Biochemical characteristics of neonatal cholestasis induced by citrin deficiency

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Abstract
AIM: To explore differences in biochemical indices between neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and that with other etiologies.

METHODS: Patients under 6 mo of age who were referred for investigation of conjugated hyperbilirubinemia from June 2003 to December 2010 were eligible for this study. After excluding diseases affecting the extrahepatic biliary system, all patients were screened for the two most common SLC25A13 mutations; the coding exons of the entire SLC25A13 gene was sequenced and Western blotting of citrin protein performed in selected cases. Patients in whom homozygous or compound heterozygous SLC25A13 mutation and/or absence of normal citrin protein was detected were defined as having NICCD. Cases in which no specific etiological factor could be ascertained after a comprehensive conjugated hyperbilirubinemia work-up were defined as idiopathic neonatal cholestasis (INC). Thirty-two NICCD patients, 250 INC patients, and 39 infants with cholangiography-confirmed biliary atresia (BA) were enrolled. Laboratory values at their first visit were abstracted from medical files and compared.

RESULTS: Compared with BA and INC patients, the NICCD patients had significantly higher levels of total bile acid (TBA) [all measures are expressed as median (inter-quartile range): 178.0 (111.2-236.4) µmol/L in NICCD vs 112.0 (84.9-153.9) µmol/L in BA and 103.0 (70.9-135.3) µmol/L in INC, \( P = 0.0001 \)]. The NICCD patients had significantly lower direct bilirubin [D-Bil 59.6 (43.1-90.9) µmol/L in NICCD vs 134.0 (115.9-151.2) µmol/L in BA and 87.3 (63.0-123.6) µmol/L in INC, \( P = 0.0001 \)]; aspartate aminotransferase [AST 34.0 (23.0-55.0) U/L in NICCD vs 134.0 (115.9-151.2) µmol/L in BA and 87.3 (63.0-123.6) µmol/L in INC, \( P = 0.0001 \)]; alanine aminotransferase [ALT 34.0 (23.0-55.0) U/L in NICCD vs 108.0 (62.0-199.0) U/L in BA and 84.5 (46.0-166.0) U/L in INC, \( P = 0.0001 \)]; aspartate aminotransferase [AST 74.0 (53.5-150.0) U/L in NICCD vs 153.0 (115.0-239.0) U/L in BA and 130.5 (81.0-223.0) U/L in INC, \( P = 0.0006 \)]; albumin [34.9 (30.7-38.2) g/L in NICCD vs 38.4 (36.3-42.2) g/L in BA and 39.9 (37.0-42.3) g/L in INC, \( P = 0.0001 \)]; glucose [3.2 (2.0-4.4) mmol/L in NICCD vs 4.57 (3.81-5.26) mmol/L in BA and 4.50 (3.24-4.74) mmol/L in INC, \( P = 0.0155 \)] levels. The D-Bil to total bilirubin (T-Bil) ratio was significantly lower in NICCD patients [all measures
INTRODUCTION

Citrin deficiency, caused by mutations in the SLC25A13 gene on chromosome 7q21.3, is an autosomal recessive disease that was first discovered in Japan and thereafter identified worldwide. At least two main phenotypes of citrin deficiency have been established: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM #605814) and adult-onset type II citrullinemia (CTLN2, OMIM #603471). The clinical features and diagnostic criteria of CTLN2 have been well established, but those of NICCD have not yet been established.

Children with NICCD usually have transient intrahepatic cholestasis that disappears by the age of 1 year with no isotope excretion was demonstrated by hepatobiliary scintigraphy, and in whom diagnosis of BA was confirmed by laparoscopic or laparotomy cholangiography were eligible for this group.

BA group: Patients with neonatal cholestasis in whom no isotope excretion was demonstrated by hepatobiliary iminodiacetic acid (HIDA) scintigraphy, and in whom diagnosis of BA was confirmed by laparoscopic or laparotomy cholangiography were eligible for this group.

INC group: Intrahepatic cholestasis was defined as con-
jugated hyperbilirubinemia following the exclusion of diseases affecting the extrahepatic biliary system (Table 1) by imaging of the hepatobiliary system. The imaging procedures included an ultrasound scan and HIDA scintigraphy in each case and laparoscopic/laparoscopic cholangiography in selected cases. Idiopathic neonatal cholestasis (INC) was defined when no specific etiological factor (Table 1) could be ascertained after a comprehensive conjugated hyperbilirubinemia test. Patients with at least single-allele mutation of SLC25A13 gene were excluded from this group as well.

