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

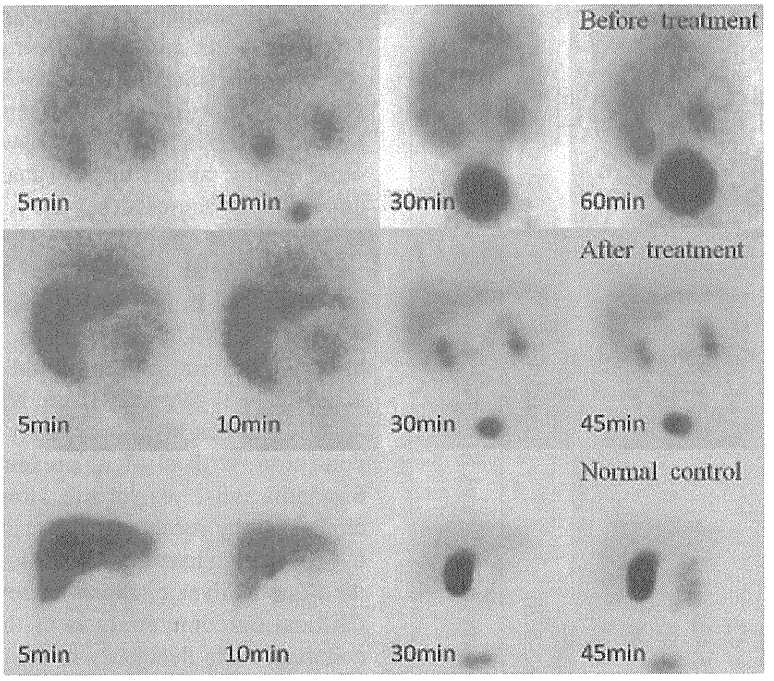

Figure 3. Hepatobiliary scintigraphic findings with Tc-99m-EHIDA as a radioactive tracer in a male infant (C0025) with citrin deficiency. Note the impaired hepatic uptake of the tracer and consequent failure of bile duct and bowl visualization before treatment (upper lane, at the age of 2.8 months). 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.

Acknowledgements

The authors are deeply grateful to all the citrin-deficient patients and their parents for their cooperation. We also appreciate Professor Nelson L.S. Tang and Dr Qun-Zhou Zhang for their critical reading and revision of this manuscript. Our research was supported financially in part by the Medical Research Fund of Guangdong Province, China (Nos.

A2008358 and A2009366); by Project 81070279 supported by the National Natural Science Foundation of China (NSFC); by Grants-in-Aid for Scientific Research (B: Nos. 16390100 and 19390096); and by the Asia-Africa Scientific Platform Program (AASPP) from the Japan Society for the Promotion of Science (JSPS).

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Molecular Genetics and Metabolism xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

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

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ARTICLE INFO

Article history:
Received 13 November 2011
Received in revised form 29 December 2011
Accepted 30 December 2011
Available online xxxx

Keywords:
Citrin deficiency
Genetic diagnosis
Rapid diagnosis
Expanded newborn screening
SLC25A13

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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doi:10.1016/j.ymgme.2011.12.024

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

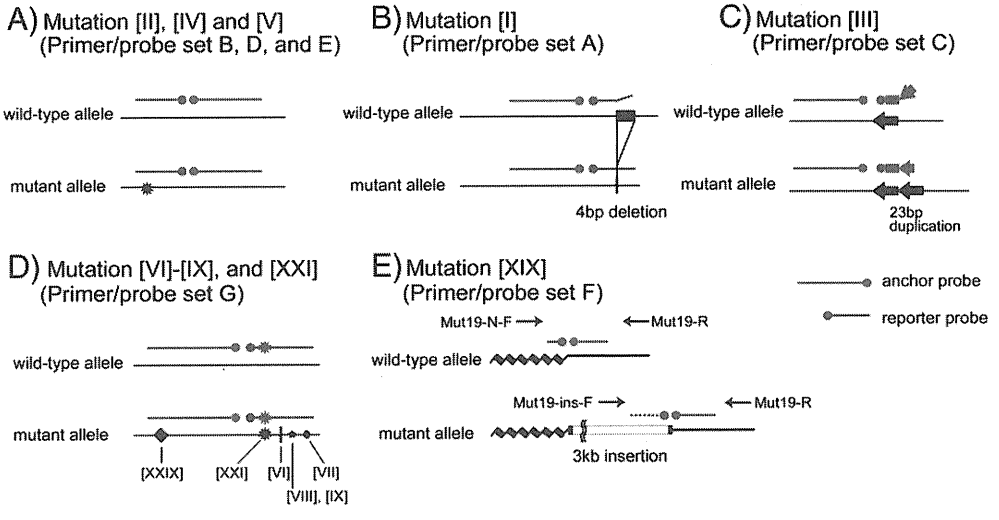

Fig. 1. Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type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

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		GGCTATACTGAAATATGAGAAatgaaaaaagggatgtttttaaatttttataatgtaaattgtaataaattggtatatttgttgcttgtgtttgtttttcccctacagac	
		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	
		tggactgtatagag**g**gttagtgccacatgctcaatacctgttaggtgaaataacactcaaaggtttggtttctcatcttagtgcctGACATGAATTAGCAAGACTG (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	
		accccctgctgatgttatcaagacgagattacaggtg	
		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		TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctattttaatggatttaatt**c**gctccttaacaac	
		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**taagtacctttttgaagctctcttcattgaaaagacttgtttcac	
		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	GGAGCTGGTGGTATGGAAatatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgtgatttctc	
		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.

