11. Tazawa Y et al. Neonatal intrahepatic cholestasis with hepatic siderosis and steatosis. Acta Paediatrica japonica 1998;40:150-4.

12. Tazawa Y et al. Idiopathic neonatal hepatitis presenting as neonatal hepatic siderosis and steatosis. Dig Dis Sci 1998;43:392-6.

13. 田澤雄作. NICCD(neonatal intrahepatic cholestasis caused by citrin deficiency)の病態と診断. 小児科診療 2007;70:913-922.

14. 田澤雄作. 新生児胆汁うっ滞—新生児肝炎及びシトリン欠損による新生児肝内胆汁うっ滞の臨床を中心として.日本小児科学会雑誌 2007;111:1493-1514.

15. 田澤雄作. 特発性新生児肝炎およびシトリン欠損による新生児肝内胆汁うっ滞. 小児内科 2008;40（増刊号）:558-64.

16. 田澤雄作. 肝内胆管減少症の概念、分類および病因. 小児内科 1987;19:73-8.

17. 田澤雄作ほか. 胆道閉鎖症の鑑別診断－特に Alagille 症候群の除外について. 小児外科 1999;31:237-41.

18. 虻川大樹ほか. Alagille 症候群の予後と肝移植の適応に関する検討. 日本小児科学会雑誌 1999;103:554-8.

19. 虻川大樹ほか. 家族性胆汁うっ滞における遺伝子異常. 小児内科 1999;31:1714-20.

CLINICAL AND LABORATORY OBSERVATIONS

# Infantile cholestatic jaundice associated with adult-onset type II citrullinemia

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Adult-onset type II citrullinemia, characterized by a liver-specific argininosuccinate synthetase deficiency, is caused by a deficiency of citrin that is encoded by the *SLC25A13* gene. Three patients with infantile cholestatic jaundice were found to have mutations of the *SLC25A13* gene. Adult-onset type II citrullinemia may be associated with infantile cholestatic disease. (J Pediatr 2001;138:735-40)

Citrullinemia is an autosomal recessive disease caused by deficiency of argininosuccinate synthetase. Classical citrullinemia has either neonatal or infantile onset, with an ASS enzyme defect in all tissues arising from mutations in the ASS gene on chromosome 9q34.[1] Adult-onset type II citrullinemia is characterized by a liver-specific ASS deficiency with no abnormalities in hepatic ASS messenger RNA or the ASS gene.[2] The biochemical abnormalities in CTLN2 include elevations of plasma ammonia and citrulline, abnormal distribution of ASS protein in the liver,[3] and elevated expression of the hepatic pancreatic secretory trypsin inhibitor gene.[4] Recently, the CTLN2 locus was identified to chromosome 7q21.3, and a novel gene, *SLC25A13*, was determined as the disease-causing gene of CTLN2.[5] Five different DNA sequence alterations in the gene have been reported.[5] Late-onset symptoms are hallmarks of CTLN2.[5,6] Significant clinical abnormalities, however, were unknown in infancy of most patients with CTLN2.

In CTLN1, focal areas of hepatocellular necrosis have been described in several cases but have been absent in others. Fatty droplets in the cytoplasm of hepatocytes were noted in several cases.[7] On the other hand, in CTLN2, fatty liver was seen in all cases. Round cell infiltration, focal necrosis, fibrosis, cirrhosis, and abnormal hepatocyte morphology have been described.[3,7] Hepatic steatosis and siderosis associated with cholestasis have been reported in an adult patient with CTLN2.[8] However, there have been no descriptions referring to liver disease in childhood CTLN2.

**See related article, p. 741.**

In Japan, neonatal cholestatic jaundice of unknown origin associated with hepatic steatosis and siderosis has been reported.[9-13] This suggests that there is an undefined metabolic disease. Here, we describe 3 cases of infantile cholestatic jaundice associated with hepatic steatosis and siderosis in which mutations of the *SLC25A13* gene were uncovered by DNA diagnosis for CTLN2.

| | |
|---|---|
| ASS | Argininosuccinate synthetase |
| CTLN2 | Adult-onset type II citrullinemia |

## CASE REPORTS

### *Patient 1*

A girl, weighing 2360 g at birth, was born to a 23-year-old primigravida at 38 weeks of gestation. Family history showed no record of hepatic or neurologic diseases, and her parents were not related. Results of neonatal screening for metabolic diseases (galactosemia,

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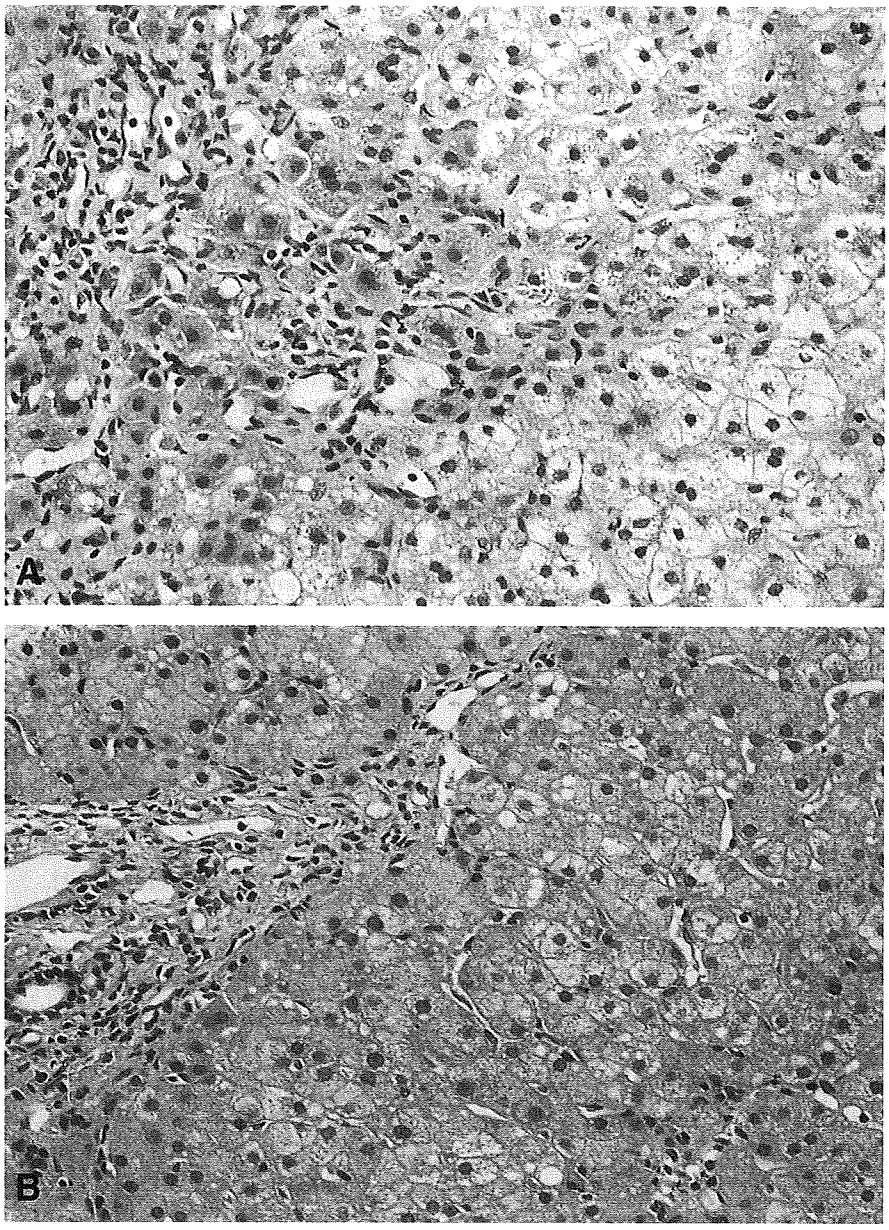


*Fig 1.* Histologic findings of the liver in patient 1. **A,** Five months of life (hematoxylin-eosin stain, original magnification ×200). **B,** Twelve months of life (hematoxylin-eosin stain, original magnification ×200).

