

## Six cases of citrin deficiency in Korea

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**Abstract.** Citrin deficiency resulting from mutations of the *SLC25A13* gene is associated with two major clinical phenotypes; neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and adult-onset type 2 citrullinemia (CTLN2). In Korea, 6 cases of citrin deficiency were diagnosed based on biochemical and molecular findings. Four NICCD patients (2 boys and 2 girls) presented high citrulline levels on a newborn screening test or neonatal cholestasis. They were associated with conjugated hyperbilirubinemia, elevated liver enzymes, hypoalbuminemia, mild hyperammonemia, elevated citrulline, methionine and threonine. All of the hepatic manifestations were resolved spontaneously at the age of 5-9 months. Mutation analysis identified them as compound heterozygotes carrying each of the c.851del4, IVS11+1G>A, IVS13+1G>A, G393S, and IVS16ins3kb mutant alleles. Two adult male CTLN2 patients were identified. They were aged 24 and 37 years, and presented sudden loss of consciousness, hyperammonemia and citrullinemia. They were compound heterozygotes with IVS13+1G>A and IVS16ins3kb, and with c.851del4 and IVS11+1G>A mutant alleles. This report describes the clinical characteristics, biochemical findings and molecular analysis of the *SLC25A13* gene of patients with citrin deficiency in Korea.

### Introduction

Adult-onset type 2 citrullinemia (CTLN2) is characterized by episodes of neurological symptoms associated with hyper-

ammonemia leading to disorientation, irritability, seizures and coma. The serum citrulline level is elevated while the activity of argininosuccinic acid synthetase (ASS) in liver tissue is decreased. CTLN2 is distinct from classical citrullinemia caused by a mutation of the *ASS* gene. In 1999, Kobayashi *et al* identified the novel *SLC25A13* gene on chromosome 7q21.3, and showed that five distinct mutations occurred in CTLN2 patients (1). Citrin, encoded by *SLC25A13*, is a liver-type mitochondrial aspartate-glutamate carrier (AGC). The deficiency in citrin causes not only CTLN2, in autosomal recessive trait, but also neonatal intrahepatic cholestasis with multiple aminoacidemia and galactosemia. This condition is named neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) (2,3).

It was previously thought that CTLN2 and NICCD occurred almost exclusively in Japan. Recently, however, several cases have been found in other Asian patients including those of Ashkenazi Jewish, Chinese, Israeli, Palestinian, Taiwanese and Vietnamese origin (4-6). Citrin deficiency is relatively common, especially in Japanese individuals, where the frequency of the *SLC25A13* mutant allele is approximately 1 in 70 (7).

In this study, we analyzed clinical characteristics and genotypes of the *SLC25A13* gene in Korean patients with NICCD and CTLN2.

### Materials and methods

**Patients.** Four NICCD (two boys and two girls) and two male CTLN2 patients were investigated in this study. Diagnosis of citrin deficiency was based on the clinical characteristics, biochemical findings and mutation analyses. Gene analysis was performed in all six patients. The following clinical and biochemical data were obtained by a retrospective medical chart review: sex, age at diagnosis, symptoms and signs, serum amino acid profiles, ammonia levels in the blood, serum galactose levels, liver transaminase levels in the blood, serum protein and albumin levels.

**Mutation analysis and DNA diagnosis of the *SLC25A13* gene.** Genomic DNA was isolated from peripheral blood leukocytes using a Puregene DNA Isolation Kit (Gentra Systems, MN). Eighteen exons of the *SLC25A13* gene and their intronic

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**Abbreviations:** CTLN2, adult-onset type 2 citrullinemia; ASS, argininosuccinic acid synthetase; AGC, aspartate-glutamate carrier; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; PCR, polymerase chain reaction