**NICCD group:** The strategy of testing for SLC25A13 gene mutations in intrahepatic cholestatic infants had been reported previously. All intrahepatic cholestasis infants with unknown causes were screened for the two most common mutations of the SLC25A13 gene in Chinese, 851de4 and 1638ins23. For patients with various aminoacidemia or patients with only single-allele mutation who were found by the above screening method, the entire 18 coding exons together with the flanking sequence of the SLC25A13 gene were amplified by polymerase chain reaction and directly sequenced. Western blotting analysis of citrin protein was performed on patients with biopsied liver specimens available. Only patients in whom homozygous or compound heterozygous SLC25A13 gene mutation and/or absence of normal citrin protein were demonstrated, for whom a definite diagnosis of citrin deficiency could be made, were regarded as NICCD patients in this study. Patients with a probable diagnosis of citrin deficiency, that is, in whom there was only a heterozygous SLC25A13 gene mutation and in whom absence of normal citrin protein could not be demonstrated by Western blotting were excluded.

**RESULTS**

**Basic information**

In total, 32 patients (19 male and 13 female) with a definite diagnosis of citrin deficiency were included in the NICCD group. Thirty-nine patients (24 male and 15 female) were included in the BA group. Two hundred and fifty patients (174 male and 76 female) were included in the INC group. The birth weight and the days at which conjugated jaundice was first noticed, and the biochemical indices at presentation, were abstracted. Liver function tests and other routine laboratory data were obtained using standard methods.

**Statistical analysis**

Statistical analysis was performed using Stata/SE 10.0 for Windows (StataCorp LP, College Station, TX, United States of America). The descriptive data of the quantitative variables were reported in box-whisker plots and compared using Kruskal-Wallis rank tests among the three groups. For results with overall statistical significance, a Mann-Whitney test with a Bonferroni correction was further performed to test the medians between a series of pairwise groups. All P values were two-sided. Results were considered statistically significant at the 0.05 level.

**Comparison of biochemical indices among three groups**

The biochemical data of the three groups were compared (Figure 1B-E, Table 2). The NICCD group had significantly lower ALT, AST, total protein, albumin, and glucose levels compared with the BA and INC groups, suggesting that the synthetic function and glucose metabolism were more severely damaged in the NICCD.
group than in the other two groups. The NICCD group also had significantly higher TBA and lower D-Bil and cholesterol levels compared with the BA and INC groups, indicating that the excretion of bile acids, D-Bil and cholesterol might be affected differently in NICCD patients. Significantly lower T-Bil and GGT levels were noticed in the NICCD group only when compared with the BA group.

Comparison of ratios of biochemical indices

To compare further the different biochemical indices, a series of ratios was calculated. The highest ratio of D-Bil to T-Bil was found in the BA group and the lowest in the NICCD group (Table 2, Figure 1F). A much higher AST/ALT ratio was found in the NICCD group compared to the INC and BA groups (Table 2).

The ratios between D-Bil, bile acids and cholesterol were also compared (Table 2). The ratio of serum TBA to D-Bil was significantly higher in the NICCD group than the ratios in the BA and INC groups (Figure 1G, \(P < 0.05\)). Significant differences were also found for the ratio of TBA to TCH between the NICCD and BA groups and between the NICCD and INC groups (Figure 1H, \(P < 0.05\)).

DISCUSSION

Citrin deficiency is one of the most common classical inborn errors of metabolism of amino acids, organic acids and fatty acid oxidation in Eastern Asia, including China. The biochemical characteristics and mechanism of cholestasis caused by citrin deficiency are still not fully understood. Although differences in some indices among patients with BA, INC and NICCD have been reported previously, the very small sample sizes of the studies precluded a definite conclusion. In the present study, the cohorts of NICCD, INC and BA had numbers large enough to test previous findings. Additionally, by comparing the elevation of D-Bil, TBA and cholesterol levels and the ratios of these compounds, it was found that the excretion of bile acids appeared to be more severely affected in NICCD than in BA and INC.

A previous study with a small number of subjects demonstrated that patients with cholestasis caused by citrin deficiency had lower ALT and AST levels and higher AST to ALT ratios compared to those with BA or idiopathic neonatal hepatitis. Low albumin and glucose levels were also associated with NICCD in a previous case series. In the present study, those findings were confirmed. Previous studies also showed that patients with NICCD had lower birth weight compared to normal controls or to the national standard. In our study, although a lower median birth weight in the NICCD group was noticed, the differences did not reach statistical significance compared to patients with BA or INC. This could be explained by the different control groups (normal control or national standard used in previous studies vs patients with cholestasis of other causes) and the large difference observed within the NICCD group in this study.

The serum TBA level in NICCD has previously been compared with that in BA and INC in a study that had very few subjects. In the present study, the serum level of TBA as well as the ratio of serum TBA to D-Bil and cholesterol levels was compared. In BA, we can suppose that excretion of D-Bil, bile acids and cholesterol is affected to the same extent in consideration of complete blockage of the biliary system. If the ratio of TBA to D-Bil in the BA group was taken as the reference value, the median for INC was found to be 1.22 (1.04/0.85) times higher.

