to be excluded. Screening investigations revealed biochemical and histopathological features of NICCD with hyperaminoacidaemia, galactosuria and hepatocellular steatosis, which prompted us to investigate further for possible citrin deficiency. The diagnosis was eventually confirmed by mutation analysis.

The clinical features of citrin deficiency are largely mild and non-specific and once neonatal cholestasis has resolved patients are generally well with no clinical or biochemical abnormalities. Unfortunately, this makes it difficult to establish a diagnosis during this phase, necessitating early investigations. Perhaps the most notable biochemical markers are increased citrulline and arginine together with galactosuria against the background of abnormalities commonly associated with liver dysfunction. However, in order to detect this, amino acid levels need to be monitored from the first 2 or 3 months of age before they fall to normal levels. At what age citrulline returns to normal is not clear (between 3 and 8 months in the patients described here), but typically by the age of 1 year all biochemistry returns to normal levels. After this a definite diagnosis can really only be made by western blot or DNA analysis. This is easier for populations where common mutations are known, notably in East Asia where 13 mutations in the *SLC25A13* gene have a carrier rate of 1/65 in Japan and China (Kobayashi et al 2003). This is not the case in the UK, or even Europe, where only a handful of cases are known. Thus at the present time sequencing of the entire *SLC25A13* gene would be required to confirm a diagnosis.

Initially it was thought that once patients recover by their first year they then remained healthy although at risk of developing features of CTLN2 any time after 11 years of age (Saheki and Kobayashi 2002; Yasuda et al 2000). However, it now appears that these patients can show some mild symptoms such as gastrointestinal symptoms, anorexia, hyperlipidaemia and seizures (Kobayashi and Saheki 2004). Episodes of hypoglycaemia as in patient 1 have also been reported (Ohura et al 2007), perhaps as a result of their dietary avoidance of carbohydrates.

Unfortunately, we still understand little about the natural history of citrin deficiency and in particular which patients will go on to develop CTLN2. Biochemical markers of CTLN2 such as amino acids, ammonia and pancreatic secretory trypsin inhibitor (Tsuboi et al 2001) may be detected only after irreversible neurological brain damage has already occurred.

Carbohydrate restriction may help prevent progression to CTLN2 (Imamura et al 2003; Yazaki et al 2005) but this is unproven and not without risk. Similarly, while liver transplantation may help avoid the onset of CTLN2 it would be hard to justify this as a pre-emptive treatment for a child with citrin deficiency.

The finding of citrin deficiency in patients other than of East Asian origin means that this disorder needs to be considered in patients of any ethnic origin. Diagnosis is difficult without early monitoring of amino acid levels and hence there are likely to be additional unidentified patients with other as yet unknown DNA mutations responsible for citrin deficiency. Careful and detailed monitoring of new NICCD patients and their subsequent progress, along with continued research, will help us better understand the effects of citrin deficiency and ways to prevent the development of CTLN2.

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INTERNAL MEDICINE

□ CASE REPORT □

Conventional Diet Therapy for Hyperammonemia is Risky in the Treatment of Hepatic Encephalopathy Associated with Citrin Deficiency

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Abstract

Citrin deficiency caused by *SLC25A13* gene mutations develops into adult-onset type II citrullinemia (CTLN2) presenting with hepatic encephalopathy. Recent studies have suggested that excessive loading of carbohydrates is harmful in citrin-deficient individuals. Here we report a CTLN2 patient who showed further deterioration of encephalopathy after the employment of conventional low-protein diet therapy for chronic liver failure. Owing to the high carbohydrate content, the conventional low-protein diet therapy should be avoided in patients with hepatic encephalopathy associated with citrin deficiency. In addition, our observation may suggest that carbohydrate-restricted diet in which the content of carbohydrate is below 50% of daily energy intake can have therapeutic efficacy in CTLN2 patients.

Key words: citrin deficiency, CTLN2, low protein diet, carbohydrate

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Introduction

Citrin is a liver-type mitochondrial aspartate (Asp)-glutamate (Glu) carrier (AGC) (1, 2), and citrin deficiency, caused by a mutation of the *SLC25A13* gene, is an autosomal recessive disorder and leads to neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and adult-onset type II citrullinemia (CTLN2) (1, 3). Patients with CTLN2 present with intractable hepatic encephalopathy, showing various neuropsychotic manifestations (3). One of the distinct features of CTLN2 is that most patients have a peculiar fondness for protein- and fat-rich foods such as beans and peanuts, and an aversion to carbohydrate-rich foods such as rice and sweets (3). It is assumed that their unusual food preferences may be directly related to the underlying pathophysiology (3). Here we report a CTLN2 patient who showed a deterioration of consciousness levels accompanied by highly elevated ammonia after conventional low-protein diet therapy for chronic liver failure. His hepatic encephalopathy was gradually ameliorated in parallel with a reduction in the carbohydrate content of his diet.

Case Report

A 51-year-old Japanese man was emergently transferred to a local hospital because of consciousness disturbance in late July, 2007. An elevated plasma level of ammonia was revealed (355 μg/dL, normal <70 μg/dL). He was regarded as having hepatic encephalopathy and was treated with an infusion of branched amino acids followed by a conventional protein-restricted diet (total calories 1,600 kcal/day, protein 40 g/day, protein, fat, and carbohydrate (PFC) ratio 10:15:75%) (Fig. 1). In addition, oral administration of lac-

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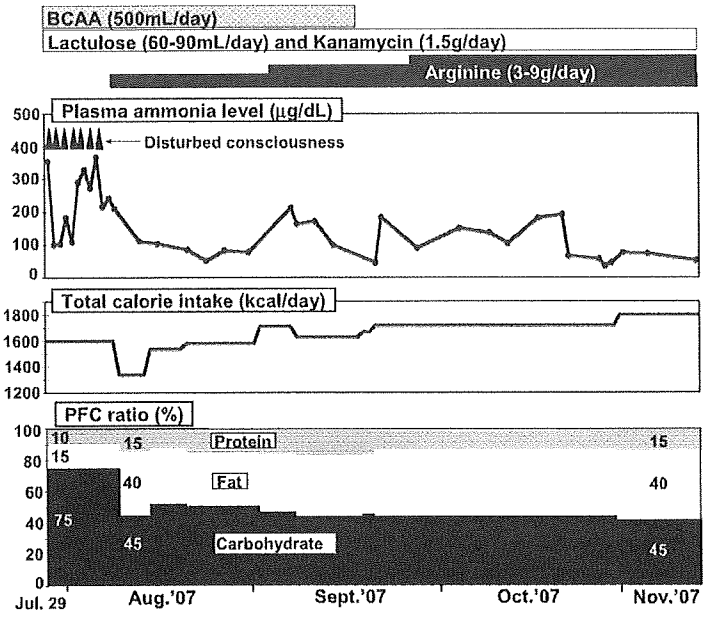

Figure 1. Clinical course of the patient showing changes in daily dietary calories and the proportion of dietary energy ratios. BCAA: branched chain amino acid fluid

tulose (60-90 mL/day) and kanamycin (1.5 g/day) was begun. After this treatment, attacks of disturbed consciousness frequently occurred (Fig. 1). An EEG showed diffuse slow waves with the appearance of triphasic waves (Fig. 2A). As his plasma levels of citrulline and arginine were raised (408.9 nmol/mL: normal <40 nmol/mL, and 186.4 nmol/mL: normal<120 nmol/mL, respectively), he was thought to have CTLN2 and referred to our hospital in early August, 2007. He had a fondness for peanuts, milk, meat and fish, and had disliked sweets from childhood. On neurological examination, he was highly irritable and confused, and showed flapping tremor in his hands. His serum transaminases were slightly high (aspartate aminotransferase: 52 IU/L, normal <37 IU/L; alanine aminotransferase: 96 IU/L, normal <45 IU/L) and the serum level of γ-glutamyltransferase was moderately elevated to 138 IU/L (normal <50 IU/L). The levels of total bilirubin, albumin, and total cholesterol, and the hepaplastin test were within normal values. The serum pancreatic secretory trypsin inhibitor (PSTI) level (4) was elevated to 40 ng/mL (normal <20 ng/mL). There was no serological evidence of hepatitis-related viral infection. The abdominal CT and MR images demonstrated no liver cirrhosis or extrahepatic portovenous shunt. There were no remarkable findings suggestive of hepatic steatosis on MR images (Fig. 2B). DNA analysis of the *SLC25A13* gene demonstrated that he was a compound heterozygote for the mutations of 851del4 and IVS13+1 G>A (1), and he was, therefore, diagnosed as having CTLN2. He was started on arginine (3 g/day) (5) and a carbohydrate-restricted diet with a high fat content (total calories 1,340 kcal/day, protein 50 g/day, carbohydrate 150 g/day, PFC 15%:40%:45%) from mid-August. His consciousness level gradually ameliorated and his plasma ammonia level also decreased (Fig. 1). The EEG recording on August 22 showed an almost normal appearance (Fig. 2A). After that, the daily dose of arginine was increased to 9.0 g/day and total dietary calories were also gradually increased to 1,800 kcal by November 2007 with the ratio of carbohydrate in the total dietary calories restricted to approximately 45% (protein 70 g/day, carbohydrate 200 g/day) (Fig. 1). The MR images in November 2007 showed mild progression of hepatic steatosis (Fig. 2C) and liver biopsy in November 2007 demonstrated relatively mild steatosis (Figs. 2D, 2E). He was discharged in November 2007 and returned to his previous work.

