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***Retrospective study***

**Autoimmune liver diseases-related autoantibodies in patients with biliary atresia**

Pang S *et al*. Autoantibodies in biliary atresia

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**Abstract**

***AIM***

To investigate the prevalence and clinical significance of autoimmune liver diseases (ALDs)-related autoantibodies in patients with biliary atresia (BA).

***METHODS***

Sera of 124 BA patients and 140 age-matched non-BA controls were assayed for detection of the following autoantibodies: ALD profile and specific anti-nuclear antibody (ANA), by line-blot assay; ANA and anti-neutrophil cytoplasmic antibody (ANCA), by indirect immunofluorescence assay; specific ANCA and anti-M2-3E, by enzyme linked immunosorbent assay. Associations of these autoantibodies with the clinical features of BA (*i.e.*, cytomegalovirus infection, degree of liver fibrosis, and short-term prognosis of Kasai procedure) were evaluated by Spearman’s correlation coefficient.

***RESULTS***

The overall positivity of serum autoantibodies in preoperative BA patients was 56.5%. ALD profile assay showed that the positive reaction to primary biliary cholangitis-related autoantibodies in BA patients was higher than to autoimmune hepatitis-related autoantibodies. Among these autoantibodies, anti-BPO was detected more frequently in the BA patients than in the controls (14.8% *vs* 2.2%, *P <* 0.05). Accordingly, 32 (25.8%) of the 124 BA patients also showed a high positive reaction for anti-M2-3E. By comparison, the controls had a remarkably lower frequency of anti-M2-3E (*P* < 0.05), with 6/92 (8.6%) of patients with other liver diseases and 2/48 (4.2%) for healthy controls. The prevalence of ANA in BA patients was 11.3%, which was higher than the disease controls (3.3%, *P* < 0.05), but the reactivity to specific ANA was only 8.2%. The prevalence of ANCAs (ANCA or specific ANCA) in BA patients was also remarkably higher than in the healthy controls (37.9% *vs* 6.3%, *P* < 0.05), yet showed no difference from that in patients with other cholestasis. ANCAs positivity was closely associated with the occurrence of postoperative cholangitis (*r* = 0.61, *P* < 0.05), whereas none of the autoantibodies showed correlation to cytomegalovirus infection or the stages of liver fibrosis.

***CONCLUSION***

High prevalence of autoantibodies in the BA developmental process strongly reveals the autoimmune-mediated pathogenesis. Serological ANCAs positivity may be a useful predictive biomarker of postoperative cholangitis.

**Key words:** Biliary atresia; Anti-nuclear antibody; Anti-neutrophilic cytoplasmic antibody; Autoimmune liver diseases; Autoantibodies

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**Core tip:** The autoimmune-mediated pathogenesis of biliary atresia (BA) is not fully understood, and non-invasive diagnostic methods cannot clearly discriminate BA from other causes of neonatal cholestasis. We investigated the prevalence and clinical significance of autoimmune liver diseases-related autoantibodies in BA patients. The overall positive rate of autoantibodies in BA was 56.5%. The data showed that frequent detection of autoantibodies in BA may strongly support the autoimmune-mediated pathogenesis. Interestingly, preoperative anti-neutrophil cytoplasmic antibodies positivity was closely associated with prediction of cholangitis occurrence after Kasai portoenterostomy.

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**INTRODUCTION**

Biliary atresia (BA) is a severe neonatal disease, characterized by a progressive inflammatory fibrosis and obliteration of both the intra-hepatic and extra-hepatic bile ducts[1,2]. Early Kasai portoenterostomy (KP), the first-line treatment of BA, may re-establish bile flow to alleviate injury of the liver caused by the cholestasis and to prolong survival with the native liver[3]. However, in the majority of BA patients, the continued existence of the bile duct injury may eventually lead to cirrhosis and need for liver transplantation[4,5]. Thus, gaining a better understanding of the pathogenic mechanisms underlying BA may facilitate early diagnosis or development of clinical therapies to halt the injury of hepatic bile ducts and to preserve liver function.

Though the etiology of BA is not fully understood, accumulated evidence in the literature supports the theory that a primary perinatal viral infection triggers an aberrant autoimmune-mediated attack on bile duct epithelia by molecular mimicry, with both the cellular and humoral immunity playing important roles in the BA autoimmune injury mechanism[6-9]. Several viruses have been proposed as the infectious agents, and perinatal infection with the cytomegalovirus (CMV) has been demonstrated as an important etiological factor for BA in China[10]. Periductal immunoglobulin (Ig) and circulating autoantibodies which might be used in the classification of autoimmune diseases have been described in both patients with BA and animal models of the disease[11,12]; unfortunately, the specificity of these autoantibodies for BA has been far less satisfying.

