	27
Phenobarbital	1	12
Fresh frozen plasma	2	3
Glucagon-insulin	0	4
Gamma globulin	0	4

NBS, Newborn Screening; pos, positive; neg, negative
MCT, medium chain triglyceride
Other formulas included phenylalanine-tyrosine-free formula, protein-free formula and low-methionine formula.
Some patients received more than one formula.

hyperammonaemia and hypermethioninaemia, respectively. On the other hand, 18 patients received no special milk formula whatsoever. Two-thirds of the patients were treated with fat-soluble vitamins to prevent the consequences of prolonged cholestasis. Ursodeoxycholic acid and phenobarbital were administered to enhance biliary excretion and control pruritus. Gamma-globulin was administered intravenously in 4 patients with suspected viral hepatitis. Four patients with severe liver damage required treatment with fresh frozen plasma or glucagon-insulin therapy.

Complications and prognosis

Complications are shown in Table 8. Cataracts were reported in 6 patients with hypergalactosaemia. Hypoglycaemia was detected in 18 patients and may be a common symptom in patients with NICCD during infancy. Mild developmental delay was reported in 2 cases, but we could not confirm whether this was the result of the citrin deficiency.

Table 8 Complications & prognosis

	No. of patients
Complications	
Cataracts	6
Hypoglycemia	18
Mild mental retardation	2
Prognosis	
Symptoms resolved	73
Progressive liver failure	2
Development of CTLN2	1

Symptoms resolved by 12 months in all but two of the patients (Table 8). The two unresolved patients reportedly suffered from progressive liver failure and underwent liver transplantation before their first birthday. Another patient was reported to have developed citrullinaemia type II at age 16 years.

Discussion

We have demonstrated that neonatal screening provides an important opportunity for the diagnosis of NICCD (Ohura et al 2001, 2003; Tazawa et al 2004). Thirty of the 75 patients in this survey were detected by elevated concentrations of galactose, methionine and/or phenylalanine. Tamamori and colleagues (Tamamori et al 2004) report that blood citrulline levels in NICCD neonates begin to increase immediately after birth and this is followed by rises in other amino acids and galactose and cholestasis due to hepatic dysfunction. A more efficient method to detect NICCD in infants would be to measure citrulline by tandem mass spectrometry during newborn screening. Shigematsu and colleagues (Shigematsu et al 2002) detected 3 patients with NICCD among 102 200 newborns screened by electrospray tandem mass spectrometry. The incidence of NICCD according to their calculations was ~1 in 34 000. The frequency of homozygotes with *SLC25A13* mutation is considerably higher, however, at an estimated 1 in 19 000 (Kobayashi et al 2003; Lu et al 2005). We speculate that newborn screenings can only detect NICCD patients if the condition manifests during the neonatal period (neonatal onset). The screen-negative patients most likely developed the condition at a later time (infantile onset). We conjecture that some of the NICCD patients might have remained apparently healthy throughout infancy.

All of the screen-positive patients manifested hypercitrullinaemia at their first hospital visits, whereas the amino acid concentrations in 6 screen-negative patients were normal. Hyperaminoacidaemia was transient and usually persisted only for couple of months in the early stage of the disease. It may be that the amino acid abnormality remains undetectable in some screen-negative patients.

An abnormal prothrombin time was a common finding in NICCD (Ohura et al 2003; Tamamori et al 2002; Tazawa et al 2004). Our data revealed that levels of vitamin K-dependent coagulation factors were low (<50% of the normal range) in 34 patients. Among these, two patients developed a critical decrease of coagulation factors (<10% of the normal range), which was probably due not only to malabsorption of vitamin K but also to severe liver damage. The decrease of vitamin K-dependent coagulation factors presents an immediate and constant risk of bleeding. The prothrombin time should be checked repeatedly while cholestatic jaundice persists, and vitamin K should be given if the prothrombin time is