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

Case	Patient	Gender	Mutations[a]	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/***V411M***	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/***G283X***	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

[a]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.

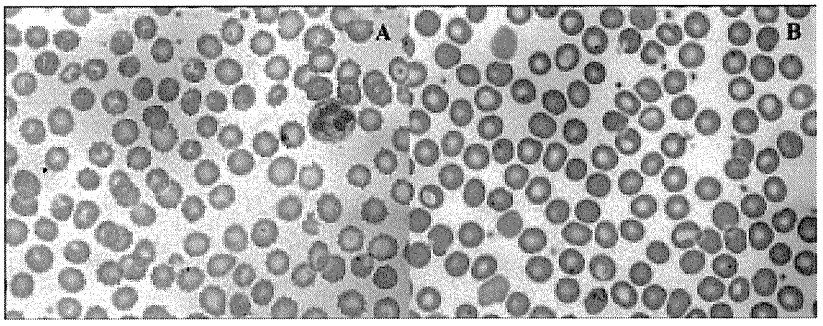

Figure 1. Light micrographs of echinocytosis in a male infant (C0016) with citrin deficiency. (A) Giemsa staining of blood smears demonstrating numerous echinocytes (x1000) at his age of 5.5 months. (B) Normalized erythrocyte morphology (x1000) when the biochemical and clinical abnormalities improved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

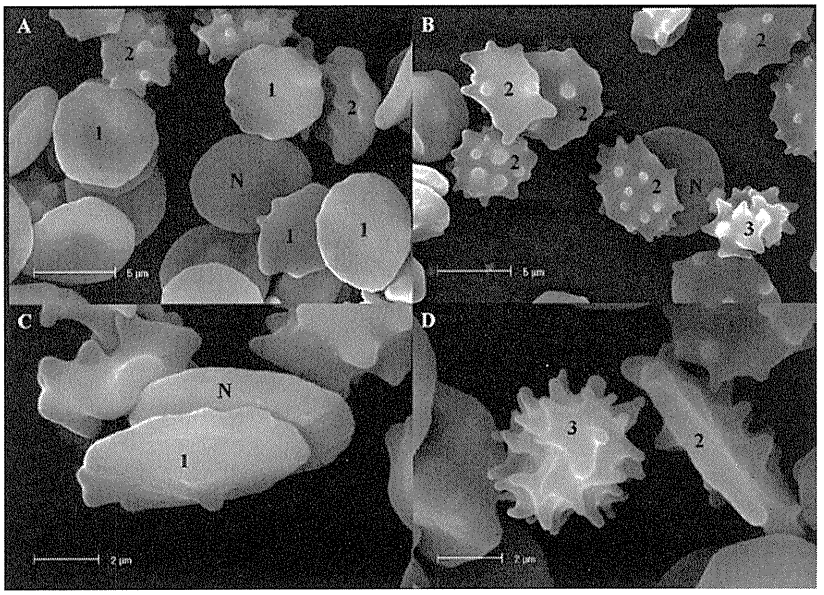

Figure 2. Scanning electron micrographs of echinocytosis in a citrin-deficient infant (C0027). The patient is a 4.5 month-old male. Numbers 1, 2 and 3 indicate echinocytes at stages 1, 2 and 3, respectively, with N representing normal erythrocytes. Magnification: x5000 in A and B, and x10000 in C and 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 χ^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 bowl 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-deficienct patients with and without echinocytosis.

Indices	Reference range	Echinocytosis[a]	Non-echinocytosis[a]	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[b]	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)[b]	7.45 (3.5, 152.9)	2.225	0.038
TBil ln(x+5)	-	3.99±1.24[b]	2.91±0.95		
DBil	0-6 μmol/l	45.2 (2.1, 129.5)[b]	3 (0.7, 98.2)	2.363	0.029
DBil ln(x+5)	-	3.63±1.26[b]	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)[b]	94.07 (2.8, 19147.13) (n=10)	2.758	0.015
AFP lg	-	3.96±0.95[b]	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[b]	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[b]	1.58±0.51	2.436	0.025
ApoB100	0.6-1.08 g/l	1.04±0.68[b]	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). [a]n=7 in the echinocytosis and 14 in the non-echinocytosis group, respectively, except where specifically indicated. [b]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