### Table 2 Comparison of birth weight, biochemical indices and their ratios among the three groups

| Reference range and unit | BA (\(n = 39\)) | INC (\(n = 39\)) | NICCD (\(n = 32\)) |
|--------------------------|-----------------|-----------------|--------------------|
| Birth weight             |                |                 |                    |
| 2.5-4.0 kg               | 3.2 ( median)   | 2.9-3.8         | 3.1 ( median)      |
| Biochemical indices      |                 |                 |                    |
| T-Bil**                  | 2.20 mmol/L     | 159.5 ( median) | 190.0 ( median)    |
| D-Bil**                  | 0.6 mmol/L      | 134 ( median)   | 115.9 ( median)    |
| ALT**< 40 IU/L           | 108 ( median)   | 133.8 ( median) | 90.1 ( median)     |
| AST**< 40 IU/L           | 153 ( median)   | 87.3 ( median)  | 60.2 ( median)     |
| GGT< 50 IU/L             | 558 ( median)   | 130.5 ( median) | 81.0 ( median)     |
| TBA**< 40 mmol/L         | 112 ( median)   | 155 ( median)   | 91.0 ( median)     |
| Total protein            | 57.4 ( median)  | 57.2 ( median)  | 52.5 ( median)     |
| Albumin                 | 112 ( median)   | 39.9 ( median)  | 37.0 ( median)     |
| Glucose**                | 4.1 ( median)   | 4 ( median)     | 3.4 ( median)      |
| TCH**2.8-5.20 mmol/L     | 4.57 ( median)  | 4 ( median)     | 3.2 ( median)      |

Ratios

| D-Bil/T-Bil**            | 0.77 ( median)  | 0.74 ( median)  | 0.59 ( median)     |
| AST/ALT**               | 1.38 ( median)  | 1.48 ( median)  | 1.10 ( median)     |
| TBA/D-Bil**             | 0.85 ( median)  | 1.0 ( median)   | 0.92 ( median)     |
| D-Bil/TCH**             | 30.2 ( median)  | 21.5 ( median)  | 16.7 ( median)     |
| TBA/TCH**               | 24.7 ( median)  | 24.2 ( median)  | 21.4 ( median)     |

\*\(P < 0.05\) between BA and INC; \(P < 0.05\) between BA and NICCD; \(P < 0.05\) between INC and NICCD. NICCD: Neonatal intrahepatic cholestasis caused by citrin deficiency; BA: Biliary atresia; INC: Idiopathic neonatal cholestasis; TBA: Total bile acid; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: \(\gamma\)-glutamyltranspeptidase; TCH: Total cholesterol.
and that of NICCD 3.95 (3.36/0.85) times higher. For the ratio of TBA to cholesterol, if the median value of the BA group was taken as a standard, the median in the INC group was nearly the same as the standard but that in the NICCD group was 2.46 (60.7/24.7) times higher. These results indicate that the excretion of bile acids is much more severely affected than the excretion of bilirubin and cholesterol in NICCD patients. As a consequence, we may speculate that the failure to excrete bile acids from hepatocytes to the canalicula is the main mechanism of cholestasis caused by citrin deficiency.

The main limitation of this study was its retrospective nature. It could be argued that some biochemical indices were affected by the drugs that patients were taking. However, prior to the determination of a clear diagnosis, the management of patients had been similar in the three groups; therefore, the patients in the different groups would have been affected by these variables in the same way. Another measure that was used to avoid sample bias was using the first available laboratory data obtained when patients were referred to us. Although significant differences in TBA and TBA ratios were found between the NICCD and other two groups, no cut-off levels can be presented at this time.
COMMENTS

Background
Citrin deficiency is one of the most common metabolic disorders in Eastern Asia. It has at least two main phenotypes: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and adult-onset type II citrullinemia. The clinical features of and the mechanism of cholestasis in NICCD have yet to be established.

Research frontiers
Some biochemical indices of patients with NICCD have been compared to those of patients with biliary atresia (BA) and of patients with idiopathic neonatal cholestasis (INC). Comparison of these biochemical indices in neonatal cholestasis cases with different etiologies will better characterize the biochemical changes of the disease and may further the understanding of the mechanism of cholestasis caused by citrin deficiency.

Innovations and breakthroughs
Apart from confirming previous findings that NICCD patients had significantly lower alanine aminotransferase (ALT) level, lower direct bilirubin (D-Bil) to total bilirubin ratio, and significantly higher aspartate aminotransferase to ALT ratio compared to the BA and INC patients, this study specifically compared the serum level of total bile acid (TBA) and its ratio to D-Bil and cholesterol, and found that NICCD patients had significantly higher TBA levels as well as higher TBA to D-Bil and TBA to cholesterol ratios than patients with BA and INC.

Applications
The excretion of TBA appears to be much more severely disturbed than that of D-Bil and cholesterol in cholestasis caused by citrin deficiency. Further study of this condition will help elucidate the mechanism of cholestasis in NICCD, and the ratios could be further developed as indices for the differential diagnosis of neonatal cholestasis.

Peer review
The authors present an interesting retrospective study comparing liver specific biochemical parameters in different groups of infants with cholestasis.

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