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Sustaining hypercitrullinemia, hypercholesterolemia and augmented oxidative stress in Japanese children with aspartate/glutamate carrier isoform 2-citrin-deficiency even during the silent period

Hironori Nagasaka [a,*], Yoshiyuki Okano [b], Hirokazu Tsukahara [c], Yosuke Shigematsu [c], Toru Momoi [d], Junko Yorifuji [d], Takashi Miida [e], Toshihiro Ohura [f], Keiko Kobayashi [g], Takeyori Saheki [h], Kenichi Hirano [i], Masaki Takayanagi [a], Tohru Yorifuji [d]

[a] Division of Metabolism, Chiba Children's Hospital, 579-1 Heta Cho, Midori-ku, Chiba 266-0007, Japan
[b] Department of Pediatrics, Osaka City Graduate School of Medicine, 1-4-3 Asahimachi, Abenoku, Osaka 545-8585, Japan
[c] Department of Pediatrics, Fukui University Hospital, 23-3 Shimoaizuki, Matsuoka, Eiheiji-cho, Yoshida-gun, Fukui 910-1193, Japan
[d] Department of Pediatrics, Kyoto University Graduate School of Medicine, 54 Shogoin Kawara-machi, Sakkyo-ku, Kyoto 606-8507, Japan
[e] Department of Clinical and Laboratory Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[f] Department of Pediatrics, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
[g] Department of Molecular Metabolism and Biochemical Genetics, Kagoshima University Graduate School of Medicine and Dental Science, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
[h] Institute of Heath Science, Tokushima Bunri University, 180 Yamashirocho, Tokushima 770-8514, Japan
[i] Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadagaoka, Suita 565-0871, Japan

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ABSTRACT

Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) shows diverse metabolic abnormalities such as urea cycle dysfunction together with citrullinemia, galactosemia, and suppressed gluconeogenesis. Such abnormalities apparently resolve during the first year of life. However, metabolic profiles of the silent period remain unknown. We analyzed oxidative stress markers and profiles of amino acids, carbohydrates, and lipids in 20 asymptomatic children with aspartate/glutamate carrier isoform 2-citrin-deficiency aged 1–10 years, for whom tests showed normal liver function. Despite normal plasma ammonia levels, the affected children showed higher blood levels of ornithine ($p < 0.001$) and citrulline ($p < 0.01$)—amino acids involved in the urea cycle—than healthy children. Blood levels of nitrite/nitrate, metabolites of nitric oxide (NO), and asymmetric dimethylarginine inhibiting NO production from arginine were not different between these two groups. Blood glucose, galactose, pyruvate, and lactate levels after 4–5 h fasting were not different between these groups, but the affected group showed a significantly higher lactate to pyruvate ratio. Low-density and high-density lipoprotein cholesterol levels in the affected group were 1.5 times higher than those in the controls. Plasma oxidized low-density lipoprotein apparently increased in the affected children; their levels of urinary oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine and acrolein-lysine were significantly higher than those in the controls. Results of this study showed, even during the silent period, sustained hypercitrullinemia, hypercholesterolemia, and augmented oxidative stress in children with citrin deficiency.

Introduction

Adult-onset type II citrullinemia (CTLN2, OMIM 603471), a disease caused by a mitochondrial transporter, is characterized by frequent bouts of hyperammonemia, liver steatosis, mental derangement, sudden unconsciousness, and ultimately death within a few years of onset [1–3]. In fact, CTLN2 results from mutations of the SLC25A13 gene on chromosome 7q21.3 encoding a calcium-binding mitochondrial protein: a liver-type aspartate/glutamate carrier isoform 2 (AGC2), so-called citrin [3–6]. In the malate-aspartate NADH shuttle and urea synthesis, AGC2 plays an important role [3,7,8]. Impairment of AGC2 function can engender an increased NADH/NAD$^+$ ratio in cytosol. Failure of the aspartate supply from the mitochondria to the cytoplasm for synthesis of argininosuccinate engenders hypercitrullinemia and hyperammonemia.

Clinical characteristics of citrin deficiency vary dramatically by age [1–6,8–12]. About half of the Japanese children diagnosed with citrin deficiency were found to have metabolic abnormalities such as hypergalactosemia, hyperphenylalaninemia, and hypermethioninemia

* Corresponding author. Fax: +81 43 292 3815.
E-mail address: nagasa-hirono@k2.dion.ne.jp (H. Nagasaka).

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by newborn mass screening (NMS) at the age of 5 days. The remaining children visited eligible hospitals to receive precise examinations for prolonged jaundice, acholic stool, and/or failure to thrive during early infancy [9–12]. These children present diverse clinical manifestations, namely neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) such as considerable liver dysfunction, along with cholestasis, citrullinemia, mild hyperammonemia, galactosemia, and hypoglycemia.

The clinical presentations of NICCD resolve from 6 months to one year of life. However, among patients who have manifested NICCD, only one-fifth of patients develop CTLN2 [8–12].

Prompted by the fact that onset of CTLN2 is fatal, we sought the metabolic profiles of affected children from the silent stage: before the onset of CTLN2. Correction of metabolic anomalies at this stage would be expected to prevent the onset of CTLN2.

For this study, we examined the profiles of amino acids, carbohydrates, and lipids. We also examined NO synthesis, which shares processes with the urea cycle [13,14]. Furthermore, the status of oxidative stress, which is related closely to the development of liver steatosis, was evaluated using biomarkers.

In this report, we describe hypercitrullinemia, dyslipidemia and augmented oxidative stress in the affected children. The metabolic abnormalities underlying the development of CTLN2 will be discussed.

Subjects and methods

Subjects and sample collection

This study enrolled 20 children with citrin deficiency (10 males and 10 females, 1 year 10 months – 10 years 5 months) and 32 age-matched healthy children (16 female and 16 males, 2 year 2 months – 9 years 5 months) as controls.

The affected children's blood levels of transaminase, gamma-glutamyl transpeptide, total bile acids, and total bilirubin at that time were entirely normal. Of the 20 affected children, 12 were found to have metabolic abnormalities (hypergalactosemia, $n = 7$; hyperphenylalaninemia, $n = 4$; hypermethioninemia, $n = 2$) by NMS performed at the age of 5 days (Table 1). Thereafter, they developed considerable liver dysfunction along with cholestasis manifesting hyperbilirubinemia, hypoproteinemia, and prolonged coagulation. Precise examination revealed that they had markedly elevated plasma citrulline levels accompanying higher plasma levels of arginine, threonine, tyrosine, and phenylalanine. The remaining eight patients developed hyperbilirubinemia and visited their respective hospitals at the ages of 1–4.5 months. Precise examination detected prominent citrullinemia accompanying higher plasma arginine, threonine, tyrosine, and phenylalanine levels.

They were diagnosed as having citrin deficiency at ages of 3 weeks – 2 years 2 months based on gene analyses for the SLC25A13 determining the genotypes as follows: [I] 851del4, [II] IVS11 + 1G > A, [III] 1638ins23, [IV] S225X, [V] IVS13 + 1G > A, [VI] 1800ins1, [VII] R605X, [VIII] E601X, [IX] E601 K, [X] *IVS6 + 5-G > A*, [XI] R184X and [XIV] IVS6 + 1G > C (Table 1) [4–6].

The liver function tests at the ages of 4–12 months yielded normal results. Their blood levels of transaminase, gamma-glutamyl transpeptidase, total bile acids, and total bilirubin at presentation were entirely normal.

Blood and urine samples were collected at 10:30–11:30 before lunch after 4–5 h fasting. The methods and purpose of the study were explained to each child's parents. Their informed consent was obtained before enrollment of the child. Approval of the project was obtained from the institutional medical ethics committee.

Methods

Estimation of amino acids metabolism in terms of urea cycle

The urea cycle is initiated by carbamoylphosphate synthesis from ammonia via carbamoylphosphate synthetase, a limiting enzyme in the urea cycle [13]. Carbamoylphosphate is subsequently transformed into citrulline, which is ultimately transformed into arginine via argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) under a supply of aspartate by citrin [7,8,13]. To estimate the urea cycle function, plasma levels of ammonia and amino acids—including citrulline, arginine and ornithine—were examined. Plasma amino acids were determined using routine ion-exchange chromatography with an auto-analyzer (L822; Hitachi High-Technologies Corp., Tokyo, Japan).

Estimation of NO pathway

Arginine and citrulline are also involved in the nitric oxide (NO) pathway [13,14]. In fact, NO is synthesized from arginine by NO

Table 1
Background and present liver functions in patients.

Pt	Age at present	M/F	Gene mutations	NMS	Age at diagnosis	Liver dysfunction	AST (IU/L)	ALT (IU/L)	Γ-GTP (IU/L)	TBA (μmol/L)
1	2y7m	F	II/II	Met	3w	3w–6m	31	13	12	3.3
2	5y3m	M	II/V	Gal	5m	1m–5m	42	23	14	4
3	5y4m	M	II/V	(–)	4.5m	4.5m–7.5m	35	22	14	7.6
4	6y7m	M	I/II	Phe	1m	1m–5.5m	34	30	16	3.4
5	4y10m	F	I/II	Phe	1m	1m–4.5m	36	15	11	4.9
6	1y8m	M	I/V	Gal	22d	3w–7m	48	20	21	3.2
7	10y5m	M	II/VIII	(–)	1m	1m–4m	26	16	10	4.9
8	5y9m	M	II/II	(–)	1y2m	1m–12m	29	16	13	16.2
9	4y1m	F	II/V	Gal	3m	1m–8m	33	23	15	3.1
10	3y3m	M	II/II	Gal	4m	3w–11m	19	27	9	6.5
11	5y6m	M	II/V	(–)	2m	2m–9m	40	35	11	7.1
12	4y9m	F	III/V	Phe	2m	1m–7m	29	29	18	5.2
13	3y7m	F	II/VI	(–)	3m	3m–11m	37	34	15	5.5
14	6y2m	M	I/I	Gal	3m	1m–6m	31	16	11	4.1
15	4y0m	M	I/VI	Met	4m	3w–10m	39	19	15	8.2
16	2y11m	F	I/II	(–)	5m	2m–7m	23	20	10	3.2
17	3y11m	F	II/II	Phe	1m	1m–4m	37	27	16	6.5
18	7y1m	M	V/XIX	(–)	2y2m	2m–11m	28	14	14	5.9
19	5y5m	F	I/II	Gal	5m	1m–7m	22	11	11	2.9
20	5y6m	M	I/I	Gal	4m	3w–8m	39	26	14	4.9

NMS, newborn mass screening at 5 days of age; AST, aspartate aminotransferase (normal range: 5–40 IU/L); ALT, alanine aminotransferase (5–40 IU/L); Γ-GTP, gamma-glutamyl-transpeptidase (5–60 IU/L); TBA, total bile acids (2–15 μmol/L); Gal, hypergalactosemia; Phe, hyperphenylalaninemia; Met, hypermethioninemia.

synthase (NOS). The availability of arginine is a rate-limiting factor in cellular NO production. Citrulline, a by-product of the NOS reaction, is recycled to arginine through successive actions of ASS and ASL, forming the citrulline-NO cycle. Therefore, in this study, blood levels of nitrite/nitrate (NO_x^-) as stable metabolites of NO and asymmetric dimethylarginine (ADMA) as a putative inhibitor of NOS were determined to estimate the NO pathway activity [15].