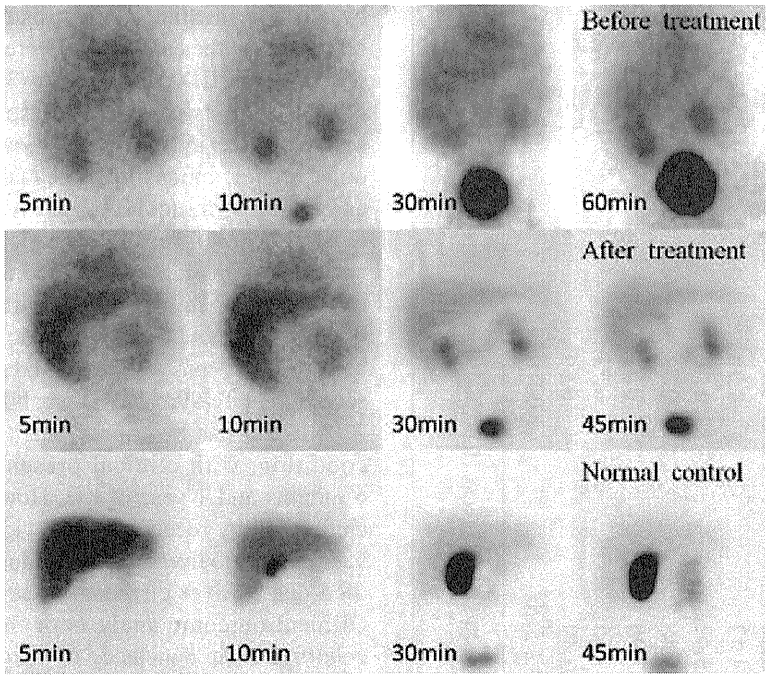

Figure 3. Hepatobiliary scintigraphic findings with Tc-99m-EHIDA as a radioactive tracer in a male infant (C0025) with citrin deficiency. Note the impaired hepatic uptake of the tracer and consequent failure of bile duct and bowl visualization before treatment (upper lane, at the age of 2.8 months). 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±6.3 and 21.9±14.4 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˚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[a])		Biochemical indices (mmol/l)			
	Weight-for-age (kg)	Height-for-age (cm)	Triglycerides	Total cholesterol	HDL-cholesterol	LDL-cholesterol
P1518 (male, 20)	**8.2** (9.4)	**72.5** (79.6)	**5.75**	**10.08**	**0.46**	2.00
P1628 (male, 22)	10.5 (9.8)	**78.0** (81.2)	0.82	**5.90**	1.37	2.80
P1643 (female, 17)	8.5 (8.2)	**74.0** (75.0)	1.14	**5.80**	1.79	3.21
P1648 (male, 18)	10.2 (9.1)	**75.0** (77.8)	**1.94**	4.42	1.76	2.11
P1933 (male, 26)	**9.0**(10.4)	**80.0** (83.6)	**2.27**	**5.42**	1.66	2.48
C0005 (male, 18)	10.5 (9.1)	**70.6** (77.8)	**2.37**	4.48	**0.72**	**3.37**
C0013 (male, 17)	**8.8** (8.9)	**75.5** (76.9)	0.69	2.96	**0.71**	1.32
C0030 (female, 13)	**5.6** (7.5)	**62.0** (70.9)	1.08	1.65	**0.71**	0.56
C0037 (male, 15)	9.5 (8.6)	**74.0** (75.0)	0.91	2.49	**0.69**	1.36
Reference range	-	-	0.39-1.70	3.12-5.20	1.00-1.55	0-3.36

[a]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 the NICCD state for this disease entity.

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Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.

1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

Abbreviations: CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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Table 1
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

Primer/probe set	Mutation		Location	Nucleotide change	Effects of mutations	Allele frequency*[19]	References
A	Mutation [I]	:851del4	exon 9	c.851_854delGTAT	p.R284fs(286X)	33.2%	[1]
B	Mutation [II]	:g.IVS11+1G>A	intron 11	c.1019_1177del	p.340_392del	37.6%	[1]
C	Mutation [III]	:1638ins23	exon 16	c.1638_1660dup	p.A554fs(570X)	3.4%	[1]
D	Mutation [IV]	:S225X	exon 7	c.675C>A	p.S225X	5.3%	[1]
E	Mutation [V]	:g.IVS13+1G>A	intron 13	c.1231_1311del	p.411_437del	8.2%	[1]
F	Mutation [XIX]	:IVS16ins3kb	intron 16	c. aberrant RNA	p.A584fs(585X)	4.6%	[19]
G	Mutation [VI]	:1800ins1	exon 17	c.1799_1800insA	p.Y600X	1.3%	[10]
	Mutation [VII]	:R605X	exon 17	c.1813C>T	p.R605X	0.90%	[10]
	Mutation [VIII]	:E601X	exon 17	c.1801G>T	p.E601X	1.2%	[11]
	Mutation [IX]	:E601K	exon 17	c.1801G>A	p.E601K	0.30%	[11]
	Mutation [XXI]	:L598R	exon 17	c.1793T>G	p.L598R	0%	[15]
						Total 95.1%	

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

2. Methods

2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

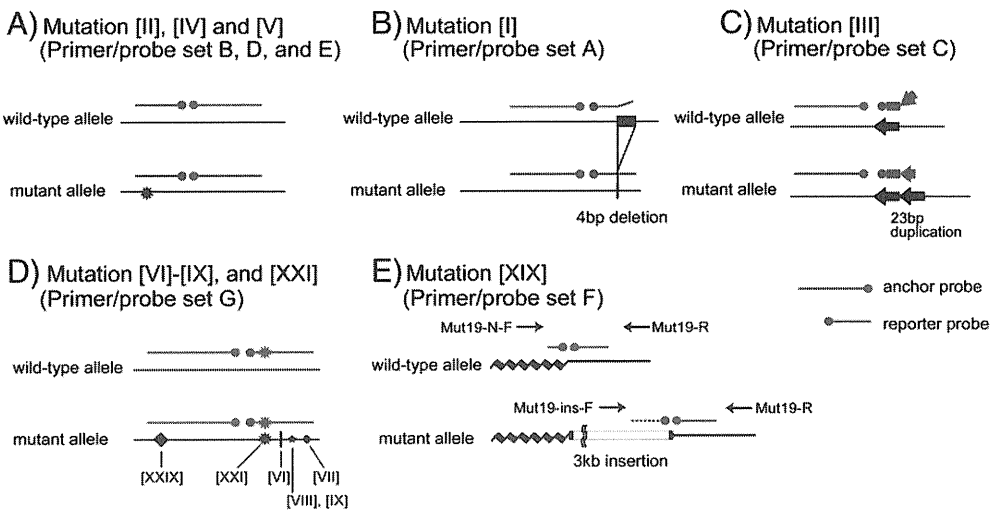

Fig. 1. Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.

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specificity (i.e., binding of the probe to a perfectly matched sequence rather than to regions with sequence mismatches).

Seven primer/probe sets were designed for this study. Fig. 1 shows a schematic diagram of the strategy for mutation detection using these primer/probe sets. Tables 1 and 2 list the primer/probe sets and corresponding sequences and primer concentrations that were used to target the 11 mutations. Primer/probe sets A, B, C, D, E, and F were designed to detect mutations [I], [II], [III], [IV], [V], and [XIX], respectively. Primer/probe set G was designed to detect the five mutations clustered on exon 17: mutations [VI], [VII], [VIII], [IX], and [XXI] (Fig. 1D). All primers and probes were synthesized based on the NCBI reference SLC25A13 gene sequence (GenBank accession no. **NM_014251**) with the exception of mutation [XIX]:IVS16ins3kb, which was designed according to [19].

Real-time PCR and subsequent melting curve analyses were performed in a closed tube using a 20-μL mixture on a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The PCR mixture contained 2.0 μL of genomic DNA (10–50 ng), 0.5 μM of forward primer, 0.5 or 0.1 μM of reverse primer, 0.2 μM of each sensor and anchor probe, and 10 μL of Premix ExTaq™ (Perfect Real Time) reagent (TaKaRa Bio Inc., Otsu, Japan).

The thermal profile conditions were identical for all seven assays and consisted of an initial denaturation step (30 s at 95 °C), followed by 45 amplification cycles with the following conditions: denaturation for 5 s at 95 °C and annealing and extension for 20 s at 60 °C. The transition rate between all steps was 20 °C/s. After amplification, the samples were held at 37 °C for 1 min, followed by the melting curve acquisition at a ramp rate of 0.15 °C/s extending to 80 °C with continuous fluorescence acquisition.