homocystinuria, phenylketonuria, and maple syrup urine disease) and endocrine diseases (hypothyroidism and adrenal hyperplasia) were negative. She had been breast fed for only the first 3 months of life. Then, jaundice was noticed by her parents at 147 days of life, and she was referred to Tottori University Medical Center for examinations. Her weight was 4.68 kg. She had mild hepatomegaly, and the liver was palpable 3 cm below the costal margin. Findings on neurologic examinations were negative. Biochemical data on admission are shown in the Table. The serum direct bilirubin level was 3.7 mg/100 mL, and the serum total bile acid level was 213 μmol/L. The blood ammonia level was within normal range. The serum protein level was 6.1 g/100 mL. Plasma amino acid analysis on admission showed hyperaminoacidemia including a citrulline level of 496 μmol/L (×12 upper limit of normal), a methionine level of 426 μmol/L (×11), a tyrosine level of 287 μmol/L (×3), and a threonine level of 479 μmol/L (×2). The serum arginine level was normal, 140 μmol/mL. A liver biopsy specimen obtained at the time of admission showed a diffuse microvesicular and macrovesicular fatty liver (Fig 1, *A*). Cholestasis was identified in liver cell cytoplasm, and dilated canaliculi containing bile were present. Moderate portal expansion with minimal cellular infiltration and portal-to-portal bridging fibrosis were seen. Cellular infiltration in the parenchyma was also minimal. Iron depositions seen in the portal and periportal areas were present in both hepatocytes and Kupffer's cells. Electron microscopy showed normal mitochondrial and canalicular appearance but increased density of mitochondrial matrix and hypertrophy of smooth endoplasmic reticulum. Restriction of protein (1.2 g/kg/d) and administration of fat-soluble vitamins, started 4 weeks after admission, resulted in normalization of plasma amino acid patterns 3 weeks later. Gradual increases of protein intake to 2.4 g/kg/d were tolerated. During protein restriction, levels of serum direct bilirubin decreased from 2.5 mg/100 mL to 1.3 mg/100 mL, but levels of serum total bile acids remained unchanged (283-326 μmol/mL). Thereafter, all abnormal biochemical data improved and normalized by 12 months of life. When she was 12 months old, 3 cytosolic enzymes of the urea cycle, measured in a liver biopsy specimen, were almost within the normal range: ASS, 0.025 U/mg protein (control, 0.025 ± 0.019; n = 7); argininosuccinate lyase, 0.034 U/mg protein (0.037 ± 0.018, n = 9); arginase, 6.2 U/mg protein (13.7 ± 3.7, n = 8). The biopsy specimen showed diffuse, mild, microvesicular fatty liver tissue with a few macrovesicular fat droplets (Fig 1, *B*). Iron deposits and mild to moderate fibrosis with minimal lymphocyte infiltrate were observed in the portal area.

At age 3 years, serum amino acids were normal. However, because it had been reported that pathologic findings in the liver of most patients with CTLN2 include fatty infiltration and mild fibrosis,[3,6-8] 5 mutations of the *SLC25A13* gene were analyzed, after informed consent had been obtained. Mutation analysis showed that the patient was a compound heterozygote for 2 mutations and that the parents were heterozygotes for mutations I and II, respectively (Fig 2, *A* and *B*).

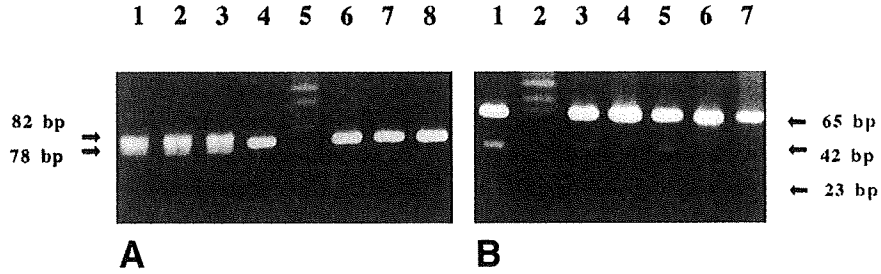


*Fig 2.* DNA diagnosis of mutated *SLC25A13* gene in patient 1 and her parents. **A,** Mutation I (851del4) analysis. *Lane 1* represents DNA of heterozygote control subject; *lane 2* shows DNA of the patient; and *lanes 3 and 4* show DNA of the patient's father and mother, respectively. *Lane 5* and *lanes 6 to 8* represent molecular markers and DNA of control subjects, respectively. The patient and the father were identified as heterozygotes for mutation I. **B,** Mutation II (IVS11+1G→A) analysis. *Lane 1* represents DNA of heterozygote control subject; *lane 2* shows molecular markers. *Lanes 3 to 5* show DNA of the patient and the patient's father and mother, respectively. *Lanes 6 to 7* represent DNA of control subjects. The patient and the mother were identified as heterozygotes for mutation II.

## *Patient 2*

A girl, weighing 2050 g at birth, was born to a 28-year-old primigravida at 37 weeks of gestation. Family history showed no record of hepatic or neurologic diseases, and her parents were not related. Results of neonatal screening were negative. During the immediate postnatal period, she had meconium aspiration syndrome but fully recovered. She had been solely breast fed. She was referred to Tottori University Medical Center because of cholestatic jaundice associated with subcutaneous hemorrhage at 132 days of life. Her weight was 5.1 kg, and she had mild hepatomegaly. Prothrombin activity was <15% (normal, ≥60%) but responded to parenteral administration of vitamin K. Findings on neurologic examination were normal. Biochemical data examined on admission are shown in the Table. The serum direct bilirubin level was 3.2 mg/100 mL, and the serum total bile acid level was 126 μmol/L. The blood ammonia level was within normal range. The serum protein level was 4.6 g/100 mL. Results of plasma amino acid analysis on admission were normal. A liver biopsy specimen obtained at the time of admission showed histologic features similar to those of patient 1. Fat-soluble vitamins were given, and abnormal biochemical values were normal at 8 months of life. At age 15 months, gene analysis with genomic DNA was performed, because her clinical presentation and liver histologic findings resembled those of patient 1, after informed consent had been obtained. DNA analysis showed that the patient was a compound heterozygote for the 2 *SLC25A13* mutations, IV and VI (a novel sixth mutation of the *SLC25A13* gene in CTLN2[13a]).

***Table.*** Biochemical data on admission in the 3 patients

| | Normal values | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|---|
| *SLC25A13* mutation | | I/II | IV/VI[a] | I/II |
| Total bilirubin level (mg/100 mL) | <1.0 | 6.2 | 7.4 | 12.6 |
| Direct bilirubin level (mg/100 mL) | <1.0 | 3.7 | 3.2 | 2.6 |
| Total bile acid level (μmol/L) | <36 | 213 | 126 | 120 |
| AST level (IU/L) | <63 | 302 | 83 | 31 |
| ALT level (IU/L) | <35 | 167 | 24 | 20 |
| ALP level (IU/L) | <450 | 1582 | 1162 | 2230 |
| γ-GTP level (IU/L) | <74 | 65 | 137 | 142 |
| Total cholesterol level (mg/100 mL) | 90-220 | 145 | 138 | 195 |
| Ammonia level (μg/100 mL) | <75 | 64 | 59 | 75 |
| Total protein level (g/100 mL) | >5.0 | 6.1 | 4.6 | 3.9 |
| Albumin level (g/100 mL) | >3.3 | 4.3 | 2.9 | 2.6 |
| Blood glucose level (mg/100 mL) | >50 | 60 | 65 | 75 |
| Prothrombin time (%) | >60 | 50 | <15 | 26 |
| BUN level (mg/100 mL) | 3-16 | 10 | 7 | 13 |
| CRP level (mg/100 mL) | <0.25 | <0.25 | <0.25 | <0.25 |
| LDH level (IU/L) | <300 | 213 | 289 | 298 |

*SLC25A13* mutations I, II, and IV were 851del4, IVS11+1G→A, and S225X, respectively.[5]
*AST,* Aspartate aminotransferase; *ALT,* alanine aminotransferase; *ALP,* alkaline phosphatase; *γ-GTP,* γ-glutamyl transpeptidase; *BUN,* blood urea nitrogen; *CRP,* C-reactive protein; *LDH,* lactate dehydrogenase.
[a]A novel sixth mutation of the *SLC25A13* gene found in CTLN2.[13a]

## *Patient 3*

This patient was previously reported[10,11]; however, at that time the diagnosis was not known. A boy, weighing 2700 g at birth, was born to a 23-year-old primigravida at term. Family history showed no record of hepatic or neurologic diseases, and his

parents were not related. Results of neonatal screening were positive for galactosemia (Paigen method, ≥8 mg/100 mL; Beulter method, normal); however, further studies could not confirm the presence of galactosemia. He was solely breast fed for the first 25 days of life and then received formula, approximately 30 mL/kg/d, together with breast milk. Jaundice was noticed by the parents, and at 39 days of life, he was referred to Akita University Medical Center. Weight was 4.04 kg. He had no hepatosplenomegaly. Edema or ascites was not observed. Findings on neurologic examination were normal. Biochemical data obtained on admission are shown in the Table. The serum direct bilirubin level was 2.6 mg/100 mL, and the serum total bile acid level was 120 μmol/L. The blood ammonia level was normal. The serum protein level was 3.9 g/100 mL. The blood galactose level was increased, 25 mg/100 mL. Results of plasma amino acid analysis on admission were normal except for a mildly increased level of tyrosine (×2). A liver biopsy specimen obtained at the time of admission showed microscopic and ultrastructural features similar to those of patient 1. Fat-soluble vitamins and galactose-free formula were administered together with breast milk; biochemical data improved and were normal by 5 months of life. A liver biopsy specimen obtained at 12 months of life showed mild residual portal fibrosis without fat or iron deposition. At age 5 years, informed consent was obtained from the parents of the patient for CTLN2 gene analysis. DNA analysis revealed that the patient was a compound heterozygote for the 2 mutations I and II.