Serum levels of (NO_x^-) and ADMA were measured using the Griess method (nitrate/nitrite colorimetric assay; Cayman Chemical, Ann Arbor, ML, USA) and a recently developed enzyme-linked-immunosorbent assay method (ADMA–ELISA; DLD Diagnostika GmbH, Hamburg, Germany) [16]. Competitive ADMA–ELISA uses the microtiter plate format. Briefly, serum samples, as well as standards and kit controls, are acetylated in 96-well plates. The acetylated samples, standards and kit controls are pipetted into the respective wells of the ADMA-coated microtiter strips and incubated with a polyclonal antibody (rabbit-anti-*N*-acetyl-ADMA). After incubation, the antiserum solution is discharged and the wells are washed with washing buffer. A peroxidase-conjugated secondary antibody is added; then all wells are washed and incubated with tetramethylbenzidine solution as the substrate for peroxidase. The enzymatic reaction is stopped using an acidic stop solution; the absorbance is then measured using a microplate reader at 450 nm. The amount of antibody bound to the solid-phase ADMA is inversely proportional to the ADMA of the sample concentration.

Estimation of carbohydrate metabolism

Carbohydrate metabolism was estimated indirectly using blood glucose, galactose, lactate, and pyruvate levels. Blood levels of glucose, lactate, and pyruvate were determined using their respective enzymatic methods. Blood galactose concentrations in dried blood spots were determined with microassay using a fluorometric microplate reader, as described by Yamaguchi and colleagues [17].

Estimation of lipid metabolism

Lipid metabolism was estimated according to the blood levels of free fatty acids (FFA), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), phospholipids (PL), triglycerides (TG), and apolipoprotein (apo) levels. Malondialdehyde-modified LDL (MDA-LDL) as oxidized LDL (Ox LDL) was also determined.

Serum levels of TC, PL, and TG were determined with commercial kits (Kyowa Medex Co. Ltd., Tokyo, Japan) using enzymatic methods. Then HDL-C was measured using 13% polyethylene glycol (PEG 300; Wako Pure Chemical Industries Ltd., Osaka, Japan.) [18]. The serum FFA level was measured using enzymatic methods (NEFA-SS kit Eiken; Eiken Chemical Co. Ltd., Tokyo, Japan). Furthermore, LDL-C was measured using an enzyme immunoassay with a commercial kit (LDL-C Daiichi; Daiichi Pure Chemicals Co. Ltd.). Serum levels of apoA-I, apo-B, apo-CII, and apo-E were determined using turbidimetric immunoassay (Apo-AI, apo-B, apo-CII, and Apo-E Auto-N 'Daiichi'; Daiichi Pure Chemicals Co. Ltd.). A sensitive enzyme-linked immunosorbent assay for detection of MDA-LDL in serum was used for determination of oxidized LDL [19]. In this assay, a monoclonal antibody interacts with MDA-apo-B.

Western blot analyses of biopsy specimens

Liver samples were obtained from two affected children (patients 19 and 20) by percutaneous liver biopsy (Table 1). Liver expressions of three important lipoprotein regulators were examined: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase as a key enzyme of cholesterol synthesis, LDL-receptor and scavenger receptor B-I (SR-BI) as a major HDL receptor. As control livers, liver fragments obtained from two non-related donors for liver transplantation (1 male and 1 female) with ages of 28 and 33 years were used; they were entirely healthy. The donors had no history of smoking.

The frozen samples (50–100 mg) were divided into cytoplasmic and nuclear fractions using nuclear and cytoplasmic extraction reagent kits (NE-PER™; Pierce Biotechnology Inc., Rockford, IL). The former was used for analyses of SR-BI, LDL-receptor, and HMG-CoA reductase.

These samples were separated using 10% SDS–polyacrylamide gel electrophoresis. Then they were transferred to nitrocellulose membranes using a semi-dry transfer unit.

After blocking with Tris-buffered saline containing 10% non-fat dried milk, the membranes were reacted with primary antibodies and then with peroxidase-conjugated secondary antibodies. After vigorous washing, the membranes were incubated with an enhanced chemiluminescence reagent (ECL; GE Healthcare Life Sciences, Tokyo, Japan), and exposed to X-ray film. The following primary antibodies were purchased from two companies: HMG-CoA reductase (mouse, polyclonal; Abcam plc., Cambridge, UK), SR-BI (goat, polyclonal; Lifespan Biosciences Inc., Seattle, WA), and LDL-receptor (chicken, polyclonal; Abcam plc.).

Estimation of oxidative stress

As the marker for oxidative stress, urinary acrolein-lysine reflecting the amounts of lipid peroxidation products in plasma and urine and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) reflecting oxidative DNA damage were examined. Furthermore, vitamin E, functioning as an antioxidant in plasma and activities of anti-oxidative enzymes such as catalase and superoxide-dismutase (SOD) in erythrocytes, was examined.

Concentrations of urinary acrolein-lysine and 8-OHdG were determined, respectively, using competitive ELISA kits: ACR-Lysine Adduct ELISA (NOF Corp., Tokyo, Japan) and 8-OHdG Check (the Institute for the Control of Aging, Shizuoka, Japan) [20]. Plasma vitamin E levels were measured using high-performance liquid chromatography (HPLC), as described in a previous report [21].

The SOD activity was determined using spectrophotometry at 505 nm (RANSOD kit; Randox Laboratories Ltd.; Antrim, United Kingdom), as described in a previous report [22]. Catalase activity was determined using the method described by Aebi [23]. In brief, we monitored the decrease in absorbance at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 M potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml.

Results

Effects on amino acids involved in the urea cycle and NO pathway

Among amino acids, despite the normal plasma ammonia level, ornithine and citrulline levels of the affected children were, respectively, 1.7 times ($p < 0.001$) and 1.4 times ($p < 0.01$) higher than those of the controls. Although their arginine level was 0.87 times as high as the controls' level, no significant difference was found between these two groups (Table 2).

The other amino acid levels of the affected patients were comparable to those of the control levels. Blood NO_x^- and ADMA levels were not different between the two groups, suggesting that the NO pathway in the affected children remained normal (Table 2).

Effects on carbohydrate metabolism

No significant difference was found between these two groups' blood glucose, galactose, lactate, or pyruvate levels at fasting

Table 2
Blood levels of amino acids involved in the urea cycle, NO_x^- and ADMA.

	Arginine (μmol/L)	Ornithine*** (μmol/L)	Citrulline** (μmol/L)	NO_x^- (μmol/L)	ADMA (μmol/L)	Ammonia (μg/dl)
20 patients	74.2(14.4)	105.1(24.2)	40.8(6.3)	31(5)	0.78(0.11)	35(14)
Ranges	45.4–137.8	65.0–193.4	25.3–56.4	22–49	0.60–1.12	20–91
32 controls	85.0(13.2)	61.3(13.6)	28.2(6.3)	30(9)	0.63(0.17)	31(9)
Ranges	52.8–106.8	40.1–90.0	14.4–41.4	22–49	0.42–0.97	18–49

NO_x^-, nitrite/nitrate; ADMA, asymmetric dimethylarginine.
Presented data are mean (SD) values and ranges.
** $p < 0.01$ versus controls.
*** $p < 0.001$ versus controls.

Table 3
Blood levels of carbohydrate at 4–5 h fasting.

	Glucose (mg/dl)	Galactose (mg/dl)	Pyruvate (mg/dl)	Lactate (mg/dl)	L/P*
Patients ($n = 20$)	84(5)	0.3(0.1)	0.8(0.3)	12(3)	15(2)
Ranges	72–95	0.1–0.6	0.2–1.7	6–26	9–18
Controls ($n = 32$)	85(5)	0.3(0.1)	0.8(0.2)	10(4)	11(1)
Ranges	76–99	0.1–0.5	0.3–1.1	7–19	7–13

L/P: ratio of lactate to pyruvate.
Presented data are mean (SD) values and the ranges.
* $p < 0.05$ versus controls.

(Table 3). The *L/P* ratio in the affected children was significantly higher than that in the controls ($p < 0.05$), suggesting a high ratio of NADH to NAD^+ and/or suppressed mitochondrial functions in the affected children [24].

Effects on lipid metabolism

Serum LDL-C and HDL-C levels in the affected children were 1.5 times higher than those in the age-matched controls, resulting in high total cholesterol levels (Table 4). Triglycerides and FFA levels were not different between these two groups. The apo-AI and apo-B levels in the affected children were apparently higher than those in the controls, respectively, reflecting the higher LDL-C and HDL-C levels (Table 4). Surprisingly, oxidized LDL levels were much higher in the affected patients.

Western blot analyses showed that liver HMG-CoA reductase expression was elevated in the two affected children, although their liver LDL-receptor and SR-BI expressions were similar to those in the control subjects (Fig. 1).

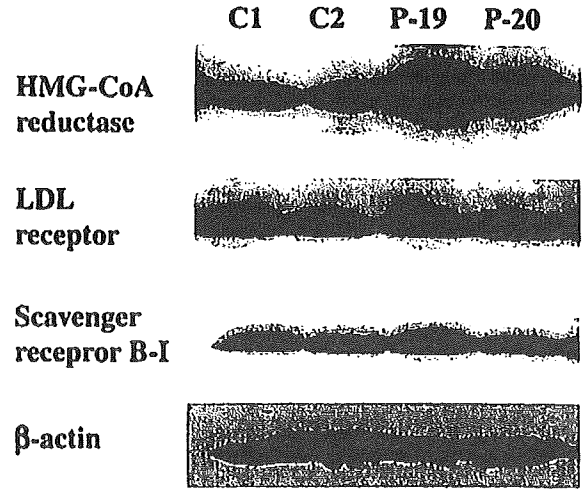

Fig. 1. Western blots of liver biopsy specimens against 3-hydroxy-3-methylglutaryl-coenzyme A reductase, low-density lipoprotein receptor and scavenger receptor B-I. HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LDL-receptor, low-density lipoprotein receptor. Liver biopsy specimens were obtained from healthy controls (C1 and C2) and two affected children (P19 and P20).