BA and autoimmune liver disease (ALD) have some similar clinical manifestations and pathological features. However, ALD-related autoantibodies have not yet been comprehensively investigated in BA patients, to the best of our knowledge. Here, we describe our investigation into the prevalence of the ALD profile and extent positivity of anti-nuclear antibodies (ANAs) and anti-neutrophilic cytoplasmic antibodies (ANCAs) in the sera of BA patients. The associations of these autoantibodies and the clinical features of BA were also assessed statistically.

**MATERIALS AND METHODS**

***Case enrollment***

A total of 124 preoperative BA patients [mean age: 2.9 mo (interquartile range, IQR: 1.9-3.0)], 92 other liver disease controls [mean age: 2.8 mo (IQR: 2.0-3.2); including 42 with choledochal cysts, 35 with transient cholestasis of unknown origin and 15 with neonatal intrahepatic cholestasis caused by citrin deficiency] and 48 healthy controls [mean age: 3.4 mo (IQR: 2.0-4.0)] were enrolled in this study. Table 1 shows the demographic and clinical features, and biochemical parameters of the study population. All study participants originated from Guangzhou Women and Children’s Medical Center (Guangzhou, China) between January 2015 and December 2016. Enrollment was proposed to all consecutive infants with diagnosed BA that had been confirmed by surgical exploration, cholangiography and histology. Diagnosis of all controls was based on the criteria published in our previous reports[13]. Clinical information was collected when available, including CMV infection, biochemical indexes, histological liver fibrosis stages and short-term outcomes. Histological liver fibrosis of BA was assessed by METAVIR fibrosis scores (F0-F4)[14,15]. Follow-up data which could evaluate persistence of jaundice (total bilirubin, TB: > 34 μmol/L), acute liver injury (alanine aminotransferase, ALT: > 35 U/L), and occurrence of cholangitis within 3-10 mo after KP were collected for analysis of short-term outcomes[16].

***Autoimmune liver diseases profile***

The line-blot ALD profile contains the primary biliary cholangitis (PBC)-related antibodies [anti-mitochondrial antibody,AMA-M2 (pyruvate dehydrogenase complex, PDC), anti-BPO (recombinant fusion proteins of the E2 subunits derived from the 2-oxo-acid dehydrogenase complex targeted by the inner mitochondrial membrane), anti-Sp100, anti-promyelocytic leukemia protein (PML) and anti-gp210], autoimmune hepatitis (AIH)-related antibodies [anti-liver-kidney microsomes type 1 (LKM-1), anti-liver cytosolic antigen type 1 (LC-1), and anti-soluble liver antigen/liver pancreas (SLA/LP)], and anti-Ro-52 antibodies. The commercially available kit (EUROIMMUN AG, Lübeck, Germany) was used according to the manufacturer’s instructions. Signal strengths of > 10 arbitrary unit (AU) were considered positive.

AMA-reactivity was confirmed, with enlarged sample size by an enhanced anti-M2-3E enzyme linked immunosorbent assay (ELISA), which mixed enveloped recombinant fusion protein BPO and natively-purified PDC from bovine heart mitochondria as antigenic targets (EUROIMMUN AG).

***Anti-nuclear antibody and specific anti-nuclear antibody***

ANA was detected by indirect immunofluorescence (IIF) using the antigen substrate panel of Hep-2 cells and primate liver. A serum titer of ≥ 1:100 was considered positive. ANA positivity was subgrouped based upon the specific fluorescence patterns. Accordingly, line-blot immunoassay was used to determine the IgG autoantibodies panel for 12 specific ANAs, which consisted of anti-nRNP/Sm, anti-Sm, anti-SS-A, anti-Ro-52, anti-SS-B, anti-Scl-70, anti-Jo-1, anti-CENP B, anti-dsDNA, anti-nucleosomes, anti-histone and anti-ribosomal phosphoprotein. Experiments were performed following the manufacturer’s instructions (EUROIMMUN AG).

***Anti-neutrophil cytoplasmic antibodies and specific anti-neutrophil cytoplasmic antibodies***

A commercially available IIF assay was used for determination of ANCA on ethanol- and formaldehyde-fixed human neutrophils (EUROIMMUN AG). A positive ANCA finding was defined as a titer of antibodies > 1:10. The ANCA findings were subgrouped into cytoplasmic (c)-ANCA, perinuclear (p)-ANCA and atypical (a)-ANCA according to the fluorescence patterns. Specific ANCA of myeloperoxidase (MPO) and proteinase 3 (PR3) were further assayed by ELISA (EUROIMMUN AG).

***Association of autoantibodies with clinical features***

To determine whether the presence of autoantibodies in BA patients was associated with worse disease progression, we compared the clinical features of the BA patients who presented with and without autoantibodies. The clinical features of 124 BA patients (mainly composed of those with CMV infection) and degree of liver fibrosis were retrospectively analyzed for the period prior to the KP; in addition, the information of short-term outcomes in 52 BA patients were collected based on the postoperative follow-up for > 3 mo, and 24 of those 52 cases were re-assessed for serum autoantibodies at the time of pre-operation and post-operation, with comparison of the change of autoantibodies over time.