**Key words:** citrin deficiency, adult-onset type 2 citrullinemia, neonatal intrahepatic cholestasis, *SLC25A13*

flanking sequence were amplified by polymerase chain reaction (PCR) with 18 sets of primers. Amplification was performed in 30 cycles; each cycle consisted of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec. The PCR reactions were carried out in reaction volumes of 20  $\mu$ l, containing 100 ng of genomic DNA template; the reactions were 1  $\mu$ M each primer, 200  $\mu$ M in each dNTPs, 1.5 mM in  $MgCl_2$ , 50 mM in KCl, 10 mM in Tris-HCl (pH 8.3). The reactions contained 0.2  $\mu$ l of Taq polymerase (Promega, Madison, WI). After amplification, PCR mixtures were electrophoresed on 1.2% agarose gel in the presence of ethidium bromide to verify the size and purity of the PCR products.

After verifying that unique PCR products were amplified, DNA sequencing was performed using the same primers used for PCR, and the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The amplification protocol consisted of 30 cycles at 94°C for 20 sec, at 55°C for 20 sec and at 72°C for 30 sec with a PTC-200 PCR machine (MJ Research, Watertown, MA). In each reaction, we used 10 ng of PCR product, treated with exonuclease I and shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Piscataway, NJ), as a template, and 10 pM of primer was added. Incorporated dyes were removed by ethanol precipitation. An ABI PRISM 3100 Genetic analyzer (Applied Biosystems) was used to derive the DNA sequence. To rule out classical citrullinemia, the ASS gene was analyzed in all patients by a direct sequencing method after PCR amplifications as described above.

Diagnoses of known mutations were also confirmed by GeneScan and the Snapshot methods (3). Detailed methods for identification and diagnosis of a novel mutation, [XIX]:IVS16ins3kb, will be described elsewhere (Tabata *et al* unpublished data). Clinical and biochemical data have been reported previously in Japanese (8) and Korean (9) NICCD patients with the [XIX] mutation.

## Results

**Clinical characteristics and genotypes of NICCD.** Patient 1 was brought to the outpatient clinic at the age of 39 days because of a high serum citrulline level of 331  $\mu$ mol/l (normal range 3-35  $\mu$ mol/l) indicated by a neonatal mass screening test. Birth weight was 2.8 kg and the boy was being fed breast milk. He had mild jaundice (total bilirubin 7.3 mg/dl), but hepatomegaly was absent. Laboratory findings showed a serum methionine level of 29.2  $\mu$ mol/l (normal range 10-60  $\mu$ mol/l), a threonine level of 547  $\mu$ mol/l (normal range 24-174  $\mu$ mol/l), a galactose level of 32.5 mg/dl (normal range 0-20 mg/dl), an ammonia level of 285  $\mu$ mol/l (normal range 10-35  $\mu$ mol/l), an alkaline phosphatase level of 757 IU/l (normal range 145-420 IU/l) and an aspartate aminotransferase level of 87 IU/l (normal range 15-55 IU/l). Urine organic acid analysis revealed no prominent abnormalities. Molecular analysis of the *SLC25A13* gene revealed that the boy was a compound heterozygote with c.1177+1G>A (IVS11+1G>A) and IVS16ins3kb. He was treated with sodium benzoate and L-arginine. All clinical and abnormal laboratory findings were resolved by 5 months of age (Figs. 1A and 2A). The