Discussion

A recent study has disclosed that carbohydrate intake was selectively reduced in the diet of most citrin-deficient subjects, compared to that of the general Japanese population (6). While the PFC ratio of the general Japanese population was 14-15%: 25-30%: 54-58%, that of the citrin-deficient subjects was 19±2%: 44±5%: 37±7% (6). This carbohydrate aversion in citrin deficiency is quite unique in contrast to the protein aversion in other urea cycle enzyme deficiencies (6). In citrin deficiency, the cytosolic NADH/NAD^+ ratio in the hepatocytes can be significantly increased in accordance with the carbohydrate metabolism, resulting in inhibition of ureagenesis by limitation of the supply of Asp for the urea cycle (3). Accumulating data have suggested that the toxicity of high carbohydrate intake, and indeed, intravenous infusion of a high glucose solution or the administration of a glycerol solution resulted in severe hyperammonemia or rapid deterioration of encephalopathy leading to death in

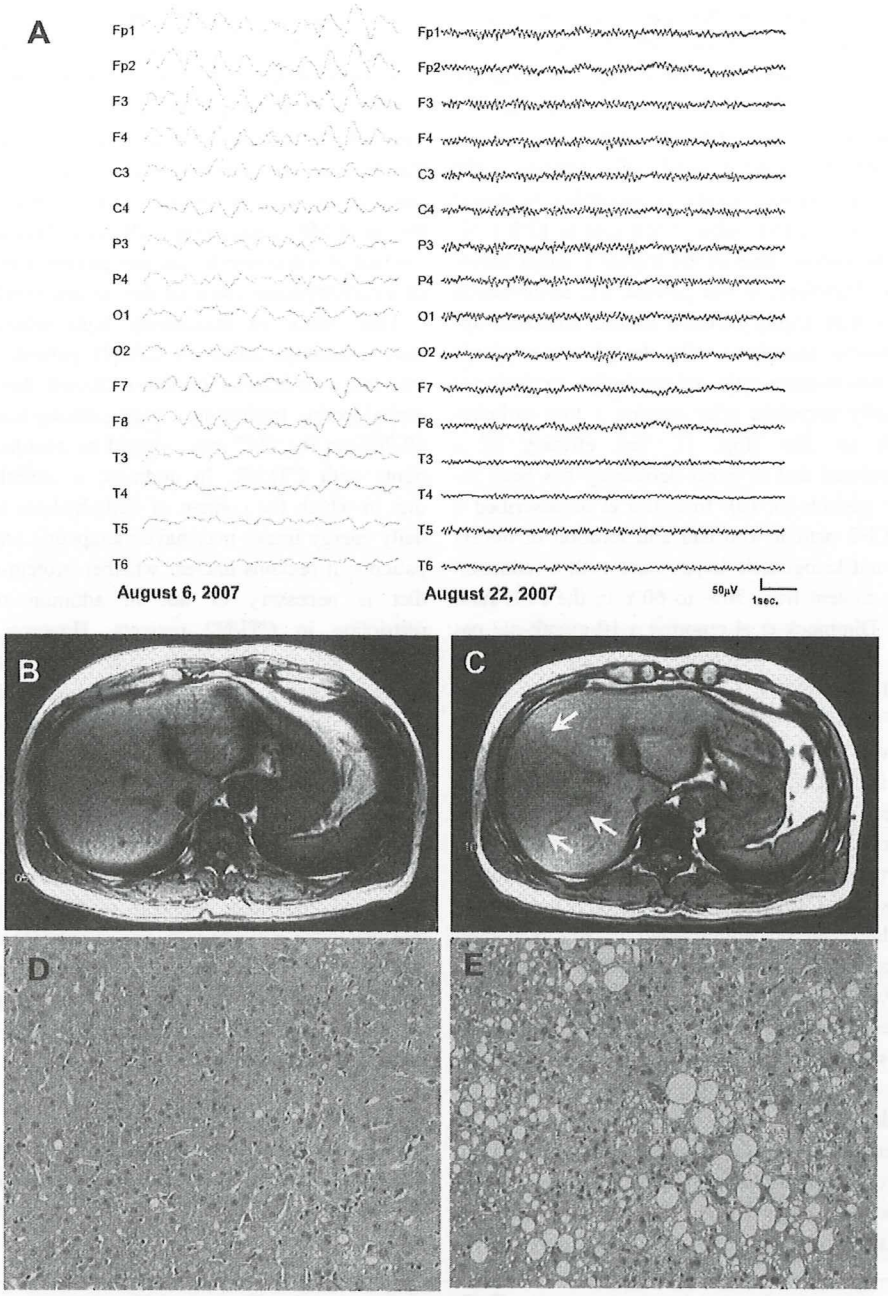

Figure 2. Serial EEG recordings (A), MR images (T1-weighted, out-of-phase images) (B,C) and histopathology of the biopsied liver (D,E) in the patient. Serial EEG recordings show a marked decrease in slow waves in the two weeks between early August, 2007 (PFC: 10%:15%:75%) and mid-August, 2007 (PFC:15%:35%:50%) (A). On MR imaging, mild progression of hepatic steatosis (white arrows) was seen between MR imaging performed in mid-August, 2007 (B) and mid-November, 2007 (C). The histopathology of the liver, which was biopsied in mid-November, 2007, demonstrated mild steatosis (D) with scattered moderate fat infiltration (E) (Hematoxylin and Eosin staining ×100).

many CTLN2 patients (7). Also, it has been found that oral sucrose administration exacerbated hyperammonemia in citrin/mitochondrial glycerol 3-phosphate dehydrogenase double-knockout mice, which are an animal model of human citrin deficiency (8). Therefore, high carbohydrate intake can deteriorate hepatic encephalopathy in CTLN2 patients.

Protein restriction has classically been considered a main-

stay of treatment in hepatic encephalopathy to reduce the nitrogen load (9). Usually, in patients with chronic liver disease, especially in liver cirrhosis, protein intake is restricted to 0.8 to 1.0 g/kg/day (9). In the conventional diet for chronic liver disorders in our institution, dairy protein intake is commonly restricted to 40 g (total daily calories 1,400 kcal). However, carbohydrate intake is inevitably increased (270 g/day) and thus the PFC ratio of this diet is 12%:11%:77%, which is the almost same as the patient's initial hospital diet (Fig. 1). Therefore, in our patient, the deterioration of consciousness with highly elevated plasma ammonia appeared to be closely associated with the protein-restricted diet with high carbohydrate ratio. Interestingly our patient's condition gradually improved after starting a low carbohydrate and high fat diet (Fig. 1). The efficacy of a carbohydrate-restricted diet in citrin deficiency has been reported in a few patients (5, 10). Imamura et al described a 37-year-old CTLN2 patient who had amelioration of hypertriglyceridemia and ketogenesis impairment with a reduction of carbohydrate content from 70% to 60% in the PFC ratio (5). In addition, Dimmock et al reported a 10-month-old patient with citrin deficiency presenting with failure to thrive, which improved after starting a high protein and low carbohydrate diet (PFC ratio 15%:50%:35%) (10).

Hepatic steatosis or steatohepatitis is one of the cardinal manifestations in CTLN2 patients and often severe fatty liver is also seen (11, 12). So far, over 10 patients with CTLN2 have undergone liver transplantation at our institution (13), and rapid progression of steatosis often occurred in some patients with CTLN2 who were given a low-protein diet (30-40 g/day), and/or administered hyperalimentation fluid over several months while waiting for liver transplantation (13, 14). Particularly, in our previously reported 32-year-old Japanese man with CTLN2 (14, 15), significant progression of steatosis, probably associated with the low-protein diet and infusion of hyperalimentation fluid, was observed in just three weeks. In the past, this progression of steatosis was thought to be due to the malnutrition caused by the excessively protein-restricted diet. However, several reviews have recently suggested that it could be directly associated with glucose metabolism in citrin deficiency: an increase of the cytosolic NADH/NAD^+ ratio following carbohydrate metabolism could activate the citrate-malate shuttle in compensation for the hepatic AGC (citrin) insufficiency, resulting in the overproduction of fatty acids in the hepatocytes (3). In the present patient, it was of particular interest that hepatic steatosis was relatively mild at biopsy and the rapid progression of hepatic steatosis was not observed in the serial MR images (Figs. 2B, 2C). This may suggest both the risk of the conventional low-protein diet and the efficacy of a carbohydrate-restricted diet as described previously (5).

Thus, since an excessively high-carbohydrate diet can have a harmful effect on CTLN2 patients, it is concluded that the conventional protein-restricted diet for hepatic encephalopathy, leading to a high carbohydrate intake of over 60-70% in the PFC ratio, should be avoided, at least in patients with CTLN2. In addition, a carbohydrate-restricted diet in which the content of carbohydrate is below 50% of daily energy intake may have therapeutic efficacy in CTLN2 patients. It remains unclear whether protein-restriction in the diet is necessary or not in addition to carbohydrate-restriction in CTLN2 patients. However, because most CTLN2 patients have urea cycle deficiency due to the decrease of hepatic argininosuccinate synthetase, high protein diet may lead to excessive nitrogen load to the urea cycle. In our patient's diet, therefore, in addition to carbohydrate-restriction, the daily protein was restricted (50 g-70 g/day).