Please cite this article as: A. Kikuchi, et al., Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*, Mol. Genet. Metab. (2012), doi:10.1016/j.ymgme.2011.12.024

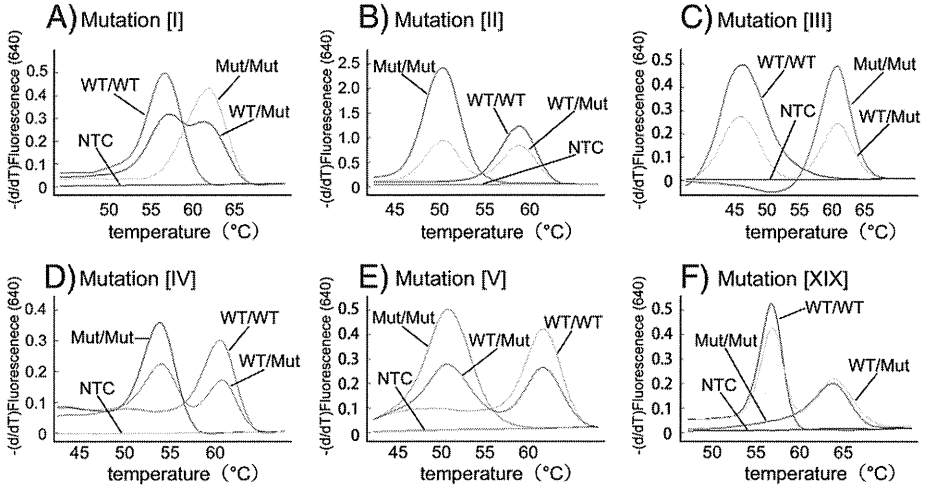

Fig. 2. Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

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

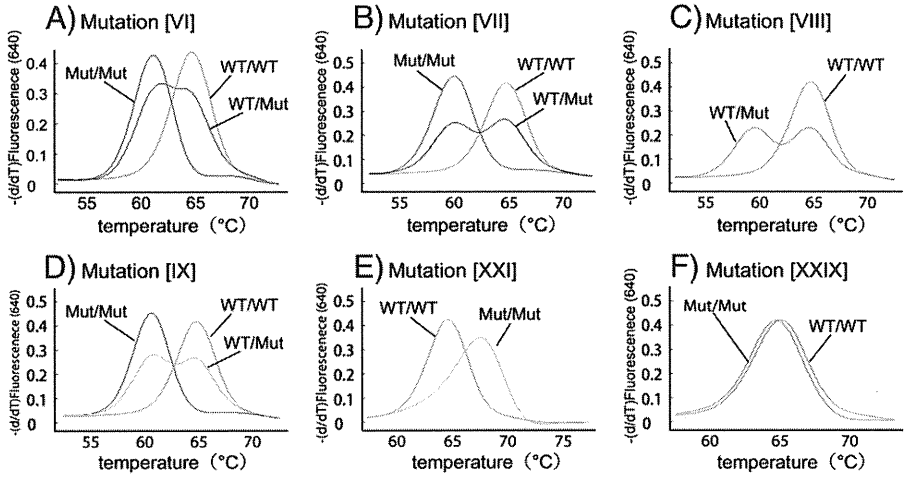

Fig. 3. Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor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

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.

Acknowledgments

The authors acknowledge the contribution of Dr. Keiko Kobayashi, who passed away on December 21th, 2010. Dr. Kobayashi discovered that the *SLC25A13* gene is responsible for citrin deficiency and devoted much of her life to elucidating the mechanism of citrin deficiency. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor, and Public Welfare.

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Please cite this article as: A. Kikuchi, et al., Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*, Mol. Genet. Metab. (2012), doi:10.1016/j.ymgme.2011.12.024

＜シンポジウム 02—5＞アカデミア発の創薬・治療研究

成人型シトルリン血症の治療
—低炭水化物食と経口ピルビン酸投与の有効性

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(臨床神経 2010;50:844-847)
Key words：成人型シトルリン血症，低炭水化物制限食，ピルビン酸ナトリウム，アルギニノコハク酸合成酵素，シトリン

はじめに

シトルリン血症は，尿素回路の律速酵素であるアルギニノコハク酸合成酵素(ASS)の異常により，高シトルリン血症・高アンモニア血症をきたす疾患である．シトルリン血症は，ASS 酵素異常により 3 型に分類され，ASS 遺伝子の異常に起因する新生児・小児期発症の古典型シトルリン血症（I 型・III 型シトルリン血症，CTLN1）と成人型シトルリン血症（II 型シトルリン血症，CTLN2）に分けられている[1)]．

CTLN2 は，ASS の肝細胞における低下(ASS 蛋白の量的低下）により高アンモニア血症・高シトルリン血症をきたす常染色体劣性遺伝性疾患で，1999 年に本疾患が *SLC25A13* 遺伝子異常によるシトリンの欠損に起因することが明らかにされた[2)3)]．従来，本疾患はまれな疾患と考えられていたが，遺伝子異常をホモ接合体で有する頻度が 1/20,000 以上と推定されており，決してまれな疾患ではないことを示している[1)]．本疾患には肝移植療法が奏功することが報告され，これまでに多くの患者が救命されている[4)]．しかしながら，移植医療はドナー不足の問題などで，全員が平等に治療を受けることができず，現在食事療法ふくめた肝移植以外の治療法の確立が急務とされている．