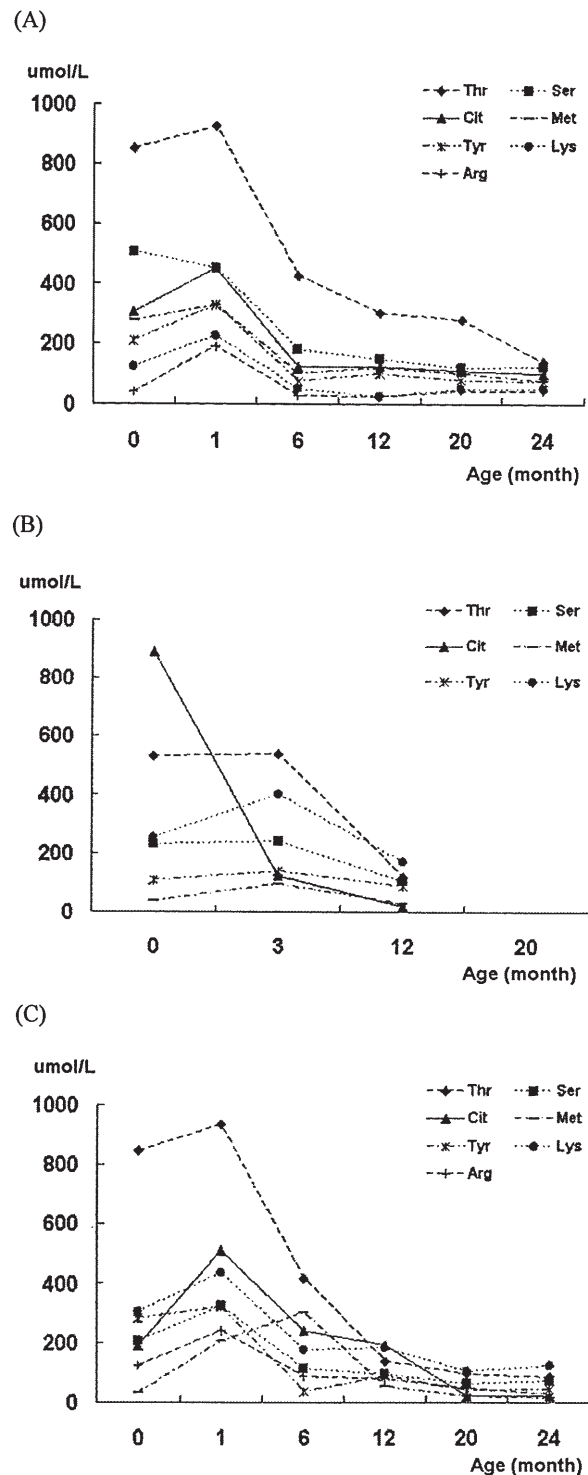
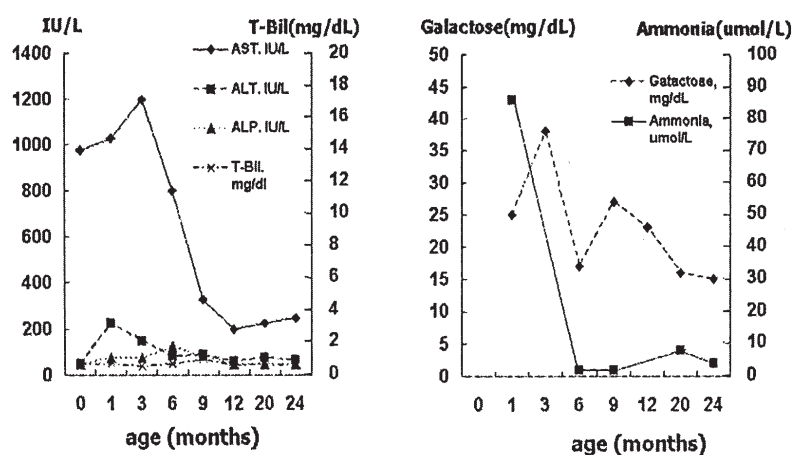


Figure 1. Sequential change of serum amino acid profiles in patient 1 (A), patient 2 (B) and patient 3 (C).

