

# Genotypic and phenotypic features of citrin deficiency: Five-year experience in a Chinese pediatric center

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**Abstract.** Citrin is a liver-type aspartate/glutamate carrier (AGC) encoded by the gene SLC25A13. Two phenotypes for human citrin deficiency have been described, namely the adult-onset citrullinemia type II (CTLN2) and the neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). However, citrin deficiency currently remains a perplexing and poorly recognized disorder. In particular, description of post-NICCD clinical presentations before CTLN2 onset is rather limited. Analysis of SLC25A13 mutations, identification of dysmorphic erythrocytes, hepatobiliary scintigraphic imaging and investigation of post-NICCD clinical presentations were performed in a citrin-deficient cohort comprised of 51 cases of children diagnosed with citrin deficiency in a Chinese pediatric center. Twelve SLC25A13 mutations were detected in this cohort, including the novel V411M and G283X mutations. Among the 51 citrin-deficient subjects, 7 cases had echinocytosis, which was associated with more severe biochemical abnormalities. Delayed hepatic discharge and bile duct/bowel visualization were common scintigraphic findings. Moreover,

9 of the 34 post-NICCD cases demonstrated concurrent failure to thrive and dyslipidemia, constituting a clinical phenotype different from NICCD and CTLN2. The novel mutations, echinocytosis, hepatobiliary scintigraphic features and the novel clinical phenotype in this study expanded the genotypic and phenotypic spectrum of citrin deficiency, and challenge the traditionally-assumed 'apparently healthy' period after the NICCD state for this disease entity.

## Introduction

Citrin is a liver-type aspartate/glutamate carrier (AGC) encoded by the gene SLC25A13 which was cloned in 1999 by Kobayashi *et al* (1). It has been well-recognized that human citrin deficiency encompasses both adult-onset type II citrullinemia (CTLN2, OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM #605814). An 'apparently healthy' period without symptoms between the state of NICCD and the onset of CTLN2 has been traditionally assumed for years (2). However, a potentially different course of citrin deficiency, including failure to thrive (FTT), hyperlipidemia, hepatoma and pancreatitis at the post-NICCD but pre-CTLN2 stage, has been proposed by Saheki *et al* (3), suggesting the existence of other post-NICCD phenotype(s) in addition to CTLN2. Although citrin deficiency was initially reported among individuals of East Asian ancestry, more and more citrin-deficient patients have been identified in other populations (4-10), suggesting it is a panethnic disease with a worldwide distribution. Further elucidation of the clinical and laboratory characteristics of this disease will facilitate its early diagnosis and appropriate management. Citrin deficiency currently remains a perplexing and poorly recognized disorder (11,12). In particular, a description of post-NICCD clinical presentations before CTLN2 onset is rather limited, although abnormal metabolic profiles at this stage have been reported by Nagasaka *et al* (13). We performed a comprehensive review in a citrin-deficient cohort obtained from a Chinese pediatric center, to explore

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**Abbreviations:** NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; CTLN2, adult-onset citrullinemia type II; Tc-99m-EHIDA, technetium-99m-N,α-(2,6-diethylacetanilide)-iminodiacetic acid; SPECT, single-photon emission computed tomography; FTT, failure to thrive; FTTDCD, failure to thrive and dyslipidemia caused by citrin deficiency

**Key words:** citrin deficiency, neonatal intrahepatic cholestasis caused by citrin deficiency, SLC25A13, echinocytosis

novel molecular, erythrocytic, scintigraphic and post-NICCD clinical features.

## Subjects and methods

**Subjects.** This citrin-deficient cohort was composed of 51 pediatric cases diagnosed between July 2005 and September 2010 in the Department of Pediatrics, the First Affiliated Hospital, Jinan University, Guangdong, China. All diagnoses were confirmed by SLC25A13 analysis, and some genotypes had been reported in our previous publications (14-18). Galactose-free and/or medium-chain triglycerides (MCTs) enriched formulas were introduced as dietary therapeutics in most cases once their diagnoses were suspected or established. In some patients with obvious jaundice and cholestatic indices, oral ursodeoxycholate and intravenous reductive glutathione were given, and intravenous arginine was administered in several cases of hyperammonemia. All subjects were followed-up by counseling at the clinic and by e-mail or telephone counseling, and the clinical information was recorded in detail.