Effects of oxidative stress

Urinary acrolein-lysine and urinary 8-OHdG in the affected children were significantly higher than those in the age-matched con-

Table 4
Blood lipid and apolipoprotein levels at a fasting state.

Lipids	TC*** (mg/dl)	LDL-C*** (mg/dl)	HDL-C*** (mg/dl)	PL*** (mg/dl)	TG (mg/dl)	FFA (mmol/L)
Patients ($n = 20$)	213(32)	116(23)	79(7)	237(40)	77(19)	0.8(0.2)
Ranges	153–319	76–196	54–108	189–333	38–124	0.4–1.2
Controls ($n = 32$)	169(22)	85(13)	54(11)	193(25)	80(25)	0.9(0.2)
Ranges	111–207	42–106	39–77	123–239	35–139	0.4–1.5
Apoproteins & Ox LDL	Apo-AI*** (mg/dl)	Apo-AII *** (mg/dl)	Apo-B*** (mg/dl)	Apo-CII* (mg/dl)	Apo-E* (mg/dl)	Ox LDL*** (U/L)
Patients ($n=20$)	169(20)	36(5)	111(22)	3.5(1.1)	5.6(1.5)	82(24)
Ranges	133–213	30–50	79–192	1.5–7.5	3.1–9.4	39–156
Controls ($n=32$)	127(16)	30(4)	78(13)	3.1(1.1)	4.6(1.1)	25(7)
Ranges	88–165	20–42	48–109	0.8–5.5	1.9–7.5	5–50

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; PL, phospholipids; TG, triglycerides; FFA, free fatty acids; Ox LDL, oxidized LDL.
Presented data are mean (SD) values and the ranges.
* $p < 0.05$ versus controls.
*** $p < 0.001$ versus controls.

Table 5
Levels of urinary biomarkers for oxidative stress, anti-oxidant enzyme activities in erythrocytes and blood vitamin E level.

	3-OHdG*** (ng/mg Cr)	Acrolein-lysine** (nmol/mg Cr)	SOD*	Cat*	Vitamin E** (mg/dl)
Patients ($n = 13$)	67(21)	481(125)	1.49(0.34)	3.60(0.52)	0.60(0.21)
Ranges	32–100	220–686	0.92–1.92	2.77–4.44	0.32–1.29
Controls ($n = 32$)	19(5)	272(90)	1.06(0.18)	2.96(0.21)	0.98(0.14)
Ranges	11–29	70–424	0.80–1.50	2.55–3.56	0.67–1.45

3-OHdG; urinary 8-hydroxy-2'-deoxyguanosine; SOD: superoxide-dismutase (U/mg protein); Cat, catalase (pmol/mg protein); Cr, creatinine.
Presented data are mean (SD) values and ranges.
* $p < 0.05$ versus controls.
** $p < 0.01$ versus controls.
*** $p < 0.001$ versus controls.

trols (acrolein-lysine, $p < 0.01$; 8-OHdG, $p < 0.001$). In contrast, blood vitamin E levels in the affected patients were significantly lower than those in the controls (Table 5). Erythrocyte SOD and catalase activities in the affected patients were significantly higher than those in the age-matched controls ($p < 0.05$) (Table 5).

These findings suggest that the affected children were substantially influenced by oxidative stress.

Discussion

Citrin deficiency manifests as NICCD during neonatal and infancy periods. Clinical manifestations of NICCD resolve at around 12 months of age, with no subsequent overt clinical presentations [1,3,6,8–12]. However, about one-fifth of affected subjects develop CTLN2 at ages of 11–79 years. It has been postulated that multiple factors involving diet can determine whether the affected patients develop CTLN2.

Results of recent studies indicate that the affected children prefer a low-carbohydrate high-fat/protein diet [25] and that a high-protein low-carbohydrate diet is effective for CTLN2 [26]. The diet patterns of the affected children enrolled in this study apparently differed from those of age-matched healthy children, although the total daily energy intake was not significantly different between these two groups. The former favored a low-carbohydrate high-lipid/protein diet: energies obtained from respective nutritional components to the total daily energy ratios were the following: carbohydrates, 35 ± 5% (control, 55 ± 3%); fat, 45 ± 4% (29 ± 2%); protein, 20 ± 3% (14 ± 2%). It is likely that their low-carbohydrate high-lipid/protein diets affected the metabolic profiles.

Results of this study suggest that the affected children at the silent stage differed from age-matched healthy children in many metabolic aspects.

Despite the high-protein intake, plasma ammonia levels in the affected children were comparable to those in healthy age-matched controls. However, plasma citrulline and ornithine levels were substantially higher in the affected children than in the healthy children. Citrulline is synthesized from ornithine and carbamoylphosphate by ornithine transcarbamylase. It is subsequently transformed by ASS into argininosuccinate under the supply of aspartate from the mitochondria by AGC2-citrin [3,7,8]. From this context, amino-profiles of affected children enabled us to assume that the supply of aspartate to cytosol from mitochondrial fraction remains at lower levels in them. Strikingly high plasma citrulline levels in NICCD and CTLN2 engender high synthesis of arginine by ASS and ASL in kidney or intestine, resulting in a high plasma arginine level [1,2,27]. As compared to NICCD and CTLN2, the increase in plasma citrulline level at the silent stage was too minute to increase plasma arginine levels.

The NO synthesis in the affected patients remained normal. Their NO_x^- and ADMA levels resembled those in the age-matched healthy controls. We recently reported that urea cycle defects exhibiting markedly abnormal arginine and citrulline levels show abnormal NO synthesis [28]. The citrulline level abnormalities in the affected children during the silent period might be too slight to affect NO synthesis.

The *L/P* ratio in the affected children was significantly higher than that in the controls, suggesting that their NADH to NAD^+ ratio remains high even during the silent period. The *L/P* ratio is determined by the NADH to NAD^+ ratio in cytosol [24]. It has been postulated that citrin plays a crucial role in the regulation of the NADH to NAD^+ ratio in the cytosol and that citrin deficiency presents a high NADH to NAD^+ ratio [3,7,8].

The affected children showed high levels of serum total cholesterol, LDL-C, and HDL-C. The mechanistic explanation for the hypercholesterolemia remains unclear. Affected patients favor a considerably high-lipid diet [25]. Accordingly, their hypercholesterolemia is expected to be at least partly attributable to such a dietary habit. Using Western blot analysis, we examined HMG-CoA reductase expressions in liver samples that had been obtained by percutaneous liver biopsies from a few affected patients. The results suggest that liver HMG-CoA reductase expression was increased in such patients, although expressions of LDL-receptor and HDL receptor such as SR-BI in their liver tissues were comparable to those in the controls (Fig. 1). For that reason, we now assume that hypercholesterolemia in the affected children was at least partly attributable to increased cholesterol synthesis.

On the other hand, their triglyceride levels remained at values comparable to those of the age-matched healthy children. As described above, our patients consumed a considerably low-carbohydrate diet during this study. Probably, such a diet prevented hypertriglyceridemia. A high-carbohydrate diet has been proven to promote production of NADH and thereby stimulate productions of triglycerides [1,8,29,30]. In particular, citrin-deficient individuals are apparently directed easily to hypertriglyceridemia [1,8,26]. We found in many cases that CTLN2 patients developed hypertriglyceridemia when consuming a high-carbohydrate diet. Furthermore, we are now following two citrin-deficit adults with postprandial hypertriglycemia but without overt liver dysfunction.

We inferred that the affected children were subjected persistently to considerable oxidative stress, although is difficult to judge the magnitude of the oxidative stress merely using oxidative stress biomarkers. Nevertheless, decreased blood vitamin E levels and increased erythrocyte anti-oxidant enzyme activities implied augmented oxidative stress in the affected patients. The increased SOD and catalase activities in erythrocytes can be interpreted as responses to increased plasma oxidants. The considerable increase in oxidized LDL supports this notion.

We speculate that the augmented oxidative stress might be partly attributable to the increased cytosolic NADH. Accumulation of cytosolic NADH has been shown to have some probability of causing oxidative stress [7,31,32].

Evidence that dyslipidemia and oxidative stress are closely related to development of liver steatosis or steatohepatitis has been accumulating [33,34]. Considering that citrin deficiency often

develops liver steatosis as a clinical presentation of CTLN2 in later life, it might be important to improve dyslipidemia and to reduce oxidative stress for management of citrin deficiency.

Results of this study show that metabolic abnormalities such as hypercitrullinemia and hypercholesterolemia were sustained in children with citrin deficiency, even during the silent stage. Results provide evidence that the affected children were subjected persistently to oxidative stress.

Further study is necessary to determine whether such sustained metabolic abnormalities might induce development of CTLN2.