## METHODS

The procedure for DNA diagnosis of *SLC25A13* mutations was previously reported in detail.[5] We used the following primers and conditions: mutation I (851del4), the primer set IVS-8F2 (5′-GGTATATTTGTTGCTTGTGTTTG-3′) and Ex-9B (5′-TCTTCCAGAGGAGCAATCCG-3′), direct electrophoresis on 4% Amplisize (Bio-Rad) agarose gel; mutation II (IVS11+1G→A), Ex-11F2 (5′-GAAAGTGCTACGCTATGAAGG-3′) and IVS-11Bm2 (5′-AGGTATTGAGCATGTGGCACTG-3′), *Sau*3AI digestion, electrophoresis on 3% Amplisize gel; mutation IV (S225X), IVS-6F (5′-GGAGAGTACAAGTTCTGGTC-3′) and IVS-7B (5′-ACTAGTTGCCTTCTTCACCC-3′), *Alu*I digestion, electrophoresis on 2.5% Amplisize gel.

In all 3 patients, known causative diseases of neonatal hepatitis were eliminated: infectious hepatitis (hepatitis A, B, and C viruses, cytomegalovirus, and Epstein-Barr virus), metabolic diseases ($\alpha_1$-antitrypsin deficiency, cystic fibrosis, Niemann-Pick disease), chemical hepatic injury, and biliary tract diseases. Results of urinary organic acid analysis were negative, and urinary bile acid analysis did not indicate the presence of abnormal bile acids. Conventional ultrasound examination showed no gallstones, sludge, or dilated bile ducts. Doppler ultrasound studies, performed for patients 1 and 3, did not show abnormal runs of portal vein and portosystemic shunts.

So far, the 3 patients have not had clinical episodes indicating recurrent cholestasis or the presence of hyperammonemia.

## DISCUSSION

*SLC25A13*, which is responsible for CTLN2, is a novel gene expressed abundantly in the liver.[5] A protein encoded by *SLC25A13*, named *citrin*, is a new member of the mitochondrial carrier protein with a possible role in urea cycle function,[5,6,14] but the function is unknown. Citrin may be associated with a member of the urea cycle, particularly with ASS itself. The absence of functional citrin might lead to a reduction of ASS protein, possibly through its destabilization or degradation, and induce hyperammonemia. A significant reduction of ASS protein in CTLN2, however, may not develop in early childhood, as shown in patient 1.

Five distinct mutations in *SLC25A13* have been reported,[5] and a novel sixth mutation has been identified.[13a] Of the 22 patients from consanguineous parents, 17 were homozygous for their respective mutation, and 5 patients were compound heterozygotes,[5,6] suggesting a high frequency of the mutated *SLC25A13* gene. Actually, the frequency of carrier status with an *SLC25A13* mutation in the Japanese population was predicted to be 1 to 2 in 100.[5,6] Kobayashi et al[2] found a discrepancy between the frequency of homozygotes with an *SLC25A13* mutation (about 1 in 20,000) and the incidence of CTLN2 calculated from proportion of consanguinity (1 in 100,000). This suggests that some homozygotes with an *SLC25A13* mutation are still healthy, have another disease, or have CTLN2. Several individuals without citrullinemia who have mutations in both alleles of the *SLC25A13* gene have been described, including adults who are siblings of patients with CTLN2[15] and infants with a neonatal hepatitis syndrome, as shown in the present study.

The most characteristic feature of CTLN2 is the late onset of serious and recurring symptoms, varying from age 11 to 72 years, although several patients with CTLN2 have difficulty during infancy or early childhood.[6] However, a pediatric liver disease presentation in patients with CTLN2 has not been described previously. The presence of neonatal liver disease in 3 patients suggests that hepatic pathology may be related to CTLN2 in the pediatric population.

In Japan, neonatal fatty liver of unknown cause has been reported.[9-13] Clinical features of the disease include cholestatic jaundice, hepatic steatosis and siderosis, hypoproteinemia, and hyperaminoacidemia. We suspected

that a metabolic error was present in the 3 patients, because their liver histologic findings, which included a fatty liver associated with cholestasis, did not show evidence of a giant-cell hepatitis, which is the usual histologic pattern in most forms of transient neonatal cholestasis.[16]

The pathogenesis of neonatal cholestasis in CTLN2 remains unsolved. Other metabolic liver diseases produce transient neonatal cholestasis, such as $\alpha_1$-antitrypsin or Niemann-Pick disease type C.[17,18] It is possible that defects in normal hepatic metabolism and energy production in combination with immaturity of bile secretion in early infancy result in cholestasis. Mitochondrial diseases are often associated with a variety of liver diseases, such as cholestasis, hepatic steatosis, and siderosis, fibrosis, and cirrhosis.[19-22] Citrin alterations might affect bile secretion via mitochondrial dysfunction in infants with CTLN2.

Results of plasma amino acid analyses at presentation of patients 2 and 3 were normal. These data are in contrast with a markedly elevated level of plasma citrulline in patient 1. The patient had exclusively received human milk for 1 month before admission. After that, she started to receive infant formula only. Patients 2 and 3, on the other hand, had received human milk exclusively or in part. Infant formula has greater protein content, approximately over twice that found in human milk.[23] The higher plasma concentrations of amino acids, methionine, threonine, and tyrosine, which were seen in patient 1, have been reported to correlate with higher total protein intake.[23,24] Restriction of protein intake in the patient resulted in normalization of plasma amino acid patterns. Thus a relatively high protein load may have caused elevated levels of plasma citrulline in these patients with CTLN2. After abnormal amino acid patterns disappeared, cholestasis improved and normalized. These data indicate that patients with CTLN2 may present with infantile cholestatic liver disease without abnormal amino acid profiles, depending on their protein intake.

The full spectrum of CTLN2 in the pediatric patient remains to be elucidated. However, this report suggests a relationship of CTLN2 with neonatal cholestasis with fatty liver. Current therapy for adults with CTLN2 has included liver transplantation in 17 patients.[2,4,8,15,25-28] All metabolic abnormalities and neurologic symptoms disappeared. We continue to search for non-transplant options for the treatment of this metabolic disease.[29]

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## REFERENCES

1. Kakinoki H, Kobayashi K, Terazono H, Nagata Y, Saheki T. Mutations and DNA diagnoses of classical citrullinemia. Hum Mutat 1997;9:250-9.
2. Kobayashi K, Shaheen N, Kumashiro R, Tanikawa K, O'Brien WE, Beaudet AL, et al. A search for the primary abnormality in adult-onset type II citrullinemia. Am J Hum Genet 1993;53: 1024-30.
3. Yagi Y, Saheki T, Imamura Y, Kobayashi K, Sase M, Nakano K, et al. The heterogenous distribution of argininosuccinate synthetase in the liver of type II citrullinemic patients. Its specificity and possible clinical implications. Am J Clin Pathol 1988;89: 735-41.
4. Kobayashi K, Horiuchi M, Saheki T. Pancreatic secretory trypsin inhibitor as a diagnostic marker for adult-onset type II citrullinemia. Hepatology 1997; 25:1160-5.
5. Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, et al. The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. Nat Genet 1999;22:159-63.
6. Kobayashi K, Iijima M, Yasuda T, Sinasac DS, Yamaguchi N, Tsui L-C, et al. Type II citrullinemia (citrin deficiency): a mysterious disease caused by a defect of calcium-binding mitochondrial carrier protein. In: Pochet R, Donato R, Haiech J, Heizmann C, Gerke V, editors. Calcium. The molecular basis of calcium action in biology and medicine. Dordrecht: Kluwer Academic Publishers; 2000. p. 557-79.
7. Walser M. Urea cycle disorders and other hereditary hyperammonemic syndromes. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS, editors. The metabolic basis of inherited disease. 5th ed. New York: McGraw-Hill Book Company; 1983. p. 402-38.
8. Todo S, Starzl TE, Tzakis A, Benkov KJ, Kalousek F, Saheki T, et al. Orthotopic liver transplantation for urea cycle enzyme deficiency. Hepatology 1992;15:419-22.
9. Nishinomiya F, Abukawa D, Takada G, Tazawa Y. Relationships between clinical and histological profiles of nonfamilial idiopathic neonatal hepatitis. Acta Paediatr Jpn 1996;38:242-7.
10. Tazawa Y, Abukawa D, Oyake Y, Nishinomiya F, Maisawa S, Aikawa J, et al. [Neonatal cholestasis associated with hypoproteinemia—hepatic steatosis and siderosis]. J Jpn Pediatr Soc 1997;101:840-4. (Jpn, abstract in Eng).
11. Tazawa Y, Nishinomiya F, Abukawa D, Aikawa J, Ohura T, Tohma M, et al. Neonatal intrahepatic cholestasis with hepatic siderosis and steatosis. Acta Paediatr Jpn 1998;40:150-4.
12. Ohura T, Abukawa D, Aikawa J, Nakagawa M, Ishizawa S, Tazawa Y, et al. [Disturbance of amino acids metabolism in infants with idiopathic neonatal hepatitis]. J Jpn Pediatr Soc 1997; 101:1522-5 (Jpn, abstract in Eng).
13. Tazawa Y, Abukawa D, Maisawa S, Nishinomiya F, Oyake Y, Takada G, et al. Idiopathic neonatal hepatitis presenting as neonatal hepatic siderosis and steatosis. Dig Dis Sci 1998;43:392-6.
13a. Yashuda T, Yamaguchi M, Kobayashi K, Nishi I, Horinouchi H, Jalil MA, et al. Identification of two novel mutations in the SLC25A13 gene and detection of seven mutations in 102 patients with adult-onset type II citrullinemia. Hum Genet 2000;107:537-45.
14. del Arco A, Agudo M, Satrustegui J. Characterization of a second member of the subfamily of calcium-binding mitochondrial carriers expressed in human non-excitable tissues. Biochem J 2000;345:725-32.