**SLC25A13 mutation analysis.** The four most frequent mutations, i.e. 851del4, 1638-1660dup, IVS6+5G>A and IVS16ins3kb, were screened by means of a routine procedure (15,16), and DNA sequencing of the 18 exons and their flanking sites in the SLC25A13 gene was performed as described in our previous investigations (17,18) in the subjects in which only one mutation was identified. Adhering to the principles of the Declaration of Helsinki, the parents of all subjects gave informed consents. This study has been approved by the Committee for Ethics of the Kagoshima University Faculty of Medicine in Japan, and by the Committee for Medical Ethics, the First Affiliated Hospital, Jinan University in China.

**Light and electron microscopy.** Blood smears were prepared with venous samples collected from the subjects. Erythrocyte morphology observation was performed after Giemsa staining by means of a previously described procedure (19). Scanning electron microscopy (SEM) was used to confirm the erythrocyte dysmorphism in some cases. SEM sample preparation, including erythrocyte fixing with glutaraldehyde, buffer washing, post-fixing with osmium tetroxide, dehydration with ethanol, depositing of a diluted sample drop, air drying and coating with gold, was carried out as previously described (20). A Nikon Eclipse 80i (Japan) microscope and a Philips ESEM XL-30 SEM instrument (The Netherlands) were used for the analysis. The echinocyte stages were classified according to Brecher *et al* (21): stage 1 is characterized by irregularity of cellular edges; stage 2 by spicules in a still flat cell; and stage 3 by spicules uniformly distributed over the surface of a round cell.

**Hepatobiliary scintigraphic imaging.** Scintigraphy of the hepatobiliary system with technetium-99m-N, $\alpha$ -(2,6-diethylacetanilide)-iminodiacetic acid (Tc-99m-EHIDA) as the tracer has been well recognized as a reliable tool for clinical investigation of the origin of jaundice. To distinguish between the icterus of a hepatocellular and an obstructive origin, Tc-99m-EHIDA was injected intravenously at the dosage of 1 mCi, and serial abdominal views were obtained by

means of a scintillation camera, during at least the first hour and up to 24 h when necessary. A single-photon emission computed tomography (SPECT) instrument (Elscent Helix, GE Healthcare) was used in this study to perform hepatobiliary imaging.

**Clinical presentations after the NICCD state.** We investigated the clinical features of the citrin-deficient subjects after the NICCD state, focusing on their anthropometric and biochemical information collected in the past five years. In this study, measurements of weight- and/or length-for-chronological age <5th percentile were defined as FTT according to Olsen *et al* (22), and the age and gender-matched 5th percentile values of the anthropometric indices were based on the WHO child growth standards (<http://www.who.int/childgrowth/standards/en/>). Furthermore, patients with serum levels of total triglycerides (TG)  $\geq 1.70$  mmol/l, and/or total cholesterol (T-Chol)  $\geq 5.18$  mmol/l, and/or high density lipoprotein (HDL)-cholesterol  $\leq 1.04$  mmol/l and/or low density lipoprotein (LDL)-cholesterol  $\geq 3.37$  mmol/l, were defined as dyslipidemic (23).

**Statistics.** Independent-sample t-tests were used to compare the differences of serum biochemical indices between different groups of patients with and without echinocytosis, and those with and without failure to thrive and dyslipidemia caused by citrin deficiency (FTTDCD). The indices following a Gaussian distribution are presented as the mean  $\pm$  SD, and those skewed are denoted as the median (minimum, maximum). Skewed raw data were logarithmically transformed before statistical assessment as indicated in the corresponding tables. The difference in the SLC25A13 mutation spectrum was examined by using the latitude of 30°N as the border line dividing the patient origin between the south or north China. The mutation spectrum difference was evaluated by means of a 2x2 table  $\chi^2$ -test with correction for continuity. A P-value <0.05 was taken to denote statistical significance.

## Results

**Patient information.** The subjects came from 13 provinces, municipalities and autonomous regions of mainland China, including Guangdong, Guangxi, Hunan, Hubei, Jiangxi, Shanghai, Jiangsu, Zhejiang, Fujian, Shandong, Shanxi, Henan and Hebei, with most of them from south China (south of 30°N) such as the Guangdong and Hunan provinces. As listed in Table I, the citrin-deficient cohort in this study was composed of 51 subjects including 17 females and 34 males, with NICCD as the main clinical presentation in most cases. Three subjects demonstrated poor outcomes and another 2 lost contact, but all the remaining 46 cases recovered or improved clinically. However, FTT and dyslipidemia were observed as rather common manifestations after the NICCD state, as described in detail below.