In East Asian countries, the frequency of heterozygotes with the mutated *SLC25A13* gene is approximately 1 in 70, suggesting that over 80,000 East Asians may be homozygotes (16), thus large numbers of patients may still be undiscovered. In patients with hepatic encephalopathy, particular those who have a peculiar food preference with a dislike of alcohol, the possibility of citrin deficiency should be considered and special care is necessary in this unique inherited hepatocerebral disorder.

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Hepatology Research

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Original Article

Histological findings in the livers of patients with neonatal intrahepatic cholestasis caused by citrin deficiency

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Aim: To characterize the histological features of the livers of patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), we studied specimens from 30 patients diagnosed with NICCD by genetically analyzing the *SLC25A13* gene.

Methods: Liver biopsy specimens were subjected to hematoxylin–eosin, Azan, and Berlin-blue staining.

Results: Most specimens showed varying degrees of fibrosis. The degree of inflammation varied among the specimens, with half showing moderate or severe inflammatory changes. Fat deposition in hepatocytes was observed in almost all of the specimens, and severe fatty liver was noted in 20 (67%) of them. There was a mixture of two types of hepatocytes with macrovesicular or microvesicular fat droplets, and cholestasis was observed at a rate of 77%. Hemosiderin deposition, mostly mild and localized in periportal hepatocytes and macrophages in portal areas, was observed in 57% of the specimens.

Conclusion: A combination of mixed macrovesicular and microvesicular fatty hepatocytes and the above-described findings, such as fatty liver, cholestasis, necroinflammatory reaction and iron deposition, are almost never observed in other liver diseases in infants and adults. We believe that NICCD is a disease with characteristic hepatopathological features.

Key words: citrin, citrullinemia, fatty liver, fibrosis, neonatal intrahepatic cholestasis caused by citrin deficiency, *SLC25A13*.

INTRODUCTION

SAHEKI *ET AL.* reported that the enzyme abnormalities of citrullinemia can be classified as qualitative, type I and type III, or quantitative, type II.[1,2] The first, the classical form (CTLN1), is found in most patients with neonatal/infantile-onset citrullinemia, and was first described by McMurray *et al.*[3] In CTLN1, the enzyme defect is found in all tissues in which argininosuccinate synthetase (ASS) is expressed.[1,2,4] The second form, type II citrullinemia (CTLN2) is an adult- or late childhood-onset liver disease characterized by a liver-specific defect in ASS, and most of these patients have a fatty liver.[5] This enzyme abnormality is caused by a deficiency in citrin, a calcium-binding

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mitochondrial solute carrier protein which is encoded by the *SLC25A13* gene.[6]

Recently, several cases of *SLC25A13* mutations have been reported in early infancy with cholestatic liver disease.[7–13] Yamaguchi *et al.*[14] designated these findings as neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). Citrin deficiency causes two age-dependent phenotypes, CTLN2 in adults and NICCD in infants.[15] Most NICCD patients showed hypoproteinemia, galactosemia, multiple aminoacidemia including citrullinemia, methionemia and tyrosinemia, cholestasis, and have a fatty liver.[7–13] Only a few papers have described the pathology of the NICCD[8,9,11,13] or CTLN2[5] liver.

Therefore, the present study was designed to clarify the histological findings of the NICCD liver.

METHODS

Patients

WE STUDIED THE liver histological findings of 30 patients aged 2.9 ± 1.7 months with a range of 1–7 months consisting of 17 men and 13 women who had been diagnosed with NICCD with *SLC25A13* mutations by genetic analysis including five patients who were documented in previous reports.[7–11] Moreover, mutations in *SLC25A13* were detected in both alleles of 29 patients and in a single allele of one patient. Mutation detection and DNA diagnosis of the *SLC25A13* gene were performed as previously described ([6,14,16] and T. Saheki et al., 2006, unpublished data). Moreover, we examined biochemical data within 1 week before or after liver biopsy for 30 patients with NICCD.

Methods

Liver biopsy specimens from 30 patients diagnosed with NICCD were subjected to hematoxylin–eosin, Azan, and Berlin-blue staining. The grading of fibrosis and inflammation was based on Ludwig's Classification with slight modifications (Table 1).[17] The other histopathological features were graded as none, mild, moderate and severe, and scored as 0, 1, 2 and 3, respectively.

Grading was independently performed by three pathologists, and the grade for each specimen was determined by consensus between two or three of them.

Relationship between age and histological findings

To clarify the relationship between age and the histological findings, the cases were divided into three groups according to their ages: group A, less than 2 months old; group B, 3–4 months old; and group C, more than 5 months old. The average of the grading score of the histological findings for each group was then obtained.

Table 1 Histological classification of liver biopsy

Stage of fibrosis	
Stage 0	No portal fibrosis
Stage 1	Mild to moderate fibrous expansion of portal tract
Stage 2	Bridging fibrosis between portal tracts without lobular distortion
Stage 3	Bridging fibrosis between portal tracts with lobular distortion
Stage 4	Liver cirrhosis
Grade of inflammation	
Grade 0	None (0)
Grade 1	Mild (1–3)
Grade 2	Moderate (4–6)
Grade 3	Severe (≥7)

Parentheses indicate scores derived by Ludwig's scoring system.

Statistical analysis

The data regarding the relationship between age and histological findings were analyzed using the Mantel–Haenszel linear trend test. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Patients

THE PROGNOSIS OF almost NICCD patients at 1 year of age was fairly well. However, some NICCD patients had developed progressive liver failure by then. For example, two patients developed liver failure by 6 months (patient 28) and 7 months (patient 30)[10] of age and one patient (patient 9) developed behavioral aberrations, which included shouting and episodes of violence, by 16 years of age.[9,18] Two patients, one with liver failure[10] and one with mental derangement,[9,18] received a living-related liver transplant. Therefore, the outcomes of the NICCD patients were not always favorable. We obtained four sets of follow-up liver biopsy specimens from patients 8, 9, 13 and 18 (data not shown).

From the clinical laboratory data, serum levels of citrulline, α-fetoprotein, ferritin and pancreatic secretory trypsin inhibitor (PSTI) were noted to have generally increased (Table 2). We also detected high serum levels of total and direct bilirubin, aspartate (AST) and/or alanine aminotransferase (ALT), total bile acids and

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Table 2 Biochemical data on liver biopsy in the 30 patients with neonatal intrahepatic cholestasis caused by citrin deficiency

Patient No.	1	2	3	4	5	6	7	8	9	10	11
Age (months)/sex	1/M	1/M	1/M	1/M	1/F	1/F	2/M	2/M	2/M	2/M	2/M
Total/direct bilirubin (mg/dL)	9.0/3.4	12.6/2.6	3.3/2.2	10.4/5.8	5.6/1.9	3.3/0.7	6.2/3.8	9.9/5.4	7.6/3.3	6.6/2.6	3.6/1.6
AST/ALT (IU/L)	96/38	31/20	115/61	121/24	62/41	43/21	112/28	109/50	41/20	100/30	190/53
Total bile acids (μM)	250	120	513	298	210	52	323	331	n.d.	240	212
γ-GTP (IU/L)	206	142	131	251	186	148	142	408	130	n.d.	125
Total cholesterol (mg/dL)	212	195	n.d.	181	161	158	175	206	133	n.d.	196
Total protein/albumin (g/dL)	4.9/3.2	3.9/2.6	5.3/4.0	4.5/3.0	5.1/3.5	4.4/3.3	4.7/2.6	-/-	3.6/1.9	-/-	4.7/2.8
Citrulline (nmol/mL)	4.3	n.d.	85.0	n.d.	40.5	149.0	74.3	12.6	n.d.	117.0	211.0
α-Fetoprotein (ng/mL)	n.d.	n.d.	n.d.	200 700	n.d.	n.d.	n.d.	n.d.	29 600	n.d.	n.d.
PSTI (ng/mL)	n.d.	n.d.	n.d.	91.0	n.d.	40.0	24.0	n.d.	n.d.	n.d.	110.0
Ferritin (ng/mL)	447	n.d.	n.d.	2656	n.d.	117	502	1830	n.d.	n.d.	n.d.
Prothrombin activity (%)	75	26	93	55	37	88	70	37	9	n.d.	76
Mutation type	V/XIX	I/II	I/I	I/II	II/II	I/V	II/V	II/V	II/II	I/V	I/V