15. Onuki J, Nishimura S, Yoshino K, Takahashi H, Suzuki T, Abe K, et al. [Genetic abnormality in 2 brothers of a case with adult onset type II citrullinemia: trial of pre-onset genetic diagnosis]. Acta Hepatologica Jpn 2000;41: 555-60. (Jpn).
16. Jacquemin EJ, Lykavieris P, Chaoui N, Hadchouel M, Bernard O. Transient neonatal cholestasis: origin and outcome. J Pediatr 1998;133:563-7.
17. Balistreri WF, Stocker JT. $\alpha_1$-Antitrypsin deficiency and neonatal cholestasis. In: Seeff LB, Lewis JH, editors. Current perspective in hepatology. New York: Plenum Publishing Corporation; 1989. p. 271-86.
18. Kelly DA, Portmann B, Mowat AP, Sherlock S, Lake BD. Niemann-Pick disease type C: diagnosis and outcome in children, with particular reference to liver disease. J Pediatr 1993;123:242-7.
19. Cormier-Daire V, Chretie D, Rustin P, Rotig A, Dubuisson C, Jacquemin E, et al. Neonatal and delayed-onset liver involvement in disorders of oxidative phosphorylation. J Pediatr 1997;130: 817-22.
20. Bakker HD, Scholte HR, Dingemans KP, Spelbrink JN, Wijburg FA, Van den Bogert C. Depletion of mitochondrial deoxyribonucleic acid in a family with fatal neonatal liver disease. J Pediatr 1996;128:683-7.
21. Bioulac-Sage P, Parrot-Roulaud F, Mazat JP, Lamireau T, Coquet M, Sandler B, et al. Fatal neonatal liver failure and mitochondrial cytopathy (oxidative phosphorylation): a light and electron microscopic study of the liver. Hepatology 1993;18:839-6.
22. Tyni T, Palotie A, Viinikka L, Valanne L, Salo MK, von Döbelln U, et al. Long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency with G1528C mutation: clinical presentation of thirteen patients. J Pediatr 1997;130:67-76.
23. Janas LM, Picciano MF, Hatch TF. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula. Pediatrics 1985;75:775-84.
24. Karlsland Åkeson PM, Axelsson IEM, Räihä NCR. Protein and amino acid metabolism in three- to twelve-month-old infants fed human milk or formulas with varying protein concentrations. J Pediatr Gastroenterol Nutr 1998;26: 297-304.
25. Yazaki M, Ikeda S, Takei Y, Yanagisawa N, Matsunami H, Hashikura Y, et al. Complete neurological recovery of an adult patient with type II citrullinemia after living related partial liver transplantation. Transplantation 1996;62:1679-81.
26. Kawamoto S, Strong RW, Kerlin P, Lynch SV, Steadman C, Kobayashi K, et al. Orthotopic liver transplantation for adult-onset type II citrullinemia. Clin Transplant 1997;11:453-8.
27. Tsuboi Y, Hori T, Matsumoto S, Takahashi M, Yamada T. [Liver transplantation in type II citrullinemia]. Clin Neurol 1999;39:1049-53. (Jpn, abstract in Eng).
28. Kasahara M, Ohwada S, Takeichi T, Kaneko H, Tomomasa T, Morikawa A, et al. Living-related liver transplantation for type II citrullinemia using a graft from heterozygote donor. Transplantation 2001;71:157-9.
29. Balistreri WF. Nontransplant options for the treatment of metabolic liver disease: saving livers while saving lives. Hepatology 1994;19:782-7.

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ORIGINAL INVESTIGATION

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# Neonatal presentation of adult-onset type II citrullinemia

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**Abstract** Adult-onset type II citrullinemia (CTLN2) is characterized by a liver-specific argininosuccinate synthetase deficiency caused by a deficiency of the citrin protein encoded by the SLC25A13 gene. Until now, however, no SLC25A13 mutations have been reported in children with liver diseases. We described three infants who presented as neonates with intrahepatic cholestasis associated with hypermethioninemia or hypergalactosemia detected by neonatal mass screening. DNA analyses of SLC25A13 revealed that one patient was a compound heterozygote for the 851del4 and IVS11+1G→A mutations and two patients (siblings) were homozygotes for the IVS11+1G→A mutation. These results suggested that there may be a variety of liver diseases related to CTLN2 in children.

## Introduction

Citrullinemia is an autosomal-recessive disease caused by a deficiency of argininosuccinate synthetase (ASS; EC 6.3.4.5). Citrullinemia has been classified into two forms according to the pathogenesis: classical citrullinemia (CTLN1; OMIM no. 215700) and adult-onset type II citrullinemia (CTLN2; OMIM no. 603471; Saheki et al. 1987). Most cases of CTLN1 follow a severe course with onset of symptoms from birth, and more than half of the cases die in the neonatal period. In CTLN1, the citrullinemia caused by mutations in the ASS gene on chromosome 9q34 (Kobayashi et al. 1995), and the enzyme defect is found in all tissues and cells in which ASS is expressed. CTLN2 is characterized by a liver-specific ASS deficiency with no abnormalities in the ASS gene (Kobayashi et al. 1993). The most characteristic feature of CTLN2 is the late onset of serious and recurring symptoms. To date, onsets have been reported in patients ranging from 11 to 72 years old (Kobayashi et al. 1997). Most CTLN2 individuals suffer from a sudden disturbance of consciousness associated with flapping tremor, disorientation, restlessness, drowsiness, and coma, and the majority die within a few years of onset, mainly because of cerebral edema. Recently, Kobayashi et al. (1999) have identified a novel SLC25A13 gene on chromosome 7q21.3 together with five different DNA sequence alterations that lie at this locus and that account for all of the mutations found in the CTLN2 patients examined. SLC25A13 is a novel gene expressed abundantly in the liver. Citrin, a protein encoded by SLC25A13, has been reported as a calcium-binding mitochondrial carrier protein, but its precise function is unknown. Until now, no CTLN2 patients have been reported in children.

We believe that the children reported herein as having SLC25A13 mutations following diagnosis by DNA analyses are the first reported cases to present neonatal intrahepatic cholestasis.

## Subjects and methods

### Patients

Patient 1, a female weighing 2958 g at birth, was born to a 30-year-old gravida II mother at 38 weeks of gestation by vacuum delivery. The newborn was referred to Tohoku University Hospital at 19 days of age because of hypermethioninemia (134 μmol/l by the Guthrie test) detected by neonatal mass screening.

Patient 2, the younger brother of patient 1, was delivered at 40 weeks of gestation by cesarean section performed because of fetal hypoxia. His birth weight was 2514 g, and the Apgar scores at 1 and 5 min were 6 and 8, respectively. Laboratory data on day 3 of life showed evidence of liver dysfunction. The concentration of total serum bilirubin was 99 μmol/l with 14 μmol/l direct bilirubin, 130 IU/l aspartate aminotransferase, and 349 IU/l alanine aminotransferase. The activities of vitamin-K-dependent coagulation

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factors were less than 10% of control (Hepaplastin test; Eisai, Tokyo, Japan; normal range: 70%–110%). This patient was treated with intravenous vitamin K injections and discharged on day 21. He was later referred to Tohoku University Hospital at 25 days of age because of hypermethioninemia (201 μmol/l) detected by metabolic screening.

The parents were not related. Both infants received milk-based formulas, and their protein intakes were estimated to be in a range from 3.4 to 3.9 g/kg per day. They manifested mild jaundice and light yellow-colored stools.

Patient 3, a female weighing 2840 g at birth, was born to a 29-year old gravida III mother at 40 weeks of gestation. She was referred to the Tohoku University Hospital at 21 days of age because of hypergalactosemia detected by neonatal mass screening (Paigen method, >1.1 mmol/l; Beutler method, normal). Her parents were unrelated, and she had been fed on mother's milk. She had no cataracts and jaundice was not noticed.

### DNA diagnosis of mutations

The procedure for DNA diagnosis of SLC25A13 mutations was as previously reported in detail (Kobayashi et al. 1999; Yasuda et al. 2000). Polymerase chain reaction (PCR) amplification was performed for detection of the 851del4 mutation by using the primer set IVS-8F2 (5'-GGTATATTTGTTGCTTGTGTTTG-3') and Ex-9B (5'-TCTTCCAGAGGAGCAATCCG-3'). PCR products were separated on 4% Amplisize (BioRad, USA) gel and visualized by ethidium bromide staining. The size of PCR product from the control allele was 82 bp and that from the mutant allele with 851del4 was 78 bp, but the heteroduplex molecules ran slowly in the gel. For the detection of the IVS11+1G→A mutation, we used primer set Ex-11F (5'-CAGCTTTGACTGTTTTAAGAAAGT-3') and IVS-11Bm2 (5'-AGGTATTGAGCATGTGGCACTG-3'). The underlined G is a mismatched base creating the site for the *Sau*3AI restriction enzyme for mutant genes. PCR products were digested with *Sau*3AI and fragments were separated on 3% Amplisize gel.