今回，CTLN2 の治療について自験例での結果を基に，主に肝移植療法，食事療法，ピルビン酸ナトリウム療法について報告する．

シトリン欠損症の病因と病態

シトリンは，ミトコンドリア内膜において aspartate/glutamate carrier (AGC) として機能し，ミトコンドリア内のアスパラギン酸を細胞質内のグルタミン酸との交換で細胞質へ輸送する[3)]．尿素回路における ASS の機能は，細胞質においてシトルリンとアスパラギン酸を基質としてアルギニノコハク酸を合成することにあり，シトリンは ASS の基質（アスパラギン酸）の供給源として重要な役割を果たしている．またシトリンは，リンゴ酸アスパラギン酸シャトル（MAS：malate aspartate shuttle)の一員として，糖代謝によって生じた細胞質内の NADH 還元当量をミトコンドリア内へ輸送する役割を担っている．このため，シトリン欠損患者では，糖分の過剰摂取やアルコールなどの摂取によって，細胞質内の NADH 上昇をきたし，尿素回路不全や，エネルギー産生障害，乳酸からの糖新生障害をきたす[1)]．また蓄積した細胞質 NADH を代償的に処理するため，リンゴ酸クエン酸シャトルが活性化され，この系は本来脂肪酸合成に働くため，脂肪肝が誘発されるものと考えられている．患者の糖質嫌いと，高蛋白・高脂質食を好んで食べるという特異な食嗜好は，このような病態を回避するために備わったものと推測される．

患者と臨床像

当院で経験した 26 名の CTLN2 患者の臨床像，治療について検討した（Table 1）．診断は，高アンモニア血症をともなう中枢神経症状（肝性脳症）の存在と，血漿シトルリン値上昇に加えて，肝組織中の ASS 活性の低下の確認，あるいは *SLC25A13* 遺伝子解析にておこなった．

患者の内訳は，男性 17 名・女性 9 名で，発症年齢は 37.4 ± 13.6 歳（12～65 歳）であった．初発症状は，意識障害が 23 名と最多で，ほかに抑うつ症状，てんかん発作，幻覚などの精神症状での発症者が存在した．平均の body mass index は 18.5 ± 2.6 と痩せた患者が多かった．ほぼ全例に，ピーナッツや大豆，肉や魚類，乳製品などの高蛋白・高脂質食を好み，糖質や飲酒を嫌う傾向がみとめられ，全例に軽度から高度の脂肪肝をみとめた．また 7 名(27%)の患者で膵炎の罹患歴を有し，2 名（8%）で肝癌を併発した．

26 名中，5 名が脳症の悪化により死亡(2 名が脳浮腫に対してグリセロールを使用[5)]），1 名が肝癌の全身転移で死亡して

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（受付日：2010 年 5 月 21 日）

Table 1 Clinical data of 26 CTLN2 patients.

	Sex	Onset	*Mutation	Onset symptoms	BMI	food fondness	Alcohol	Pancreatitis	Hepatoma	Fatty liver	Liver transplant	Arginine	Low-carbohydrate diet	Na-Pyr	prognosis (yrs after LT)
1	M	24	I/I	consciousness disturbance	18.4	+	−	−	−	+	+	−	−	−	alive (15)
2	M	44	V/VI	consciousness disturbance	17.1	+	−	+	−	+	+	−	−	−	alive (14)
3	F	23	I/II	consciousness disturbance	15.1	+	−	+	−	+	+	−	−	−	alive (12)
4	F	12	I/II	depression	15.7	+	−	−	−	+	+	−	−	−	alive (11)
5	F	17	I/II	epilepsy	23.6	+	−	−	−	+	+	−	−	−	alive (11)
6	M	20	I/I	consciousness disturbance	18.8	+	−	+	−	+	+	−	−	−	alive (10)
7	F	51	I/II	consciousness disturbance	21.5	+	−	−	−	+	+	−	−	−	alive (9)
8	M	32	IV/IV	consciousness disturbance	18	+	−	−	−	+	+	+	−	−	alive (7)
9	F	40	IV/IV	consciousness disturbance	16.2	+	−	−	−	+	+	+	−	−	alive (7)
10	M	47	I/II	consciousness disturbance	16.1	+	−	−	−	+	+	+	−	−	alive (6)
11	M	35	I/XXXVII	consciousness disturbance	18.1	+	−	−	−	+	+	+	−	−	alive (5)
12	F	45	I/I	consciousness disturbance	20.3	+	−	−	−	+	+	+	+	−	alive (3)
13	M	24	unknown	consciousness disturbance	15.7	+	−	−	−	+	−	−	−	−	dead
14	M	29	unknown	consciousness disturbance	19.7	+	−	−	−	+	−	−	−	−	dead
15	M	25	I/II	consciousness disturbance	20.6	+	−	+	−	+	−	+	−	−	dead
16	F	39	II/II	consciousness disturbance	14.8	+	−	−	+	+	−	+	−	−	dead
17	M	25	II/XXII	halcination	14.9	+	−	+	−	+	−	+	−	−	dead
18	M	50	II/II	consciousness disturbance	22	+	−	−	+	+	−	+	+	−	dead
19	M	42	V/V	consciousness disturbance	18.6	+	−	−	−	+	−	+	+	−	alive
20	M	65	IV/XVI	consciousness disturbance	17.7	+	−	−	−	+	−	+	+	−	alive
21	M	51	I/V	consciousness disturbance	22.3	+	−	−	−	+	−	+	+	+	alive
22	M	48	I/I	consciousness disturbance	16.6	+	−	−	−	+	−	+	+	+	alive
23	M	52	I/II	consciousness disturbance	16	+	−	−	−	+	−	+	+	+	alive
24	F	47	I/II	consciousness disturbance	21.3	+	−	+	−	+	−	−	+	+	alive
25	F	49	II/IV	consciousness disturbance	22.3	+	−	+	−	+	−	+	+	+	alive
26	M	35	II/XXIX	consciousness disturbance	22.3	+	−	−	−	+	−	+	+	+	alive
mean		37.4			18.5			27%	8%	100%					
SD		13.6			2.6										