12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
2/M	2/F	2/F	2/F	2/F	3/M	3/M	3/M	3/F	3/F	4/M	4/M	4/F	4/F	5/M
10.2/3.9	11.1/3.6	13.0/8.5	6.9/2.7	5.3/2.4	6.1/3.5	6.0/3.8	9.6/2.7	8.8/3.2	12.0/2.6	5.1/2.5	6.7/3.7	5.4/3.5	6.2/2.4	15.0/10.1
106/22	86/23	133/45	78/25	74/44	98/36	232/48	85/44	95/39	75/19	95/90	295/105	208/100	83/24	146/66
240	320	172	290	143	302	269	205	389	157	283	172	253	127	355
213	132	78	209	160	124	249	n.d.	149	198	145	270	132	90	129
n.d.	n.d.	204	232	n.d.	194	n.d.	140	223	256	128	169	n.d.	n.d.	n.d.
4.9/3.7	4.0/3.5	3.8/2.6	4.1/2.7	n.d.	5.3/3.9	n.d.	5.7/3.8	5.1/3.1	4.8/3.0	4.2/3.5	4.8/3.1	5.5/3.5	n.d.	4.0/2.8
242.0	478.0	581.0	n.d.	291.7	839.1	208.0	n.d.	32.2	392.0	675.0	524.0	27.5	28.4	5.8
n.d.	n.d.	87 000	n.d.	91 940	n.d.	n.d.	n.d.	n.d.	n.d.	75 300	n.d.	n.d.	n.d.	10 578
n.d.	24.0	n.d.	n.d.	57.0	n.d.	n.d.	n.d.	62.0	12.9	12.5	n.d.	n.d.	n.d.	188.0
743	n.d.	775	n.d.	1651	n.d.	n.d.	n.d.	n.d.	200	n.d.	n.d.	503	n.d.	n.d.
87	n.d.	n.d.	25	51	43	n.d.	66	50	75	29	39	69	15	15
IV/IV	II/II	II/V	II/II	II/II	II/III	I/I	II/II	VI/VI	I/II	I/-	I/II	VIII/X	IV/VI	II/V

27	28	29	30	Mean ± SD	Range	Normal range
5/F	6/M	6/F	7/F			
5.8/3.4	5.5/3.9	6.2/2.0	5.9/2.9	7.6 ± 3.0/3.6 ± 2.0 ($n = 30$)	3.3–15.0/0.7–10.1	0.2–1.1/0.0–0.4
260/169	123/87	127/38	191/67	120.3 ± 63.7/49.2 ± 33.3 ($n = 30$)	31–295/20–169	6–40/5–40
213	n.d.	150	168	241.3 ± 96.1 ($n = 28$)	52–513	5–25
67	149	65	292	168.6 ± 75.0 ($n = 28$)	65–408	5–32
n.d.	148	n.d.	168	183.1 ± 34.8 ($n = 19$)	133–256	130–220
6.4/4.7	4.5/3.0	4.6/2.7	6.0/3.2	4.7 ± 0.7/3.2 ± 0.6 ($n = 25$)	3.6–6.4/1.9–4.7	6.5–8.3/3.7–5.2
48.2	11.0	41.3	86.8	179.1 ± 199.2 ($n = 25$)	4.3–291.7	17–43
n.d.	11 000	329 000	207 000	115 790.9 ± 108 111.0 ($n = 9$)	11 000–329 000	<10 000
n.d.	n.d.	21.9	n.d.	58.5 ± 53.6 ($n = 11$)	12.5–188.0	22–46
n.d.	n.d.	n.d.	197	874.6 ± 816.3 ($n = 11$)	117–2656	12–80
88	9	41	29	51.3 ± 26.0 ($n = 25$)	9–93	70–140
I/II	I/II	I/II	I/II			

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; PSTI, pancreatic secretory trypsin inhibiter; M, male, F, female; n.d., not done; I, 851del4; II, IVS11 + 1G > A; III, 1638ins23; IV, S225X; V, IVS13 + 1G > A; VI, 1800ins1; VIII, E601X; X, IVS6 + 5G > A; XIXIVS16ins3kb; -, unknown; SD, standard deviation.

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γ-glutamyl transpeptidase. Prothrombin activity, total protein and albumin were decreased. The mutation types were 851del4/IVS11 + 1G > A throughout most of late infancy, being more than 5 months of age in patients 27, 28, 29 and 30.

Histological findings

Histological findings of the 30 patients are shown in Table 3. The results of the fibrosis staging and inflammation grading are shown in Figure 1.

Fibrosis

Most specimens showed varying degrees of fibrosis; 35% of all specimens were classified as stage 0, while stages 1 and 2 together accounted for 50%. However, there was a wide spectrum of fibrosis: more advanced liver lesions with distorted lobular architecture (stage 3) (Fig. 2) and cirrhosis were observed in four and one specimens, respectively. One patient with cirrhosis developed hepatic failure. Therefore, this patient underwent a living-related liver transplant. One patient with cirrhosis developed at 10 months of age.[10]

Inflammatory reaction

The degree of inflammation varied with the specimens, where half showed moderate or severe inflammatory changes. Inflammatory cell infiltration in the portal tracts and piecemeal necrosis were observed (Fig. 3). Inflammatory cells present in the portal tracts were predominantly lymphocytes. Focal necrosis and acidophilic bodies in the parenchyma were seen in 23 (77%) and 12 (40%) specimens, respectively. The sinusoids showed the proliferation of mononuclear cells with scarce neutrophils and the activation of Kupffer cells.

Fat deposition in hepatocytes

Fat deposition in hepatocytes was observed in all specimens except one and severe fatty liver was noted for 20 (67%) specimens (Fig. 4a). Fat droplets deposited in the cytoplasm of hepatocytes varied in size, and fat-laden hepatocytes were classified as those with macrovesicular fat droplets, those with foamy, microvesicular fat droplets, and those with mixed macrovesicular and microvesicular fat droplets. Hepatocytes with microvesicular fat droplets had a centrally located nucleus. In 80% of 29 specimens with fat deposition including all 20 specimens which showed severe fatty livers, there was a mixture of macro- and microvesicular fat droplets (Fig. 4b,c). Macrovesicular and microvesicular fatty liver alone accounted for three (10%) and one (4%) specimens, respectively. A moderate and severe fatty liver with an inflammatory reaction and lipogranuloma were diagnosed as steatohepatitis, which accounted for 60% of the patients. The histopathological findings in this disease were different from those in non-alcoholic steatohepatitis. The clinical features of one patient who had no fat deposition in hepatocytes did not differ from that of other patients with such fat deposition.

Cholestasis

Cholestasis was observed in 77% of the specimens and was severe in 57%. The acinar arrangement of hepatocytes was prominent in specimens with severe cholestasis (Fig. 5) and multinucleated giant cell transformation was found in one case (Fig. 6).

Hemosiderin deposition

Hemosiderin deposition, mostly mild and localized in periportal hepatocytes and macrophages in portal areas (Fig. 4b), was observed in 57% of the specimens.

A combination of all five features, fatty liver, inflammatory cell infiltration, fibrosis, cholestasis and hemosiderin deposition was observed in the same liver biopsy specimen in 12 (40%) of the total specimens.

Relationship between the age and the histological findings

The mean score of each histological finding in each of groups A, B and C are summarized in Table 4. The degree of fibrosis, necroinflammatory reaction such as focal necrosis and acidophilic bodies, acinar arrangement of hepatocytes, cholestasis and steatohepatitis of infants more than 3 months old (groups B and C) were more accentuated than those of the early infants of group A. Conversely, hemosiderosis and extramedullary hematopoiesis in groups B and C were less pronounced than in group A. The staging score of fibrosis, grade of inflammation and steatohepatitis were significantly higher in the older than in the younger group in order of group A, B and C.

Histological findings of follow-up biopsy

Follow-up biopsies were conducted for patients 8, 9, 13 and 18, and the findings were as follows: patients 8, 9 and 13 showed histological deterioration of cholestasis and fatty change. Of note, patient 9 underwent a liver transplant at the age of 16 years because of hepatic failure. The findings for the explant liver were

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Table 3 Histological findings of liver biopsy in the 30 patients with neonatal intrahepatic cholestasis caused by citrin deficiency

Patient no.	1	2	3	4	5	6	7	8	9	10
Stage of fibrosis	0	0	1	0	0	0	0	0	3	2
Grade of inflammation	1	2	2	1	1	1	2	1	1	1
Focal necrosis[a]	1	1	2	0	0	0	1	0	0	1
Acidophilic body[b]	0	1	0	2	0	1	0	1	0	0
Acinar arrangement[c]	0	1	3	3	0	1	0	1	2	1
Cholestasis[d]	0	3	3	3	1	0	1	2	3	1
Degree of fat deposit[e]	1	3	3	3	3	3	2	3	3	3
Type of fat deposit[f]	1	3	0	3	3	3	1	3	0	0
Steatohepatitis[g]	0	1	1	1	0	1	1	1	0	2
Hemosiderosis[h]	0	2	1	2	0	0	1	2	0	2
Extramedullary hematopoiesis[i]	0	2	0	3	2	1	0	2	0	0
Patient no.	11	12	13	14	15	16	17	18	19	20
Stage of fibrosis	0	2	2	1	0	0	3	2	1	1
Grade of inflammation	1	1	1	2	1	2	2	2	3	1
Focal necrosis	1	0	1	1	1	2	1	1	3	0
Acidophilic body	1	0	0	1	0	0	1	0	0	0
Acinar arrangement	2	0	0	2	2	1	1	1	2	1
Cholestasis	3	0	0	3	3	2	2	2	3	3
Degree of fat deposit	3	0	2	2	3	2	3	3	2	3
Type of fat deposit	3	0	2	3	3	3	3	3	3	3
Steatohepatitis	2	0	0	1	1	1	1	1	2	1
Hemosiderosis	2	0	1	0	2	1	1	0	2	1
Extramedullary hematopoiesis	0	0	0	3	2	0	1	0	0	0
Patient no.	21	22	23	24	25	26	27	28	29	30
Stage of fibrosis	2	2	0	2	2	3	1	3	3	4
Grade of inflammation	3	2	1	2	3	2	1	2	3	3
Focal necrosis	1	2	1	1	3	1	1	1	2	1
Acidophilic body	1	2	0	1	1	1	0	0	0	2
Acinar arrangement	3	2	0	2	2	1	2	1	3	2
Cholestasis	3	3	0	3	0	3	3	3	3	3
Degree of fat deposit	3	3	3	3	1	3	2	3	3	3
Type of fat deposit	3	3	3	3	1	3	3	3	3	3
Steatohepatitis	0	3	2	1	0	2	1	3	3	3
Hemosiderosis	3	1	1	1	0	1	1	0	0	0
Extramedullary hematopoiesis	1	0	1	1	2	0	0	0	1	0