**SLC25A13 mutations.** A total of 12 mutations including the 7 previously reported mutations (14-18), 851del4, IVS6+5G>A, 1638-1660dup, IVS16ins3kb, G333D, A541D and R319X, and 5 additional ones, IVS11+1G>A, R360X, R467X, V411M and G283X, were found in this Chinese cohort, as shown

Table I. General information and SLC25A13 mutations in the citrin-deficient cohort.

Case	Patient	Gender	Mutations <sup>a</sup>	Major presentations	Clinical outcomes
01	P1071	Male	851del4/1638-1660dup	NICCD	Normal
02	P1194	Female	851del4/A541D	NICCD	Normal
03	P1194S	Female	851del4/A541D	NICCD	Normal
04	P1443	Male	IVS6+5G>A/R319X	NICCD	Died of ICI
05	P1478	Female	851del4/851del4	NICCD	Normal
06	P1482	Male	851del4/851del4	NICCD	Lost contact
07	P1495	Female	851del4/G333D	NICCD	Normal
08	P1513	Female	851del4/IVS16ins3kb	NICCD	Normal
09	P1628	Male	851del4/IVS6+5G>A	NICCD	FTT, dyslipidemia
10	P1638	Male	851del4/1638-1660dup	NICCD	FTT
11	P1643	Female	851del4/?	NICCD	FTT, dyslipidemia
12	P1644	Female	851del4/IVS6+5G>A	NICCD	Lost contact
13	P1648	Male	851del4/851del4	NICCD	FTT, dyslipidemia
14	P1751	Male	IVS6+5G>A/?	NICCD	Died of DIC
15	P1752	Female	851del4/851del4	NICCD	Normal
16	P1863	Male	851del4/IVS6+5G>A	NICCD	Normal
17	P1883	Male	851del4/IVS16ins3kb	NICCD	FTT
18	P1933	Male	851del4/IVS16ins3kb	NICCD	FTT, dyslipidemia
19	P1945	Female	IVS6+5G>A/?	NICCD	Normal
20	P1946	Male	851del4/851del4	NICCD	Normal
21	P1947	Male	851del4/851del4	NICCD	Normal
22	P1518	Male	851del4/851del4	FTT, dyslipidemia	Normal
23	C0002	Male	IVS11+1G>A/R360X	NICCD	Normal
24	C0004	Female	851del4/851del4	NICCD	Normal
25	C0005	Male	851del4/IVS6+5G>A	NICCD	FTT, dyslipidemia
26	C0006	Male	851del4/R467X	NICCD	Normal
27	C0009	Male	851del4/851del4	NICCD	Normal
28	C0010	Male	1638-1660dup/IVS6+5G>A	NICCD	Normal
29	C0012	Female	851del4/ <b>V411M</b>	NICCD	Improved cholestasis, FTT
30	C0013	Male	851del4/851del4	Liver cirrhosis, FTT, GDD	Dyslipidemia, died of hepatic encephalopathy
31	C0016	Male	851del4/851del4	NICCD	FTT, Transient GDD
32	C0018	Female	851del4/ <b>G283X</b>	NICCD	Normal
33	C0019	Male	851del4/R467X	NICCD	Motor retardation, dyslipidemia
34	C0020	Male	851del4/851del4	NICCD	Normal
35	C0021	Male	851del4/IVS16ins3kb	NICCD	Normal
36	C0025	Male	851del4/851del4	NICCD	Improved, FTT
37	C0027	Male	851del4/851del4	NICCD	Improved cholestasis
38	C0028	Male	851del4/851del4	NICCD	Improved cholestasis
39	C0029	Male	851del4/851del4	NICCD	Improved cholestasis
40	C0030	Female	851del4/851del4	NICCD	FTT, dyslipidemia
41	C0031	Male	1638-1660dup/IVS16ins3kb	NICCD	Improved cholestasis
42	C0032	Male	851del4/1638-1660dup	NICCD	Improved cholestasis
43	C0033	Female	851del4/851del4	NICCD	Improved cholestasis
44	C0035	Male	851del4/IVS16ins3kb	NICCD	Improved cholestasis
45	C0036	Male	851del4/851del4	NICCD	Improved cholestasis
46	C0037	Male	851del4/851del4	NICCD	FTT, dyslipidemia
47	C0041	Male	851del4/1638-1660dup	NICCD	Improved cholestasis
48	C0042	Male	851del4/1638-1660dup	NICCD	Improved cholestasis
49	C0043	Female	851del4/?	NICCD	Improved cholestasis
50	C0044	Female	851del4/851del4	NICCD	Improved cholestasis
51	C0046	Female	1638-1660dup/IVS6+5G>A	NICCD	Improved cholestasis