*SLC25A13 gene mutation number: Tabata et al. J Hum Genet 53:534-545,2008

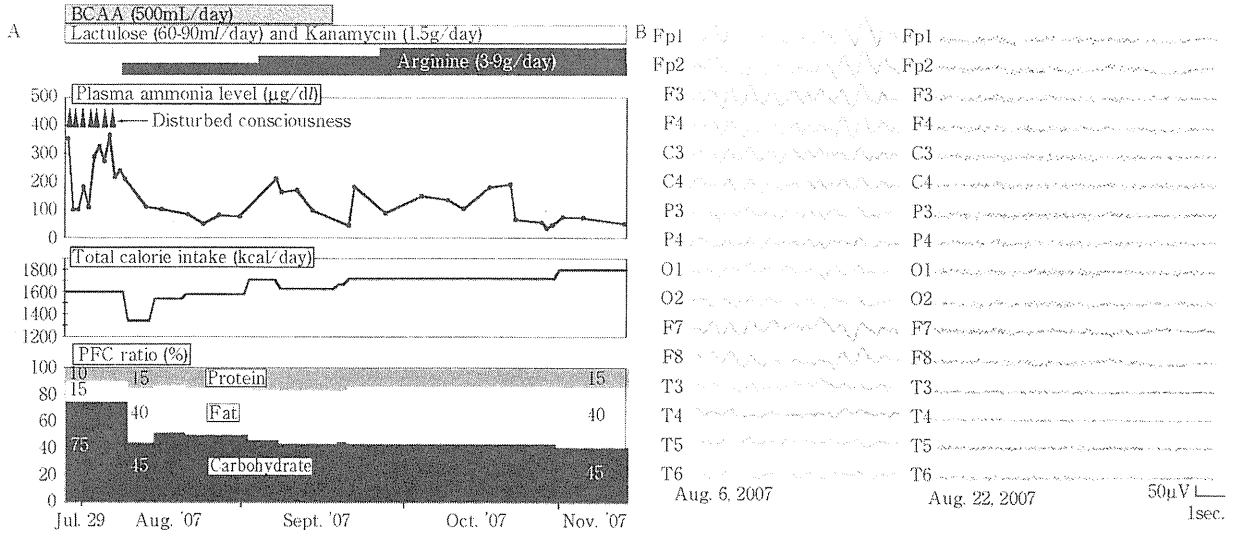

Fig. 1　炭水化物制限食が有効であった自験例[8]．
（A）自験例患者（51歳男性）の臨床経過，血漿アンモニア値，食事熱量，PFC比の推移．
（B）炭水化物制限食導入前後の経時的脳波所見．
当初，病院肝臓食（蛋白40g制限食）を食べていたが，炭水化物制限食（PFC比で炭水化物を45%に制限）を導入後，脳症が治まり，アンモニアも低下した．また炭水化物制限食に変更後，脳波所見も正常化した．
BCAA：分岐鎖アミノ酸製剤

いる（全例肝移植未施行）．

治療について

1）肝移植療法

当院では，1995年から現在まで12名の患者に対し生体部分肝移植療法を施行してきた[4)6)]．全例で術前みとめられた神経症状は術後消失し，血漿アンモニア・アミノ酸異常，脳波異常もすみやかに是正されている．また全例が，術後社会復帰されており，本症の再燃による脳症の再発はみとめていない．

2）内科的治療

食事療法（低炭水化物食）

Saheki ら[7)]は，シトリン欠損患者の食事調査から，蛋白・脂質・炭水化物の熱量比（PFC比）が19±2%：44±5%：37±7%であり，一般日本人のPFC比（14～15%：25～30%：54～58%）にくらべて，明らかに炭水化物を嫌忌していることを報告している．当科では，以前は患者に対して食事中の蛋白を一日40～50gに制限する肝臓食（肝不全食）を適用していたが，この病院セット食は，食事中の炭水化物含量がいちじるしく高く（PFC比10%：15%：75%），病態を悪化させる可能性が考えられた．実際に病院肝臓食で脳症を頻回におこしていた51歳男性患者に，低炭水化物・高脂肪食（PFC比15%：40%：45%）としたところ，血漿アンモニア値も低下し，脳症のコントロールが良好となった患者を経験した（Fig. 1AB）[8)]．この結果をふまえて現在は，少なくともPFC比で，炭水化物比を40～50%程度に制限する炭水化物制限食を推奨している[8)]．炭水化物を制限することによる熱量不足は，まずは高脂質とすることで補うようにして，徐々に状態をみながら蛋白量を増やすようにしている．