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Liver, Pancreas and Biliary Tract

Neonatal intrahepatic cholestasis caused by citrin deficiency: Clinical and laboratory investigation of 13 subjects in mainland of China☆

Y.-Z. Song[a,d,*], B.-X. Li[a], F.-P. Chen[b], S.-R. Liu[c], J.-S. Sheng[d], M. Ushikai[d], C.-H. Zhang[e], T. Zhang[f], Z.-N. Wang[g], K. Kobayashi[d], T. Saheki[d,h], X.-Y. Zheng[i]

[a] Department of Pediatrics, First Affiliated Hospital, Jinan University, Guangzhou 510630, China
[b] Department of Laboratory Science, First Affiliated Hospital, Jinan University, Guangzhou 510630, China
[c] Medical Imaging Center, First Affiliated Hospital, Jinan University, Guangzhou 510630, China
[d] Department of Molecular Metabolism and Biochemical Genetics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan
[e] Matsumoto Institute of Life Science International, Kanazawa 921-8154, Japan
[f] Capital Institute of Pediatrics, Beijing 100020, China
[g] Department of Obstetrics and Gynecology, First Affiliated Hospital, Jinan University, Guangzhou 510630, China
[h] Institute for Health Sciences, Tokushima Bunri University, Tokushima 770-8514, Japan
[i] Institute of Population Research, Peking University, Beijing 100871, China

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ABSTRACT

Background: Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) is a novel inborn error of metabolism due to dysfunction of citrin protein, and much more information about this new disease is still needed for its clinical management.
Aims: To investigate in detail the clinical and laboratory features of NICCD.
Patients: 13 NICCD subjects in mainland of China diagnosed in our department since 2006.
Methods: The anthropometric parameters of the patients at birth were compared with controls, representative biochemical changes and metabolome findings were investigated cross-sectionally, and mutations in the causative gene *SLC25A13* were analyzed by protocols established previously.
Results: The patients showed reduced birth weight, length and ponderal index. Main clinical manifestations consisted of jaundice, hepato/hepatosplenomegaly and steatohepatosis on ultrasonography. Biochemical analysis revealed intrahepatic cholestasis, delayed switch of AFP to albumin, and elevated triglyceride, total cholesterol and LDL-cholesterol together with reduced HDL-cholesterol. Metabolome findings included co-existence of markers for galactosemia and tyrosinemia in urine, and elevated Cit, Met, Thr, Tyr, Lys, Arg and Orn in blood. Mutations of 851-854del, IVS6+5G>A, 1638-1660dup, A541D, IVS16ins3kb, R319X and G333D were detected in the gene *SLC25A13*.
Conclusions: The diagnosis of NICCD cannot be established based just on the numerous but non-specific clinical manifestations and biochemical changes. The relatively specific metabolome features provide valuable tools for its screening and diagnosis, while *SLC25A13* mutation analysis should be taken as one of the reliable tools for the definitive diagnosis. The body proportionality at birth, steatohepatosis on ultrasonography, delayed switch of AFP to albumin, dyslipidemia pattern, urinary metabolome features and the novel mutation G333D expanded the clinical spectrum of NICCD.

1. Introduction

Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM 605814) is a novel entity of inherited metabolic disease due to dysfunction of citrin, a liver-type aspartate/glutamate carrier protein located within mitochondrial inner membrane [1,2]. The causative gene *SLC25A13* locating on chromosome 7q21.3 was identified by means of homozygosity mapping and positional cloning by Kobayashi's group in 1999 [3]. NICCD was first described in Japanese nearly at the same time in the year 2001 [4–6]. Since then more and more NICCD patients were diagnosed,

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* Corresponding author at: Department of Pediatrics, First Affiliated Hospital, Jinan University, No.613, Huang Pu Da dao Xi, Guangzhou 510630, Guangdong, China. Tel.: +86 20 38688642; fax: +86 20 38688000.
E-mail address: songyuanzong@tom.com (Y.-Z. Song).

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however, most of them are Japanese [7–9] although some cases in such East Asians as Chinese, Vietnamese and Korean have also been reported in recent years [8–17]. Furthermore, NICCD is now recognized as a novel disease with the feature of world-wide distribution, since some Caucasian, Ashkenazi Jewish, Pakistani, and Israeli patients in USA, UK, Netherland, Czech Republic and Israel, although still quite rare in number, have also been identified by mutation analysis in our group [8,9,18–20]. In 2006, we reported a NICCD patient in mainland of China by international collaboration [11], and from then on, 13 such cases from 12 families have been definitely diagnosed by analysis of the gene *SLC25A13*. More recently, our selective screening investigation of inborn errors of metabolism revealed a second highest positive rate of NICCD just behind methylmalonic aciduria in high-risk Chinese population [21]. However, it should be recognized that much more clinical and laboratory information about NICCD still need further accumulation and exploration at current stage, which is absolutely necessary for pediatricians to make a correct diagnosis and therapeutic measures at an earlier stage. In this paper, we investigate the clinical manifestations of the 13 NICCD patients and report their *SLC25A13* mutations.

2. Subjects and methods

2.1. Subjects

Since 2006, 13 NICCD patients have been diagnosed in our department, all with origin of Chinese Han population. Eleven patients came from south China, including nine from Guangdong, one from Zhejiang and one from Shanghai, respectively. There are only two patients from north China, with one from Shandong and one from Hebei, respectively. None of the 13 subjects have parental consanguineous marriages or similar patients in the same family except P1194. After referral to our hospital, most patients were diagnosed clinically as NICCD according to the major evidences summarized in reference [11], however, three patients were, at first stage, misdiagnosed as tyrosinemia, galactosemia, and CMV hepatitis, respectively, with NICCD diagnosed after *SLC25A13* gene mutation analysis. After establishment of the diagnosis, therapeutic interventions were performed, and their prognosis followed up for at least 6 months by clinical visits, telephone and email.

2.2. Case-control study and statistics

We compared the anthropometry parameters at birth, including weight, length and ponderal index [PI, represented as weight (g) × 100/length(cm)^3] of 12 NICCD patients with that of 12 gestational age (GA), gender and deliver pattern-matched normal neonates. All parameters were presented as mean ± S.D., and Student test (*t*-test) was used to evaluate the significance of the differences between the patient and control groups.

2.3. Cross-sectional investigation

The major clinical manifestations, representative blood biochemical changes and metabolome findings were collected, analyzed, and described cross-sectionally. Some data were collected from the copies of clinic or hospitalization records in other hospitals, provided by parents of the patients at their referrals to the authors for diagnosis, treatment and genetic counseling.

2.4. Mutation analysis

Dried blood spots on filter paper of the subjects were collected with the informed consents of their parents. DNA was extracted and 13–17 known mutations in the gene *SLC25A13* were screened by means of PCR without or with restriction endonuclease digestion (PCR-RFLP), GeneScan and SNaPshot established and described previously by our group [3,8,10,22–24]. We used direct sequencing of DNA fragments amplified by genomic DNA-PCR to identify novel mutation in the gene *SLC25A13* [8]. Furthermore, we developed a protocol of PCR-RFLP for the molecular diagnosis of the mutation G333D. Sequences of the forward and backward primers for PCR amplification of the mutation are 5′-TGCCTGGCCTCAGTGATGTT-3′ and 5′-CCTGTCTTTGGAAGGCCTGA-3′, respectively. The restriction endonuclease is Mbo I. This study was approved by the Committee for Ethics of Kagoshima University Faculty of Medicine, and adheres to the principles of the Declaration of Helsinki.

3. Results

3.1. General clinical presentations

The NICCD subjects were comprised of seven females and six males, with onset ages as early as 15 (3–42) days (n = 13), but

Table 1
Anthropometric parameters at birth and *SLC25A13* mutations in the NICCD subjects.

Patients	Gender	Gestational age (weeks)	Birth weight (g)	Birth length (cm)	Ponderal Index	Mutations
P1071	Male	39.0	3050	49.0	2.59	851del4/1638-1660dup
P1194	Female	40.0	3250	50.0	2.60	851del4/A541D
P1194S	Female	40.0	2800	48.0	2.53	851del4/A541D
P1443	Male	40.0	3000	50.0	2.40	IVS6+5G>A/R319X
P1478	Female	40.0	2800	50.0	**2.24**	851del4/851del4
P1482	Male	unknown	unkown	unknown	unknown	851del4/851del4
P1495	Female	39.3	3350	50.0	2.68	851del4/G333D
P1513	Female	37.7	**2250**	46.0	2.31	851del4/IVS16ins3kb
P1628	Male	40.1	2550	49.0	**2.17**	851del4/IVS6+5G>A
P1638	Male	38.6	**2310**	45.0	2.53	851del4/1638-1660dup
P1643	Female	40.9	**2225**	49.0	**1.89**	851del4/?
P1644	Female	38.0	2750	48.0	2.49	851del4/IVS6+5G>A
P1648	Male	38.9	**2400**	46.0	2.47	851del4/851del4
Patients group (n = 12)		39.4 ± 1.0	2728 ± 387$^{\Delta}$	48.3 ± 1.8*	2.41 ± 0.22*	–
Control group (n = 12)		39.4 ± 1.0	3202 ± 254	49.8 ± 1.1	2.60 ± 0.14	–

The birth weight values in bold black indicate IUGR, while ponderal index (PI) values in bold black suggest asymmetric body proportionality. P1194S is the elder sister of P1194, and the mutations in the 24 *SLC25A13* alleles from the 12 families were listed.

* $p < 0.05$ compared with control.

$^{\Delta}$ $p < 0.01$ compared with control.

Table 2
Major clinical manifestations and representative blood biochemical changes in the NICCD subjects.

Major clinical manifestations			Tested cases	Positive cases	Positive rate (%)
Jaundice (prolonged/late-onset/recurrent)			13	11 (5/3/3)	84.6
Hepato/hepatosplenomegaly			13	6/2	61.5
Failure to thrive (FTT)			13	7	53.8
Prolonged prothrombin time			8	4	50.0
Steatohepatosis on ultrasonography			11	5	45.5
Diarrhea			13	4	30.8
Poor appetite			12	3	25.0
Anemia			13	3	23.1
Positive ketonuria			11	1	9.1
Digestive tract hemorrhage			13	1	7.7
Representative blood biochemical changes			Tested cases	Positive cases	Positive rate (%)
Parameters	Mean ± S.D.	Reference range			
ALT (U/L)	38.8 ± 15.1	5–40	11	4↑	36.4↑
AST (U/L)	90.9 ± 54.6	5–40	11	10↑	90.9↑
GGT (U/L)	251 ± 96	8–50	11	11↑	100↑
ALP (U/L)	908 ± 315	20–220	11	11↑	100↑
LDH (U/L)	476 ± 161	50–240	7	6↑	85.7↑
TBil (μmol/L)	149.3 ± 80.7	2–19	11	11↑	100↑
DBil (μmol/L)	68.0 ± 46.7	0–6	11	11↑	100↑
TBA (μmol/L)	223.9 ± 88.1	0–10	11	11↑	100↑
TP (g/L)	48.2 ± 8.8	60.0–83.0	11	10↓	90.9↓
ALB (g/L)	30.6 ± 3.5	35.0–55.0	11	10↓	90.9↓
GLB (g/L)	17.7 ± 5.6	20.0–30.0	11	9↓	81.8↓
A/G	1.83 ± 0.38	1.5–2.5	11	2↓	18.2↓
AFP (ng/mL)	74705.8(52.0–319225.7)	0–28	8	8↑	100↑
Lac (mmol/L)	2.9 ± 0.8	0.5–2.0	5	4↑	80↑
Ammonia (μg/dL)	101.8 ± 40.2	<91	9	5↑	55.6↑
BS (mmol/L)	2.80 ± 1.44	3.9–6.1	10	4↓#	40↓
TG (mmol/L)	2.22 ± 1.56	0.39–1.10	11	10↑	90.9↑
Tchol (mmol/L)	4.04 ± 1.37	3.12–5.20	11	2↑	18.2↑
HDL-Chol (mmol/L)	0.90 ± 0.42	1.00–1.55	11	7↓	63.6↓
LDL-Chol (mmol/L)	2.18 ± 1.41	0–3.36	10	2↑	20↑