<sup>a</sup>The mutations in cases 1-22, 30 and 31 have been previously reported (14-18). Bold italic letters indicate the two novel mutations; the bold question marks indicate the unknown mutations. NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; FTT, failure to thrive; ICI, intracranial infection; DIC, disseminated intravascular coagulation; GDD, gross developmental delay.

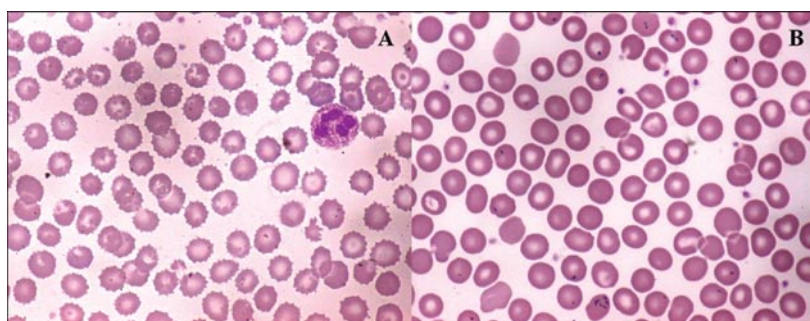


Figure 1. Light micrographs of echinocytosis in a male infant (C0016) with citrin deficiency. (A) Giemsa staining of blood smears demonstrating numerous echinocytes (x1000) at his age of 5.5 months. (B) Normalized erythrocyte morphology (x1000) when the biochemical and clinical abnormalities improved 2 months later.

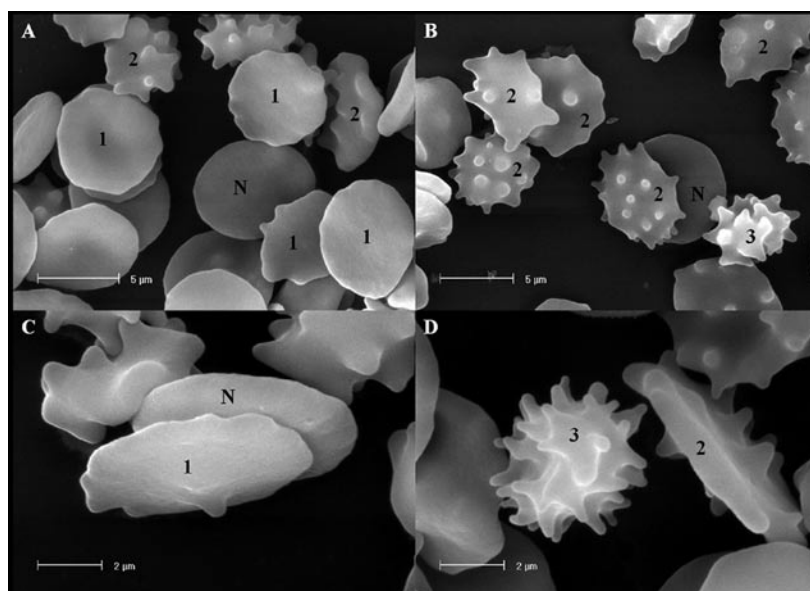


Figure 2. Scanning electron micrographs of echinocytosis in a citrin-deficient infant (C0027). The patient is a 4.5 month-old male. Numbers 1, 2 and 3 indicate echinocytes at stages 1, 2 and 3, respectively, with N representing normal erythrocytes. Magnification: x5000 in A and B, and x10000 in C and D.

in Table I. As far as we know, V411M and G283X are novel mutations never reported before. With regard to the frequency of the mutations, the 4 most frequent mutations 851del4, IVS6+5G>A, IVS16ins3kb and 1638-1660dup took account for 87%, while the remaining mutations occupied only 13% of the total 100 mutant SLC25A13 alleles (P1194 and P1194S from the same family). The distribution of SLC25A13 mutations in north and south China was compared using the latitude 30°N as the dividing line, and the 4 most frequent mutations occupied a proportion of 92.8% vs. 58.5% of the total amount of SLC25A13 mutations identified in citrin-deficient patients from south and north China, respectively. The distribution difference was significant statistically, with  $\chi^2$ -value of 11.53 and  $P < 0.005$ .