Table 2
Primers, probes and target amplicon sequences, target mutation sites, and primer concentrations.

Primer/probe set	Name	Sequences of PCR products, primer locations, probe sequences, and mutation sites (5′ to 3′)	Concentration (μmol/L)
A		GGCTATACTGAAATATGAGAAatgaaaaagggatgtttttaaatttttataatgtaaattgtaataaattggtatatttgttgcttgtgtttgtttttcccctacagac	
		gtatgaccttagcagacattgaacggattgctcctctggaagagggaactctgccCTTTAACTTGGCTGAGG (181 bp)	
	Mut1-F	GGCTATACTGAAATATGAGAA	0.5
	Mut1-R	CCTCAGCCAAGTTAAAG	0.5
	Mut1-UP	ATGTAAATTGTAATAAATTGGTATATTTGTTGCTTGTGTT-FITC	
	Mut1-DW	LC Red640-GTTTTTCCCCTACAGAC**GACC**-P	
B		GAATGCAGAACCAACGAtcaactggctcttttgtgggagaactcatgtataaaaacagctttgactgttttaagaaagtgctacgctatgaaggcttctt	
		tggactgtatagaggttagtgccacatgctcaatacctgttaggtgaaataacactcaaaggtttggtttctcatcttagtgcctGACATGAATTAGCAAGACTG (205 bp)	
	Mut2-F	GAATGCAGAACCAACGA	0.5
	Mut2-R	CAGTCTTGCTAATTCATGTC	0.1
	Mut2-UP	ACCTAACAGGTATTGAGCATGTG-FITC	
	MUt2-DW	LC Red640-CACTAA**C**CTCTATACAGTCCA-P	
C		GCAGTTCAAAGCACAGTTATTtttatatagtgagaatgtgaccagactgagatggtgttgtgtctctcctgcaggtatgcctgcagcatctttagtg	
		accctgctgatgttatcaagacgagattacaggtg	
		gctgcccggg(**gagatta**caggtggctgcccggg)ctggccaaaccaCTTACAGCGGAGTGATAGAC (175 bp)	
	Mut3-F	GCAGTTCAAAGCACAGTTATT	0.5
	Mut3-R	GTCTATCACTCCGCTGTAAG	0.5
	Mut3-UP	ACCCCTGCTGATGTTATCAAGACGAGATTACAGGT-FITC	
	Mut3-DW	LC Red640-GCTGCCCGGG**GAGATTA**-P	
D		TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctatttt**aatggatttaattcg**ctcctttaacaac	
		atggaactcattagaaagatctatagcactc	
		tggctggcaccaggaaagatgttgaagtGACTAAGGGTGAGTGAGAA (164 bp)	
	Mut4-F	TCAATTTATTTGAGGCTGC	0.5
	Mut4-R	TTCTCACTCACCCTTAGTC	0.5
	Mut4-UP	AATGGATTTAATT**C**GCTCCTTAACA-FITC	
	Mut4-DW	LC Red640-ATGGAACTCATTAGAAAGATCTATAGCACTC-P	
E		TGCACAAAGATGGTTCGgtcccacttgcagcagaaattcttgctggaggctgc**g**taagtacctttt**g**aagctctcttcattgaaaagacttgtttcac	
		atatatatcactaccatggtcaacaggtgtggactaaggcttctgttTAACCACAGATCCTGCA (162 bp)	
	Mut5-F	TGCACAAAGATGGTTCG	0.5
	Mut5-R	TGCAGGATCTGTGGTTA	0.5
	Mut5-UP	GTGAAACAAGTCTTTTCAATGAAGAGAGCTTC-FITC	
	Mut5-DW	LC Red640-AAGGTACTTA**C**GCAGCCTC-P	
F	normal allele	GGAGCTGGTGGTATGGAAataatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgtgatttctc	
		catttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaatttt	
		GGCTGCTGCTAATGCTC (244 bp)	
	insertion allele	CCATCTTCCTCCTCCCTTggcagccccgccccccgatttctccatttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgatttt	
		ggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (196 bp)	
	Mut19-N-F	GGAGCTGGTGGTATGGAA	0.5
	Mut19-ins-F	CCATCTTCCTCCTCCCTT	0.5
	Mut19-R	GAGCATTAGCAGCAGCC	0.5
	Mut19-UP	ACCAAACTGGGGTGAGGATCGAAATACACGAGCTTTAAAAAAATG-FITC	
	Mut19-N-DW	LC Red640-AGAAATCACAGATATAATTAGATATTT-P	
	Mut19-ins-DW	LC Red640-AGAAATCGGGGGGCGGGG-P	
G		TCTTAACTAACTCTTTGGTATCAGGTaaatttttaaaatatctaattatatctgtgatttctccatttttttaaagctcg	
		tgtatttcgatcctcaccccagtttggtgtaactttgc**t**gactt**a(a)cg**aattgctacag**c**ga	
		tggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (217 bp)	
	Mut6-9, 21-F	TCTTAACTAACTCTTTGGTATCAGGT	0.5
	Mut6-9, 21-R	GAGCATTAGCAGCAGCC	0.5
	Mut6-9, 21-UP	TGTATTTCGATCCTCACCCCAGTTTGGTGTAACTT-FITC	
	Mut6-9, 21-DW	LC Red640-GC**G**GACTT**ACG**AATTGCTACAG**C**GA-P	

Upper case and underlined letters indicate the locations of primers and probes, respectively. Inserted DNA is shown in parenthesis. Nucleotides in boldface were used for mutation detection.
F: forward, R: reverse, UP: upstream, DW: downstream, N: normal allele, ins: insertion allele, FITC: fluorescein isothiocyanate, P: phosphate.