↑Beyond the upper limit; ↓Beyond the lower limit, except # <2.2mmol/L as the criterion for hypoglycemia.

ascertainment of NICCD achieved at 0.87 (0.36–4.25) years. Three cases were once misdiagnosed as tyrosinemia type Ib (P1194), galactosemia type II (P1482) and CMV hepatitis (P1495), respectively. The birth weight, length and PI in NICCD subjects were all reduced, compared with GA, gender and delivery pattern-matched normal controls (Table 1). Most of the patients were symmetric subjects at birth with PI > 2.5, except three asymmetric cases (P1478, P1628 and P1643) with PI < 2.5. Four subjects (P1513, P1638, P1643 and P1648) presented as Intrauterine Growth Restriction (IUGR). Jaundice at different pattern takes the top place in the list of major clinical manifestations (Table 2), followed by hepato/hepatosplenomegaly, prolonged prothrombin time and steatohepatosis on ultrasonography, a novel imaging feature for NICCD which has been further confirmed in three cases (P1194, P1648 and P1513) by MRI, CT and biopsy, respectively. Fig. 1 illustrated steatohepatosis on ultrasonography and MRI in P1194. Once diagnosis of NICCD was established, breast feeding was stopped and galactose-free or limited and/or MCT-enriched formula introduced. In some cases, fat-soluble vitamins and arginine were also given orally. All individuals were followed up for at least 6 months, and, except one (P1443) who passed away due to severe intracranial infection at age 9 months and another one (P1482) lost to follow-

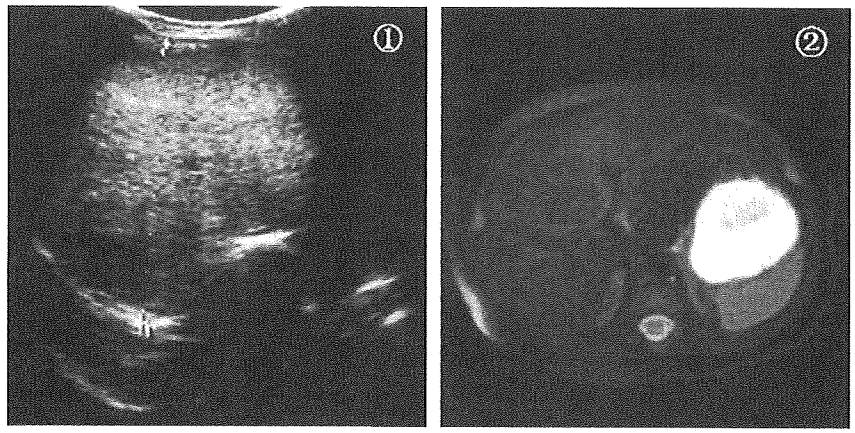

Fig. 1. Images of liver scanning in P1194. Ultrasonography (①) shows increased echogenicity and slight deep attenuation, while T2 weighting imaging with fat saturation in MRI (②) illustrates uniform and diffuse lower signal intensity in the liver than in the spleen. Both of the images suggest existence of steatohepatosis.

Table 3
Results of urinary analysis by GC–MS in the NICCD subjects.

Parameters	Procedure of sample preparation before GC–MS analysis						
	Urease pretreatment		Organic extraction		In summary		
	Tested cases	Positive cases	Tested cases	Positive cases	Tested cases	Positive cases	Positive rate (%)
↑Galactose	9	8	–	–	9	8	88.9
↑Galactitol	9	8	–	–	9	8	88.9
↑Galactonate	9	7	–	–	9	7	77.8
↑Tyr	9	5	–	–	9	5	55.6
↑4-HPL	9	7	3	3	12	10	83.3
↑4-HPP	9	6	3	2	12	8	66.7
↑Thr/Ser	9	5	–	–	9	5	55.6
↑Lys	9	3	–	–	9	3	33.3
↑Orn	9	3	–	–	9	3	33.3
↑Met	9	1	–	–	9	1	11.1
↑Asn	9	1	–	–	9	1	11.1
↑Glycerol	9	1	–	–	9	1	11.1

4-HPL: 4-hydroxyphenyllactate; 4-HPP: 4-hydroxyphenylpyruvate; "–": the substrates were lost due to the organic extraction procedure before sample analysis.

up during recovery stage, all subjects showed a benign prognosis. Most of the manifestations in Table 2 resolved gradually within 1 year of age, but FTT still remains in some individuals.

3.2. Biochemical changes

The representative biochemical data were collected and analyzed in 11 NICCD cases, and the medin age at blood testing was 1.6 (1.0–6.0) months. As shown in Table 2, 100% of our subjects present with elevated levels of hallmarks for intrahepatic cholestasis, such as GGT, ALP, TBil, DBil and TBA as well. Elevated AST was found in 90.9% of the patients while ALT in 36.4%. In addition, such non-specific metabolic derangements as hypoproteinemia, hyperlactic acidemia and hyperammonemia and hypoglycemia were also detected, suggesting a significant role of citrin protein in various biochemical pathways. It is noteworthy that albumin was decreased in 90.9% of cases, while its homologous protein alpha-fetoprotein (AFP) increased dramatically beyond its upper limit in all tested cases. And in particular, besides hypertriglyceridemia (90.9%) and hypercholesterolemia (18.2%), we further observed reduced levels of HDL cholesterol in seven of 11 patients (63.6%) and elevated levels of LDL cholesterol in two of 10 cases (20%), respectively.

3.3. Metabolome findings

Metabolome investigations were conducted in 12 patients. As shown in Table 3, GC–MS analysis of urine samples revealed high frequency of existence of the markers for galactosemia (including galactose, galactitiol and galactonate) and tyrosinemia [such as 4-hydroxyphenyllactate (4-HPL) and 4-hydroxyphenylpyruvate (4-HPP)], together with changes of some amino acids. Fig. 2 showed a representative TIC profile on GC–MS analysis of the urine components in P1643. Regarding blood sample analysis by MS–MS or HPLC (Table 4), the NICCD patients presented with characteristic amino acid pattern, i.e. elevated levels of Cit, Met, Thr, Tyr, Lys, Arg and Orn, and reduced level of Gln, Val and Leu.

3.4. Mutations in SLC25A13 gene

As shown in Table 1, 851–854del (851del4) is the most common mutation type (in 14 of 24 alleles from the 12 families), followed by IVS6+5G>A (3/24) and 1638-1660dup (2/24). Other mutations include A541D (1/24), IVS16ins3kb (1/24), R319X (1/24) and G333D (1/24), and there is another novel mutation needing identification in one allele. So far as we know, G333D is a novel mutation never reported in other paper, and the sequencing result and PCR-RFLP diagnostic approach were illustrated in Fig. 3. The mutation resulted in formation of a novel digestive site for the restriction endonuclease Mbo I, producing a mutation-specific band of 376 bp at gel running.

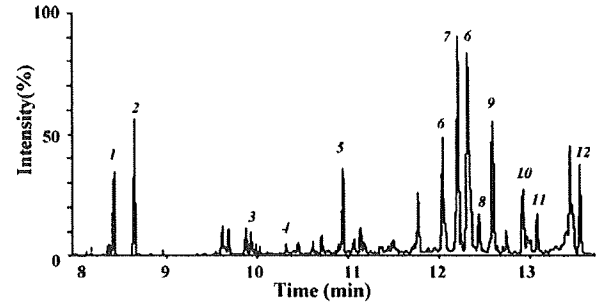

Fig. 2. Representative Total Ion Current profile of the urine components in P1643 on metabolome analysis by means of GC–MS after an urease pretreatment procedure. Peaks identifications are: (1) Serine (Ser), (2) Threonine (Thr), (3) Creatinine (Cr), (4) Ornithine (Orn), (5) Lysine (Lys), (6) Galactose, (7) 4-hydroxyphenyllactate (4-HPL), (8) Tyrosine (Tyr), (9) Galactitol, (10) Galactonate, (11) 4-hydroxyphenylpyruvate (4-HPP) and (12) internal standard, respectively.

4. Discussion

The reduced weight, length and PI at birth, and the existence with 4 IUGR subjects with NICCD suggest that the intrauterine fetal development has been impaired by the inherited disease. PI is a measure of body proportionality at birth, distinguishing symmetric from asymmetric growth restriction [25,26], and symmetric IUGR suggests etiologic factors from early pregnancy while asymmetric suggests factors at late pregnancy [27]. The finding that most of the NICCD patients are symmetric subjects at birth suggests that citrin deficiency restricted fetal growth from early pregnancy. Steatohepatosis on ultrasonography in this paper is a newly uncovered imaging feature for NICCD, which can be judged comprehensively by enlarged liver size, hepatorenal contrast, increased echogenicity with deep attenuation, and vascular blurring [28,29]. The detailed mechanism for the development of steatohepatosis has not been clarified in NICCD, but in our latest study on CTLN2, another phenotype of citrin deficiency usually occurring in adults, the overproduction of fatty acid in cytosol and its enhanced uptake into hepatocytes were proposed to be associated with steatogenesis in the liver [30]. Dramatically elevated AFP simultaneously with low levels of albumin was quite common in our NICCD patients even beyond the neonatal age. AFP and albumin have highly homolo-

Table 4
Analysis of blood amino acid spectrum by MS–MS or HPLC in the NICCD subjects.