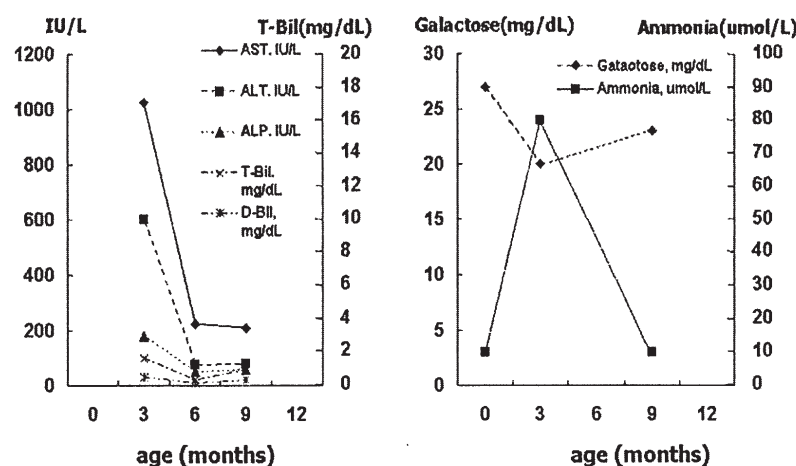
boy is now aged 20 months and developing normally with a preference for soy milk.

Patient 2 was brought to our hospital at the age of 30 days because of a high serum citrulline level (888  $\mu$ mol/l) on a neonatal mass screening test. She was born by normal vaginal delivery with body weight of 2.36 kg and was being fed breast milk. Jaundice (total bilirubin 9.8 mg/dl) and mild hepatomegaly were detected by physical examination. Laboratory findings showed a direct bilirubin level of 2.4 mg/dl (normal

(A)



(B)



(C)

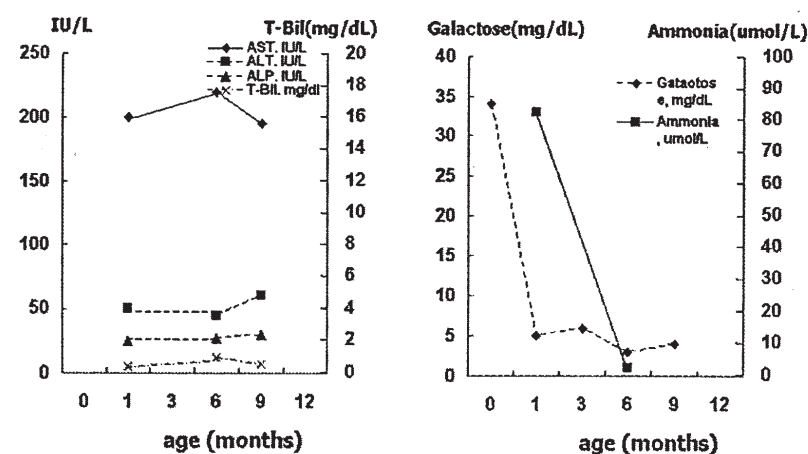


Figure 2. Sequential change in liver function, plasma galactose and ammonia in patient 1 (A), patient 2 (B) and patient 3 (C).

range  $<0.2$  mg/dl) and a serum albumin level of 2.2 g/dl (normal range 3.9-5.0 g/dl). Serum methionine ( $94.5 \mu\text{mol/l}$ ), threonine ( $534.6 \mu\text{mol/l}$ ), galactose ( $24.3$  mg/dl), ammonia ( $201 \mu\text{mol/l}$ ) and alkaline phosphatase ( $1044$  IU/l) levels were elevated at the time of diagnosis. c.1230+1G>A (IVS13+1G>A) and IVS16ins3kb mutations in the *SLC25A13* gene were identified by molecular analysis. Treatment with

sodium benzoate and L-arginine was attempted. All clinical and abnormal laboratory findings were resolved at the age of 9 months. The girl is now 20 months of age and developing normally with a preference for egg and ham (Figs. 1B and 2B).

