INTRODUCTION

The SLC25A13 gene on chromosome 7q21.3 is known to be responsible for adult-onset type II citrullinemia (CTLN2). The SLC25A13 gene consists of 18 exons and encodes a liver-type mitochondrial aspartate-glutamate carrier named citrin (1, 2), which plays an important role in malate-aspartate NADH shuttling, urea synthesis, and gluconeogenesis. CTLN2 is characterized by late-onset hyperammonemia and neuropsychiatric symptoms, such as disorientation, delirium, delusion, and disturbed consciousness (3). Ohura et al. (4) and Tazawa et al. (5) demonstrated that mutations in the SLC25A13 gene cause neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). Two of these three showed failure to thrive. The laboratory findings showed hypoproteinemia and hyperammonemia, and liver biopsies revealed micro-macronodular fatty liver and cholestasis. The three patients each harbored compound heterozygous 1,638-1,660 dup/ S225X mutation, compound heterozygous 851del4/S225X mutation, and heterozygous 1,638-1,660 dup mutation, respectively. With nutritional manipulation, liver functions were normalized and catch-up growth was achieved. NICCD should be considered in the differential diagnosis of cholestatic jaundice in Korean infants.

Key Words : Cholestasis; Citrin; Citrullinemia; SLC25A13; Mutation

MATERIALS AND METHODS

Patients

Between 2005 and 2006, 47 patients were admitted to the Department of Pediatrics in Seoul National University Children's Hospital for neonatal cholestasis. Examinations included abdominal ultrasonography, duodenal intubation, a hepatobiliary scan, and liver biopsy. Total parenteral nutrition, drug-related cholestasis, and cholestasis secondary to sepsis were excluded.

NICCD was diagnosed based on the presence of hyperaminoacidemia, galactosemia, fatty liver, and on the results of the genetic study detailed below. Patients with NICCD were followed until at least 18 months of age.

**Neonatal Intrahepatic Cholestasis Caused by Citrin Deficiency in Korean Infants**

Citrin is a liver-type mitochondrial aspartate-glutamate carrier encoded by the SLC25A13 gene, and its deficiency causes adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). Here, the authors investigated clinical findings in Korean infants with NICCD and performed mutation analysis on the SLC25A13 gene. Of 47 patients with neonatal cholestasis, three infants had multiple aminoacidemia (involving citrulline, methionine, and arginine) and galactosemia, and thus were diagnosed as having NICCD. Two of these three showed failure to thrive. The laboratory findings showed hypoproteinemia and hyperammonemia, and liver biopsies revealed micro-macronodular fatty liver and cholestasis. The three patients each harbored compound heterozygous 1,638-1,660 dup/ S225X mutation, compound heterozygous 851del4/S225X mutation, and heterozygous 1,638-1,660 dup mutation, respectively. With nutritional manipulation, liver functions were normalized and catch-up growth was achieved. NICCD should be considered in the differential diagnosis of cholestatic jaundice in Korean infants.

Key Words : Cholestasis; Citrin; Citrullinemia; SLC25A13; Mutation

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# Genetic study

Genomic DNA was extracted from peripheral blood using Wizard genomic DNA purification kits, according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Informed consent was obtained from all parents. Mutations of the SLC25A13 gene were detected by using previously described primers (11, 12). Seven different primer sets were used to detect the 12 known mutations of SLC25A13 by polymerase chain reaction (PCR), namely, IVS8F2 and Ex9B, mutation [I] (851del4); Ex11F2 and IVS11Bm2, mutation [II] (IVS11+1G>A); IVS15F2 and Ex16B, mutation [III] (1,638-1,660 dup); IVS6F and IVS7B, mutation [IV] (S225X); Ex13F and IVS13Bm, mutation [V] (IVS13+1G>A); IVS16F and IVS17B, mutation [VI] (1,800ins1), mutation [VII] (R605X), mutation [VIII] (E601X), and mutation [IX] (E601K); IV55NF and IV56NB, mutation [X] (IV56+5G>A), mutation [XI] (R184X), and mutation [XIV] (IV56+1G>C). PCR was performed using 1 cycle at 94 °C for 30 sec, followed by 72 °C for 5 min. Forward and reverse strands of PCR products were direct-sequenced using the above-mentioned PCR primers on the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Sequences obtained were compared with the reference sequence NM-014251 registered in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

## Table 1. Differential diagnosis of neonatal cholestasis

| Diagnosis                                | No (%) |
|------------------------------------------|--------|
| Extrahepatic biliary atresia             | 11 (23) |
| TORCH infection                          | 4 (9)  |
| NICCD                                    | 3 (6)  |
| Progressive familial intrahepatic cholestasis | 2 (4)  |
| ARC syndrome                             | 2 (4)  |
| Dubin-Johnson syndrome                   | 2 (4)  |
| Alagille syndrome                        | 1 (2)  |
| Non-syndromic bile duct paucity          | 1 (2)  |
| Idiopathic                               | 21 (45) |

NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; ARC syndrome, arthrogryposis, renal dysfunction, cholestasis syndrome.