## Results

### Clinical data on admission

The laboratory data obtained from these patients (Table 1) was similar and suggested intrahepatic cholestasis. Complete blood counts, blood ammonia determinations, and TORCH antibody titers (toxoplasma, rubella, cytomegalovirus, and herpes) were all normal. The blood galactose and galactose-1-phosphate levels in patient 3 were elevated to 0.88 mmol/l and 0.19 mmol/l, respectively, but the activities of galactose-1-phosphate uridyltransferase and UDP galactose-4-epimerase were within the normal range. Hepaplastin test findings were low in patient 1 (30.8%) and within the normal range in patient 3 (80.0%). Plasma amino acid analysis showed significant elevation of citrulline and methionine in all three patients. The concentrations of threonine, tyrosine, lysine, and arginine were also 2–4 times higher than the control levels. Other amino acid concentrations were within or near the normal range. An analysis of organic acids in the urine of patient 1 by using gas chromatography showed significant elevation of p-hydroxyphenyllactic acid and p-hydroxyphenylpyruvic acid. Succinylacetone was not detected.

### Histology

Patient 1 underwent liver biopsy at 55 days of age. The specimen showed no giant-cell transformation of hepatocytes, although diffuse fatty change of hepatocytes including micro- or macrovesicular fat droplets, mild inflammatory infiltration, and portal fibrosis were noted.

### Clinical course

Without specific treatment other than feeding with formulas containing middle-chain triglycerides (for patients 1 and 2) or lactose-free formula (for patient 3) and supplementation of fat-soluble vitamins, all three patients had favorable clinical courses. Abnormal aminograms persisted for a couple of months but then improved, and 2–3 months later, the cholestasis improved. All biochemical abnormalities normalized by the age of 12 months. Follow-up has been maintained for 18 months to 8 years. All patients are alive and show no developmental delay or neurologic abnormalities.

**Table 1** Biochemical data on the three patients

| Parameter | Patient 1 35 days[a] | Patient 2 25 days[a] | Patient 3 26 days[a] | Normal range |
|---|---|---|---|---|
| Total bilirubin (μmol/l) | 101 | 63 | 41 | 3–21 |
| Direct bilirubin (μmol/l) | 31 | 41 | 22 | <12 |
| Alkaline phosphatase (IU/l) | 859 | 524 | 1439 | 22–125 |
| Gamma-glutamyltranspeptidase (IU/l) | 191 | 253 | 220 | 1–49 |
| Aspartate aminotransferase (IU/l) | 74 | 75 | 64 | 4–30 |
| Alanine aminotransferase (IU/l) | 42 | 43 | 42 | 3–28 |
| Total bile acids (μmol/l) | 256 | 196 | 230 | <10 |
| Threonine (μmol/l) | 681 | 619 | 415 | 65–153 |
| Citrulline (μmol/l) | 547 | 286 | 485 | 5–37 |
| Methionine (μmol/l) | 465 | 229 | 240 | 13–32 |
| Tyrosine (μmol/l) | 264 | 143 | 185 | 34–94 |
| Lysine (μmol/l) | 347 | 220 | 384 | 102–203 |
| Arginine (μmol/l) | 262 | 148 | 315 | 28–99 |

[a]Age at examination

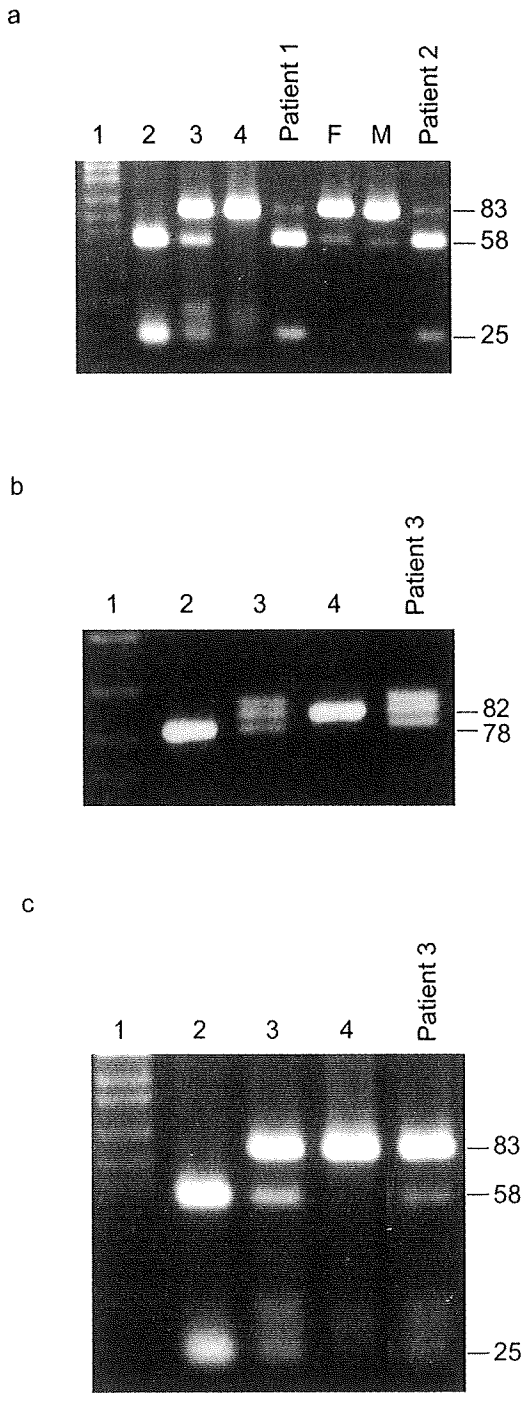


**Fig. 1a–c** Identification of the SLC25A13 mutation. *Lane 1* Molecular markers, *lanes 2–4* represent the homozygote control, heterozygote control, and normal control, respectively. **a** The PCR product was 83 bp long and was cut into 58-bp and 25-bp bands by *Sau*3AI digestion in patients 1 and 2. These siblings were homozygotes for the IVS11+1G→A mutation, and their parents (*lanes F, M*) were heterozygotes for this mutation. **b** Patient 3 was a heterozygote for the 851del4 mutation. The PCR products for the normal allele and mutant allele were 82 bp and 78 bp, respectively. **c** Patient 3 was a heterozygote for the IVS11+1G→A mutation. The presence of heteroduplex molecules in opposite strands in the IVS11+G→A mutation results in a failure of cleavage at either site (**a**, **c**). The three bands observed in the heterozygote with the 851del4 mutation result from the formation of heteroduplexes (**b**)

DNA diagnosis

The principal features of the present cases were abnormal aminograms showing more than 10-fold increases in citrulline concentrations beyond the upper limit of normal. We suspected that these abnormalities might be related to CTLN2, so we obtained informed consent from the parents to perform genetic diagnosis. DNA analysis of SLC25A13 showed that patients 1 and 2 were homozygous for a known IVS11+1G→A mutation (Kobayashi et al. 1999; Yasuda et al. 2000), that both their parents were heterozygous for this mutation (Fig. 1a), and that patient 3 was a compound heterozygote for the 851del4 and IVS11+G→A mutations (Fig. 1b, c). These mutations were confirmed by direct sequencing of genomic DNA from the patients (data not shown).

## Discussion

We treated three patients who presented with adult-onset type II citrullinemia in the neonatal period. In a previous report (Ohura et al. 1997), we described seven patients (including patients 1 and 2) with neonatal intrahepatic cholestasis. The clinical features of the other five patients were similar to those of the present cases, i.e., abnormal results in neonatal mass screening (2 were positive for hypermethioninemia, 2 for hyperphenylalaninemia, 1 for both hypermethioninemia and hypergalactosemia), intrahepatic cholestasis, transient hypercitrullinemia, and fatty liver. We can conjecture that these clinical features represent a neonatal manifestation of CTLN2, but molecular analyses are still pending because of the inavailability of DNA samples from these patients.

Overall, CTLN2 has been diagnosed exclusively in Japan. Its prevalence has been reported to be approximately 1 in 100,000 (Kobayashi et al. 1993), but molecular analysis of SLC25A13 suggests that the actual prevalence of the disease is likely to be higher (Kobayashi et al. 1999; Yasuda et al. 2000). We presume, therefore that some individuals with CTLN2 may not present symptoms during life or may receive an erroneous diagnosis, such as chronic hepatitis. Patients 1, 2, and 3 are now 8, 4, and 1.5 years old, respectively, and their laboratory data, including aminograms, are all within the normal range. We shall have to continue to observe these patients regularly to see whether they develop symptoms of CTLN2.