Parameters	Analytical Method						
	MS-MS		HPLC		In summary		
	Tested cases	Positive cases	Tested cases	Positive cases	Tested cases	Positive cases	Positive rate (%)
↑Cit	5	5	5	5	10	10	100
↑Met	5	5	5	4	10	9	90
↑Thr	5	3	5	5	10	8	80
↑Tyr	5	3	5	2	10	5	50
↑Lys	5	1	5	4	10	5	50
↑Arg	5	2	5	2	10	4	40
↑Orn	5	1	5	2	10	3	30
↑Phe	5	0	5	1	10	1	10
↑His	5	0	5	1	10	1	10
↑Asn	5	0	5	2	10	2	20
↑Gly	5	1	5	0	10	1	10
↑Hcy	5	1	5	0	10	1	10
↓Gln	5	2	5	4	10	6	60
↓Val	5	1	5	1	10	2	20
↓Leu	5	1	5	0	10	1	10

gous and conserved primary structures, with genes belonging to the same family locating at human chromosome 4. Similar to the switch from HbF to HbA, there is a switch from AFP to albumin in humans and AFP decreases dramatically and immediately after birth, resulting in the substitution of AFP by albumin [31]. Since the expression pattern of the proteins mirror development and maturation of the liver [32], the delayed switch of AFP to albumin may reflect the immature liver development in NICCD. Actually, the bile acids metabolism in NICCD patients also showed an immature pattern as in the fetal period [33]. Therefore, we speculate that the delayed maturation of liver function is, at least partly, involved in the development of NICCD, although other factors such as hepatocellular destruction and regeneration are also likely to contribute to low albumin and high AFP. Besides hypertriglyceridemia and hypercholesterolemia which have been described in CTLN2 [30], reduced levels of HDL cholesterol and increased levels of LDL cholesterol were also observed in our NICCD subjects. Although we have speculated on the pathogenesis of fatty liver and hyperlipidemia [1,34], the mechanism for such a dyslipidemia pattern in NICCD remains unclear. As far as we know, this paper is the first direct evidence revealing such a dyslipidemia pattern in NICCD, further expanding its clinical spectrum.

It is indeed difficult for pediatric physicians to make the exact diagnosis of NICCD based just on the numerous clinical manifestations and laboratory changes listed in Table 2. These findings are rather non-specific since many other congenital or acquired factors can cause similar presentations of intrahepatic cholestasis [35–39]. However, metabolome investigation provides relative characteristic clues for NICCD diagnosis. Actually, on urinary analysis by GC–MS following urease-pretreatment procedure, co-existence of the markers for galactosemia (galactose, galactitol and galactonate) and tyrosinemia (4-HPL and 4-HPP) is quite common a phenomenon. Three patients in Table 3 only showed elevated 4-HPL and/or 4-HPP, but their pretreatment procedure was performed with organic acid extraction, losing such substrates with strong polarity as galactose, galactitol, galactonate, amino acids and glycerol [40]. Neither galactosemia nor tyrosinemia indices were detected in one patient, however, her age at sampling was

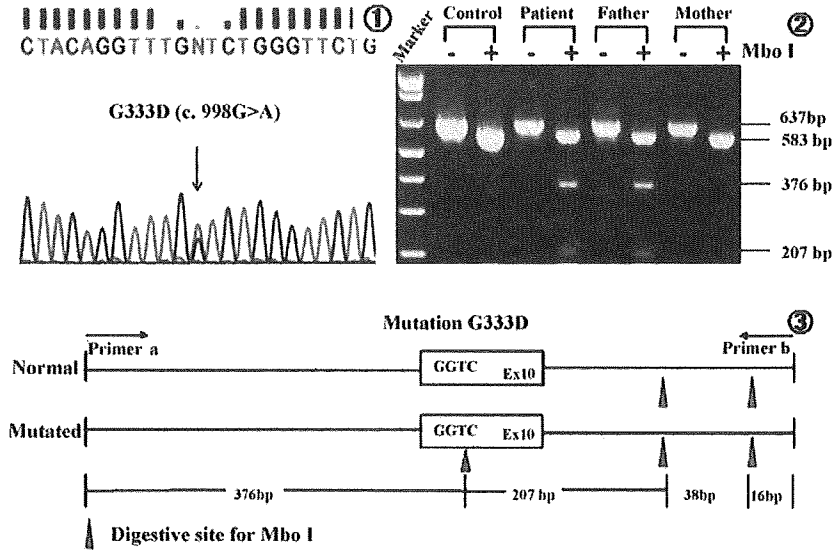

Fig. 3. G333D (c.998G>A), a novel mutation identified in the *SLC25A13* gene of P1495. (①) Partial sequencing result of Exon 10 and its flanking sequence revealed the mutation (c.998G>A; G333D). (②) Genetic diagnosis of the mutation G333D by gel running after PCR-RFLP with restriction enzyme Mbo I. (③) The schematic diagram for the genetic diagnosis of the novel mutation. Sequences of the primers "a" and "b" are 5′-TGCCTGGCCTCAGTGATGTT-3′ and 5′-CCTGTCTTTGGAAGGCCTGA-3′, respectively.

11 months, beyond the period with typical NICCD phenotype. Moreover, we have encountered some cases of galactosemia and tyrosinemia [21], but none of them had such a feature in the same analytic conditions. The characteristic amino acid spectrum (Table 4) in NICCD is another characteristic metabolome feature, which can be partly explained. Deficiency of citrin will affect the liver cytosol concentration of aspartate, one of the substrates for argininosuccinate synthetase (ASS), a key enzyme in urea cycle, leading to accumulation of citrulline (substrate of ASS) and ornithine (upstream substrate in the same cycle). Although liver urea cycle is affected in NICCD, four patients showed elevated levels of arginine, a downstream product of ASS in the urea cycle. Arginine is mainly synthesized in kidney and small intestine from citrulline formed also in small intestine during the neonatal period, and accumulation of citrulline in NICCD accelerates this process [1].

There are NICCD patients without the metabolome features above [19,41], and moreover, an infant who presented with some metabolome changes suggestive of citrin deficiency was not a case of NICCD (but a case of galactose-1-phosphate uridyltransferase deficiency) [42], and the clinical presentations and biochemical changes in NICCD, as mentioned above, are numerous but non-specific, so the definitive diagnosis of this inherited disease relies on the mutation analysis of the causative gene *SLC25A13* [8], although measurement of citrin protein in lymphocytes isolated from peripheral blood has been proposed as an alternative diagnostic method [43]. Our latest results revealed that IVS11+1G>A and 851del4 are quite common in Japanese, while 851del4 is the most common in Chinese, followed by IVS6+5G>A, 1638–1660dup (1638ins23) and IVS16ins3kb [8]. We confirmed the diagnosis of NICCD by *SLC25A13* analysis in this paper, and found that except 851–854del (851del), IVS6+5G>A, 1638ins23 and IVS16ins3kb, mutations A541D, R319X and the novel one G333D were never encountered previously in Japanese, although most recently we found a Japanese NICCD with R319X (Kobayashi et al. unpublished data). The fact that there is difference in mutation distribution between the two populations who share the quite common mutation 851del4 suggests that, with 851del4 as the founder mutation from the same predecessor individual, various mutations arose during the long history of population migration in East Asia. We have approved that IVS16ins3kb is a retrotransposal insertion showing an antisense strand of processed complementary DNA (cDNA) from a gene on chromosome 6 (C6orf68), and, although we have no detailed epidemiological information about it yet, the mutation exists in Korean population [8,15] besides Japanese and Chinese, further supporting our speculation that IVS16ins3kb and 851del4 are both relative earlier mutations occurring in East Asia [8]. Actually, molecular analysis of the gene *SLC25A13* works both as a patent tool for the definitive diagnosis of NICCD but also as a valuable approach for the epidemiological investigation on citrin deficiency in special population.

Based on the findings above, we concluded that (1) the diagnosis of NICCD cannot be established based only on the numerous but non-specific clinical manifestations and biochemical changes; (2) Metabolome investigation showed relatively specific features, providing valuable tools for the selective screening and clinical diagnosis of NICCD; (3) *SLC25A13* mutation analysis should be considered as a reliable tool for definitive diagnosis; (4) The information of body proportionality at birth, steatohepatosis on ultrasonography, delayed switch from AFP to albumin and dyslipidemia pattern on blood biochemistry, metabolome features on urinary analysis by GC–MS and the novel mutation G333D in *SLC25A13* further expanded the clinical spectrum of NICCD.

Conflict of interest statement
None declared.

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Short communication

A case of adult onset type II citrullinemia with portal-systemic shunt

D. Noto [a,*], K. Takahashi [a,b], T. Hamaguchi [a], K. Inamura [c], K. Nobata [d], M. Yazaki [e], S. Ikeda [e], K. Kobayashi [f], M. Yamada [a]

[a] Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan
[b] Department of Neurology, National Hospital Organization Iou National Hospital, Kanazawa, Ishikawa, Japan
[c] Department of Internal Medicine, Tonami General Hospital, Tonami, Toyama, Japan
[d] Department of Radiology, Tonami General Hospital, Tonami, Toyama, Japan
[e] Third Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Nagano, Japan
[f] Department of Molecular Metabolism and Biochemical Genetics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

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ABSTRACT

A 48-year-old woman who had conscious disturbance and abnormal behaviors had been misdiagnosed as having hepatic encephalopathy due to hyperammonemia and portal-systemic shunt, and retrograde transvenous obliteration of the shunt did not improve her symptoms. Thereafter, analyses of plasma amino acids and citrin gene revealed a diagnosis of adult onset type II citrullinemia (CTLN2). She underwent auxiliary partial orthotopic liver transplantation (APOLT) using a left lobe graft from her brother, and her symptoms as well as hyperammonemia improved. Our case demonstrates the importance of CTLN2 as a differential diagnosis in patients with hyperammonemia and consciousness disturbance, even if they present with a portal-systemic shunt.

1. Introduction

Adult onset type II citrullinemia (CTLN2) is a metabolic disorder characterized by increased plasma concentrations of citrulline and ammonia. Patients with CTLN2 display various neurological symptoms, such as coma, seizures, and abnormal behaviors, which are the same as shunt encephalopathy caused by portal-systemic shunt. We report a patient with both CTLN2 and portal-systemic shunt.