## RESULTS

Among the 47 patients with neonatal cholestasis presented during the two-year period, extrahepatic biliary atresia was diagnosed in 11, congenital infection with TORCH in 4, progressive familial intrahepatic cholestasis (PFIC) in 2, ARC (arthrogryposis, renal dysfunction, cholestasis) syndrome in 2, neonatal Dubin-Johnson syndrome in 2, Alagille syndrome in 1, and non-syndromic bile duct paucity in 1. No etiology was identified in 21 patients (Table 1). NICCD was diagnosed in three patients. Table 2 summarizes the genotype and clinical and laboratory findings of these three patients. Birth weights ranged from 2.7 to 3.1 kg. The chief complaint was cholestatic jaundice in all patients, and all were referred for suspected neonatal hepatitis or biliary atresia. Two infants were referred at 3 months of age and one infant at 7 months. Two infants showed failure to thrive at initial presentation. Newborn tandem mass screening performed on case 2 at 1 week of age was normal. Total serum protein levels were reduced in two patients. Blood ammonia levels were slightly increased in all three, and gamma glutamyltranspeptidase and alkaline phosphatase were markedly elevated in all three, whereas alanine aminotransferase levels were slightly increased in two. Hypoglycemia was present in case 1. Alpha-fetoprotein levels were high and galactosemia was detected in all patients. Plasma amino acid analyses showed that citrulline, methionine, and arginine were significantly elevated in all three. Plasma tyrosine, threonine, and lysine were elevated in two (Table 3). No patient had a bleeding tendency or a cataract.

## Table 2. Clinical and laboratory findings, and the genotypes of the 3 NICCD patients

| Case | Age (mo) | Weight (kg) | TB/DB (mg/dL) | AST/ALT (IU/L) | γ-GT (IU/L) | Protein (g/dL) | NH₃ (µg/dL) | α-fetoprotein (ng/mL) | Genotype |
|------|----------|-------------|---------------|----------------|-------------|----------------|-------------|-----------------------|----------|
| 1    | 3        | 5.7         | 5.4/3.2       | 145/48         | 135         | 5.2            | 109         | 84,000                | III(-)   |
| 2    | 3        | 4.6         | 5.2/3.2       | 238/158        | 263         | 5.1            | 89          | 29,500                | IV       |
| 3    | 7        | 6.4         | 4.5/2.7       | 115/70         | 148         | 6.1            | 76          | 44,700                | II/IV    |
| Normal value | <1.2/0.5 | <40/40       | <63            | >6.0          | >75         | <55           |            |                       |          |

NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; TB, total bilirubin; DB, direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

SLC25A13 mutations I, III, and IV are 851del4, 1,638-1,660 dup, and S225X, respectively.

# Table 3. Plasma amino acid and galactose levels in the 3 NICCD patients

| Case | Citrulline | Methionine | Threonine | Arginine | Tyrosine | Lysine | Galactose |
|------|------------|------------|-----------|----------|----------|--------|-----------|
| 1    | 301        | 73         | 553       | 397      | 215      | 332    | 162       |
| 2    | 186        | 106        | 216       | 215      | 248      | 246    | 14        |
| 3    | 93         | 64         | 168       | 120      | 100      | 166    | 26        |

NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; amino acid, t/mL; galactose, mg/dL.
Liver biopsy was performed in two patients. Cholestasis and micro-macrowesicular fatty change were observed in these two, and moderate periportal fibrosis was present in case 3. Five of the six alleles (83%) examined showed mutations. In detail, compound heterozygous 1,638-1,660 dup/S255X mutation (mutation [III]/[IV]), compound heterozygous 851del4/ S225X mutation (mutation [I]/[IV]), and heterozygous 1,638-1,660 dup mutation (mutation [III]) were found in each patient. The allele frequencies of mutations [I], [III], and [IV] were 17%, 33%, and 33%, respectively.