[a]Focal necrosis was graded from 0–3 based on the number of counts per 10 fields at a magnification of ×40. A score of denotes 0 is none, 1 denotes 1–2; 2 denotes up to 4, and 3 denotes >4.
[b]Acidophilic bodies were counted and graded from 0–3, as similar to that for focal necrosis.
[c]The acinar arrangements of the hepatocytes were graded 0–3. A rating of 0 denotes none, 1 denotes involvement up to 30% of the hepatocytes, 2 denotes 30–60%, and 3 denotes >60%.
[d]Cholestasis was graded from 0–3. A score of 0 denotes none, 1 denotes cholestasis without a bile plug, 2 denotes scattered bile plugs, and 3 denotes frequent bile plugs.
[e]The degree of fat deposition in hepatocytes was graded from 0–3 based on the percentage of hepatocytes in the biopsy involved. A rating of 0 denotes none; 1 denotes up to 30%, 2 denotes 30–60%, and 3 denote >60%.
[f]The type of fat deposit was classified as being between 0–3. A score of 0 denotes no fatty change, 1 denotes predominantly macrovesicular fat droplets, 2 denotes predominantly microvesicular fat droplets, and 3 denotes mixed microvesicular and macrovesicular fat droplets.
[g]Steatohepatitis was graded from 0–3, where 0 denotes none, 1 denotes steatosis involving up to 60% and intra-acinar inflammation with no or mild portal inflammation, 2 denotes steatosis (>66%) with both acinar and portal inflammation, and 3 denotes panacinar steatosis with intra-acinar inflammation and piecemeal necrosis.
[h]Hepatocellular iron was graded between 0–3, where 0 denotes none, 1 denotes localized deposition in the portal and /or periportal area; 2 denotes iron deposition involving up to 60% of the parenchyma, and 3 denotes >60%.
[i]Extramedullary hematopoiesis was graded between 0–3, similar to that for focal necrosis.

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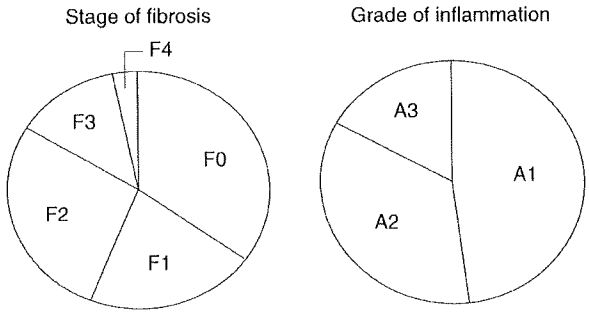

Figure 1 Results of fibrosis and the grade of necroinflammation.

more pronounced than those of the biopsy. Patient 8 showed progression of fibrosis from stage 1–3 and more pronounced portal inflammation. In contrast, patient 18 showed marked improvement of every

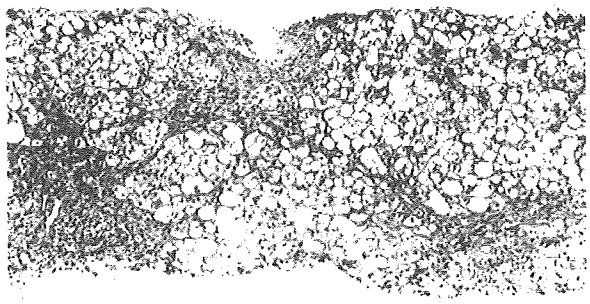

Figure 2 Severe fatty liver with distorted lobular architecture due to extensive fibrosis in stage 3 with portal inflammation (hematoxylin–eosin, original magnification ×50).

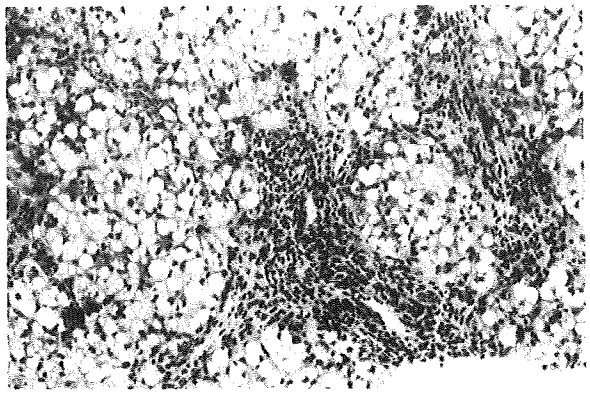

Figure 3 Fatty liver with moderate inflammatory cell infiltration in the portal tract and parenchyma, which is indicative of steatohepatitis (hematoxylin–eosin, original magnification ×100).

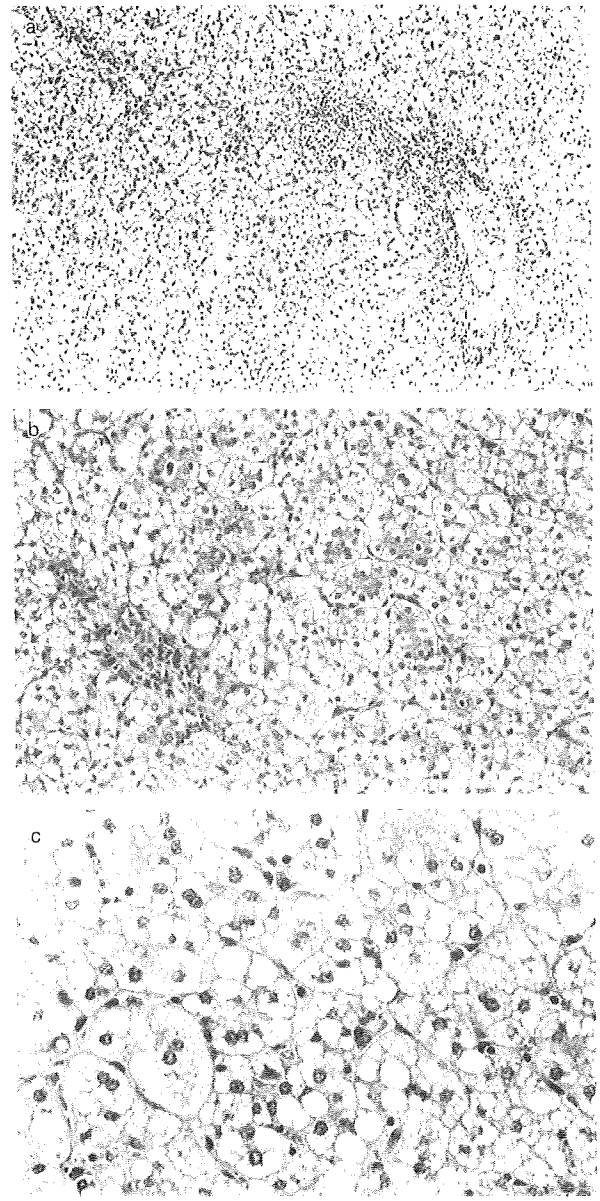

Figure 4 (a) Severe fatty liver with cholestasis. The portal tracts show mild inflammatory cell infiltration (hematoxylin–eosin [HE], original magnification ×50). (b) Pseudo-acinor transformation with bile plugs is observed. Hemosiderin-laden macrophages are present in a portal tract (HE, original magnification ×100). (c) Macro- and microvesicular-type fatty droplets. Some of the swollen hepatocytes have a foamy appearance and their cytoplasm packed with micro-fat droplets. Kupffer cells are activated (HE, original magnification ×200).

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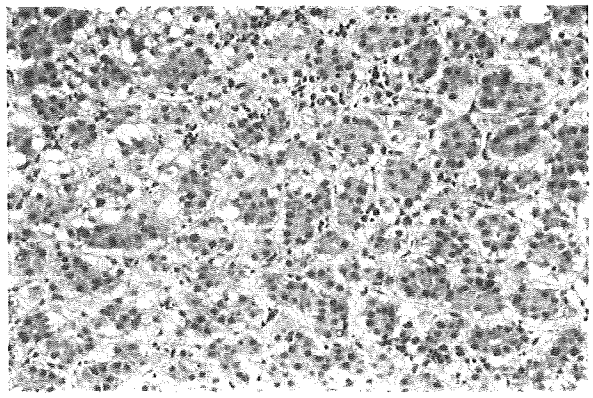

Figure 5 Striking pseudo-acinor transformation of the hepatic cords containing bile plugs. Small fatty droplets are present at the periphery of hepatocytes arranged in an acinar fashion (hematoxylin–eosin, original magnification ×100).