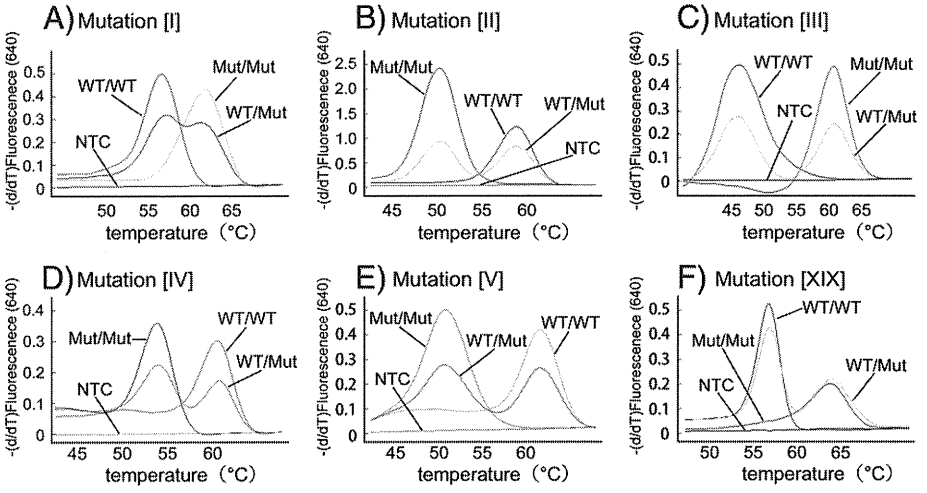

Fig. 2. Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

2.3. Validation of the mutation detection system

After establishing the protocol for detecting the 11 prevalent mutations, 50 DNA samples from patients' blood were sent from Kagoshima University to Tohoku University for the validation of this system in a single-blind manner. Similarly, 26 DNA samples purified from paper-filter blood samples were analyzed in the same manner as the blood DNA samples.

2.4. Estimation of the carrier frequency

For the estimation of the heterozygous carrier frequency, 420 genomic DNA samples from healthy volunteers were screened using the HybProbe analysis for the 11 prevalent mutations. All detected mutations were confirmed by direct sequencing.

2.5. Ethics

This study was approved by the Ethical Committees of Tohoku University School of Medicine and Kagoshima University. Written informed consent was obtained from all participants or their guardians.

3. Results

3.1. Development of the mutation detection system

In primer/probe sets B, D, and E, the reporter probes were designed to be complimentary to the wild-type allele (Fig. 1A). To allow for an improved detection of the mutations, primer/probe sets A and C were designed to be complementary to the mutant allele (Figs. 1B, C). In the primer/probe set F, two forward PCR primers, which were specific to the wild-type and the mutant alleles, were used with a common reverse primer for the co-amplification of the wild-type and 3-kb insertion alleles (Fig. 1E). Two reporter probes, which had a common anchor probe, were used for the detection of the wild-type and mutant alleles. Because the two reporter probes had different melting temperatures, we were able to identify the allele that was amplified. Fig. 2 shows representative results of the melting curve analyses using the primer/probe sets A–F, in which all of the mutant alleles generated distinct peaks corresponding to the wild-type alleles.

In the primer/probe set G, we used a reporter probe that was complementary to the mutant [XXI] allele (Fig. 1D). All five mutations in exon 17 were successfully differentiated from the wild-type allele (Figs. 3A–E). The [XXIX] mutation is an additional mutation in exon

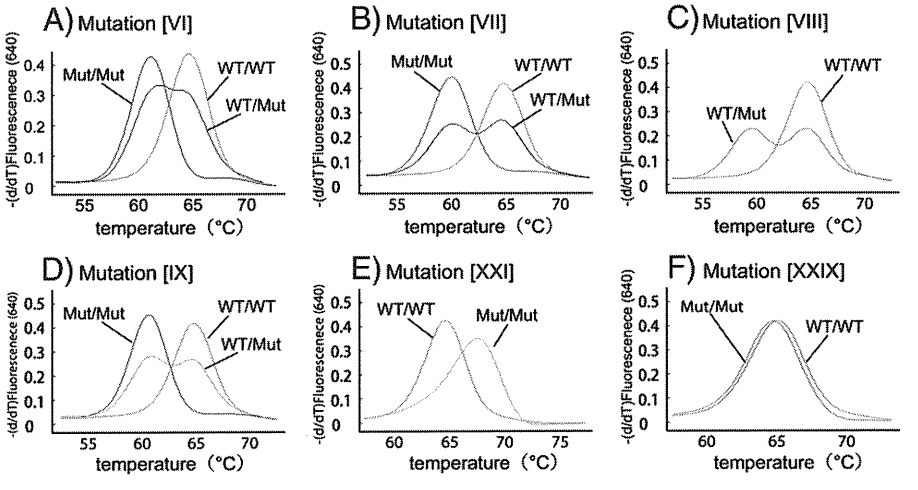

Fig. 3. Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor probe. WT: wild-type allele, Mut: mutant allele.

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17 that is not listed in Table 1. The [XXIX] mutation is located in the anchor-probe binding site and not on the reporter-probe binding site (Fig. 1D). To examine the effect of mutations on the anchor probe, we genotyped a patient with a heterozygous [XXIX] mutation using primer/probe set G (Fig. 3F). We found no change in the melting curves between the wild-type allele and the [XXIX] allele, thereby suggesting that point mutations within the anchor probe sequence have little effect on the melting curve analysis.

3.2. Validation

The genotypes determined at Tohoku University using the proposed method and those determined at Kagoshima University using a previously published method were identical for the 11 common mutations (Table S1 in supplementary material). We performed a similar test using DNA samples purified from filter-paper blood samples to determine if this method could be used for newborn screening. The genotypes determined in both laboratories were identical for all 26 DNA samples (Table S2 in supplementary material).

3.3. Frequency of eleven prevalent mutations

We found four heterozygous carriers of mutation [I], three of mutation [II], and two of mutation [V]. In addition, primer/probe set G detected one heterozygous mutation, which was confirmed as mutation [VIII] by direct sequencing. Altogether, 10 mutations were detected in 420 Japanese healthy controls.

4. Discussion

We developed a simple and rapid genetic test using real-time PCR combined with the HybProbe system for the 11 prevalent mutations in *SLC25A13*: mutations [I], [II], [III], [IV], [V], [VI], [VII], [VIII], [IX], [XIX], and [XXI]. This genetic test is a closed-tube assay in which no post-PCR handling of the samples is required. In addition, the genotyping is completed within 1 h. This test can utilize DNA samples purified from both peripheral blood and filter-paper blood. The reliability of the test was confirmed by genotyping 76 blind DNA samples from patients with citrin deficiency, including 50 peripheral blood and 26 filter-paper blood DNA samples. Because screening for the 11 targeted mutations would identify 95% of mutant alleles in the Japanese population [19], both, one, and no mutant alleles are expected to be identified in 90.4%, 9.3%, and less than 0.3% of patients, respectively. This genetic test would be useful not only in Japan but also other East Asian countries, including China, Korea, Taiwan and Vietnam, in which the same mutations are prevalent. Our test is expected to detect 76–87% of the mutant alleles in the Chinese population [12,19,25], 95–100% in the Korean population [12,19,26], 60–68% in the Taiwanese population [27,28], and 100% in the Vietnamese population [12,19]. If we were to prepare a primer/probe set for mutation [X]:g.IVS6+5G>A [12], which is prevalent in Taiwan, the estimated sensitivity would exceed 90% in the Taiwanese population [27,28].