Patient 3 was brought to the hospital at the age of 37 days. The presenting symptoms were intermittent vomiting and a high citrulline level ( $189 \mu\text{mol/l}$ ) on a neonatal mass

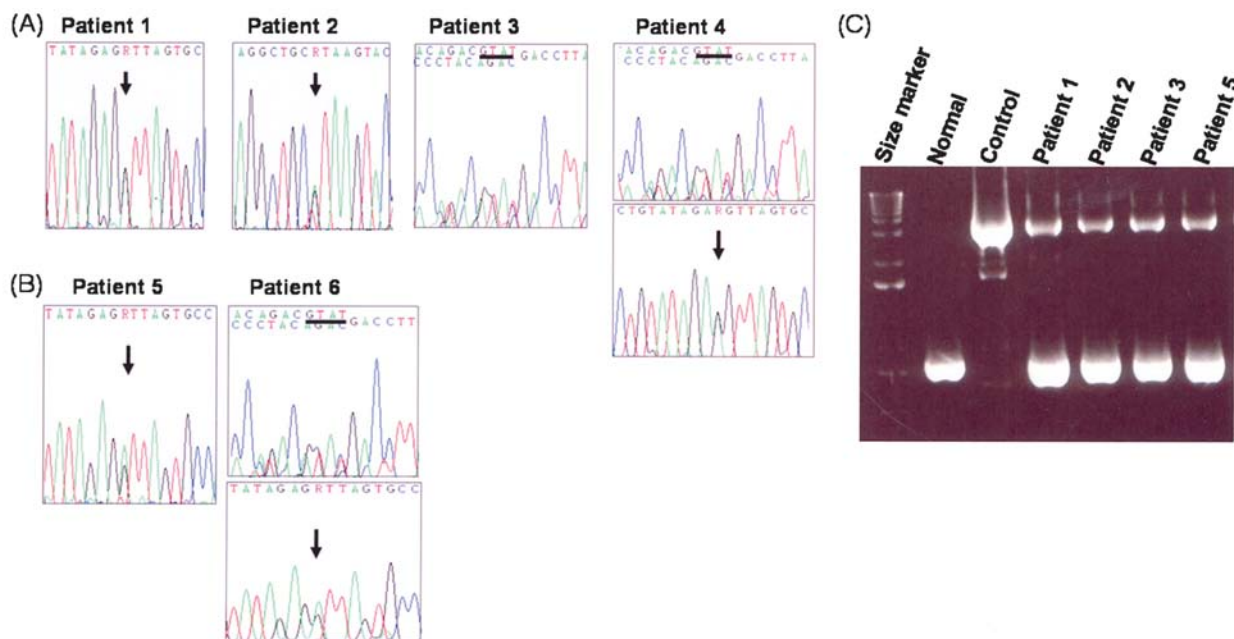


Figure 3. The mutation sequences of *SLC25A13* for patients with (A) NICCD and (B) CTLN2, were performed in the ABI PRISM 3100 Genetic Analyzer with BigDye v3.0. The 'R' means a heterozygosity of nucleotide 'A' and 'G'. To identify the IVS16ins3kb mutation, PCR analysis was carried out using the primer set spanning exon 16-17. PCR products were electrophoresed on 1.2% agarose gel (C). Approximately 3.5 kb of insertion mutation allele were revealed with 881 bp normal allele.

screening test. Mild jaundice (total bilirubin 8.1 mg/dl) and mild hepatomegaly (3 finger breadths below the right costal margin) were detected by physical examination. Serum threonine (844.1  $\mu\text{mol/l}$ ), tyrosine (282.8  $\mu\text{mol/l}$ ; normal range 22-108  $\mu\text{mol/l}$ ), galactose (42.6 mg/dl) and ammonia (129  $\mu\text{mol/l}$ ) levels were elevated at diagnosis. Serum alkaline phosphatase level (1251 IU/l) and  $\gamma$ -glutamyl transferase (141 IU/l; normal range 12-123 IU/l) levels were elevated. The boy was shown to be a compound heterozygote with c.851\_854del (c.851del4) and IVS16ins3kb mutations of the *SLC25A13* gene. Treatment with sodium benzoate and L-arginine was attempted. All clinical and abnormal laboratory findings were resolved by the age of 6 months. The boy is now 33 months old and developing normally with a preference for beef and ham (Figs. 1C and 2C).