**Echinocytosis.** Microscopic observation of the morphology of erythrocytes was conducted in 22 citrin-deficient children, and echinocytosis was found in 7 cases. Echinocytosis was transient and resolved along with their biochemical and clinical improvement in 6 cases but one toddler (C0013) with persistent echinocytosis had a lethal outcome at 1 year and 10 months of age due to cirrhosis. Representative micrographic

changes of echinocytosis in a citrin-deficient subject (C0016) are illustrated in Fig. 1, and in Fig. 2, echinocytes at different stages in another citrin-deficient infant (C0027) are illustrated as the means of SEM. We compared the serum biochemical indices between the citrin-deficient subjects with and without echinocytosis. As shown in Table II, patients with echinocytosis demonstrated more severe biochemical abnormalities, including higher serum levels of AST, TBil, DBil, AFP and ApoB100 and lower levels of HDL-Chol and ApoA1.

**Tc-99m-EHIDA scintigraphic findings.** We describe the features of hepatobiliary scintigraphy performed in 8 NICCD subjects (P1513, P1945, C0002, C0025, C0032, C0037, C0042 and C0046). Patient C0025 (Fig. 3) demonstrated impaired hepatic uptake of Tc-99m-EHIDA and consequently a failure of bile duct and bowel visualization before treatment. Delayed hepatic discharge and delayed/weak bile duct and bowel visualization still existed regardless of the significant improvement in the hepatic uptake at his discharge. Similar findings were observed in patient C0046. The remaining 6 citrin-deficient patients did not present with impaired hepatic uptake, however, delayed hepatic discharge and delayed/

Table II. Comparison of serum biochemical indices between citrin-deficient patients with and without echinocytosis.

Indices	Reference range	Echinocytosis <sup>a</sup>	Non-echinocytosis <sup>a</sup>	t	P
Age (months)	-	7.3 (2.7, 18.4)	13.6 (3.5, 90)	2.006	0.059
Age ln(x+10)	-	2.8±0.3	3.3±0.6		
ALT	5-40 U/l	50±17	31±21	2.043	0.055
AST	5-40 U/l	126±65 <sup>b</sup>	67±41	2.563	0.019
GGT	8-50 U/l	87 (39, 429)	23.5 (11, 753)	1.63	0.069
GGT lg	-	2.04±0.37	1.59±0.54		
ALP	20-220 U/l	562±178	382±205	1.976	0.163
LDH	50-240 U/l	355±116	321±70	0.862	0.399
CHE	4600-12000 U/l	5897±3314	9393±3657 (n=13)	2.103	0.050
ADA	4-24 U/l	21±13	16±6	1.312	0.205
TP	60.0-83.0 g/l	58.86±9.73	67.03±8.73	1.949	0.066
ALB	35.0-55.0 g/l	38.40±9.10	44.24±4.58	1.983	0.062
GLB	20.0-35.0 g/l	20.46±6.97	22.57±5.89	0.730	0.474
TBil	2-19 µmol/l	65.9 (4.5, 173.7) <sup>b</sup>	7.45 (3.5, 152.9)	2.225	0.038
TBil ln(x+5)	-	3.99±1.24 <sup>b</sup>	2.91±0.95		
DBil	0-6 µmol/l	45.2 (2.1, 129.5) <sup>b</sup>	3 (0.7, 98.2)	2.363	0.029
DBil ln(x+5)	-	3.63±1.26 <sup>b</sup>	2.48±0.93		
IBil	2.56-20.9 µmol/l	20.7 (2.4, 66.5)	4.15 (2.1, 54.7)	1.792	0.089
IBil ln(x+5)	-	3.15±0.90	2.50±0.71		
TBA	0-10 µmol/l	162.2 (8.2, 328.1)	9.85 (1.6, 174)	1.878	0.076
TBA lg	-	1.86±0.69	1.22±0.75		
AFP	0-10 ng/ml	8069 (575.41, 157736) <sup>b</sup>	94.07 (2.8, 19147.13) (n=10)	2.758	0.015
AFP lg	-	3.96±0.95 <sup>b</sup>	2.21±1.47 (n=10)		
TG	0.39-1.70 mmol/l	1.35 (0.61, 4.11)	1.375 (0.53, 2.37)	1.201	0.244
TG ln(x+10)	-	2.48±0.10	2.44±0.044		
T-Chol	3.12-5.20 mmol/l	4.00±1.62	3.89±1.31	0.172	0.865
HDL-Chol	1.00-1.55 mmol/l	0.72±0.39 <sup>b</sup>	1.30±0.64	2.163	0.044
LDL-Chol	0-3.36 mmol/l	2.23±1.44	1.85±0.93	0.743	0.467
ApoA1	1-1.6 g/l	0.92±0.71 <sup>b</sup>	1.58±0.51	2.436	0.025
ApoB100	0.6-1.08 g/l	1.04±0.68 <sup>b</sup>	0.60±0.21	2.119	0.047
Lpa	0-300 mg/l	37 (20, 94)	118.5 (11, 658)	1.651	0.115
Lpa lg(x+10)	-	1.69±0.20	2.00±0.46		
ApoE	27-49 mg/l	81.45±60.74 (n=4)	65.41±42.73	0.536	0.604

