Application of Mutation Analysis for the Previously Uncertain Cases of Adult-Onset Type II Citrullinemia (CTLN2) and Their Clinical Profiles

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TANAKA, T., NAGAO, M. and TSUTSUMI, H. *Application of Mutation Analysis for the Previously Uncertain Cases of Adult-onset Type II Citrullinemia (CTLN2) and Their Clinical Profiles.* Tohoku J. Exp. Med., 2002, **198** (2), 89–97 — Type II citrullinemia (CTLN2) is characterized by a deficiency of argininosuccinate synthetase (ASS) in the liver. Mutation analysis of the *SLC25A13* gene, which is responsible for CTLN2, provides a rapid and accurate diagnosis. We describe clinical, biochemical and histologic features of two patients, whose diagnosis was finally made by mutation analysis. They initially presented with symptoms related to hyperammonemia at 16 to 22 years of age. A patient had shown mental retardation and growth failure from early childhood. Laboratory findings including amino acids, were characteristic, such as elevated citrulline, arginine, and lysine concentrations, but definitive diagnosis had not been made. The patients died of liver cirrhosis and hepatoma at 31 and 34 years old, respectively. Fatty change in the hepatocytes was commonly observed in the autopsied specimens. ASS activity was decreased in the liver of both patients, and a concomitant decrease of arginase activity was found in one case. Investigation for the *SLC25A13* mutation revealed that one patient was homozygous for IVS11+1G>A, and the other was compound heterozygote (851del4/S225X). Comparison of genetic, enzymatic and biochemical data among various cases of CTLN2 will be essential to understand the real nature of the disease. ——— type II citrullinemia; *SLC25A13*; citrin; hyperammonemia
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Citrullinemia is an autosomal recessive disease characterized by deficiency of argininosuccinate synthetase (ASS, EC 6.3.4.5). Classical citrullinemia (CTLN1, OMIM 215700) is caused by an ASS gene mutation, and more than half of CTLN1 cases experience a severe clinical course with death in the neonatal period. In contrast, a distinct late-onset form of citrullinemia (CTLN2, OMIM 603471) is caused by a mutation in the *SLC25A13* (OMIM 603859) gene (Kobayashi et al. 1999). It is associated with decreased ASS activity and protein in the liver but normal ASS levels in other tissues such as kidney, brain, and fibroblasts (Saheki et al. 1981, 1982, 1983).

SLC25A13 is a novel gene expressed abundantly in the liver (Kobayashi et al. 1999; Iijima et al. 2001). Citrin, which is encoded by the gene, is a new member of the mitochondrial carrier proteins with a possible role in the urea cycle (Kobayashi et al. 1999). Diagnostic analysis for the nine different mutations in the *SLC25A13* gene accounted for more than 80% of the cases of CTLN2 (Yamaguchi et al. 2002). No cross-reactive immune materials were detected in the liver of CTLN2 patients with any of these mutations (Yasuda et al. 2000). However, the mechanism of ASS deficiency remains unknown.

CTLN2 has been mostly found in Japanese patients, most of whom had uneventful infancies and early childhoods. Delayed mental and physical development was rare. Generally, significant clinical abnormality developed between age 20 to 40 years. Typical symptoms were recurrent vomiting, abnormal behavior, and the sudden onset of disturbances of consciousness, such as disorientation, restlessness, and coma. Patients may die with cerebral edema within a few years of onset. Recently, three infants were reported to have *SLC25A13* mutations, who presented as neonates with hypermethioninemia or hypergalactosemia detected by neonatal mass screening (Ohura et al. 2001). They are classified as neonatal intra-

hepatic cholestasis caused by citrin deficiency (NICCD) (Yamaguchi et al. 2002), but some of them developed to CTLN2 later in life (Tomomasa et al. 2001). The full spectrum of citrin deficiency is not well understood despite detection of the disease-causing mutations.

In this article we describe the clinical, biochemical, histologic and genetic features of two patients with CTLN2, who were hospitalized because of severe motor and intellectual disorders. They were found to have citrullinemia and hyperammonemia during the course of the disease, but their clinical pictures were heterogeneous. Therefore the diagnosis as CTLN2 was uncertain only by the clinical findings. In this study, we demonstrated decreased ASS activity in the liver specimens obtained by autopsy in the beginning. Liver samples were also examined histologically. The definitive diagnosis of type-II citrullinemia was finally made by genetic analysis of the *SLC25A13*.