Recently, the high resolution melting (HRM) method was reported to be suitable for the screening of mutations in the diagnosis of citrin deficiency [28]. HRM analysis is a closed-tube assay that screens for any base changes in the amplicons. The presence of SNPs anywhere on the amplicons can affect the melting curve, thereby suggesting that HRM is not suitable for screening for known mutations, but rather, is best suited to screening for unknown mutations. When we detected one heterozygous prevalent mutation, we performed HRM screening for all 17 exons of *SLC25A13*. After HRM screening, only the HRM-positive exons were subjected to direct sequencing analysis. Several mutant alleles were identified using this approach.

The frequency of homozygotes, including compound heterozygotes, presenting *SLC25A13* mutations in the population at Kagoshima (a prefecture in the southern part of Japan) has been calculated to be 1/17,000 based on the carrier rate (1/65) [19]. The prevalence of NICCD has been also reported to be 1/17,000–34,000 [29]. In this study, the carrier rate in Miyagi (a prefecture in northern Japan) was 1/42 (95% confidential interval, 1/108–1/26), thereby yielding an estimated frequency of patients with citrin deficiency of 1/7,100. Our result, together with the previous report [19], suggests that a substantial fraction of the homozygotes or compound heterozygotes of SLC25A13 mutations was asymptomatic during the neonatal period.

The early and definitive diagnosis of citrin deficiency may be beneficial for patients with citrin deficiency by encouraging specific dietary habits and avoiding iatrogenic worsening of brain edema by glycerol infusion when patients develop encephalopathy [30,31]. Because the screening of blood citrulline levels by tandem mass analysis at birth does not detect all patients with citrin deficiency, the development of a genetic test would be welcomed. In this study, we demonstrated that genomic DNA extracted from filter paper blood samples was correctly genotyped, thereby indicating the feasibility of newborn screening using this genetic test. If 100,000 babies in the northern part of Japan were screened by this method, we would detect 14 homozygotes or compound heterozygotes with *SLC25A13* mutations and 2400 heterozygous carriers. In 2400 heterozygous carriers, we would expect to observe only 1 to 2 compound heterozygotes with one target and one non-target mutation. The estimated frequency of babies with two non-target mutations is 0.04/100,000. Our genetic method would therefore allow us to screen newborn babies efficiently. If we performed this genetic test in a high-throughput real-time PCR system, such as a 384- or 1,536-well format, the cost per sample could be lowered.

In conclusion, we have established a rapid and simple detection system using the HybProbe assay for the 11 prevalent mutations in *SLC25A13*. This system could be used to screen newborns for citrin deficiency and may facilitate the genetic diagnosis of citrin deficiency, especially in East Asian populations.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2011.12.024.

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9. 代　謝

尿素回路障害2：citrin欠損症（NICCD，CTLN2）

citrin deficiency（NICCD，CTLN2）

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Citrin欠損症の病態像は多彩であるため，分子遺伝学的な確定診断が必要であるが，特異な食癖も有効な診断マーカーとなる．従来の治療法・対症療法の中に，症状の増悪化に繋がるものがあるので注意を要する．

診断のポイント

Citrin欠損症診断のフローチャートを図1[1]に示す．肝内胆汁うっ滞性新生児肝炎（neonatal intrahepatic cholestasis caused by citrin deficiency：NICCD）の40%は新生児マス・スクリーニング陽性で，半数はおもに1～4カ月齢に遷延性黄疸，成長障害，凝固異常などの症状を呈することで気づかれる．いずれも一過性のシトルリン（citrulline：Cit）血症を示すので，タンデムマス法などの導入により血中Citが測定できれば，多くの症例の早期発見に繋がる．しかし，citrin欠損症では多種多様な検査所見から診断に苦慮する症例が少なくないため，遺伝子検索による確定診断の意義は大きい．多くのNICCDでは，臨床症状・異常検査データが生後6カ月には正常化するので，生後4カ月くらいまでにアミノ酸分析を行うことが重要である．また1歳以降では，問診により特異な食癖（糖質を嫌い，蛋白質・脂質を好む）の有無を必ず把握すべきである．

成人発症Ⅱ型Cit血症（adult-onset type Ⅱ citrullinemia：CTLN2）の診断も，本疾患を念頭においていない限り大変むずかしく，他疾患と誤診する場合も多い．しかし，血中アンモニア・血漿Cit・血中PSTI（pancreatic secretory trypsin inhibitor）などの測定，脂肪肝の有無などに加え，特異な食癖を聴き出すことで容易にCTLN2を診断することができる（図1）[1]．

重症度評価

NICCDでは，これまで5例が肝移植手術を受けているが，重篤になる要因は明らかでない．一方CTLN2では，肝臓特異的にASS（argininosuccinate synthetase）蛋白が低下する症例が多いため，高アンモニア血症をきたし重篤な症状の出現に至る．この肝ASS蛋白低下の原因はcitrin欠損に基づく二次的変化であること以外，いまだに解決されていない．しかし，肝ASS蛋白・活性の残存量は，治療法を検討するうえで重要な要因になると考えられる．

基本病態

図2に示すように，citrin欠損症の病態は実に多彩である．二つの基本病態（NICCDとCTLN2）以外に，見かけ上，健康と考えられていた時期においてもさまざまな症状が出現していることもわかってきている．また一方，健康に過ごすcitrin欠損症例も存在すると考えられるので，citrin欠損症の詳細な自然歴の検討が必要である．