The indices that followed a Gaussian distribution are presented as the mean ± SD, and those skewed as the median (minimum, maximum). <sup>a</sup>n=7 in the echinocytosis and 14 in the non-echinocytosis group, respectively, except where specifically indicated. <sup>b</sup>p<0.05, compared with the counterpart in the non-echinocytosis group. ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ-glutamyl transferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CHE, choline esterase; ADA, adenosine deaminase; TP, total protein; ALB, albumin; GLB, globulin; TBil: total bilirubin; DBil, direct bilirubin; IBil, indirect bilirubin; TBA, total bile acid; AFP, α-fetoprotein; TG, triglyceride; Chol, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apoprotein; Lpa, lipoprotein a; lg, common logarithm; ln, natural logarithm.

weak bile duct and bowel visualization were their common scintigraphic findings. Specifically, radioactivity could still be detected in the liver of patient P1945 even 24 h after intravenous injection of Tc-99m-EHIDA. These findings indicate impaired hepatocyte uptake and/or excretion in particular, of the tracer Tc-99m-EHIDA in citrin-deficient patients.

*Clinical phenotypes after the NICCD state.* By the end of September 2010, 34 of the 51 citrin-deficient subjects

were beyond the age of one year. Fifteen patients after the NICCD state showed feeding problems including poor appetite and picky habits, while 13 demonstrated FTT. Dyslipidemia was observed in 25 patients after the NICCD state, among whom 9 cases including 2 females and 7 males (18.4±3.8 months of age) presented with concurrent FTT and dyslipidemia (Table III), constituting a novel clinical phenotype, namely FTT and dyslipidemia caused by citrin deficiency (FTTDCD). This phenotype is quite different from