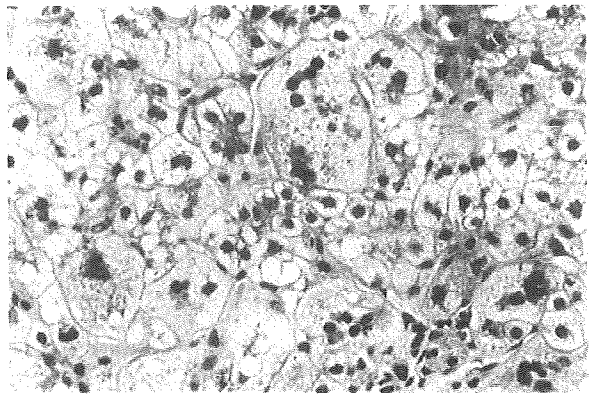

Figure 6 Giant cell hepatitis and cholestasis. Multinucleate giant cells contain several nuclei (hematoxylin–eosin, original magnification ×200).

histological finding, including decreased portal fibrosis and inflammation.

DISCUSSION

THE CAUSE OF liver dysfunctions such as fatty liver, hypoglycemia and galactosemia in this disease is as follows.[15] Citrin deficiency blocks the malate aspartate shuttle, which may increase the ratio of cytosolic nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide ($NADH/NAD^+$), which in turn is associated with the inhibition of glycolysis and makes reduced alcohol metabolism. This may be why CTLN2 patients dislike carbohydrates and cannot drink alcohol, and why alcohol consumption often results in psychiatric symptoms. An increased $NADH/NAD^+$ ratio is also characteristic of the inhibition of gluconeogenesis involving reduced substrates.[19] This, together with the reduction in alanine metabolization to urea and glucose due to citrin deficiency may cause hypoglycemia in NICCD patients. Although NICCD patients suffer from galactosemia, which sometimes even leads to the development of cataracts, no abnormalities in the enzymes involved in galactose metabolism have been found.[20] Because uridine diphosphate-glucose epimerase which requires NAD as a cofactor is strongly inhibited by NADH,[21] galactosemia in NICCD may represent a high NADH level in the cytosol of the liver.

From the biochemical data of this study, 50% of the high level of total bilirubin was associated with direct bilirubin, but it was not always dominant. The levels of AST were increased to more than twice the levels of ALT. Low levels of total protein, albumin and prothrombin

Table 4 Relationship between age and histological changes

Pathological findings	Group A ($n = 16$) <2 months	Group B ($n = 9$) 3–4 months	Group C ($n = 5$) >5 months	*P*-value
Stage of fibrosis	0.69 ± 1.01	1.67 ± 0.87	2.80 ± 1.10	$P = 0.001$
Grade of inflammation	1.31 ± 0.48	2.11 ± 0.78	2.20 ± 0.84	$P = 0.004$
Focal necrosis	0.75 ± 0.68	1.44 ± 1.01	1.20 ± 0.45	$P = 0.063$
Acidophilic body	0.44 ± 0.63	0.67 ± 0.71	0.60 ± 0.89	$P = 0.523$
Acinar arrangement	1.19 ± 1.05	1.56 ± 0.88	1.80 ± 0.84	$P = 0.172$
Cholestasis	1.75 ± 1.29	2.11 ± 1.27	3.00 ± 0.00	$P = 0.059$
Degree of fat deposit	2.44 ± 0.89	2.67 ± 0.71	2.80 ± 0.45	$P = 0.333$
Steatohepatitis	0.81 ± 0.66	1.22 ± 0.97	2.40 ± 0.89	$P = 0.008$
Hemosiderosis	1.00 ± 0.89	1.11 ± 0.93	0.40 ± 0.55	$P = 0.356$
Extramedullary hematopoiesis	0.94 ± 1.18	0.67 ± 0.71	0.20 ± 0.45	$P = 0.297$

The data are expressed as means ± standard deviation.
P-values are by the Mantel–Haenszel linear trend test.

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activity and high levels of citrulline, α-fetoprotein, ferritin and PSTI were observed as previously described in NICCD patients.[6–13] However, 11 patients showed high levels of ferritin, which were not observed in previous reports on NICCD patients. Therefore, the pediatric hepatologist should suspect NICCD when a neonatal cholestatic patient has high levels of AST of more than twice the ALT value, citrulline, α-fetoprotein and ferritin, and low levels of total protein and prothrombin activity.

The histological findings in this study such as a fatty liver, cholestasis, necroinflammatory reaction and iron deposition are not pathognomonic findings because they occur in various liver diseases.[22] However, the combination of mixed macrovesicular and microvesicular fatty hepatocytes and these histological findings are almost never observed in other liver diseases in infants and adults. Microvesicular fatty deposition was found in NICCD, this type of fatty change is a characteristic feature of Reye syndrome[23] and other rare conditions.[22] However, the histogenesis of the microvesicular fatty deposition in NICCD is unclear. It might reflect the acute impairment of β-oxidation of fatty acid due to mitochondrial dysfunction as in Reye syndrome.

Although our series of NICCD patients shared common liver histological findings as described above, there seemed a tendency that late infants of group C had more advanced fibrosis and more accentuated inflammation than those of early infants of group A. The duration of illness may be an aggravating factor of the progression of the disease in some cases. There was no difference between the liver histological findings and mutation type. Interestingly, however, the mutation type of patients with severe fibrosis who were 6 and 7 months of age was 851del4/IVS11 + 1G > A. Because evidence for this relationship between the mutation type and the progression of fibrosis is not clear, further investigation is needed. Moreover, in the follow-up liver biopsy patients, we observed improvements in their liver histological findings as the liver dysfunction was ameliorated. Therefore, we speculate that the correlations between the stage of the liver histological findings and the biochemical test data exist.

This study found that NICCD is a disease with characteristic hepatopathological features. If NICCD is suspected in the presence of cholestasis during infancy, a liver biopsy should be performed to screen for liver diseases. We believe that a liver biopsy is of high diagnostic value for NICCD, and is useful for accurately assessing inflammation and the degree of the progression of fibrosis.

Although we were not able to elucidate the natural history of the disease, we previously found that despite a benign course in the majority of the patients, it leads to the development of liver cirrhosis in some patients with CTLN2.[5,10] This suggests that it involves the risk of progressive fibrosis and eventually leads to the development of cirrhosis. This possibility is suggested by the above histopathological findings characteristic of NICCD in the patients who progressed to stage 3 chronic hepatitis and cirrhosis. Although the process responsible for the progression of liver lesions is not clear, some patients with steatohepatitis including nonalcoholic steatohepatitis (NASH) progress to cirrhosis.[24] In this study, steatohepatitis was found in 60% of the specimens. It is likely that, in NICCD, steatohepatitis repeatedly deteriorates, persists and progresses.

In conclusion, if NICCD is suspected in the presence of cholestasis during infancy, a liver biopsy should be performed to screen for other liver diseases. NICCD is a disease with characteristic hepatopathological features, such as the combination of mixed macrovesicular and microvesicular fatty hepatocytes, cholestasis, necroinflammatory reaction and iron deposition. Therefore, it is possible to diagnose NICCD based on histological liver findings in most cases. However, when cirrhosis with fat deposition is detected in hepatocytes in liver specimens, the patient should be carefully observed, because the prognosis of NICCD patients is not always fair, with some developing progressive liver failure by 1 year of age. Finally, we could not infer the development of CTLN2 from the histological findings of the patients with NICCD who were examined in this study.

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Citrin deficiency and current treatment concepts

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ABSTRACT

In this paper, we describe the historical aspects of citrin and citrin deficiency, characteristic food preference and food aversion of citrin-deficient subjects, and carbohydrate toxicity in relation to ureogenesis and issues of the conventional treatment procedures for hyperammonemia in citrin deficiency, leading to current treatment concepts for citrin deficiency. We also emphasize the importance of a citrin deficiency mouse model in elucidating the pathophysiology and developing novel therapeutics based on the pathophysiology, such as sodium pyruvate.

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Historical aspect citrin and citrin deficiency

Human citrin deficiency is a newly-established disease entity [1,2] that encompasses both adult-onset type II citrullinemia (CTLN2) and neonatal intrahepatic cholestasis (NICCD), and results from mutations in the *SLC25A13* gene that encodes citrin [3]. It was first described in Japan and East Asia, but is now a panethnic disease [4]. Citrin and the closely-related protein aralar (encoded by *SLC25A12*) [5] are isoforms of the mitochondrial aspartate (Asp)–glutamate (Glu) carrier (AGC) in the inner mitochondrial membrane and are responsible for the exchange of matrix Asp for cytosolic Glu and a H^+ ion, which is an electrogenic process [6]. Citrin is predominantly found in liver, kidney, heart and small intestine, while aralar is found in brain, skeletal muscle, kidney and heart [3,7,8]: citrin can be thought of as the liver-type, while aralar as the brain- and muscle-type, AGC. The function of the AGC is to participate in gluconeogenesis from lactate and transporting cytosolic NADH-reducing equivalents into mitochondria as part of the malate Asp shuttle, in addition to providing Asp from mitochondria to the cytosol for the synthesis of proteins, nucleotides and urea (Fig. 1).

Citrullinemia is caused by a deficiency of the urea cycle enzyme, argininosuccinate synthetase (ASS), which catalyzes the ligation of citrulline (Cit) and Asp to form argininosuccinate at the expense of ATP utilization. Saheki et al. [9] classified citrullinemia into classical or type one citrullinemia (CTLN1) caused by mutations in the *ASS* gene [10,11] and CTLN2 caused by mutations in *SLC25A13* [3]. CTLN2 is characterized by a liver-specific decrease in ASS protein [12] without any detectable abnormalities in the *ASS* gene or hepatic *ASS* mRNA levels [13,14]. The hepatic loss of ASS protein in CTLN2 patients is secondary to citrin deficiency [15], although its cause still remains to be clarified. Patients with CTLN2 suffer from recurring neuropsychiatric symptoms associated with hyperammonemia, including disorientation, delirium, seizures, and coma that can lead to death from brain edema [1,2,12].