MATERIALS AND METHODS

Patients

Case 1. This male patient was first hospitalized because of consciousness disturbance at 22 years of age. He had been healthy and no neurological abnormality had been noted. He showed drowsiness and delirium for a few weeks, but the symptoms resolved with conservative treatment. Similar episodes occurred several times until he was 26 and his mental status deteriorated, but the diagnosis was not made. Hyperammonemia was first noticed at 27 years of age, his protein intake was restricted to 1 g/kg/day and sodium benzoate was administered. Amino acid analysis, which was first performed at 28 years of age, showed elevation of citrulline, arginine and lysine. Although the patient's condition improved after treatment, plasma concentrations of citrulline and ammonia remained elevated. Ataxia and mild hyperreflexia of all extremities were noticed on neurological examination. He

was then admitted to a local sanatorium for mentally handicapped patients without further investigation on the pathogenesis. He again became critically ill because of hyperammonemia a few months later, and a liver biopsy taken on that occasion revealed cirrhosis and fatty infiltration. Hepatoma was diagnosed at 31 years of age and he died of ruptured esophageal varices. He was considered to be "Juvenile hepatocerebral disease."

Case 2. This male patient exhibited anorexia and vomiting in infancy. He was admitted several times to a local hospital because of malnutrition. At 9 years of age he weighed only 15 kg and was apparently mentally retarded. With time, attacks of coma and convulsions increased in frequency, to about once a week. He recovered from these symptoms with a low protein diet and an intravenous glucose infusion. He was hospitalized because of mental and neurological symptoms at 18 years old. Laboratory examination revealed elevation of citrulline, lysine and ammonia levels in his blood for the first time. Urinary excretion of homocitrulline was also increased. He was treated with a low protein diet and administration of sodium benzoate, however the levels of ammonia and citrulline fluctuated. An elevation of α -fetoprotein was first found at 32 years of age, and the liver and spleen were markedly enlarged. Pathological examination revealed the presence of liver cirrhosis and hepatoma. He died of massive bleeding from esophageal varices at 34 years of age. His clinical diagnosis was an "atypical citrullinemia" since the level of plasma lysine and urinary excretion of homocitrulline were constantly high, suggesting impairment of lysine metabolism.

DNA diagnosis

Genomic DNA was isolated from peripheral leukocytes or cultured skin fibroblasts after obtaining informed consent from the patients or their family. This study was approved by the

Institutional Review Boards of the National Nishi-Sapporo Hospital. For mutation detection of *SLC25A13*, we amplified DNA fragments using the primers and conditions described by Kobayashi et al. (1999) and Yasuda et al. (2000). We first investigated the presence of five major mutations (851del4, IVS11+1G>A, 1638ins23, S225X, IVS13+1G>A) in each patient. The 851del4 and 1638ins23 mutations can be detected by direct electrophoresis on 4% Amplisize (Bio-Rad, Hercules, CA, USA) agarose gel. The IVS11+1G>A, S225X and IVS13+1G>A mutations were detected by the PCR-RFLP method, using *Sau*3A1, *Alu* I and *Pst* I digestion, respectively.

Enzyme activities

Liver tissues were obtained for enzymatic analysis from the two patients by autopsy. The activities of the urea cycle enzymes in the liver were measured by the method of Schimke (carbamylphosphate synthetase, ornithine transcarbamylase and arginase; 1962), and of Su et al. (argininosuccinate synthetase and argininosuccinate lyase; 1981).

Histologic examination

In addition to hematoxylin-eosin-stained slides, each case included a variable panel of special stains, including PAS, trichrome, azan, reticulin and oil red-O. Fresh tissue for electron microscopy was immediately fixed in 2% glutaraldehyde and was available for examination.

RESULTS

Laboratory findings

The major biochemical data of the patients at hyperammonemic episode (case 1 at 28 years old and case 2 at 18 years old, respectively) are summarized in Table 1. The abnormalities in the values related to liver function were not remarkable, except for the elevation of ammonia. The plasma citrulline concentrations were increased to 7 to 9 times of control, and the

TABLE 1. *Laboratory findings at the episodes of hyperammonemia*

	Case 1 (28 year)	Case 2 (18 year)	Control
AST	25	28	10-40 (IU/liter)
ALT	73	24	5-45 (IU/liter)
Total protein	6.7	7.4	6.7-8.3 (g/100ml)
Albumin	4.1	4.3	3.8-5.3 (g/100ml)
BUN	18	13	8-23 (mg/100ml)
Ammonia	295	315	< 75 (μ g/100ml)
Amino acids			
Cit	254	253	28-40 (nmol/ml)
Arg	182	220	33-99 (nmol/ml)
Lys	321	381	142-208 (nmol/ml)
Thr/Ser	2.3	2.0	1.0
Homocitrulline (urine)	588	3613	< 34 (μ mol/g creatinine)