Patient 4, a 5-month-old female, was admitted for the investigation of prolonged jaundice and hepatomegaly. The liver was palpable 4 cm below the right costal margin. Laboratory data showed an aspartate aminotransferase level of 89 IU/l, an alanine aminotransferase of 35 IU/l (normal range 5-45 IU/l), a  $\gamma$ -glutamyl transferase of 218 IU/l, a serum albumin of 2.2 g/dl and a total/direct bilirubin level of 5.7/3.1 mg/dl. Serum amino acid analysis revealed a methionine level of 49.8  $\mu\text{mol/l}$ , a threonine of 497.3  $\mu\text{mol/l}$ , a citrulline of 90.4  $\mu\text{mol/l}$  and a lysine level of 247.3  $\mu\text{mol/l}$  (normal range 52-196  $\mu\text{mol/l}$ ). An abdominal ultrasonogram revealed an enlarged and homogeneous liver with increased echogenicity. Liver histology obtained by needle biopsy showed diffuse microvesicular or macrovesicular fatty changes with periportal fibrosis. Mutation analysis of the *SLC25A13* gene identified a known mutation of c.851\_854del (c.851del4) and a novel mutation of c.1177G>A (p.Gly393Ser). The girl improved with time; the jaundice and hepatomegaly becoming less problematic. She is now 8 months of age.

**Clinical characteristics and genotypes of CTLN2.** Patient 5, a previously healthy 24-year-old man, was brought to the hospital due to abdominal discomfort, vomiting and disorientation. He was semicomatose and his blood ammonia level was 537  $\mu\text{mol/l}$ . His mental status improved by fasting and with frequent lactulose enemas. The serum ammonia level fluctuated after he was placed on a protein-restricted diet. Serum citrulline (286  $\mu\text{mol/l}$ ) and lysine (411  $\mu\text{mol/l}$ ) levels were markedly elevated. Serum methionine (53  $\mu\text{mol/l}$ ), arginine (134  $\mu\text{mol/l}$ ), threonine (190  $\mu\text{mol/l}$ ), and serine (94  $\mu\text{mol/l}$ ) levels were within normal limits. The citrulline to serine ratio was 3.04 and the threonine to serine ratio was 2.02. No mutation was detected in the *ASS* gene but c.1230+1G>A (IVS13+1G>A) and IVS16ins3kb mutations were identified by *SLC25A13* gene analysis. The man has been doing well without symptoms of hyperammonemia and prefers protein-rich foods.

Patient 6, a man aged 37 years, developed recurrent attacks of altered consciousness and seizure. His first attack occurred after his divorce at 36 years of age, and the episode recurred five times a year. The episodes were particularly associated with alcohol ingestion. Laboratory findings revealed a high citrulline level (282.8  $\mu\text{mol/l}$ ), normal levels of methionine (24.3  $\mu\text{mol/l}$ ), arginine (106.4  $\mu\text{mol/l}$ ), threonine (99.4  $\mu\text{mol/l}$ ) and a low serine level (59.5  $\mu\text{mol/l}$ ; normal range 60-181  $\mu\text{mol/l}$ ). The citrulline to serine ratio was 4.75 and the threonine to serine ratio was 1.67. A  $\gamma$ -glutamyl transferase level of 213 IU/l and an  $\alpha$ -fetoprotein level of 38.4 ng/ml (normal range <10 ng/ml) were measured. Hepatosplenomegaly was absent and a mild porto-periportal activity with fatty change was observed in a liver biopsy specimen. An electroencephalogram and brain magnetic resonance imaging showed normal findings. Mutation analysis of the *SLC25A13* gene identified a compound

Table I. Summary of citrin deficiency cases in Korea.