2. Case report

The patient was a 48-year-old woman. She had disliked sweets and carbohydrate-rich meals since childhood. In October 2006, she complained of dizziness and headache. On October 21, she fell into a delirious state, and was hospitalized at a psychiatric department in a general hospital (Fig. 1A). She was diagnosed as having hepatic encephalopathy on the basis of triphasic waves on electroencephalogram and hyperammonemia (187 μg/dl, normal <66 μg/dl), but physical signs suggestive of liver cirrhosis, such as vascular spiders and red palms were absent. The number of platelets, serum albumin level, and prothrombin time was normal. Abdominal CT revealed a portal-systemic shunt from the superior mesenteric vein to the inferior vena cava, but no liver cirrhosis, splenomegaly, or other porto-systemic shunts (Fig. 1B and C). On November 14, she underwent a retrograde transvenous obliteration of the shunt (Fig. 1D). However, in spite of the successful operation, the hyperammonemia never improved. Thereafter, analysis of amino acids in her blood revealed elevation of the plasma citrulline level (778.8 nmol/ml, normal <40.8 nmol/ml). With suspicion of citrullinemia, oral administration of lactulose (27 g/day) was started. However, she again developed delirium, and was transferred to our hospital on January 26, 2007. On admission, she was alert with no abnormality on neurological examination except for slightly exaggerated deep tendon reflexes. Blood analysis revealed elevation of plasma citrulline (1288 nmol/ml) and threonine/serine ratio (2.04, healthy control 1.17 ± 0.13 [1]). Brain MRI was normal. DNA analysis of the citrin gene revealed a homozygous change for SLC25A13 mutation 851del4, which led to the diagnosis of CTLN2 [2]. For prevention of encephalopathy, she was treated with protein restriction (protein:fat:carbohydrate ratio = 10:45:45), oral administration of L-arginine (7.5 g/day), sodium benzoate (10 g/day) and L-carnitine (900 mg/day), and intravenous or oral administration of branched chain amino acids. However, plasma ammonia levels were elevated and fluctuated (64–500 μg/dl) and she frequently complained of dizziness and headache. We considered liver transplantation as the only effective therapy. We evaluated her older brother as an organ donor, and he presented with normal plasma ammonia levels and citrin gene heterozygous for the 851del4 mutation. On August 7, 2007, she

* Corresponding author. Department of Neurology and Neurobiology of Aging, Kanazawa University, Graduate School of Medical Science, 13-1, Takara-machi, Kanazawa 920-8640, Japan. Tel.: +81 76 265 2292; fax: +81 76 234 4253.
E-mail address: d-noto@med.kanazawa-u.ac.jp (D. Noto).

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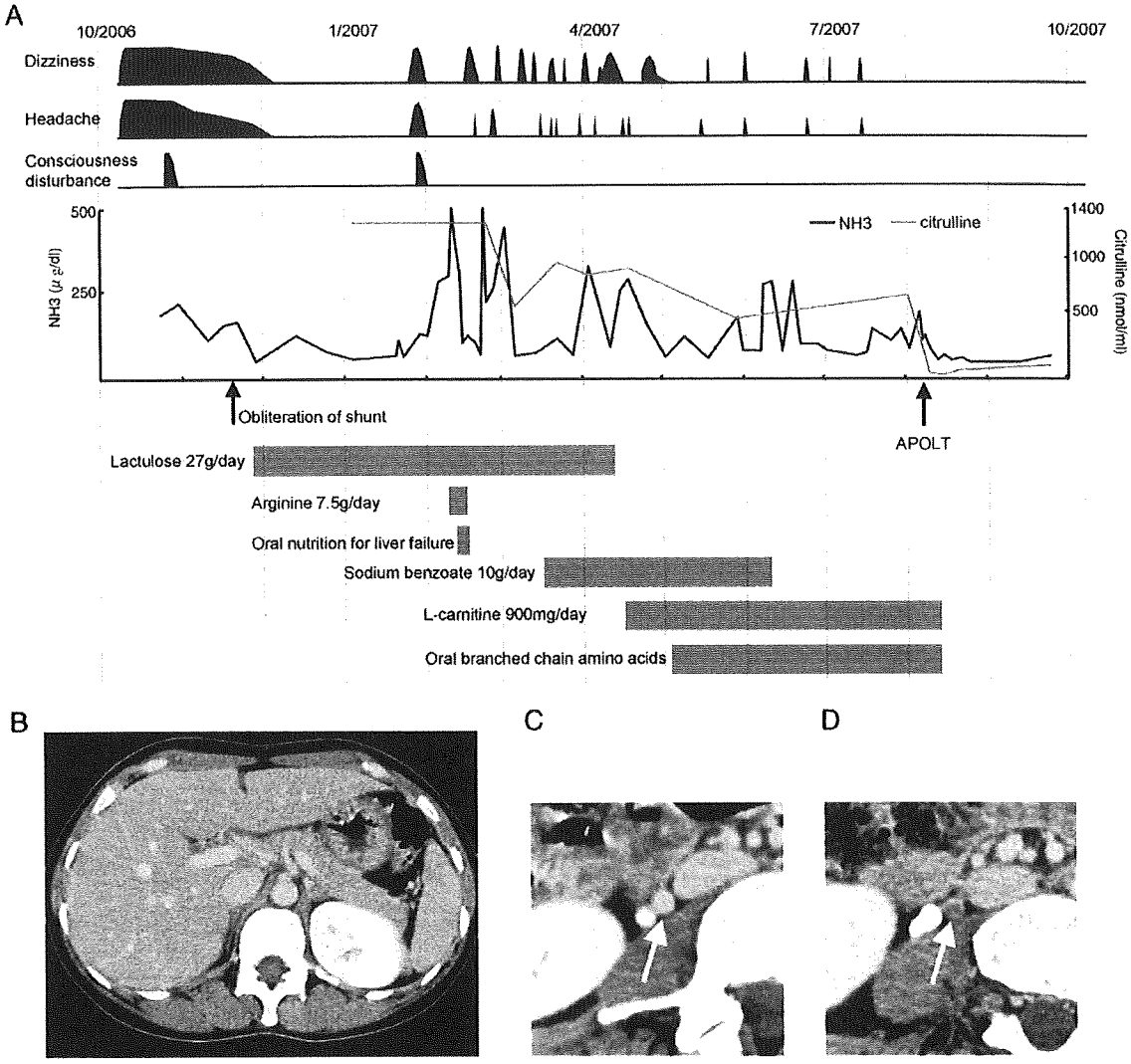

Fig. 1. (A) Clinical course of the patient. APOLT, auxiliary partial orthotopic liver transplantation. (B) Abdominal CT shows no liver cirrhosis and splenomegaly. (C) Abdominal CT reveals the abnormal shunt vessel (arrow) near the inferior vena cava. (D) Abdominal CT after operation. The shunt vessel is occluded by the thrombus (arrow).

underwent auxiliary partial orthotopic liver transplantation (APOLT) using a left lobe graft from her brother, because of insufficient graft liver volume [3]. After the liver transplantation, her plasma level of ammonia and citrulline markedly improved (ammonia, 40–70 μg/dl, citrulline, 33.9 nmol/ml), and she became symptom-free without diet restriction or oral medication for hyperammonemia.

3. Discussion

CTLN2 is caused by mutations in the SLC25A13 gene encoding citrin, which is a mitochondrial aspartate glutamate carrier and a member of the malate-aspartate shuttle [4]. In CTLN2 patients, the supply of aspartate to the cytosol by citrin is disturbed, and urea synthesis is inhibited. As a result, hyperammonemia and citrullinemia are induced. Additionally, the disturbance of the malate-aspartate shuttle caused by citrin deficiency induces an increase of the cytosolic reduced nicotinamide adenine dinucleotide to oxidized nicotinamide adenine dinucleotide ratio ($NADH/NAD^+$ ratio), the increased $NADH/NAD^+$ ratio inhibits glycolysis and causes dislike of carbohydrate-rich foods [5]. It is reported that a carbohydrate-rich diet and glycerol therapy worsen hyperammonemia and cause critical brain edema [6,7]. A combination therapy of a carbohydrate-restricted diet (protein:fat: carbohydrate ratio = 20:45:35) which is preferred by CTLN2 patients, arginine and sodium pyruvate is expected to be effective [8,9].

CTLN2 is characterized by recurring episodes of neurological and psychotic symptoms including delirium, abnormal behaviors, disorientation, loss of memory, convulsive seizures, and coma. Because of various psychotic symptoms, CTLN2 is often misdiagnosed as epilepsy, depression, schizophrenia, or acute alcohol poisoning. On the other hand, hepatic encephalopathy induced by a portal-systemic shunt without liver cirrhosis is a rare disease characterized by hyperammonemia and various neurological and psychotic symptoms, which are the same as in CTLN2. In our patient, despite successful operation for the portal-systemic shunt, her symptoms and hyperammonemia never improved. Therefore, we considered that the portal-systemic shunt had little or no effect on hyperammonemia and her symptoms.

Patients with CTLN2 may be diagnosed with other liver diseases. It was reported that a patient with an initial diagnosis of non-alcoholic fatty liver disease (NAFLD) was later revealed to be the carrier of a citrin gene mutation [10]. Three patients with CTLN2 were reported with liver histologies of steatohepatitis [11]. It was suggested that patients with NAFLD unrelated to obesity and metabolic syndrome might have citrin deficiency [12]. On laboratory findings, the ratio of threonine to serine (The/Ser) was reported to be higher in CTLN2 than

that in controls, or patients with chronic hepatitis and liver cirrhosis [1]. The existence of NAFLD and elevated Thr/Ser ratio may lead to early diagnosis of CTLN2.

In Japan, the frequency of heterozygotes for citrin mutation is reported to be 1 in 65, and that of homozygotes is estimated to be 1 in 17,000 [13]. Outside Japan, it has been recently reported that heterozygotes for citrin mutations are distributed with high frequency in China (1/65) and Korea (1/112) [14]. Further, individuals with citrin mutations have been identified in Israel, the USA, and the United Kingdom, and patients with citrin deficiency have been recently reported in Caucasians [15]. Citrin deficiency may be complicated by other conditions such as a portal-systemic shunt, as found in our patient, because of its high prevalence. CTLN2 is treatable with liver transplantation. Without liver transplantation, however, patients with CTLN2 show a rapid and progressive course and sometimes suddenly develop fatal brain edema [16], and mostly die within 2 years after onset [17]. Our case indicates that CTLN2 should be considered as a differential diagnosis in patients with hyperammonemia and consciousness disturbance, even if they present with a portal-systemic shunt. As the history of dislike of sweets and carbohydrate-rich meals is important for early diagnosis of CTLN2, preference of foods should be carefully surveyed.