TABLE 2. *Urea cycle enzyme activities in the liver*

Enzyme	Patient		Control (n = 12)
	Case 1	Case 2	Mean $\pm$ s.d.
CPS	0.050	0.041	0.036 $\pm$ 0.013
OTC	0.61	0.44	0.88 $\pm$ 0.35
ASS	0.0083	0.0072	0.033 $\pm$ 0.012
ASL	0.048	0.089	0.052 $\pm$ 0.025
Arginase	13.3	3.2	15.8 $\pm$ 3.1

CPS, carbamoylphosphate synthetase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; N.D., not determined.

arginine and lysine levels were concomitantly increased. The threonine to serine ratios were apparently high in two cases. Urinary excretion of homocitrulline was markedly increased.

Enzyme activities in the liver

Argininosuccinate synthetase activity was specifically decreased in two subjects. Arginase activity was also decreased to 20% of control in case 2. Other urea cycle enzymes did not show significant changes compared to the control (Table 2).

Mutation analysis

Fig. 1 shows the results of mutation analy-

sis in the *SLC25A13* gene. Case 1 was homozygous for the IVS11+1G>A mutation, which is most common among Japanese CTLN2 patients. Case 2 was a compound heterozygote for two mutations, 851del4 and S225X, which have the second and third frequency, respectively (Yamaguchi et al. 2002).

Liver pathology

In case 1 and 2, autopsied livers were histologically evaluated. They both showed marked fatty change with severe fibrosis, and also hepatoma cells were evident in some regions (Figs. 2A and 2B). A prominent feature of the liver in light microscopy specimens was deposition of fat droplets in the entire lobule. There were no findings of an inflammatory response or cholestasis, but fibrosis and increased lipofuscin granules suggested degeneration of hepatocytes. In the electron microscopy specimens, various size and shape of mitochondria with decreased cristae were observed characteristically (Fig. 2C).

DISCUSSION

Since our patients were initially followed as severe motor and intellectual disabilities, their clinical courses and laboratory findings

Fig. 1. Mutation analysis of the *SLC25A13* gene.

A: Case 1 was homozygote for the IVS11+1G>A mutation. PCR product (83 bp) from control was not digested by *Sau3A1*, but that from the patient was cleaved to two bands (58 and 25 bp).

B: Case 2 was a compound heterozygote for the 851del4 and S225X mutations. 851del4 was detected by direct electrophoresis of PCR product on 4% Amplisize (Bio-Rad) agarose gel. The other variant allele, which bears S225X mutation, was diagnosed by PCR coupled with *Alu I* digestion. The control was cleaved to two bands (369 and 57 bp). Since S225X mutation creates a new *Alu I* site, the 369 band was further divided into 214 and 155 bp.

had not been well evaluated. They were at last diagnosed as having citrin deficiency caused by a *SLC25A13* mutation in this study. The results of the enzymatic, genetic and histologic investigations are summarized in Table 3.

The mean age of onset is usually between ages of 20 to 40 (Yasuda et al. 2000), and it still remains to be elucidated why the clinical symptoms are delayed until adulthood. Once it appears, the encephalopathy generally follows a rapid and progressive course. It is therefore desirable to make the diagnosis of CTLN2

before the typical symptoms appear. Hyperammonemia may be the sole clue to the presence of the CTLN2 in many cases. Histological investigation and enzymatic studies are rarely performed in the initial stage. One of the characteristic laboratory findings is an elevated serum citrulline ranging from 100 to 1000 nmol/ml, which is less than in CTLN1 (Saheki et al. 1986; Kobayashi et al. 1997). Our patients had values around 250 nmol/ml at the hyperammonemic episodes. The citrulline level sometimes was only slightly elevated during an

Fig. 2. Histologic findings of the liver biopsy specimen.

A: Case 1. Cluster of hepatocellular carcinoma cells in the lobule (H & E, $\times 40$).

B: Case 2. Marked fatty degeneration of hepatocytes. The arrow indicates severe fibrosis between lobules and in Glisson's sheath (Azan stain, $\times 40$).

C: Case 2. Electron-microscopic examination. The hepatocytes contain mitochondria (M) of various size and shape, fat (F), lipofuscin granule (L), rough endoplasmic reticulum (E). Smooth endoplasmic reticulum (S) have a lot of lipid droplets inside. Uranyl acetate and lead citrate stain, original magnification $\times 6000$.