Elevation of serum methionine, tyrosine, or galactose has been reported in liver diseases, such as neonatal hepatitis syndrome (Goodman and O'Brien 1968; Balistreri 1985; Mowat 1987). Hereditary tyrosinemia was unlikely in the present cases because their hypertyrosinemia was transient. Liver function tests revealed that they had severe liver damage. Histological analysis of a biopsied liver sample showed fatty liver, round cell infiltration, and mild fibrosis, all of which are consistent with pathological findings for CTLN2 (Walser 1983). We speculated that hypermethioninemia, hypertyrosinemia, and hypergalactosemia were secondary abnormalities caused by liver

dysfunction attributable to CTLN2. The pathogeneses of transient and self-limited cholestasis in these infants with SLC25A13 mutations remain unsolved. The function of citrin should be clarified in order to understand these phenomena.

The aminogram of our cases showed that not only methionine, but also threonine, citrulline, and arginine levels were significantly elevated. Saheki et al. (1986) analyzed serum amino acid patterns of CTLN2 patients and showed the following features: (1) serum arginine levels were higher than in controls and were significantly correlated with serum citrulline levels; (2) serum alanine, serine, glycine, and branched chain amino acids were lower than in controls, whereas threonine was somewhat higher. These characteristics are unique to CTLN2 and consistent with our results obtained from infants with SLC25A13 mutations.

In summary, we reported the first known CTLN2 cases manifesting symptoms in early infancy. The identification of the SLC25A13 gene provides us with a powerful tool for detecting CTLN2 in children with idiopathic hepatobiliary diseases and for clarifying the mechanisms of various phenomena found in CTLN2 patients. Neonatal screening for hypermethioninemia or galactosemia provides an important opportunity for diagnosis of CTLN2 in infancy.

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## References

Balistreri WF (1985) Neonatal hepatitis. J Pediatr 106:171–184

Goodman SI, O'Brien D (1968) Transient infantile hypermethioninemia. Pediatrics 42:528–529

Kobayashi K, Shaheen N, Kumashiro R, Tanikawa K, O'Brien WE, Beaudet AL, Saheki T (1993) A search for the primary abnormality in adult-onset type II citrullinemia. Am J Hum Genet 53:1024–1030

Kobayashi K, Kakinoki H, Fukushige T, Shaheen N, Terazono H, Saheki T (1995) Nature and frequency of mutations in the argininosuccinate synthetase gene that cause classical citrullinemia. Hum Genet 96:454–463

Kobayashi K, Horiuchi M, Saheki T (1997) Pancreatic secretary trypsin inhibitor as a diagnostic marker for adult-onset type II citrullinemia. Hepatology 25:1160–1165

Kobayashi K, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, Yasuda T, Ikeda S, Hirano R, Terazono H, Crackower MA, Kondo I, Tsui LC, Scherer SW, Saheki T (1999) The gene mutated in adult-onset type II citrullinemia encodes a putative mitochondrial carrier protein. Nat Genet 22:159–163

Mowat AP (1987) Liver disorders in childhood, 2nd ed. Butterworths, London, pp 37–71

Ohura T, Abukawa D, Aikawa J, Nakagawa M, Ishizawa S, Tazawa Y, Narisawa K, Iinuma K (1997) Disturbances of amino acid metabolism in infants with idiopathic neonatal hepatitis (in Japanese with an English abstract). J Jpn Pediatr Soc 101:1522–1525

Saheki T, Kobayashi K, Miura T, Hashimoto S, Ueno Y, Yamazaki T, Araki H, Nara H, Shiozaki Y, Sameshima Y, Suzuki M, Yamauchi Y, Sakazume Y, Akiyama K, Yamamura Y (1986) Serum amino acid pattern of type II citrullinemic patients and effect of oral administration of citrulline. J Clin Biochem Nutr 1:129–142

Saheki T, Kobayashi K, Inoue I (1987) Hereditary disorders of the urea cycle in man: biochemical and molecular approaches. Rev Physiol Biochem Pharmacol 108:21–68

Walser M (1983) Urea cycle disorders and other hereditary hyperammonemic syndromes. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds) The metabolic basis of inherited disease, 5th edn. McGraw-Hill, New York, pp 402–438

Yasuda T, Yamaguchi N, Kobayashi K, Nishi I, Horinouchi H, Jalil MA, Li MX, Ushikai M, Iijima M, Kondo I, Saheki T (2000) Identification of two novel mutations in SLC25A13 gene and detection of seven mutations in 102 patients with adult-onset type II citrullinemia. Hum Genet 107:537–545

[5] Yeaman SJ. The 2-oxo acid dehydrogenase complexes: recent advances. Biochem J 1989;257:625–32.
[6] Provenzano G, Diquattro O, Craxi A, et al. Immunoblotting as a confirmatory test for antimitochondrial antibodies in primary biliary cirrhosis. Gut 1993;34:544–8.
[7] Kitami N, Komada T, Ishii H, et al. Immunological study of anti-M2 in antimitochondrial antibody-negative primary biliary cirrhosis. Intern Med 1995;34:496–501.
[8] Inoue K, Hirohara J, Nakano T, et al. Prediction of prognosis of primary biliary cirrhosis in Japan. Liver 1995;15:70–7.
[9] Grimbert S, Johanet C, Bendjaballah F, Homberg JC, Poupon R, Beaugrand M. Antimitochondrial antibodies in patients with chronic hepatitis C. Liver 1996;16:161–5.
[10] Berg PA, Klein R. Mitochondrial antigens and autoantibodies: from anti-M1 to anti-M9. Klin Wochenschr 1986;64:897–909.
[11] Tsuruya T. Detection of anti-pyruvate dehydrogenase complex antibody in primary biliary cirrhosis by an enzyme-linked immunosorbent assay. Gastroenterol Jpn 1990;25:471–7.
[12] Ishikawa E, Imagawa M, Hashida S, Yoshitaka S, Hamaguchi Y, Veno T. Enzyme labeled of antibodies and their fragments for enzyme immunology and immunohistochemical staining. J Immunoassay 1983;4:209–27.
[13] Mosbach K. Methods in Enzymology, vol. 135. New York: Academic Press, 1987.
[14] Stanley CJ, Perham RN. Purification of 2-oxo-acid dehydrogenase multienzyme complexes from ox heart by a new method. Biochem J 1980;191:147–54.
[15] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[16] Schneider WC, Hogeboom GH. Intracellular distribution of enzymes: further studies on the distribution of cytochrome in rat liver homogenate. J Biol Chem 1950;183:123–8.
[17] Towbin H, Steahelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350–4.
[18] Mutimer DJ, Fussey SPM, Yeaman SJ, Kelly PJ, James OFW, Bassendine MF. Frequency of IgG and IgM autoantibodies to four specific M2 mitochondrial autoantigens in primary biliary cirrhosis. Hepatology 1989;10:403–7.
[19] Miyakawa H, Kawaguchi N, Kikuchi K, et al. Combination assays for IgG class and IgM class anti-pyruvate dehydrogenase complex (PDC)-E2 by ELISA using recombinant autoantigen to diagnose primary biliary cirrhosis. Hepatol Res 1998;12:140–50.

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# An undescribed subset of neonatal intrahepatic cholestasis associated with multiple hyperaminoacidemia

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**Abstract**

Five patients of cholestatic jaundice and multiple hyperaminoacidemias were uncovered during neonatal mass screening for homocystinuria. All five patients had increased plasma levels of methionine, citrulline, tyrosine, threonine, phenylalanine, lysine and arginine. Compared with those of age-matched cholestatic disease controls, idiopathic neonatal hepatitis ($n = 9$) and biliary atresia ($n = 14$), plasma levels of three amino acids, citrulline, methionine, and threonine, were significantly greater, respectively ($P < 0.01$). Liver biopsies examined in four patients uniformly showed diffuse hepatic fatty liver with micro- and macrovesicular droplets without giant cell transformation. Administration of fat-soluble vitamins and formula milk containing middle-chain triglyceride resulted in normalization of amino acid profiles by 6 weeks after the treatment. All liver function tests normalized by 17 months of age. 

*Keywords:* Neonatal cholestasis; Neonatal mass screening; Hypermethioninemia; Hypercitrullinemia; Fatty liver

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## 1. Introduction

Elevated levels of methionine and galactose in the blood are utilized to screen for homocystinuria and galactosemia, respectively. Such screening tests occasionally disclose subclinical liver diseases in neonates [1,2]. In neonatal cholestatic liver diseases, such as idiopathic neonatal hepatitis (INH) or biliary atresia (BA), hypertyrosinemia and hypermethioninemia are commonly encountered [3]. On the other hand, neonatal fatty liver has been described in a variety of neonatal diseases, particularly in metabolic diseases including galactosemia or hereditary tyrosinemia [3]. We report an undescribed subset of neonatal intrahepatic cholestasis associated with unusual hyperaminoacidemias and diffuse fatty liver, which was uncovered by neonatal screening for homocystinuria.