Lactose-free soy formula and a medium chain triglyceride (MCT) formula were fed to all patients. Fat-soluble vitamins were added. Within 2 months of diagnosis, plasma amino acid profiles were normalized, whereas liver functions were nearly normalized at the age of 9 to 13 months. At the age of 18 months, catch-up growth had been achieved by all three patients.

**DISCUSSION**

Citrullinemia is categorized as CTLN1 (classical neonatal onset) or as CTLN2 (adult onset). CTLN1 is an autosomal recessive disease that is caused by a deficiency of arginosuccinate synthetase, and is characterized by a neonatal-onset and severe hyperammonemia, irritability, lethargy, poor feeding, and tachypnea (13). On the other hand, CTLN2 is an autosomal recessive disease caused by citrin deficiency, and is characterized by late onset (11 to 79 yr), frequent attacks of hyperammonemia, and mental derangement, and leads to death within a few years from the onset (3). The SLC25A13 gene, which encodes citrin, has been found to be defective in CTLN2. In the liver, citrin participates in the urea cycle by supplying aspartate from mitochondria to the cytosol for the incorporation into argininosuccinate. Lack of aspartate for argininosuccinate synthesis under citrin deficiency probably causes citrulline accumulation (14). Previous DNA analyses have shown that some infants with idiopathic neonatal hepatitis harbor the same mutations in SLC25A13 as CTLN2 patients (4, 5), and these infants had cholestasis, hyperammonemia, galactosemia, hypoproteinemia, and hepatic steatosis. On newborn mass screening, about a half of NICCD patients did not show an abnormal amino acid profile on newborn mass screening at 7 days of age. Tandem mass screening data in Japan suggest that the incidence of NICCD is approximately 1 in 34,000 (15), but the frequency of homozygotes with mutated SLC25A13 has been estimated to be 1 in 19,000 (17). Tamamori et al. (16) demonstrated that NICCD patients with negative newborn mass screening findings have lower total amino acid levels, and that citrulline/total amino acids ratios can offset the effect of low total amino acids.

This is the first report of genetically confirmed NICCD in a Korean cohort. The mutation rate observed in three NICCD patients was 83%, which is similar to that reported in Taiwanese patients (18). In the present study, the allele frequencies of mutation [I], [III], and [IV] were 17%, 33%, and 33%, respectively. Screening of 12 mutations in Korean control individuals showed that mutation [I] and [II] were found to be most common, and mutation [III] accounted for 5% of total mutations, but mutation [IV] was absent (11). A Japanese study found that the frequency of mutation [IV] differs in NICCD patients and controls (6). However, since the number of patients with NICCD was comparatively small in the present study, further mutation studies are needed. To date only one adult case of CTLN2 has been reported in Korea (19); the patient presented with insomnia and lethargy at 55 yr of age. However, the theoretical frequency of homozygotes calculated from carrier rates in Koreans is 1 in 50,000 (11), which suggests that NICCD and CTLN2 are substantially under diagnosed in Korea.

The management of NICCD is directed toward treating the consequences of cholestasis and galactosemia. MCT formula and supplementation with fat-soluble vitamins have been used to prevent complications of prolonged cholestasis. It should be noted that lactose may be toxic to NICCD patients and should be avoided while cholestasis persists (20).
The prognosis of our patients was good because liver functions were normalized before 13 months of age and catch-up growth was achieved at 18 months. The amelioration of NICCD symptoms over a year suggests hepatocyte maturation and some adaptation or compensation by other mitochondrial carriers (14). One of our three patients showed moderate portal fibrosis on liver histology. Mild to moderate portal fibrosis has been previously observed in NICCD (5, 7). Liver function was normalized in this patient, but his clinical course needs to be carefully monitored. One NICCD patient developed liver failure and underwent liver transplantation at 11 months of age (21). It should be noted that some NICCD patients develop CTLN2 at their twenties or even several decades later (7, 8). Although the prognosis of CTLN2 is poor, liver transplantation is remarkably effective (5). As yet, it cannot be determined which NICCD patients will later develop CTLN2, and thus, all NICCD patients should be followed for signs of development of CTLN2.

The present study indicates that NICCD should be considered in the differential diagnosis of cholestatic jaundice in Korean infants, and that plasma amino acid analysis should be included in the evaluation of infantile intrahepatic cholestasis. Moreover, the findings of SLC25A13 genetic studies were found to be useful for confirming the diagnosis of NICCD.

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