TABLE 3. Summary of CTLN2 patients

	Sex	M.R.	ASS activity (% of control)	Genotype	Liver histology
Case 1	Male	—	25%	IVS11+1G>A, IVS11+1G>A	Cirrhosis, hepatoma Fatty degeneration
Case 2	Male	+	22%	851del4, S225X	Cirrhosis, hepatoma Fatty degeneration

M.R., mental retardation in childhood.

episode of unconsciousness, therefore evaluation of other diagnostic markers is important. The high plasma arginine and lysine concentrations and an increase in the ratio of plasma threonine to serine were major differences between CTLN2 and CTLN1 (Saheki et al. 1986; Kobayashi et al. 1997). Since increased excretion of homocitrulline was observed in both cases, it was important to differentiate from hyperornithinemia, hyperammonemia, homocitrullinuria (H.H.H) syndrome. Recently an increase of PSTI (pancreatic secretory trypsin inhibitor) is considered to be characteristic in CTLN2 (Kobayashi et al. 1997). An accurate diagnosis is now possible using these laboratory findings and the DNA test for the *SLC25A13* without measuring ASS activity in the liver.

In case 2, anorexia, frequent vomiting and developmental delay was already noticed during childhood. Hyperammonemia might have caused his mental and neurological symptoms in his childhood although blood ammonia was not measured until 18 years of age. Gradual brain damage may be associated with repeated hyperammonemic attacks and the underlying metabolic disorder. It is also important to investigate the relationship between mutation genotype and the brain pathology among such patients with motor and intellectual disorders. The compound heterozygote of S225X/851del4 make up less than 5% of CTLN2 patients according to the results of Yamaguchi et al. (2002). Clinical features with the genotype should be investigated in a large number of CTLN2 patients.

There is a discrepancy between the incidence of CTLN2 (1 in 100 000) (Kobayashi et al. 1993) and the carrier frequency (1 to 2 in 100) (Yasuda et al. 2000). Some cases are known to show fluctuation in plasma citrulline level or atypical symptoms (Takenaka et al. 2000; Maruyama et al. 2001), which may lead to misdiagnosis. Patients with developmental disorders tend to be missed without sufficient investigation, if metabolic abnormalities are not noticed. In addition, there are asymptomatic individuals with mutations in the family. The gene tracking in another CTLN2 family revealed that the 17-year-old sister was the same compound heterozygote (IVS11+1G>A/IVS13+1G>A) as the index case, although she is now quite healthy and has no abnormality in her laboratory examinations (Nagao et al. 2002). Since the most characteristic feature is the late onset of serious and recurring symptoms, anytime from adolescence to old age, regular examination including liver function tests and amino acids is necessary. Recently some cases of neonatal liver disease with cholestatic jaundice (NICCD) have also been found to have CTLN2 mutations (Ohura et al. 2001; Tazawa et al. 2001; Tomomasa et al. 2001). Although most of the clinical abnormalities disappear within a year of birth, we should extend the follow-up of these patients until adulthood because late-onset symptoms of CTLN2 may occur.

Most CTLN2 patients showed less than 20% of normal ASS activity in the liver, but a few patients showed 50% to 80% of control

(Yasuda et al. 2000). A patient, who was homozygous for the IVS11+1G>A mutation, suffered from citrullinemia with hepatocellular carcinoma. The ASS activity in the liver was 79% of control (Yasuda et al. 2000). Therefore, the severity of each patient cannot be evaluated only by decreased activity of ASS in the liver. On the other hand, histologic findings in the liver seem to reflect the pathophysiology at the time of diagnosis. The association of cirrhosis or hepatoma as in cases 1 and 2 may lead not only the loss of ASS protein but also of other liver functions. Many patients showed fatty change of hepatocytes from an early stage of the disease (Yagi et al. 1988). Progression of fat deposition and fibrosis in hepatocytes is considered to be serious, because long-term metabolic injury is probable. Citrin deficiency impairs the malate aspartate shuttle, which may increase cytosolic NADH and promote fatty degeneration and hyperlipidemia (Saheki and Kobayashi 2002). Structural changes in mitochondria as shown in Fig. 2 may also be caused by the elevated cytosolic NADH level.

Various conservative therapies, such as a low protein diet, arginine supplementation, sodium citrate or sodium benzoate administration cannot completely prevent serious complications. Peritoneal dialysis or hemodialysis has also been performed in several patients (Oshiro et al. 2002). Although some of these treatments have contributed to a temporary alleviation of hyperammonemia and neurological problems, they have failed to improve the long-term prognosis. Current therapy for adults with CTLN2 includes liver transplantation, when patients are critically ill with progressive encephalopathy. All the metabolic abnormalities were corrected and the neurological symptoms disappeared (Yazaki et al. 1996; Ikeda et al. 2001). We should establish effective therapeutic protocols to prevent the progress of liver and central nervous system disorders.

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