## 2. Case report

During the past 12 years, five patients, 24–61 days of life, were referred to Tohoku University Medical Center, because of hypermethioninemia found by neonatal metabolic screening for homocystinuria, and subsequently subclinical liver dysfunction was disclosed. All five patients except one, case 2, were girls. Two patients, cases 1 and 2, simultaneously had elevated levels of serum galactose, 6–16 mg/100 ml. However, case 1 had negative results for hypergalactosemia on further studies at 44 days of life, and cases 2 showed no hypergalactosemia on subsequent re-examinations. All patients were born at full term, bottle-fed, and had normal-birth-weight except for case 5, 2382 g. They had no perinatal episodes, including asphyxia or sepsis. There was no consanguinity, but two patients, cases 2 and 3, were siblings. All patients had jaundice, dark urine, and light yellow-colored stool, but had no hepatosplenomegaly. Serum biochemical studies showed total bilirubin, 3.7–8.4 mg/100 ml, direct bilirubin, 1.9–5.3 mg/100 ml (normal, $< 1.2$), total bile acids, 156–370 μmol/l ($< 38$), alkaline phosphatase, 621–1306 IU/l ($< 394$), and γ-glutamyltranspeptidase, 121–253 IU/l ($< 74$). Serum AST and ALT activities were 62–179 IU/l ($< 63$) and 22–93 IU/l ($< 35$), respectively. All patients had low activities of vitamin K-dependent coagulation factors, 31–49% of the normal ($> 66\%$). No patients had hyperammonemia, hypoproteinemia, hypoglycemia, metabolic acidosis, or elevated levels of serum galactose in blood. Serum BUN levels were within normal ranges. Viral infections, metabolic diseases, and hepato-biliary diseases of known causes were all ruled out by a battery of examinations for neonatal cholestasis. Urinary organic acid and bile acid analyses were negative. Ultrasound studies showed no gallstone, sludge, dilated bile ducts, patent ductus venosus, abnormal runnings of portal vein, and portosystemic shunts. Without specific treatment, except for substituting formulas containing middle-chain triglycerides and supplementation of fat soluble vitamins, all patients had favorable clinical courses. The formulas and fat soluble vitamins had been given until jaundice subsided and cholestasis disappeared, respectively. Results of biochemical studies completely normalized between 5 and 17 months of age. A follow-up study

from 3 to 12 years showed that all patients are alive without recurrent episodes of liver dysfunction or hyperaminoacidemias.

### 3. Plasma amino acid profiles

Plasma amino acids, which were examined by an autoanalyzer (Amino Acid Analyzer 835, Hitachi Co. Ltd., Japan), showed multiple hyperaminoacidemias, elevated levels of methionine, citrulline, threonine, tyrosine, arginine, phenylalanine, and lysine (Fig. 1). In particular, plasma levels of methionine, citrulline, and threonine were remarkable. Compared with those in age-matched cholestatic disease controls, INH ($n = 9$) and BA ($n = 14$), plasma levels of the three amino acids in the five patients were significantly greater ($P < 0.01$, Fig. 2). Statistical comparisons were performed by Mann–Whitney's $U$-test. Without specific treatment, except for substituting formulas containing middle-chain triglycerides and supplementation of fat soluble vitamins, plasma amino acid profiles normalized within 2 months.

### 4. Liver histology

Four patients, cases 1 and 3–5, underwent percutaneous liver biopsies on admission. The specimens showed similar histological features. Diffuse fatty change

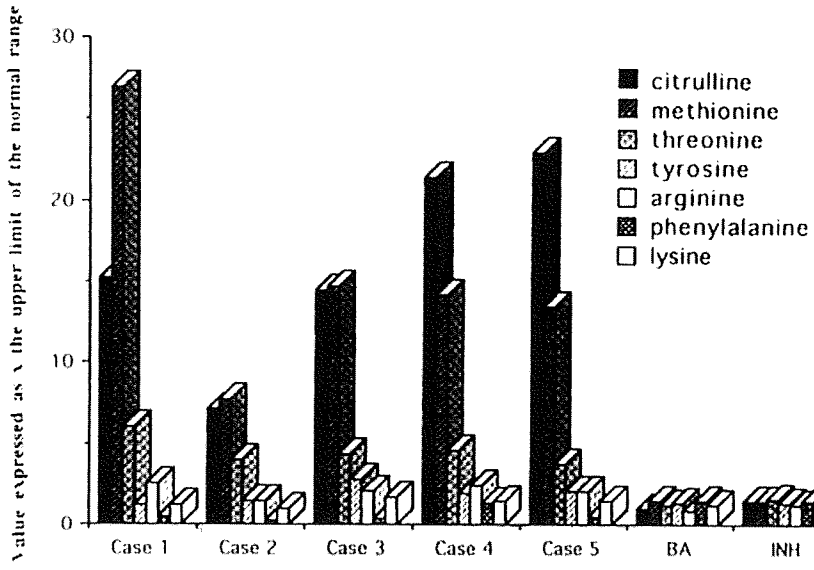


Fig. 1. Plasma amino acid levels of the five patients in comparison with mean values of those of neonatal cholestatic diseases. BA, biliary atresia; INH, idiopathic neonatal cholestasis.

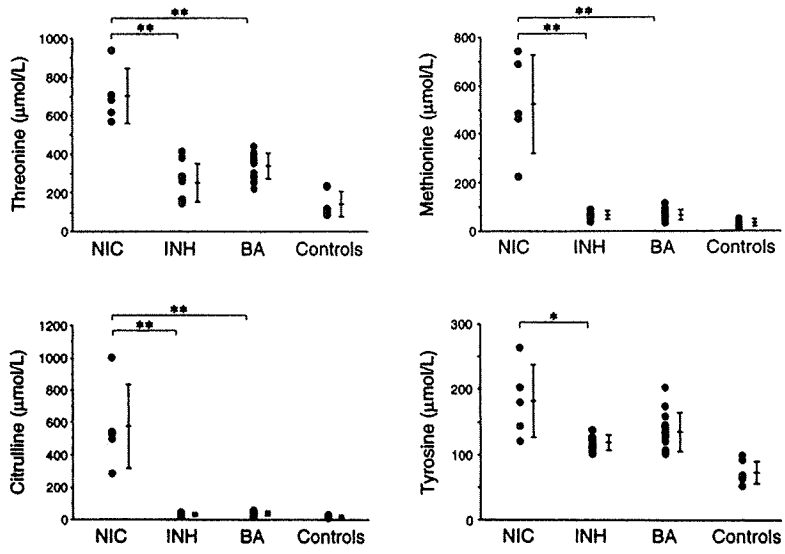


Fig. 2. Plasma levels of threonine, citrulline, methionine and tyrosine in the five patients, compared with those of patients with neonatal cholestatic diseases. NIC, neonatal intrahepatic cholestasis with multiple hyperaminoacidemias and diffuse fatty liver ($n = 5$); INH, idiopathic neonatal hepatitis ($n = 9$); BA, biliary atresia ($n = 14$); **, $P < 0.01$; *, $P < 0.05$.

of hepatocytes with micro- and macrovesicular fat droplets was uniformly present. Cholestasis was identified in the liver cell cytoplasma, and dilated canaliculi containing bile were seen. Minimal to mild fibrosis and inflammatory infiltration in the portal area were observed. Extramedullary hematopoietic foci were occasionally found (Fig. 3).

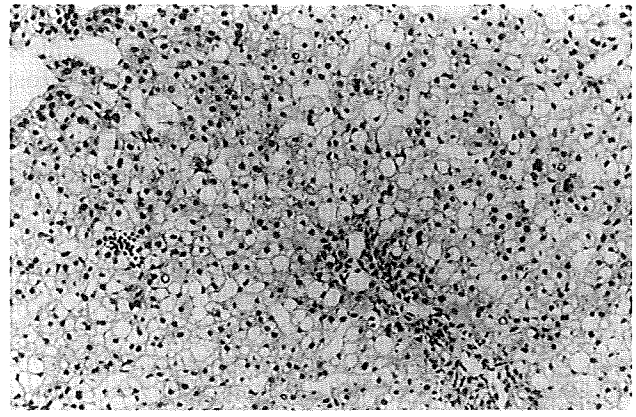

Fig. 3. Histologic findings of the liver in case 1 (HE stain, × 200).

## 5. Discussion

Neonatal mass screening for some metabolic diseases, such as homocystinuria and galactosemia, occasionally and unexpectedly uncovers neonatal liver diseases [1,2]. In case of either elevated levels of methionine or galactose in the blood, neonatal liver diseases should be considered as possible candidates.

Hypermethioninemia found in neonatal mass screening has been reported to be caused by neonatal liver diseases or protein overload [1]. Plasma amino acid patterns of common neonatal cholestatic diseases, such as INH and BA, are characterized by elevated levels of tyrosine and methionine [3], as shown in this study. The present patients, however, had an unusual multiple hyperaminoacidemias, including raised levels of methionine, citrulline, threonine, and tyrosine. In bottle-feeding, overfeeding may occur, which can lead to protein overload. All five patients had received formula milk, which is greater in protein content, approximately over twice that found in human milk. Protein overloading and metabolic immaturity in neonates during the first weeks of life may contribute to the occurrence of the unusual amino acid pattern. In extreme protein overnutrition, normal infants had increased levels of a majority of plasma amino acid, particularly methionine, proline, and branched chain amino acids, and the greatest elevations occur in the levels of methionine, reaching 35 times the normal average value [4]. Methionine, the most hepatotoxic amino acid produces cholestasis [5]. Furthermore, methionine is a substrate in the synthesis of glutathione in the liver. It is necessary in its reduced form to protect the liver from oxidative injury. The disturbance of methionine metabolism and subsequent glutathione deprivation may cause intrahepatic cholestasis. Diffuse fatty liver, however, has never been reported [1,5]. The higher plasma concentrations of threonine and phenylalanine were further reported to correlate with higher total protein intake [6]. Higher plasma concentrations of methionine, threonine, and tyrosine in bottle-fed infants, compared with those in breast-fed infants, have been also reported [7]. Hypercitrullinemia, however, cannot be explained by protein overload. Hypercitrullinemia is observed in classical citrullinemia, citrullinemia type I, which is ruled out by absence of the hyperammonemia [8].