これまで10名の患者に炭水化物を制限する食事を導入したが，肝癌の転移で死亡した患者を除いては，死亡患者は出ていない．

経口ピルビン酸ナトリウム

最近，肝細胞に蓄積したNADHを再酸化させる目的で[9)]，ピルビン酸ナトリウムの有効性が着目されており，実際にモデルマウスや13歳の小児患者での有効性も報告されている[9)10)]．しかしながら，成人患者での有効性は明らかではない．当科では，これまで6名の患者に対して，信州大学医学部倫理委員会の承認と，患者と家族への充分な説明と同意取得後に，ピルビン酸ナトリウム4～9g/日の投与をおこない，その有効性について検討中である．最長で3年半投与を継続しているが，6名中5名で，明らかに脳症発作の頻度が減少・消失し，内服治療でも長期コントロールが可能であることが示唆された．

ま と め

病因・病態が明らかになるにつれ，炭水化物制限による食事療法，あるいはピルビン酸ナトリウム療法などのシトリン欠損病態に則した治療法が確立されつつある．今後，多くの患者が肝移植療法を回避できるように，これらの治療法の有効性・安全性など検討していきたいと考えている．

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Abstract

Therapeutic approaches for patients with adult-onset type II citrullinemia (CTLN2): effectiveness of treatment with low-carbohydrate diet and sodium pyruvate

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Adult-onset type II citrullinemia (CTLN2) is an autosomal recessive disease characterized by highly elevated plasma levels of citrulline and ammonia due to the urea cycle dysfunction associated with citrin deficiency. Patients with CTLN2 show various neurological symptoms with hyperammonemia closely resembling those of hepatic encephalopathy. Since 1990, 26 CTLN2 patients (17 males and 9 females) have been admitted and treated at Shinshu University Hospital. Twelve of the 26 patients received living related partial liver transplantation (LRLT). After LRLT, neurological symptoms soon disappeared, and all patients returned to their previous social lives. Among the 14 patients that did not undergo LRLT, 6 died of intractable encephalopathy or the development of hepatic cancer, but 8 patients have had relatively good clinical courses (follow-up range 0.5-8 years) with oral intake of L-arginine and low-carbohydrate and relatively protein-rich diet. Six patients have been also given sodium pyruvate and the frequency of attacks of encephalopathy markedly decreased in 5 of 6 patients. Our observations indicated that liver transplantation is a very promising type of therapy but that other therapeutic approaches, including low-carbohydrate diet and pyruvate, are being established.

(Clin Neurol 2010;50:844-847)

Key words: adult-onset type II citrullinemia, low carbohydrate diet, Sodium pyruvate, argininosuccinate synthetase, citrin

シンポジウム：先天代謝異常症マス・スクリーニングのこれから

シトリン欠損症マス・スクリーニングの可能性

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要旨　シトリン欠損は新生児肝内胆汁うっ滞症（NICCD）と成人発症Ⅱ型シトルリン血症（CTLN2）という年齢依存性の二つの臨床像を呈する。保因者頻度から患者は1/17,000人の頻度と計算されるが、実際の発見率はCTLN2が約1/100,000人であり、未発症例も多いと考えられている。我が国では200名以上のNICCDが既に診断されており、今後CTLN2の発症予防法の確立が急務となっている。最近本症に対して低炭水化物食事療法やピルビン酸投与が有効であるとの報告があり、早期発見することで予後の改善が期待されているが、生化学的検査法によるマス・スクリーニングでは見逃し例が多い。遺伝子スクリーニングでは患者変異アリルの91%が検出可能であり、スクリーンニングへの応用も期待される。しかし、未発症例に対する遺伝子を用いたスクリーニング検査は倫理的問題を含め、解決すべき問題が残されている。

Key words: シトリン，NICCD，CTLN2，マス・スクリーニング，遺伝子診断

はじめに

シトリン欠損症はわが国では頻度が高く、最近有効な治療法、発症予防法が報告されたことより、早期発見・早期治療介入が求められるようになってきた。本シンポジウムではシトリン欠損症のマス・スクリーニングが可能か検討を行う。

シトリン欠損症とは

1999年Kobayashiらにより成人発症Ⅱ型シトルリン血症の原因遺伝子SLC25A13が報告され、その遺伝子産物はシトリンと名付けられた[1]。現在その機能はアスパラギン酸・グルタミン酸輸送体であることが明らかにされている。その後脂肪肝、特異なアミノ酸異常、ガラクトース血症を伴う新生児肝炎例においても本遺伝子異常が報告され、CTLN2と異なる臨床像であることよりシトリン欠損による新生児肝内胆汁うっ滞症（NICCD）と命名された[2-4]。NICCDと診断された患児の大部分は1年以内に軽快し、検査値も正常化する。その後見かけ上健康な時期が続くが、成人期以降にCTLN2を発症する（表1）。見かけ上健康とされている時期は特異な食癖以外には無症状と考えられていたが、最近では低血糖、高脂血症、膵炎などを合併する例も報告されている。保因者頻度から推定されるわが国での患者頻度は約1/17000人であるが、CTLN2の発症頻度は約10万人に一人と推定されており、未発症例や他疾患として治療されている例も存在していると考えられている[5]。現在わが国では200例以上のNICCDが遺伝子診