本疾患のもっとも際立った特徴のひとつは，甘い

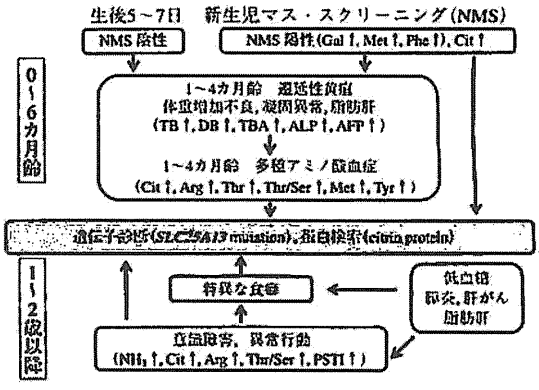

図1　Citrin欠損症診断のフローチャート
文献1）より引用，一部改変

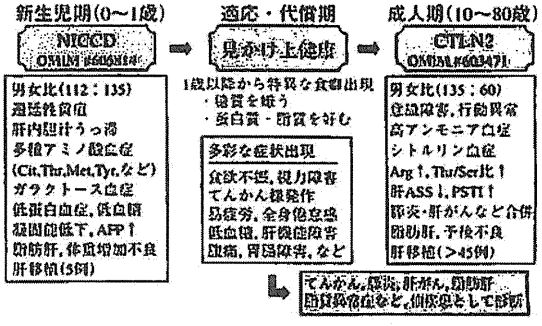

図2　Citrin欠損症の病態像

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ものを嫌い，糖質を投与すると高アンモニア血症を増悪化させることである．この常識を外れた病態のため，後述するように，従来の高アンモニア血症治療法はむしろ危険でさえあり，これまで医原性（iatrogenic）に予後不良な状態にしていたと考えられる．CTLN2 はもはや，きわめて重篤な疾患ではなくなりつつあることを特記しておく．

治療の実際

NICCD では，新生児肝炎・ガラクトース血症・チロシン血症などに対する対症療法として，中鎖脂肪酸（MCT）強化・乳糖除去・Phe/Tyr 除去ミルクなどに加えて，脂溶性ビタミン，ウルソデオキシコール酸，フェノバルビタールなどが用いられてきた．

これまで CTLN2 に対して行われてきた治療は，高アンモニア血症に対する対症療法と肝移植である．しかし，肝型アスパラギン酸・グルタミン酸輸送体である citrin 欠損症では，一般的な高アンモニア血症に対する治療〔低蛋白食・高カロリー輸液・脳浮腫治療薬のグリセオール®（禁忌薬剤）[2]の投与〕は病態発症を誘導・増悪化する因子となりうる．

最近，citrin 欠損症では，患者の好む（後述するような糖質が少なく，蛋白質と脂質の多い）食事組成にするという治療方針が取り入れられてきている．これは，NICCD の成長障害と出血傾向の改善においても有効であった[3]．一方，アルギニンは血中アンモニアと血清トリグリセリドの低下を誘導した[4]．さらに現在，細胞質で NADH を消費する作用をもつピルビン酸の投与効果が検討されつつある[5]．また肝移植は，citrin 欠損症の代謝異常矯正には非常に有効である．

最新ガイドライン/エビデンス

Citrin 欠損症 18 名（NICCD 経験者およびその同胞と父親）で実施した定量的栄養調査では，PFC 比（蛋白質，脂肪，炭水化物のエネルギー比）が，17～21：40～47：33～40 となり，厚生労働省調査の一般集団のデータ 14～15：25～30：54～58 に比べて，炭水化物の摂取を避けているという結果が得られた[6]．一方，病院食摂取時の経験[7]から推察すると，蛋白質および脂肪が炭水化物の毒性を和らげている可能性があることもわかってきた．

近年のトピックス

CTLN2 未発症の citrin 欠損症例（13 歳女子）はピルビン酸治療[5]の開始前に，検査入院で病院食（PFC 比 10～15：20～25：55～65）を 1 日半食べただけで血中アンモニアと Cit が上昇し，自分の好きな食事（PFC 比 20：45：35）に切り換えるとそれらの上昇は消失した[7]．特異な食癖は自ら生み出した知恵（治療法）と解釈できるので，学校給食など日本人特有の食事の強要は避けるべきである．

私の治療方針

「Citrin 欠損症は，これまでの一般的な概念では通用しないこともおこりうる疾患である」ことを，医療関係者のみならず患者自身と家族が頭に入れておくことと，早期の確定診断が誤治療防止に繋がり，最大の治療・予防法になると考える．

ピットフォールと対策

通常，嘔吐下痢症では食事制限と乳製品を与えない指導が行われる．しかし，蛋白質と脂肪を必要とする citrin 欠損症例に対して，糖も制限して水分のみ投与すると，低ナトリウム血症，脱水の持続，低血糖，エネルギー不足を招き危険である．糖の投与は，肝臓での取り込み・代謝を必要とするほどの大量になると危険となるが，血糖を維持する程度の投与には問題はないことを強調したい．

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◆◆一般演題：原著◆

先天性代謝疾患と一酸化窒素, 酸化ストレス

Nitric oxide and oxidative stress in inherited metabolic disorders

長坂博範[1)]、塚原宏一[2)]

はじめに

先天性の代謝性疾患において蓄積する代謝産物はその有害性から多種多様な症状を引き起こす。これらの産物はまたreactive oxygen species (ROS) を発生させることが想定されているが、実証された疾患は、まだ少ない。先天性尿素サイクル異常症では、一酸化窒素nitric oxide (NO) 合成の基質アミノ酸であるarginineやcirtullineの血中濃度の著明な変化を伴うことから、NO合成が変化することが想定される (図1) [1)]。酸化ストレスとNO代謝は多くの生体反応に影響を与えることから、先天性代謝疾患においても、これらを評価することは病態を理解するうえで重要である[2)-7)]。

先天性尿素サイクル異常症では、血中arginineとcitrulline濃度の著明な異常が特徴的である (図1)。その病態は単にammoniaが高いというだけで説明できない部分が多いとされている。citrin欠損症は尿素サイクル関連疾患とされているが、そのclinical pictureは極めて特徴的である (図2) [8)9)]。乳児期は胆汁鬱滞、ないしは、乳児肝炎様症状を呈し、大部分の症例が1歳までに症状は軽快する。しかし、一部の症例は思春期ないしはそれ以降に、著明な脂肪肝および肝不全 (成人発症高シトルリン血症；CTLN) を発症する。先天性門脈大循環shuntは、新生児mass-screeningで高galactose血症などにより、しばしば発見される[10)-12)]。正確な予後は不明であるが最近、10歳以降に肺高血圧症を呈した症例のreportsが増えている。NOは血管内皮機能endothelial functionのhomeostasisを維持するという極めて生体にとって重要な役割を担っていることから、先天性門脈大循環shuntにおいてもNO合成を評価することは必要と思われる。