Identification of mutations in *SLC25A13* of CTLN2 patients led to the discovery that patients with a type of neonatal hepatitis were also caused by the same mutations [16–18]. Since the neonatal symptoms were markedly different from those of adult CTLN2 patients, we named the neonatal presentation NICCD (neonatal intrahepatic cholestasis caused by citrin deficiency) [1,19]. Patients with NICCD show multiple metabolic abnormalities, including aminoacidemias (Cit, threonine, methionine, tyrosine, and arginine) accompanied by an increased threonine/serine ratio, galactosemia, hypoproteinemia, cholestasis, and fatty liver [2]. Human citrin deficiency therefore results in NICCD during the first few months of life, with symptoms usually self-resolving by the first year in most cases [20]. Following an apparently healthy period that can last from one to several decades, some patients with human citrin deficiency go on to develop severe CTLN2 [1,17,21].

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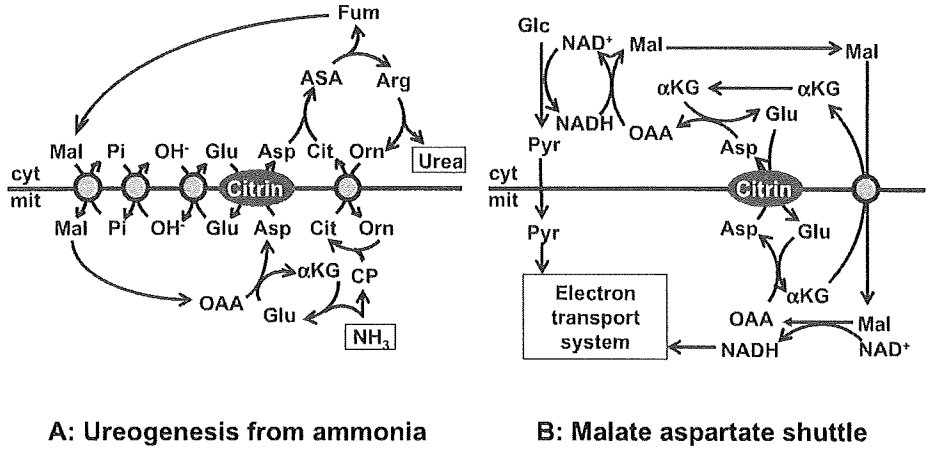

Fig. 1. Functions of citrin (liver-type AGC). (A) The role of citrin in ureogenesis from ammonia [35], and (B) the role of citrin in malate aspartate shuttle. Abbreviations are, Arg, arginine; ASA, argininosuccinate; Asp, aspartate; αKG, α-ketoglutarate; Cit, citrulline; CP, carbamoyl phosphate; cyt, cytosol; Fum, fumarate; Glc, glucose; Glu, glutamate; Mal, malate; mit, mitochondria; OAA, oxaloacetate; Orn, ornithine; Pyr, pyruvate.

Now, we propose a potentially different course of citrin deficiency (Fig. 2), because a number of Japanese subjects with citrin deficiency suffer from various kinds of disorders during the so-called "Apparently Healthy Period" [2,22–27]. There have been a few patients with NICCD that have required liver transplantation due to severe, prolonged symptoms [2,28,29], and patients presenting with CTLN2 typically continued to worsen unless also treated by liver transplantation [1,2,21,30,31]. The reason for the deterioration will be discussed later.

Characteristic food preference/food aversion of citrin-deficient subjects

It has been long known that CTLN2 patients have peculiar preference for protein- and fat-rich food, such as peanuts and soy beans. Recently, we noticed that aversion to carbohydrate-rich food, such as cooked rice and sweet things, was the other side of their food preference. Saheki et al. [32] performed nutritional assessment of 18 Japanese citrin-deficient subjects in the age ranged from 1 to 33 years old, who have been diagnosed as carrying mutation(s) in both alleles: some had suffered from NICCD, some are siblings or a father of NICCD patients, and one was at the early stage of CTLN2. The results (Fig. 3) clearly showed a marked decrease in carbohydrate intake in viewpoint of a smaller proportion of carbohydrates contributing to the total energy distribution (protein/fat/carbohydrate: PFC ratio), a reduced net intake relative to age- and sex-matched controls and a shift towards a lower centile distribution for carbohydrate intake. This unique food preference of citrin-deficient subjects is markedly different from the well-known aversion to protein among the patients with the other late-onset urea cycle enzymopathy or lysinuric protein intolerance. This unique food taste suggests some correlation of the tendency to the pathogenesis and pathophysiology of citrin deficiency.

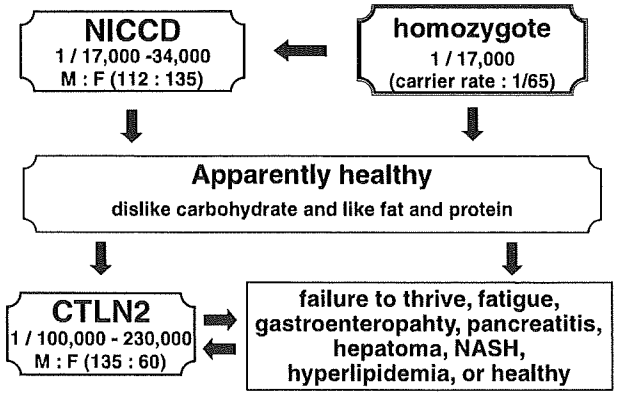

Fig. 2. Prognosis or life cycle of Japanese citrin-deficient subjects. M:F means numbers of male and female patients diagnosed. Incidence and prevalence were shown in the figure.

Carbohydrate toxicity in citrin deficiency

Tamakawa et al. [33] reported an interesting and important case with CTLN2. A 52-year-old woman fell into coma associated with hyperglycemia and hyperammonemia after receiving an infusion first composed of high dose of glucose (120 g/700 ml) and amino acids because of intractable pain and no appetite after operation for breast cancer. After recovering well, she received again a high-dose glucose infusion without amino acids. Again, she fell into coma with hyperammonemia.

Many CTLN2 patients died in a few weeks or months after infusion of glyceol composed of 10% glycerol and 5% fructose for the treatment of brain edema caused by hyperammonemia. Yazaki et al. [34] summarized reports in which the CTLN2 patients were treated with glyceol (8 cases), glyceol plus mannitol (4 cases), or mannitol (2 cases) for brain edema. All except one treated with glyceol alone or glyceol plus mannitol worsened after the treatment. Two CTLN2 patients treated with mannitol alone survived.

Concerning carbohydrate toxicity, we present a girl (P557S2) who was found to be a compound heterozygote carrying two different mutations in *SLC25A13* at 10 years old [26,32], together with her elder sister (P557) who suffered from CTLN2 and was treated with liver transplantation. Since at 13 years old, P557S2 complained of skinniness, fatigue and abdominal disorders, we examined her in connection with food intake (Figs. 3 and 4) [26,32]. As shown in Fig. 4, when she took hospital meals with high-carbohydrate energy ratios, she showed significant increases in plasma ammonia and Cit, and became drowsy. Therefore, we allowed her to take her favorite meals with fat- and protein-rich, and carbohydrate-low meals, resulting in only a slight increase in plasma ammonia with no increase in Cit. In this examination, we noticed a linear relation between plasma glucose and ammonia concentra-

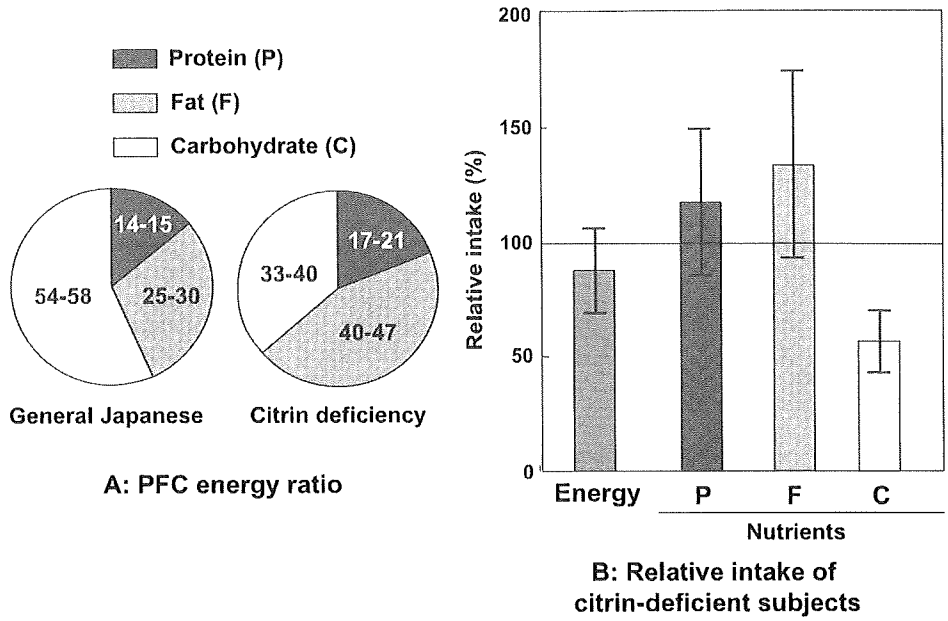

Fig. 3. Nutritional assessment of 18 citrin deficiency subjects revealed their characteristic dietary intakes. (A) Energy ratio of protein, fat and carbohydrate (PFC) of control general Japanese (left) and of citrin deficiency subjects (right), and (B) intake of energy and nutrients relative to age- and sex-matched controls [32].