断されている。これらの患児が将来CTLN2を発症しない様にするにはどうすればよいか、その予防法の確立が急務となっている。

現行の新生児マス・スクリーニングで発見可能か？

我々はNICCDと診断された75例の臨床像を検討したところ、現行のNBSでガラクトース、メチオニンやフェニルアラニン陽性を契機に発見された症例が30例（40%）存在した[6]。NBS陽性例で胆汁うっ滞、肝障害を認める場合はNICCDを疑う必要があるが、NICCD発症例の40%を発見出来るにすぎない。

タンデムマス法を用いたスクリーニングの場合は、最も鋭敏な指標であるシトルリン値を測定することが出来る。しかし、この方法でも発見率は1/80,000であり[7]、予想される頻度の1/4程度である。生化学的検査を指標とするスクリーニング法の問題点として、NICCDは必ずしも新生児期早期から発症するわけではなく、検査異常も一過性であることが挙げられる。その為日齢5前後で行うNBSでは発症例の内半分以上が見逃される。また、変異ホモ接合体であっても未発症例が多く存在することも考えられ、この場合は生化学的指標で発見することは不可能である。

治療法の進歩～早期診断の重要性

シトリンの機能はアスパラギン酸・グルタミン酸輸送体であり、シトリン欠損症の病態は肝細胞内cytosolでのアスパラギン酸欠乏、NADH/NAD+比の上昇によるものであることが明らかとなった。その為、cytosolのNADHを増加させる高カロリー輸液や脳圧降下剤であるグリセオール注®投与は禁忌であること、アルコールや過剰な糖質の摂取を控え、低炭水化物食投与を行うことがCTLN2の治療、発症予防に有効であることなどが明らかとなってきた。またcytosolのNADHの酸化を促すピルビン酸ナトリウムなどの臨床投与も進められている[8]。

これらの研究成果よりシトリン欠損症は低炭水化物食事療法、薬物療法などによりCTLN2の発症を予防できる可能性が出てきた。また、病気のことを知っておくことで、間違った食事を避け、禁忌となる薬剤の投与を防ぐことが出来る。その意味でシトリン欠損症を早期に診断することは、有意義であると考えられる。

迅速遺伝子診断法

前述のごとく、NICCDの生化学的異常は一過性であり、生化学的検査で診断できるのは一部の典型例のみである。NICCDが疑われた場合、確定診断法として最も信頼性が高いのは原因遺伝子SLC25A13の解析である。我々はReal-time PCR法を用いて日本人における6種類の高頻度変異を簡便にスクリーニングするシステムを開発した[9,10]。表2に対象となる高頻度変異を示したが、

表1. シトリン欠損症の臨床像

NICCD 新生児～乳児	→ 見かけ上健康 →	CTLN2 11～79才
黄疸、胆汁鬱滞 肝障害、脂肪肝 凝固障害，Cit ↑ 低蛋白血症 NBS陽性(40%) 肝移植	特異な食癖 豆類を好む 糖質を好まない 無症状(?) 易疲労感 低血糖、膵炎 高脂血症、肝癌	意識障害 失見当識 精神症状、脂肪肝 NH3↑，Cit↑ PSTI ↑ 肝ASS活性低下 予後不良、肝移植

NICCD: Neonatal intrahepatic cholestasis caused by citrin deficiency.
CTLN2: Adult onset type 2 citrullinemia.
Cit: シトルリン、NBS: Newborn screening、NH3: アンモニア、PSTI: 膵分泌性トリプシンインヒビター、
ASS: アルギニノコハク酸合成酵素

表2. シトリン欠損症の遺伝子検査～日本人高頻度変異～

遺伝子変異	遺伝子変異	変異部位	アレル頻度
I	c.851del4	Exon 9	32.8 %
II	g.IVS11+1G>A	Intron 11	36.3 %
III	c.1638ins23	Exon 16	4.6 %
IV	p.S225X	Exon 7	3.2 %
V	g.IVS13+1G>A	Intron 13	9.8 %
XIX	g.IVS16ins3kb	Intron 16	4.3 %
			計 91.0%

Tabata et al. J Hum Genet. 2008;53(6):534-45.