今回、我々は、これらの疾患におけるNO代謝、酸化ストレスの評価を試みたので報告する。

対象と方法

以下の3疾患を対象にして、NOの代謝産物nitrite/nitrate (NOx^-) およびNO合成酵素 (NOS) の抑制物質であるaymmetric dimethylarginine (ADMA)の血清濃度を調べた[13)-15)]。酸化ストレスに関しては尿中酸化ストレスマーカーである、8-hydroxy-2'-deoxyguanosine (8-OHdG) および acrolein-lysine、血中酸化LDLなどにより評価した。また、superoxide dismutase (SOD), catalaseなどのanti-oxidants enzymesの赤血球での活性や抗酸化作用を有するビタミンEの血中レベルを測定した。先天性門脈大循環shuntではvasoconstrictorであるendothelin-1 (ET-1) の血漿中濃度も調べた[16)17)]。

尿素サイクル異常 (図1)：ornithine transcarbamylase defect (OTCD) 7名 (女/男, 5/2; 1－7歳) Arginosuccinate synthase defect (ASSD) 5名 (女/男, 4/1; 9－19歳), Arginosuccinate lyase defect (ASLD) 3名 (女/男, 1/2; 5－8歳)。類縁疾患として、リジン尿性蛋白不耐症lysinuric protein intolerance (LPI) 患者3名 (女/男, 1/2; 21－34歳)。Citrin欠損症：1－10歳の肝機能ほぼ正常化している20名 (女/男10/10)。先天性門脈大循環

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shunt：1－5歳の14名（女/男6/8）の肝内門脈肝静脈shuntの症例。

結　果

尿素サイクル異常症患者ではL-arginine投与中であり血中arginine濃度は正常群よりやや高めである。OTCD患者のNOx⁻は同年代正常群に較べて有意に高かったが、ADMA値は正常群同様であった。さらに、NOx⁻は血中arginine値と有意な正の相関をしていた。ASSD患者は、NOx⁻は同年代正常群に較べて有意に低く、ADMA値は高値であった。ASLD患者は、NOx、ADMA値ともに正常群と有意差示さなかった。ASSD, ASLDはともにcitrulline値が極めて高く(特にASSD)、citrulline濃度はADMAと正の相関、NOx濃度と負の相関を示した。LPI患者では、arginine低値とNOx高値が目立った (図3、表1, 2)。

Citrin欠損症患者では、NOx⁻, ADMA濃度は正常群と有意差はなかった。しかし、尿中8-OHdGおよびacrolein-lysine、血中酸化LDLは有意に高かった (表3, 4)。

先天性門脈大循環shuntでは、血中アミノ酸濃度に変化はなかったが、NOx⁻は正常群より有意に低値、ADMA濃度は有意に高値を呈していた。血漿中ET-1濃度は、正常群より有意に高かった。また、尿中8-OHdGおよびacrolein-lysine、血中酸化LDLなどの酸化ストレスマーカーの有意に上昇しており、逆にvitamin E濃度は低下していた (図4)。

考　察

尿素サイクル異常症では、血中arginineないしはcitrulline濃度がNO代謝にリンクしていることが示された。ASSD、ASLDでは極めて高い血中citrulline値がNO産生を抑制していると解釈された。その際、ADMAはNOxと反対にcitrullineと強い正の相関がみられたことから、ADMA増加を介したNO産生の抑制という解釈もできよう。LPIは腎、腸管上皮における二塩基アミノ酸(lysine, arginine, ornithine) トランスポーターである、SLC7Aの欠損により起こる疾患である[18]。この疾患ではNOxの増加は顕著であった。しかしながら、ADMAの増加はみられず、NOとADMAとの間で前者優位の不均衡が生じていると考えられた。本疾患の随伴症状としてmacrophage-activated hemophagocytosisが有名であるが、NOの過剰産生がその要因になっている可能性がある。Arginineが細胞内に停滞することによりNOが異常に産生されるという報告もある[19]。

Citrin欠損症ではsilent stageといえども酸化ストレスがenhanceされた状態であることが推察された。酸化ストレスとCTLN-2の関係をより詳細に検討する必要があると考えられた。特に、CTLN-2の脂肪肝はしばしば顕著であり何らかの機序で肝臓にかかる酸化ストレスがenhanceされた状態が続くと考えられる[8) 9)]。

先天性門脈大循環shuntでは、NOとET-1産生のアンバランスがあることが推察された。これが思春期以降の肺高血圧発症に関与しているかどうか、これから検証しなければならない[12]。また、酸化ストレスマーカーが有意に増加していることから、酸化ストレスもまた肺高血圧発症に関与している可能性が考えられた。NO産生は酸化ストレスと密接に関係している（図5）。酸化ストレスが遷延する場合にはNOS構造が変化し、またNO産生に必須のBH4が枯渇してNOの代わりにsuperoxideが過剰産生されることがある[4]。さらに酸化ストレスは内皮細胞でのET-1産生を亢進させる[7]。先天性門脈大循環shuntのNOとET-1産生のアンバランスは、酸化ストレスが介在するこれらの機序からくるのかもしれない。特にshunt率が40%を超えると、このような異常が顕著になってくることから、shunt率が高い場合、肺高血圧発症に注意して、心電図、心エコー、血中BNPなどのモニターが必須であると考える。

以上、酸化ストレス、NO代謝異常が先天代謝異常症の病態を多面にわたり修飾していることが強く示唆された。

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表1 Levels of plasma arginine and citrulline and serum nitrite/nitrate and asymmetric dimethylarginine in urea cycle defects