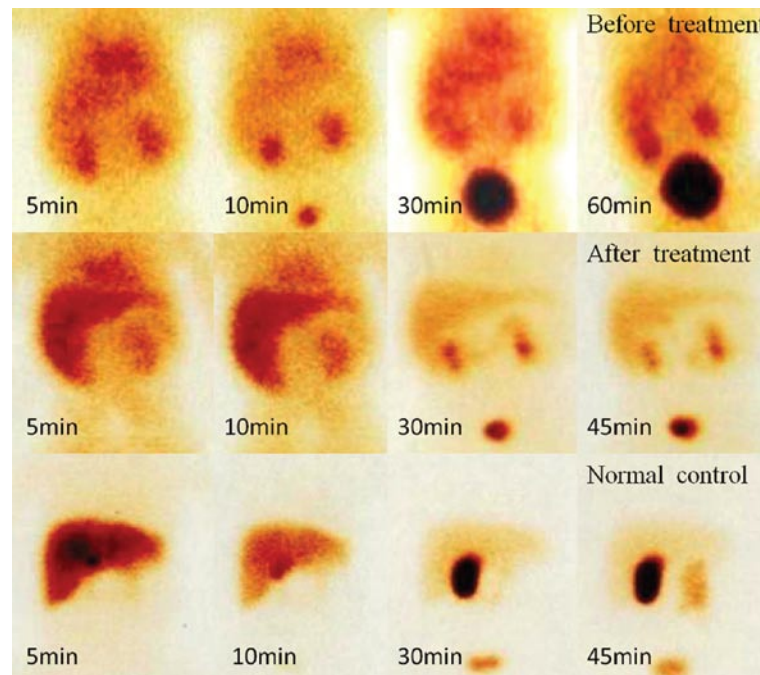


Figure 3. Hepatobiliary scintigraphic findings with Tc-99m-EHIDA as a radioactive tracer in a male infant (C0025) with citrin deficiency. Note the impaired hepatic uptake of the tracer and consequent failure of bile duct and bowel visualization before treatment (upper lane, at the age of 2.8 months). Hepatic uptake was improved significantly in the same children after treatment, however, delayed hepatic discharge and delayed/weak bile duct and bowel visualization (middle lane, at the age of 3.3 months) were still observed, compared with the normal control (lower lane) that demonstrated good hepatic uptake at 5 min, clear bowel visualization as well as liver parenchymatous discharge at 45 min.

NICCD and CTLN2, the two well-recognized citrin-deficient phenotypes. Serum biochemical indices in Table II were also compared in post-NICCD subjects with and without FTTDCD. No significant differences ( $t=0.075$ ,  $P=0.488$ ) were found between the ages of the FTTDCD and non-FTTDCD groups ( $19.8\pm6.3$  and  $21.9\pm14.4$  months, respectively). In addition, no statistically significant differences were observed for the biochemical indices, except for a higher total bile acid (TBA) level in the FTTDCD group ( $t=2.304$ ,  $P=0.034$ ), which suggested increased intrahepatic cholestasis.

## Discussion

SLC25A13 gene analysis in this 51-case cohort confirmed the diagnosis of citrin deficiency in all subjects. Previously identified SLC25A13 mutations in our department were updated in this study, reaching twelve types in total, with V411M and G283X being two novel mutations. Most of the patients diagnosed with citrin deficiency were from south rather than from north China, consistent with the finding that the carrier frequency of SLC25A13 mutations in south China is higher than that in the north (24). The distribution difference of the SLC25A13 mutations in south and north China might be attributed to the heterogeneity of the Chinese nation. The modern Chinese population is believed to have been originated from two distinct populations, one originated in the Yellow River valley and the other in the Yangtze River valley during early Neolithic times (3,000-7,000 years ago), with the latitude of  $30^{\circ}\text{N}$  as the most likely border line (25). The detailed reason why citrin deficiency is so common in south China remains an issue that has not been elucidated. However, glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is another

common genetic disease in the same area, and G-6-PD deficiency confers protection against malaria infection maybe by increasing oxidative stress in erythrocytes (26). Interestingly, augmented oxidative stress in citrin deficiency which has been described very recently (13) may also be a reason for high prevalence of this disease in south China.

Echinocytosis could occur in many conditions, such as hyperbilirubinemia (20,27), uremia (28), liver diseases of varying severity (29) as well as splenic hemangiomas (30). The findings in this study suggest that citrin deficiency is an additional novel echinocytogenic condition. Various mechanisms for echinocytosis have been proposed in other disorders, including abnormal HDL molecules (29), increased intracellular calcium (31) and high plasma pH levels (32). Since citrin-deficient patients with echinocytosis presented with more severe biochemical abnormalities (Table II), echinocytosis in our NICCD patients may be attributed to the interaction of these various biochemical factors. Although the clinical significance of echinocytosis in citrin deficiency still remains an unresolved issue, our findings (Table II) and the lethal outcome of patient C0013 strongly suggest that echinocytosis could be a marker of severe impairment of liver function and prolonged echinocytosis may be a poor prognostic indicator in citrin deficiency.

In this study, the scintigraphic manifestations in the NICCD patients were reported for the first time. The results suggest impaired hepatocyte uptake and/or excretion of Tc-99m-EHIDA in the citrin-deficient liver, although we have no direct evidence to clarify the detailed mechanism(s) at the current stage. Iminodiacetic (IDA) analogs undergo the same metabolism as bilirubin and other organic anions. After hepatocyte uptake, the analogs are excreted into the biliary tree

Table III. Anthropometric and biochemical indices in the 9 post-NICCD patients with failure to thrive and dyslipidemia caused by citrin deficiency.