Elevated levels of galactose in blood are found in portosystemic shunt, particularly in infancy [3,9]. Furthermore, fatty liver can develop in portosystemic shunt. Severe fatty liver has been reported in three brothers with patent ductus venosus [10]. Portosystemic shunt usually accompanies hyperbileacidemia or hyperammonemias [9]. Three patients presented in this study, who had transient increases of galactose in blood, had hyperbileacidemias. The patients, however, had neither hyperammonemias nor patent ductus venosus.

Neonatal fatty liver may be a primary cause. Neonates experience a high oxygen environment immediately after birth, and fatty liver may occur as a consequence of oxidative injury to the liver. Fatty liver in neonates was observed directly by histological studies in mice, and indirectly by ultrasonography in humans [11]. Diffuse fatty depositions in the liver might be insufficient to handle either galactose or amino acids, leading to elevated levels of either galactose or multiple hyperaminoacidemias.

We report an undescribed subset of neonatal cholestasis, characterized by unusual, multiple hyperaminoacidemias and diffuse fatty liver. Greater application of neonatal screening may increase the discovery of this disease. However, the pathegenesis remains unsolved. Further investigation needs to search for unknown causes.

## References

[1] Whiteman PD, Clayton BE, Ersser RS, Lilly P, Seakins JWT. Changing incidence of neonatal hypermethioninemia: implications for the detection of homocystinuria. Arch Dis Child 1979;54:593–8.
[2] Mizoguchi N, Kawaguchi H, Eguchi T, Matsumoto T, Sakura N, Ueda K. Four cases of hepatic hemangiomas detected by mass-screening for galactosemia. J Jpn Pediatr Soc 1994;98:1392–9 Japanese with English abstract.
[3] Balistreri WF. Neonatal cholestasis. J Pediatr 1985;106:171–84.
[4] Snyderman SE, Holt LE, Norton PM, Roitman E, Phansalker SV. I. Influence of the level of protein intake and a comparison of whole protein and amino acid diets. Pediatr Res 1968;2:131–44.
[5] Moss RL, Haynes AL, Pastuszyn A, Glew RH. Methionine infusion reproduces liver injury of parenteral nutrition cholestasis. Pediatr Res 1999;45:664–8.
[6] Janas LM, Picciano MF, Hatch T. Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow's milk formula. Pediatrics 1985;75:775–84.
[7] Karlsland Akeson PM, Axelsson IEM, Raiha NCR. Protein and amino acid metabolism in three- to twelve-month-old infants fed human milk or formulas with varying protein concentrations. J Pediatr Gastroenterol Nutr 1998;26:297–304.
[8] Walser M. Urea cycle disorders and other hereditary hyperammonemic syndromes. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS, editors. The Metabolic Basis of Inherited Disease, Fifth. New York: McGraw-Hill Book Company, 1983:402–38.
[9] Sakura N, Mizoguchi N, Eguchi T, et al. Elevated plasma bile acids in hypergalactosemic neonates: a diagnostic clue to portosystemic shunts. Eur J Pediatr 1997;156:716–8.
[10] Uchino T, Endo F, Ikeda S, et al. Three brothers with progressive hepatic dysfunction and severe hepatic steatosis due to a patent ductus venosus. Gastroenterlogy 1996;110:1964–8.
[11] Kawamura T, Toyabe S, Moroda T, et al. Neonatal granulocytosis is a postpartum event which is seen in the liver as well as in the blood. Hepatology 1997;26:1567–72.

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# Comparison of the dot immunobinding assay and two enzyme-linked immunosorbent assay kits for the diagnosis of liver cystic echinococcosis

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**Abstract**

The dot immunobinding assay for the detection of hydatid antigen-specific antibodies (HA-DIA) was evaluated in patients with liver cystic and alveolar echinococcosis in comparison to two commercial ELISA kits. In 30 patients, *E. granulosus* infection (CE) was confirmed by histopathology or by the presence of parasite protoscoleces and/or hooks or specific antigen 5 (Ag5) in cyst fluid samples obtained by the fine needle aspiration biopsy (FNAB). Infection of *E. multilocularis* (AE) was diagnosed in two patients by the detection of specific anti-Em2$^{plus}$ ELISA and -Em18 Western blot antibodies and finally confirmed by histopathology. The HA-DIA using bovine hydatid antigens showed a high sensitivity in serum samples from CE patients; specific antibodies were found in 29 of 30 CE patients (96.7%). One negative result has been observed in a patient 2.6 years after radical surgery with a subsequent albendazole chemotherapy. The Echinococcosis ELISA® (Dialab Diagnostic) was positive in 23 CE cases (76.7%). The correlation between the HA-DIA and the Echinococcosis ELISA® was statistically significant. By contrast, *Echinococcus granulosus* IgG ELISA® (Bordier Affinity Products) gave positive results in only 12 of 30 CE patients (40.0%). Sera from two AE patients were high positive in all three methods analysed in our study. In non-endemic areas, due to the between-strains variations and differences in cyst immunogenic activity, related to the natural history of the parasite, a choice of an optimal method for a diagnosis of liver cystic echinococcosis has been discussed.

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ORIGINAL PAPER

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# A novel inborn error of metabolism detected by elevated methionine and/or galactose in newborn screening: neonatal intrahepatic cholestasis caused by citrin deficiency

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**Abstract** Adult-onset type II citrullinaemia, caused by deficiency of the citrin protein encoded by the *SLC25A13* gene, is characterised by a liver-specific argininosuccinate synthetase deficiency. DNA analysis for citrin deficiency revealed that *SLC25A13* mutations are the cause of a particular type of neonatal intrahepatic cholestasis. We retrospectively investigated nine infants with cholestatic jaundice of unknown origin, detected by newborn screening over a period of 17 years, to determine the role of *SLC25A13* defects in children. The results of the newborn screening were varied; four neonates were positive for hypermethioninaemia, two for hyperphenylalaninaemia, one for hypergalactosaemia and two for both hypermethioninaemia and hypergalactosaemia. Clinical characteristics of the patients were severe intrahepatic cholestasis, hypercitrullinaemia, and fatty liver. The symptoms resolved in all patients by 12 months of age without special treatment other than nutritional management. Although five patients were lost to follow-up, we detected *SLC25A13* mutations in the remaining four patients examined. *Conclusion*: the differential diagnosis of cholestatic jaundice of unknown origin in infants should therefore include citrin deficiency. In this paper, we stress the importance of newborn screening to detect infants with neonatal intrahepatic cholestasis caused by citrin deficiency.

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**Keywords** Citrin deficiency · Citrullinaemia · Fatty liver · Intrahepatic cholestasis · Newborn screening

**Abbreviations** *ASS* argininosuccinate synthetase · *ALT* alanine aminotransferase · *CTLN1* citrullinaemia type I · *CTLN2* citrullinaemia type II · *Gal* galactose · *γ-GTP* γ-glutamyl transpeptidase · *Met* methionine · *NICCD* neonatal intrahepatic cholestasis caused by citrin deficiency · *PCR* polymerase chain reaction · *Phe* phenylalanine

## Introduction

Citrullinaemia is an autosomal recessive inborn error of the urea cycle caused by deficient argininosuccinate synthetase activity (ASS; EC 6.3.4.5). It is classified into two groups: classical or type I/III citrullinaemia (CTLN1; McKusick 215700) and adult-onset type II citrullinaemia (CTLN2: McKusick 603471) [12]. CTLN1, in which ASS activity is almost completely absent in every cell where the ASS gene is expressed, is caused by mutations in the ASS gene on chromosome 9q34 [4]. The most common clinical presentation is the neonatal form, characterised by striking, life-threatening hyperammonaemia during the neonatal period. CTLN2 is characterised by a liver-specific ASS deficiency and most CTLN2 patients suffer from sudden disturbances of consciousness with hyperammonaemia after 20 years of age, with the majority dying within a few years of onset, mainly due to cerebral oedema. CTLN2 has been diagnosed exclusively in Japan [3]. Kobayashi et al. [5] identified a novel gene, *SLC25A13* on chromosome 7q21.3, as the gene responsible for CTLN2. The *SLC25A13* gene encodes a 3.4 kb transcript which is expressed most abundantly in the liver, but also in the kidneys, heart and other tissues [5]. Citrin, a protein encoded by *SLC25A13*, has been identified as a mitochondrial aspartate glutamate carrier protein [10]. To date, nine *SLC25A13* mutations have been reported in CTLN2 patients [17] but very little is known about the