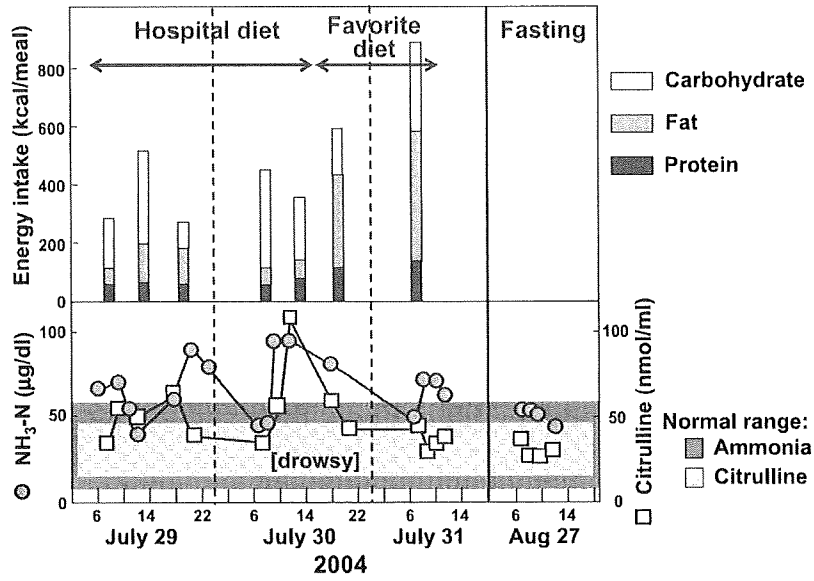

Fig. 4. Laboratory examination of 13-year-old girl (P557S2) at the early stage of CTLN2: relation between diets and plasma ammonia and citrulline. The upper panel depicts energy intake of each meal together with protein (black): fat (grey): carbohydrate (white) energy ratio. Arrows in the figure indicate duration of the hospital or her favorite meals taken. The lower panel, plasma ammonia (circle) and citrulline (square) concentrations with their control ranges (heavy and light grey, respectively).

tions after she took various kinds of breakfast (Fig. 5). All these results suggest carbohydrate toxicity in CTLN2. This can be explainable by metabolic disturbances in citrin deficiency as follows.

Ureogenesis in citrin-deficient state and its relation to carbohydrate toxicity

As described by Williamson in 1976 [35], ureogenesis under control states, liver-type AGC, citrin, plays a role in ureogenesis from ammonia as a nitrogen source (Fig. 1A). Under citrin deficiency without AGC (Fig. 6), Glu formed from ammonia, instead of Asp, goes out of mitochondria, converted to Asp by the action of cytosolic aspartate aminotransferase, and formed Asp is used for ASS reaction, indicating that urea may be synthesized in citrin deficiency. But in this metabolic pathway, oxaloacetate as an amino donor should be formed from fumarate via malate in the cytosolic compartment, which generates NADH. If the generated NADH is oxidized, urea can be synthesized under citrin deficiency. Under enhanced carbohydrate metabolism, liver plays a role in glucogenesis and fat synthesis. During the carbohydrate metabolism from glucose, fructose, glycerol and so on, NADH is formed and should be oxidized to continue aerobic glycolysis. The resultant NADH

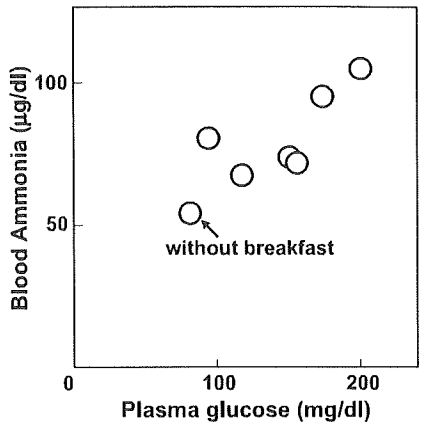

Fig. 5. Relation between plasma ammonia and glucose of a citrin-deficient patient (P557S2) after taking various kinds of breakfast. Plasma ammonia and glucose concentrations were measured 1 h and 2 h after taking various kinds of breakfast, and the maximum value of each breakfast was plotted.

accumulation or increase in NADH/NAD^+ ratio in the cytosol inhibits ureogenesis in citrin deficiency. Entry of a large amount of carbohydrate into the citrin-deficient liver may result in inhibition of glycolysis: accumulation of NADH inhibits glyceraldehyde 3-phosphate dehydrogenase and causes accumulation of glycerol 3-phosphate [data not shown], leading to the inhibition of phosphofructokinase step [36,37]. It may also cause energy deficit. These metabolic disturbances in the liver of citrin deficiency may mediate some kind of signal to the brain to avoid eating carbohydrate.

Animal model for citrin deficiency

It is important to generate animal models for elucidating the pathogenesis and pathophysiology of a disease and for developing new therapeutics based on the pathogenesis. First, we generated a citrin knockout (KO) mouse by homologous recombination to destroy *Slc25a13*. The resultant citrin KO mice were useful for analysis of ureogenesis in the perfused liver [38,39], but did not show any significant phenotype or symptoms (Table 1). This is apparently because mouse liver contains another active NADH shuttle, glycerophosphate shuttle, which consists of two glycerol 3-phosphate dehydrogenases from cytosol (cGPDH) and mitochondria (mGPDH). The citrin/mGPDH double KO mice we generated [40] revealed a number of human citrin deficiency symptoms, such as hyperammonemia under fed conditions, citrullinemia, hypoglycemia and so on (Table 1). The hepatic lactate/pyruvate ratio was high and increased by the administration of sucrose in the double KO mice. The most prominent characteristics of the mice are the hyperammonemia enhanced by the administration of sugars, confirming the carbohydrate toxicity in citrin deficiency.

Since sweet taste is a signal of an energy source, even mice like sweet taste. The citrin/mGPDH double KO mice, however, do not like a sweet taste. Mice from all genotypes (wild type, citrin KO, mGPDH KO mice) except the double KO mice voluntarily took a large amount of sucrose solution [data not shown]: namely, the double KO mice revealed a tendency similar to the citrin-deficient subjects.

Issues of the conventional treatment procedures for hyperammonemia in citrin deficiency

Some of the conventional treatment procedures for hyperammonemia may be very harmful for citrin deficiency. Low-protein and high-carbohydrate meals are standard for the therapy of urea cycle enzymopathy and lysinuric protein intolerance. Such meals, however, may cause disturbances such as hyperammonemia and hypertriglyceridemia in citrin deficiency [41]. In a similar sense, infusion of high concentration of sugar solution and glycerol solution for brain edema may cause deterioration [33,34,42]; many yet-unreported CTLN2 patients in Japan have been treated with such therapy, resulting in rapid death. Since high-carbohydrate meals are common in Japan (Fig. 3) even for hospital meals (Fig. 4), some citrin-deficient subjects should have become hyperammonemic for the first time when they were admitted to a hospital, as reported [25]. Similarly, some citrin-deficient subjects should have become ill after the entrance into primary school [26], where all schoolchildren had to take a high-carbohydrate lunch provided from school.

Possible treatment procedures for citrin deficiency

The most effective treatment procedure for citrin deficiency so far is liver transplantation [1,2,21,28–31]; more than 50 cases of CTLN2 and five cases of NICCD have been treated successfully.

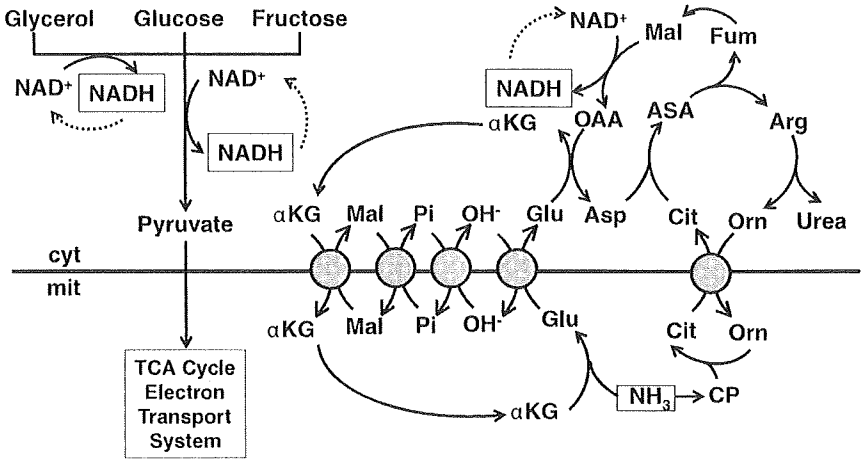

Fig. 6. Ureogenesis under citrin deficiency is inhibited by the accumulation of cytosolic NADH generated by the metabolism of carbohydrate. Abbreviations are shown in Fig. 1.