Patients (Gender, months of age at examination)	Anthropometric indices (5th percentile <sup>a</sup> )		Biochemical indices (mmol/l)			
	Weight-for-age (kg)	Height-for-age (cm)	Triglycerides	Total cholesterol	HDL-cholesterol	LDL-cholesterol
P1518 (male, 20)	<b>8.2</b> (9.4)	<b>72.5</b> (79.6)	<b>5.75</b>	<b>10.08</b>	<b>0.46</b>	2.00
P1628 (male, 22)	10.5 (9.8)	<b>78.0</b> (81.2)	0.82	<b>5.90</b>	1.37	2.80
P1643 (female, 17)	8.5 (8.2)	<b>74.0</b> (75.0)	1.14	<b>5.80</b>	1.79	3.21
P1648 (male, 18)	10.2 (9.1)	<b>75.0</b> (77.8)	<b>1.94</b>	4.42	1.76	2.11
P1933 (male, 26)	<b>9.0</b> (10.4)	<b>80.0</b> (83.6)	<b>2.27</b>	<b>5.42</b>	1.66	2.48
C0005 (male, 18)	10.5 (9.1)	<b>70.6</b> (77.8)	<b>2.37</b>	4.48	<b>0.72</b>	<b>3.37</b>
C0013 (male, 17)	<b>8.8</b> (8.9)	<b>75.5</b> (76.9)	0.69	2.96	<b>0.71</b>	1.32
C0030 (female, 13)	<b>5.6</b> (7.5)	<b>62.0</b> (70.9)	1.08	1.65	<b>0.71</b>	0.56
C0037 (male, 15)	9.5 (8.6)	<b>74.0</b> (75.0)	0.91	2.49	<b>0.69</b>	1.36
Reference range	-	-	0.39-1.70	3.12-5.20	1.00-1.55	0-3.36

<sup>a</sup>The age and gender-matched anthropometric values were based on the WHO Child Growth Standards (<http://www.who.int/childgrowth/standards/en/>). HDL, high density lipoprotein; LDL, low density lipoprotein. The anthropometric and biochemical indices in bold indicate failure to thrive and dyslipidemia, respectively.

by a carrier-mediated organic-anion pathway (33,34). Since bilirubin has the capacity to decrease the uptake and excretion of Tc-99m-EHIDA in the liver (35,36), hyperbilirubinemia in NICCD patients is a possible explanation of the scintigraphic manifestations in NICCD. Moreover, secretion of bilirubin and other organic anions by the canalicular multispecific organic anion transporter (cMOAT) are ATP-dependent in hepatocytes (37-39). Therefore, the secretion of Tc-99m-EHIDA, as a typical IDA analog widely used in clinical practice, may also consume ATP in hepatocytes. Since the energy production is inhibited by NADH accumulation in citrin-deficient hepatocytes (3), this may be the second reason causing the impaired secretion of Tc-99m-EHIDA in NICCD subjects.

NICCD has been previously reported as a self-limiting condition, with clinical presentations resolving between 6 months and 1 year of life. However, this concept has been challenged by recent clinical evidence. Some NICCD infants had to undergo liver transplantation (4,40,41) while some others died due to liver cirrhosis or severe infections (4,15,16,18). Clinical outcome analysis of the hitherto largest Chinese cohort of citrin deficiency in this study revealed an additional toddler (C0013) with a lethal outcome after the NICCD state, due to liver cirrhosis. Moreover, Lee *et al* (6) reported two citrin-deficient teenage siblings presenting with non-alcoholic fatty liver disease, growth retardation and abnormal serum lipid levels before CTLN2 onset. The pre-CTLN2 clinical manifestations in the siblings were similar to the phenotype described as FTTDCD by our group (14). In this paper, we identified more citrin-deficient children who demonstrated FTTDCD features after the NICCD state, once again challenging the traditionally-assumed 'apparently healthy' period in citrin-deficient subjects after the NICCD state (2). Since FTT and dyslipidemia are not trivial health issues in children, more emphasis should be placed on this yet poorly-understood period after NICCD in future studies of citrin deficiency.

In summary, we performed molecular, erythrocytic, scintigraphic and clinical investigations in a citrin-deficient cohort comprised of 51 patients in a pediatric center in south China. SLC25A13 mutations analysis in all cases revealed 12 mutations including two novel mutations, V411M and G283X. We further revealed that citrin deficiency caused echinocytosis that was associated with more severe biochemical abnormalities. For the first time, we described the hepatobiliary imaging feature of this disease with Tc-99m-EHIDA as the scintigraphic tracer. Furthermore, this cohort analysis revealed FTTDCD as a novel clinical phenotype for human citrin deficiency after the NICCD state. The findings in this paper further expanded the genotypic and phenotypic spectrum of citrin deficiency, providing direct evidence to challenge the traditionally-assumed 'apparently healthy' period after the NICCD state for this disease entity.

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