この方法を用いれば患者の変異アリルの91%が検出可能となる。従って、本症患児の約83%は両アレルの遺伝子変異が検出され、確定診断可能と推察される。残り約16%の患児は一方のアレルのみ変異が検出され、保因者との鑑別が問題となる。この場合、臨床症状の有無、他のスクリーニングの結果（メチオニン、フェニルアラニン、ガラクトース、シトルリンなど）を合わせて判断するのが実際的であろう。また、患児の約1%は両アレルとも高頻度変異を持たないため、本法では検出出来ない。

遺伝子診断の利点と欠点

症状よりNICCDが疑われ、遺伝子診断により確定した場合は、積極的に栄養療法・薬物療法などの治療介入が出来るため患者にとっては極めて有用である。それでは遺伝子変異をスクリーニングすることで未発症の患者を発見する場合はどうであろうか。

利点としてはシトリン欠損症と診断されることで過剰な糖質（ジュース、ジャム、アルコールなど）摂取を避け、CTLN2発症のリスクを軽減できる点があげられる。さらにピルビン酸ナトリウム投与による発症予防効果も期待されている。また、高カロリー輸液、グリセオール®の使用は本症に禁忌であるという情報を医療関係者に伝えることができることも重要である。

一方、欠点としてはシトリン欠損症と診断されることで、将来CTLN2を発症する不安、精神的ストレスを被ることや、保険加入、就職、結婚に際しての問題があげられる。遺伝子診断を実際に行うに当たっては、遺伝カウンセリングを行い病気の説明のみならず、利点、欠点についても十分説明した上で同意を取ることが重要である。

表3. シトリン欠損症の遺伝子診断の対象者（案）

1. High riskスクリーニング
   原因不明の胆汁うっ滞症、肝障害
2. 家族内検索
   家族がシトリン欠損と診断された場合
3. 化学診断スクリーニングで陽性（未発症）
   一過性高シトルリン血症
4. 新生児マススクリーニング
   新生児期に診断された場合、治療介入が可能か？

遺伝子診断の対象者

小児科領域でSLC25A13遺伝子の解析が有用な場合を表3に示した。乳児期の原因不明の肝内胆汁うっ滞症例など症状よりNICCDが疑われた場合は遺伝子診断の最も良い対象である（High riskスクリーニング）。また、シトリン欠損患児の無症状の同胞例やタンデムマス・スクリーニングでシトルリンが高値であるが無症状である例なども対象となるであろう。

新生児マス・スクリーニングへの導入に関しては発症前診断となるため、倫理的問題も含め未解決の問題が残されている。また、NICCDもしくはCTLN2の発症にはSLC25A13遺伝子異常に加えて素因（他遺伝子の関与？）や環境因子などの関与

があると想定されている。実際、変異遺伝子をホモ接合体として持っていても発症するのは一部であり、生涯発症しないで過ごす場合も多いと想像される。発症に関与する因子が解明されていない時点では、新生児マス・スクリーニングに遺伝子診断を導入するのは時期尚早であると考えられる。

結　論

生化学的検査法を用いたマス・スクリーニングは多くの見逃し例が生じるため不適当である。6種類の高頻度変異を簡便にスクリーニングするシステムは変異アレルの91%が検出でき、High riskスクリーニングなどに極めて有用である。しかし、新生児スクリーニングに導入するには時期尚早であると考えられる。

最　後　に

シトリン遺伝子の発見をはじめ、本研究に中心的役割を果たされました小林圭子先生が2010年12月に急逝されました。心よりご冥福をお祈りします。

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シンポジウム：先天代謝異常症マス・スクリーニングのこれから

ガラクトース血症マス・スクリーニングの現状と課題

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要旨　ミルクの主成分である乳糖は小腸粘膜上皮の刷子縁にある乳糖分解酵素によってグルコースとガラクトースに分解、吸収された後、門脈を通過して肝臓へ運ばれ、代謝を受ける。上記の経路に異常がある場合に血中ガラクトースは高値となる。1）肝臓への輸送経路の異常：門脈大循環シャント、肝内血管腫、2）肝細胞内への取り込みの異常:Fanconi-Bickel 症候群、3）肝臓での処理能力の異常:ガラクトース代謝異常症、シトリン欠損症、高チロシン血症、新生児肝炎、胆道閉鎖症などがある。新生児マススクリーニングによるガラクトース血症要精査患者では上記疾患について鑑別診断をガラクトースとその代謝産物、ガラクトース代謝酵素、肝機能検査、アミノ酸分析、尿検査、画像検査、遺伝子検査等で行う。ガラクトース代謝産物では、ガラクトース血症 I, III 型でガラクトース -1- リン酸の増加が、ガラクトース血症 II 型とその他の疾患ではガラクトースの増加が特徴である。

Key words: 新生児マススクリーニング，ガラクトース血症，シトリン欠損症，門脈大循環シャント，Fanconi-Bickel 症候群

序　　言

新生児期から乳児期の栄養の大部分はミルクであり、その主成分は乳糖である。乳糖は小腸粘膜上皮の刷子縁にある乳糖分解酵素によってグルコースとガラクトースに分解、吸収され、門脈を通過して肝臓へ運ばれ、代謝を受ける。血中ガラクトース高値は上記の経路に異常がある場合に生じる。持続性高ガラクトース血症を来す原因としては表1に示すような各種疾患が知られている[1]。ガラクトースは新生児期には体重 (kg) あたり1日5g 以上が摂取されている。大量のガラクトース負荷がかかる新生児期には、種々の原因で容易にガラクトースは上昇する。本稿では、新生児期にガラクトースまたはガラクトース - 1 - リン酸（Gal-1-P）が上昇する疾患として、遺伝性ガラクトース血症、シトリン欠損症、Fanconi-Bickel 症候群、門脈大循環シャントについて述べる。