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SHORT REPORT

Neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) as a cause of liver disease in infants in the UK

T. Hutchin · M. A. Preece · C. Hendriksz · A. Chakrapani · V. McClelland · F. Okumura · Y.-Z. Song · M. Iijima · K. Kobayashi · T. Saheki · P. McKiernan · U. Baumann

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Summary Citrin deficiency is a disorder with two phenotypes: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), and adult-onset type II citrullinaemia (CTLN2). NICCD presents in the first few weeks of life with prolonged cholestasis and metabolic abnormalities including aminoacidaemia (notably citrulline, tyrosine, threonine, arginine and methionine) and galactosuria. Symptoms resolve within the first year of life, thus making a diagnosis difficult after this time. Although patients subsequently remain generally healthy, some may develop more severe symptoms of CTLN2, characterized by neurological changes, one or more decades later. To date more than 400 cases have been reported, almost all from East Asia (mainly Japan). Here we describe the first two cases of NICCD in infants from the UK, one of caucasian origin and one of Pakistani origin. Both showed typical clinical and biochemical changes with a diagnosis confirmed by the presence of previously unreported mutations in the *SLC25A13* gene. The presence of citrin deficiency in other ethnic groups means that NICCD needs to be considered in the diagnosis of any neonate with an unexplained cholestasis. We discuss both the difficulties in diagnosing these patients in populations where very few DNA mutations have been identified and the problems faced in the management of these patients. These findings also raise the possibility of adults with CTLN2 in whom a diagnosis has yet to be made.

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References to electronic databases: Citrullinaemia, type II, adult onset (CTLN2): OMIM 603471. Citrullinaemia, type II, neonatal-onset (neonatal intrahepatic cholestasis caused by citrin deficiency, NICCD): OMIM 605814. *SLC25A13*: OMIM 603859. GenBank: AJ496569; NM_014251; AF118838.

T. Hutchin (✉) · M. A. Preece
Department of Clinical Chemistry,
Birmingham Children's Hospital,
Whittall Street,
Birmingham B4 6NL, UK
e-mail: tim.hutchin@bch.nhs.uk

C. Hendriksz · A. Chakrapani
Department of Inherited Metabolic Disease,
Birmingham Children's Hospital,
Birmingham, UK

V. McClelland
Department of Neurology, Willemenia Children's Hospital,
London, UK

F. Okumura · Y.-Z. Song · M. Iijima · K. Kobayashi
Department of Molecular Metabolism and Biochemical Genetics, Kagoshima University Graduate School of Medical and Dental Sciences,
Kagoshima, Japan

K. Kobayashi · T. Saheki
Institute for Health Sciences, Tokushima Bunri University,
Tokushima, Japan

P. McKiernan · U. Baumann
The Liver Unit, Birmingham Children's Hospital,
Birmingham, UK

Abbreviations

ALT	alanine aminotransferase
AP	alkaline phosphatase
AST	aspartate aminotransferase
CTLN2	citrullinaemia type II
γGT	gamma-glutamyl transpeptidase
LFT	liver function tests
NICCD	neonatal intrahepatic cholestasis caused by citrin deficiency

Introduction

Citrin is a mitochondrial aspartate glutamate carrier which acts as part of the malate-aspartate shuttle in the liver and is thus important for aerobic glycolysis and gluconeogenesis (Saheki and Kobayashi 2002). In addition, citrin also plays a role in the urea cycle, protein and nucleotide synthesis (Saheki and Kobayashi 2002). Citrin deficiency is a disorder with two phenotypes: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and adult-onset citrullinaemia type II (CTLN2) (Fiermonte et al 2008; Ohura et al 2001; Tamamori et al 2002).

NICCD has a varied clinical phenotype ranging from presentation in the first few weeks of life with neonatal cholestasis to metabolic abnormalities mimicking galactosaemia, non-specific failure to thrive in infancy and ketotic hypoglycaemia with hepatomegaly (Dimmock et al 2007; Hachisu et al 2005; Tazawa et al 2004).

Following presentation in infancy, symptoms usually resolve themselves and only 2 children out of a case series of 75 required liver transplantation for progressive liver failure before they were 1 year old (Ohura et al 2007; Tamamori et al 2002). Most patients with resolving symptoms generally remain healthy, but some individuals develop CTLN2 one or more decades later, characterized by neurological deterioration.

To date more than 400 cases have been reported and until recently it was thought to be a disease almost exclusively found in individuals of East Asian ancestry. However, cases have been reported in subjects of different genetic backgrounds (Ben-Shalom et al 2002; Dimmock et al 2007, 2009; Fiermonte et al 2008; Tabata et al 2008). Here we describe two cases of NICCD, one of caucasian background and one of Pakistani origin from the UK. Citrin deficiency has become an additional differential diagnosis in patients with neonatal cholestasis in Europe.

Case 1

The patient was a 7-week-old boy admitted for investigation of neonatal jaundice and pale stool from day 2 of life. On admission his total bilirubin was 172 μmol/L (normal 0–15 μmol/L) and γGT was 181 IU/L (normal 0–25 IU/L). The first child of a non-consanguineous caucasian couple, he was born at term following an uneventful pregnancy with a birth weight of 2.72 kg (<9th centile) and had been noted to be jaundiced from day 2 of life. Ultrasonography performed on day 61 demonstrated normal liver parenchyma and a normal-sized gallbladder. Blood glucose concentrations during admission were normal, as were most screening investigations for neonatal cholestasis. Abnormal results in blood included plasma amino acids showing elevated levels of citrulline, methionine, threonine, arginine and lysine (Table 1). Urine also revealed elevated amino acids (citrulline 213 μmol/mmol creatinine) and galactosuria (>10 mmol/L).

He was treated with fat-soluble vitamins, ursodeoxycholic acid and lactulose, and discharged from hospital. Shortly thereafter he developed occasional abdominal pains and frequently vomited, particularly after feeding.

Further investigations at 3 months of age showed that plasma amino acids were still elevated but less so than a month earlier (Table 1). Likewise, liver function tests were beginning to return to normal, but he remained icteric. Blood ammonia remained normal and alpha-fetoprotein was grossly elevated at 7571 kU/L (normal 0–10 kU/L). A liver biopsy showed severe mixed micro- and macrovesicular steatosis, ballooning of hepatocytes and focal hepatocyte rosetting, suggestive of metabolic liver disease.

At 4 months of age, despite a reluctance to breast feed, he was making good weight gain (5.95 kg, 9th centile) and showed no signs of jaundice, with near-normal LFTs. Plasma amino acids continued to fall

Table 1 Plasma amino acids (μmol/L) in patient 1

Amino acid	Age				Upper limit of normal
	2 months	3 months	8 months	1 year	
Citrulline	512[a]	111[a]	35	20	40
Arginine	166[a]	141[a]	128	52	110
Threonine	692[a]	429[a]	218	93	220
Methionine	139[a]	41	46	24	40
Tyrosine	90	42	133	89	100
Lysine	280[a]	166	209	168	240

[a] Significantly elevated.

towards normal, with citrulline concentrations decreased to normal.

The findings of raised amino acids, particularly citrulline, abnormal LFTs, galactosuria and a fatty liver in a small child with persistent neonatal cholestasis and jaundice, all of which except fatty liver resolved by 12 months of age, are typical of NICCD.

Western blot analysis of fibroblasts revealed levels of citrin protein that were 22% of normal. Analysis of the *SLC25A13* gene showed the presence of a heterozygous mutation resulting in a cysteine to arginine change at amino acid residue 489 (p.C489R) of the citrin protein. The second mutation has not been identified but appears to prevent the production of stable mRNA from the second allele, which would explain the decreased protein levels seen in fibroblasts. Taken together with the clinical and biochemical data, these findings confirm a diagnosis of citrin deficiency in this child.

He continued to improve and, apart from a disruptive sleeping pattern, by 1 year of age showed no abnormalities either biochemically (Table 1) or clinically. At the age of 5 years he developed episodes of abdominal pain and associated ketotic hypoglycaemia. A special emergency regime consisting of more protein- and fat-based components has been effective in controlling his hypoglycaemia. He remains well at 5 years of age with normal developmental progress and routine MRI imaging was normal.

Case 2

The patient, a girl, was the second child of consanguineous parents of Pakistani origin and was born following an uneventful pregnancy weighing 2.83 kg (10th centile). She was admitted at 5 weeks for persistent neonatal cholestasis and a history of recurrent vomiting, jaundice and poor weight gain from 2 weeks of age. On admission she weighed 3.18 kg and, apart from icterus, systemic examination was unremarkable. Initial investigations revealed conjugated hyperbilirubinaemia, total bilirubin 126 μmol/L (normal 0–15 μmol/L), abnormal liver enzymes (AP 2142 IU/L (normal 250–850 IU/L), ALT 51 IU/L (5–45 IU/L), AST 164 IU/L (normal 20–60 IU/L), γGT 166 IU/L (normal 0–25 IU/L)), elevated plasma lactate (3.7 mmol/L), mildly elevated ammonia levels at 53 μmol/L and abnormal liver synthetic function. Further investigations excluded usual inborn errors of metabolism and infective causes of cholestasis. Liver ultrasound findings were unremarkable. Plasma amino acids showed elevated levels of citrulline, threonine, arginine and methionine (Table 2) and urine investigations detected increased levels of citrulline and galactose. The patient was discharged on treatment with ursodeoxycholic acid, Gaviscon and fat-soluble vitamins.

Further plasma amino acid analyses indicated persistent abnormalities that resolved over time (Table 2). A low-carbohydrate, high-protein diet was commenced and cholestasis resolved by 5 months of age, although intermittent vomiting was reported and there were mild persistent abnormalities of AST and γGT. By 9 months of age, LFTs had completely normalized. At 26 months her growth and development remain normal and she has started self-selecting a low-carbohydrate diet with specific avoidance of fruit juices and sweets.

Investigations showed citrin protein was only 4% of normal levels in fibroblasts by western blotting and mutation analysis of the *SLC25A13* gene confirmed she was homozygous for a c.1610–1612delTAGinsAT mutation, which causes a premature termination of the protein.

Discussion

The two patients reported here presented with typical non-specific clinical features of neonatal cholestasis when treatable conditions such as biliary atresia need

Table 2 Plasma amino acids (μmol/L) in patient 2

Amino acid	Age					Upper limit of normal
	1 months	2 months	5 months	9 months	1 year	
Citrulline	908[a]	125[a]	458[a]	14	31	40
Arginine	188[a]	116[a]	211[a]	74	86	110
Threonine	1131[a]	632[a]	570[a]	126	226	220
Methionine	533[a]	38	490[a]	27	31	40
Tyrosine	176[a]	60	108	51	86	100
Lysine	388[a]	424[a]	287	161	244	240

[a] Significantly elevated.