Case no.	Clinical phenotype	Age at diagnosis/sex	Presenting symptoms	Biochemical findings	Mutation of <i>SLC25A13</i>	Outcome
1	NICCD	Newborn/M	Picked up by MS/MS	Hyperammonemia, hypercitrullinemia, galactosemia	IVS11+1G>A IVS16ins3kb	Resolved by 5 months
2	NICCD	Newborn/F	Picked up by MS/MS	Hyperammonemia, hypercitrullinemia, galactosemia, cholestasis, hypoproteinemia	IVS13+1G>A IVS16ins3kb	Resolved by 9 months
3	NICCD	Newborn/M	Picked up by MS/MS	Hyperammonemia, hypercitrullinemia, galactosemia, cholestasis, hypoproteinemia	851del4 IVS16ins3kb	Resolved by 6 months
4	NICCD	3 months/F	Cholestatic jaundice, gray-colored stool	Hyperammonemia, hypercitrullinemia	851del4 G393S	Resolved by 5 months
5	Adult CTLN2	28 years/M	Loss of consciousness, vomiting, seizures	Hyperammonemia, hypercitrullinemia	IVS13+1G>A IVS16ins3kb	Recovered without sequelae
6	Adult CTLN2	37 years/M	Altered consciousness, lethargy	Hyperammonemia, hypercitrullinemia	IVS11+1G>A 851del4	Recovered without sequelae

MS/MS, mass spectrometry/mass screening.

heterozygote of c.1177+1G>A (IVS11+1G>A) and c.851\_854del (c.851del4). The man recovered without neurological sequelae and had a special fondness for a protein-rich diet such as eel and meat.

The six cases of citrin deficiency in Korea are summarized in Table I.

## Discussion

CTLN2 is caused by a defect in the *SLC25A13* gene; the wild-type gene encodes citrin, a calcium-binding mitochondrial AGC (1). After the discovery of the *SLC25A13* gene, the disease spectrum has been explored. Mutations in the *SLC25A13* gene have been found in neonates suffering from cholestasis and multiple aminoacidemia, involving elevated levels of citrulline, threonine, methionine and tyrosine (2,10). This disease has been designated as NICCD. Liver function tests in NICCD patients show various degrees of hepatocyte damage. Liver biopsies from NICCD patients show fatty change, round cell infiltration, and mild fibrosis; all of these findings are consistent with the pathology of CTLN2. The clinical phenotypes of NICCD are diverse, ranging from asymptomatic elevation of citrulline levels to severe tyrosinemia-like features. Biochemical findings of NICCD include significantly high levels of galactose, bile acids,  $\gamma$ -glutamyl transferase and alkaline phosphatase. Hypoproteinemia and mildly elevated aminotransferase levels have also been detected (8). The symptoms of NICCD are resolved by 12 months of age without special treatment. The diet should contain lactose-free milk, fat-soluble vitamins and medium-chain triglycerides. One patient with NICCD was

reported, however, to develop progressive liver failure and subsequently underwent liver transplantation (11). After NICCD is resolved, patients seem healthy. Information on the natural history of the disease during this adaptation period is, however, scanty. Much later in life, some of these NICCD patients may develop CTLN2 (12,13). Thus, some type of adaptation or compensation improves the symptoms in childhood and adolescence (14).

A characteristic feature of CTLN2 is the late onset of serious and recurrent neurological symptoms such as sudden disturbances of consciousness and abnormal behaviors, when patients are aged 20-50 years. The hepatic ASS enzyme activity is reduced, although there is no mutations of the ASS gene. Hyperammonemia in CTLN2 patients causes brain edema and may be fatal. Hepatocellular carcinoma without cirrhosis, hyperlipidemia and pancreatitis have also been reported in CTLN2 patients (15-17).

In this study, 3 out of the 4 NICCD patients were detected incidentally by neonatal tandem mass screening. No subjective symptoms were present, and one NICCD patient had cholestatic jaundice. All two CTLN2 patients complained of an abrupt or recurrent onset of neurological symptoms. Most patients with citrin deficiency had peculiar dietary habits, with a preference for protein- and fat-rich food, such as ham, beef and soy, and a dislike for high carbohydrate diets. A similar observation has been made of Japanese patients (14).