I．ガラクトース代謝と病型（図1）

ガラクトースは六炭糖であり、生体内では多糖類、糖蛋白質、糖脂質、プロテオグリカンのような複合糖質の形で神経、結合織などに存在している。ガラクトースはガラクトキナーゼ（GALK）によって Gal-1-P に代謝される。Gal-1-P は UDP- グルコース (UDP-Glu) の存在下で、ガラクトース－1－リン酸ウリジルトランスフェラーゼ（GALT）によってグルコース -1- リン酸（Glu-1-P）と UDP-ガラクトース (UDP-Gal) に転換される。本酵素の反応によって生成された UDP-Gal はウリジン2リン酸ガラクトース－4`－エピメラーゼ（GALE）によって UDP-Glu に再び転換される。この反応は可逆性で、生理的には UDP-Glu 生成の方向に働いている。しかしガラクトース摂取制限下では UDP-Gal 生成の方向に働き、生体構成成分にガラクトースを供給する役割を果す。

ガラクトース血症には上記の3種類の酵素欠損症、すなわち、I 型：GALT 欠損症 (OMIM: 230400)、II 型：GALK 欠損症 (OMIM: 230200)、III 型：GALE 欠損症 (OMIM: 230350) が知られている[2]。いずれも常染色体劣性遺伝疾患で、新生児マススクリーニングで早期発見、早期診断がな

されている。わが国での頻度は、GALT欠損症で1/920,000と欧米の約15分の1、GALK欠損症で1/1,000,000と欧米とほぼ同じである。GALE欠損症は1/50,000から1/70,000とされている。GALK、GALT、GALEのいずれも、赤血球、白血球、皮膚線維芽細胞を含む多くの組織に存在している。

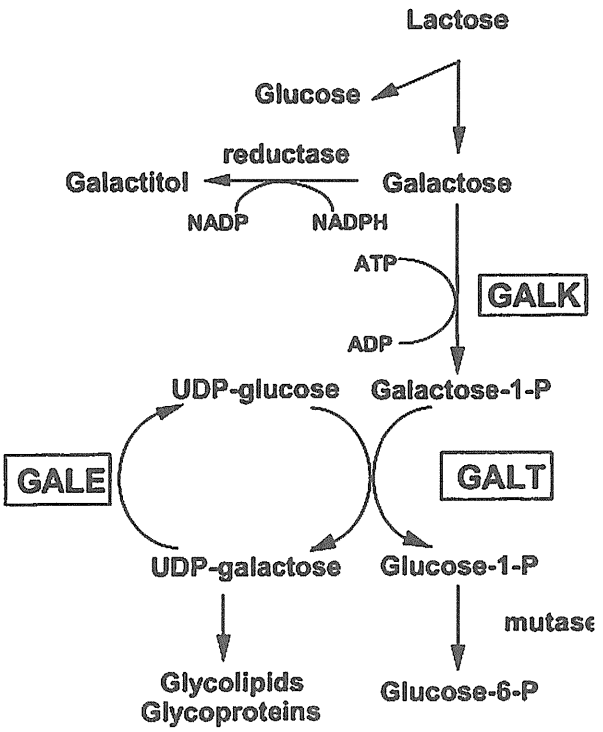

図1. ガラクトース代謝マップ

Ⅱ. 新生児マススクリーニング

わが国ではガラクトース血症の新生児マススクリーニングが1977年より全国で開始されている。GALT活性を測定するBeutler法と血中ガラクトース濃度を測定するPaigen法もしくは酵素法が併用されている[3]。施設により測定方法が異なるため、その値の解釈には注意が必要である。Paigen法で測定されたガラクトース値にはガラクトース、Gal-1-P、UDP-Galを含む。酵素法ではアルカリフォスファターゼ処理をしたもの（ALP＋）はガラクトースとGal-1-Pの合計値を示し、アルカリフォスファターゼ未処理のもの（ALP－）はガラクトースのみの値を示す。

Ⅲ. 鑑別診断

ガラクトース血症はガラクトース代謝酵素のGALT、GALK、GALE欠損症だけでなく、乳糖が小腸で分解吸収され、肝で代謝を受ける経路の異常によっても生じる（表1）。そのため、肝機能検査、総胆汁酸、アミノ酸分析、尿検査、血糖、画像診断、遺伝子検査等が必要である。Beutler法での陽性結果はGALT欠損症以外にも溶血性貧血を発症するグルコース-6-リン酸脱水素酵素異常症をも考慮する[4]。

1）血中ガラクトース値と酵素活性

遺伝性ガラクトース血症の場合には総ガラクトース値は20 mg/dlを超えていることが多い。GALK欠損症ではGal-1-Pの非検出とガラクトース

表1. ガラクトース血症の原因

1）肝臓への輸送経路の異常 門脈大循環シャント	肝内シャント：静脈管開存症、肝内門脈・肝静脈ろう、肝血管種 肝外シャント：門脈無形成、門脈大循環短絡、門脈左腎短絡
2）肝細胞内への取り込みの異常	Fanconi-Bickel症候群
3）肝臓での処理能力の異常 遺伝性ガラクトース血症	I型　galactose-1-phosphate uridyltransferase欠損症 II型 galactokinase欠損症 III型 UDP galactose 4-epimerase欠損症
肝内胆汁うつ滞症	先天代謝異常症：シトリン欠損症、高チロシン血症など 特発性新生児肝炎 ウィルス性新生児肝炎 肝内胆管低形成、減少症 進行性家族制肝内胆汁うつ滞症