Deficiency (Age at present)	Arginine (μmol/L)	Citrulline (μmol/L)	NOx^- (μmol/L)	ADMA (μmol/L)	ADMA/NOx^-
OTC, *n*=7 (1.7–7.8 years)	185 ± 45 [c] (129–278)	8.4 ± 2.1 [c] (5.9–9.6)	67 ± 23 [a] (35.3–108)	0.860 ± 0.271 (0.367–1.556)	0.015 ± 0.007 (0.003–0.027)
ASS, *n*=5 (9.3–17.1 years)	229 ± 19 [c] (204–256)	2867 ± 864 [c] (1768–3870)	22 ± 7 [b] (13.8–32.1)	1.422 ± 0.257 [b] (1.090–1.872)	0.061 ± 0.021 [c] (0.034–0.082)
ASL, *n*=3 (5.6–8.8 years)	259 ± 14 [c] (235–276)	562 ± 84 [c] (457–688)	49 ± 2 (45.1–50.8)	1.125 ± 0.064 (1.032–1.225)	0.024 ± 0.002 (0.024–0.027)
LPI, n=3 (21.5–34.8 years)	36 ± 5 [c] (31–41)	44 ± 2 (41–46)	110 ± 21 [c] (75–145)	0.583 ± 0.111 (0.423–0.698)	0.004 ± 0.004 [c] (0.002 –0.009)
Controls, *n*=36 (1.1–19.9 years)	99 ± 19 (66–156)	36 ± 8 (16–56)	39 ± 16 (17.9–80.1)	0.513 ± 0.116 (0.199–0.875)	0.019 ± 0.008 (0.006–0.036)

OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; NOx^-, nitrite/nitrate; ADMA, asymmetric dimethylarginine; LPI, lysinuric protein intolerance. Data are presented as mean ± SD and range. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs. controls. In the control group, no significant correlation was found between the age and the variables.

表2 Correlation (*r* values) in pairs of variables in children with urea cycle defects

Deficiency	Arginine vs. NOx^-	Arginine vs. ADMA	Citrulline vs. NOx^-	Citrulline vs. ADMA	NOx^- vs. ADMA
OTC	0.99 [b]	−0.47	0.08	−0.14	−0.39
ASS	−0.75	0.55	−0.97 [a]	0.93 [a]	−0.96 [a]
ASL	0.67	−0.75	−0.98 [a]	0.99 [a]	−0.75

OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; NOx^-, nitrite/nitrate; ADMA, asymmetric dimethylarginine.

[a] $p < 0.05$, [b] $p < 0.01$ (Pearson's correlation test).

表3 Blood levels of amino acids, NOx and ADMA in citrin-deficit children

	Arginine (μmol/L)	Ornithine*** (μmol/L)	Citrulline** (μmol/L)	NOx^- (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

NOx^-, nitrite/nitrate; ADMA, asymmetric dimethylarginine

Presented data are mean (SD) values and ranges.

** p <0.01, *** p <0.001 versus controls

表4 Levels of urinary biomarkers for oxidative stress, anti-oxidant enzyme activities in erythrocytes and blood vitamin E level in citrin-deficit children

	8-OHdG*** (ng/mg Cr)	Acrolein-lysine** (nmol/mg Cr)	SOD* (U/mg prot)	Cat* (pmol/mg prot)	Vitamin E** (mg/dl)
13 patients	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
32 controls	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

8-OHdG; urinary 8-hydroxy-2'-deoxyguanosine; SOD: superoxide dismutase; Cat, catalase; Cr, creatinine

Presented data are mean (SD) values and ranges.

* p <0.05, ** p <0.01, *** p <0.001 versus controls

図1. Urea cycle and NO pathway

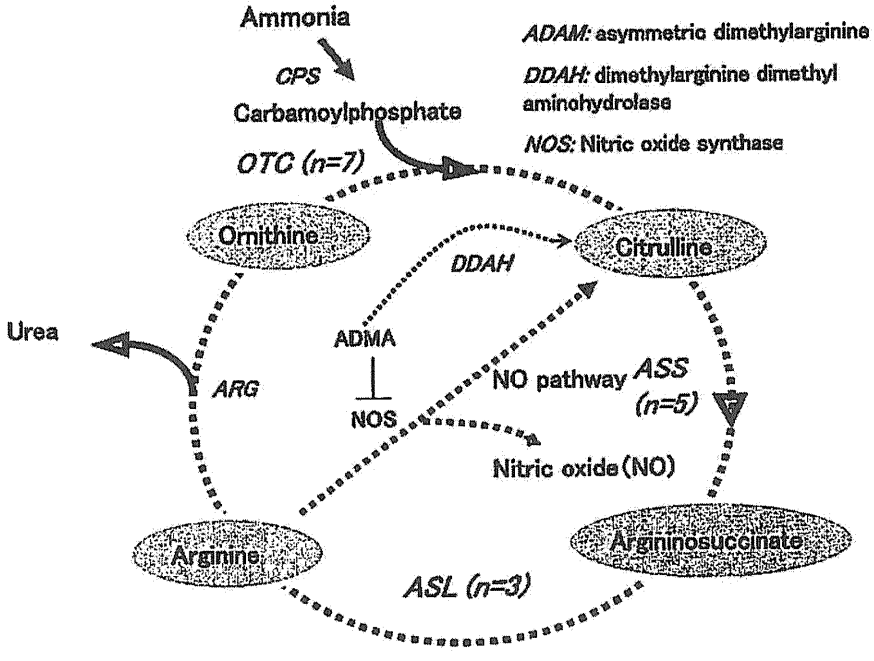
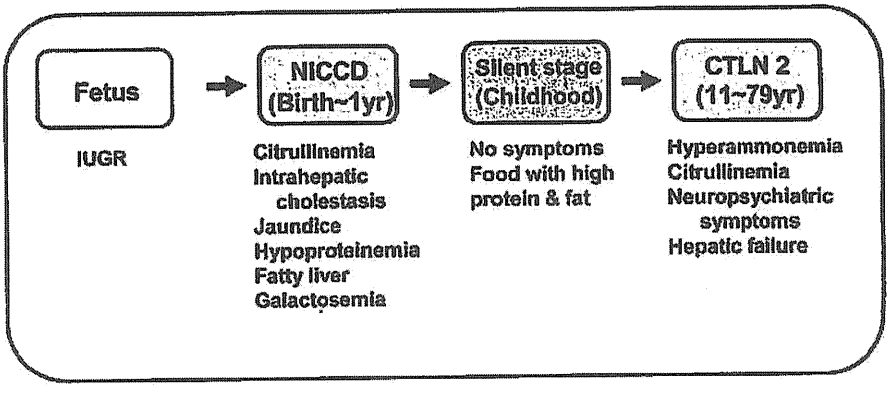


図2. Clinical course of citrin deficiency



We examined the profiles of amino acids, carbohydrates, and lipids, NO synthesis, and the status of oxidative stress.