Citrin is an AGC, encoded by the *SLC25A13* gene on chromosome 7q21.3, and consists of 18 exons and 675 amino acid residues. The gene spans 200 kb. The enzyme is relatively liver-specific. AGC plays a role in urea metabolism, protein and nucleotide synthesis, and gluconeogenesis from lactate

(16). Since the identification of the *SLC25A13* gene in CTLN2 patients, 13 common mutations have been described in Japanese patients: [I]:851del4, [II]:IVS11+1G>A, [III]:1638ins23, [IV]:S225X, [V]:IVS13+1G>A, [VI]:1800ins1, [VII]:R605X, [VIII]:E601X, [IX]:E601K, [X]:IVS6+5G>A, [XI]:R184X, [XIV]:IVS6+1G>C, and [XX]:Ex16+74\_IVS17-32del516 (1,3,18-20). Other rare mutations, [XV]:Ex15dup (IVS14\_15) and [XXI]:L598R, have been detected in various ethnic groups (4,21). Four mutations, [I], [II], [V] and [XIX]:IVS16ins3kb, found in this study have been already found in Japanese patients (1,8) and a novel mutation (p.G393S) was identified in patient 4.

Frequency analysis of 12 mutant *SLC25A13* alleles of overseas residents in Japan, using DNA samples from healthy controls and neonatal mass screening filter papers, showed mutant allele frequencies of 1/112 in Korean, 1/69 in Japanese, 1/940 in northern Chinese, 1/48 in southern Chinese of the Yangtze River, and 1/57 in Taiwanese subjects (5,20). These results suggest that the incidence of citrin deficiency is 1/50,000 in Korea and 1/19,000 in Japan. However, only eight subjects have been diagnosed by molecular genetic analysis in Korea to date. These data indicate that the disease is being under-diagnosed in Korea, in part because physicians are not familiar with the condition. Three of our four NICCD patients were initially suspected to have citrullinemia from data provided by a newborn screening test using tandem mass spectrometry. Only a few patients can be diagnosed by a newborn screening test because not all NICCD patients show highly elevated plasma citrulline levels.

Several studies have sought to develop methods for early diagnosis and neonatal screening of NICCD. Because most mutations in the *SLC25A13* gene are nonsense mutations, frameshift mutations or cause aberrant splicing, large *SLC25A13* proteins are not produced and most gene products are cross-reacting immunological material (CRIM)-negative (18). Therefore, screening could be performed by Western blotting using an anti-citrin antibody (22). Another method is tandem mass screening, where high citrulline levels ( $>30 \mu\text{mol/l}$ ), and a high ratio of citrulline to serine ( $>0.34$ ) and threonine to serine ( $>1.19$ ) are identified (23). Additional findings such as high methionine, arginine, phenylalanine and galactose levels support the diagnosis and enhance the positive predicted value of the test (24). To accurately measure the incidence of citrin deficiency in the Korean population, large-scale population-based studies and additional tests for searching for rare or novel mutations are needed.

Many issues in NICCD patient management require attention. Dietary protein restriction is not usually recommended. Rather, adequate protein intake and arginine supplementation should be encouraged, except in the case of symptomatic hyperammonemia. Carbohydrate intake must be restricted, because NICCD is considered a form of carbohydrate poisoning (25). A diet high in carbohydrates and low in protein raises NADH levels in the cytoplasm through glycolysis and consumption of lactic acid. The process of NADH reoxidation is impaired in citrin-deficient patients. It is well-known that these patients detest sweet foods such as rice or fruit, and prefer meat, egg or soy. An encouraging report, describing the therapeutic benefit of sodium pyruvate

in citrin-deficient mice, has been published (26). Various clinical treatment modalities remain valid, because the natural course of this disease is not fully understood.

All NICCD and CTLN2 patients discussed in this report were on low protein diets with arginine supplementation. In addition, hemodialysis was performed in two adult CTLN2 patients because of hyperammonemic encephalopathy.

In conclusion, the NICCD/CTLN2 disease incidence, the associated genotypes, the natural history, and the response to treatment, all remain poorly understood in Korean patients with citrin deficiency.

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