***Statistical analysis***

Normally distributed variables were represented as mean ± SD, and non-normally distributed variables as median (IQR). Categorical data were described as frequencies and/or percentages. For continuous variables, between-group differences were compared using theStudent’s *t*-test or the Mann-Whitney *U* test. For categorical variables, the *χ 2* test or Fisher’s exact test was used to compare the prevalence between groups when appropriate. Correlation was evaluated by the Spearman’s correlation coefficient. SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Armonk, NY, United States) was used to perform all statistical analyses. *P* values < 0.05 were considered statistically significant.

**RESULTS**

***Autoimmune liver diseases profile in patients with biliary atresia compared to controls***

Sera of 81 postoperative BA and 88 non-BA controls were evaluated for ALD profile (Table 2). The 88 non-BA controls consisted of 40 healthy controls and 48 disease controls, including 22 with choledochal cysts, 14 with neonatal intrahepatic cholestasis caused by citrin deficiency and 12 with transient cholestasis of unknown origin. One or more of the PBC-related antibodies was detected in 18.5% (15/81) of the patients with BA. For the PBC-related antibodies, a positive reaction to AMA-M2, anti-BPO, anti-Sp100, anti-gp210 and anti-PML in BA patients was found in 1.2%, 14.8%, 1.2%, 2.5% and 3.7% respectively. Among these autoantibodies, anti-BPO was detected more frequently in the BA patients than in the non-BA controls (14.8% *vs* 2.2%, *P* < 0.05) or the healthy controls (14.8% *vs* 0%, *P* < 0.05). The prevalence of any other antibodies was not different between the BA and non-BA groups. Only 1 in 81 of the BA patients (1.2%) showed positivity for both AMA-M2 and anti-BPO (Figure 1). For the AIH-related antibodies, low positivity was found in both the BA patients and non-BA controls (7.4% and 6.8% respectively, *P* > 0.05). Among the total 81 postoperative BA patients, 5 (6.2%) and 1 (1.2%) showed positivity for anti-LC-1 and anti-SLA/LP.

With enlarged sample size, AMA reactivity was confirmed by an enhanced anti-M2-3E ELISA. As presented in Figure 2, 32 of the 124 BA patients showed a higher positive reaction to anti-M2-3E compared to the disease controls and the healthy controls (25.8% *vs* 8.6%, *P* < 0.05 and 25.8% *vs* 4.2%, *P* < 0.05 respectively).

***Anti-nuclear antibody and specific anti-nuclear antibody in patients with biliary atresia*** ***compared to controls***

ANA exhibited a higher prevalence in 124 BA patients compared to 92 patients with other liver diseases (11.3% *vs* 3.3%, *P* < 0.05), but showed no difference from that in 48 healthy controls (4.2%). Nuclear homogeneous, speckled and nucleolar types were the main fluorescence patterns of ANA positivity in BA (Table 3). Patterns involving nuclear dots, centrosome, cytoplasm, ring or rod Golgi, and centromere were present occasionally.

The specific ANA findings of the BA patients evaluated in this study were as follows (Table 3): positivity for anti-SSA in 1, for anti-Ro-52 in 5, for anti-CENP B in 4, and for anti-dsDNA in 3. At least one autoantibody was present in 8.9% (11/124) of the BA patients, which was a high rate compared to the non-BA controls, but the difference did not reach the threshold for statistical significance. No other specific ANA was found in the sera of BA patients.

***Anti-neutrophil cytoplasmic antibodies and specific anti-neutrophil cytoplasmic antibodies in patients with biliary atresia compared to controls***

Twenty-nine percent (36/124) of BA patients were positive for ANCA. The prevalence was similar to that of the patients with other liver diseases (25.0%, *P* > 0.05), and was higher than that of the healthy controls (4.2%, *P* < 0.05) (Table 4). As shown in Figure 3, p-ANCA and c-ANCA were present more frequently than a-ANCA in BA patients (20.2% and 8.1% compared to 0.8% respectively)

Anti-MPO and anti-PR3, alone or associated, were found in 23/124 (18.0%) of BA patients, whose positivity rates were higher than those in healthy controls (*P* < 0.05). Among the 124 BA patients, 7 showed positive reactions for both anti-MPO and anti-PR3. The prevalence of ANCAs (ANCA, anti-MPO or anti-PR3) in BA patients was higher than that in healthy controls (37.9% *vs* 6.3%, *P* < 0.05).

***Association of autoantibodies with clinical features***

In this study, anti-M2-3E, ANA and ANCAs had higher prevalence in 124 BA patients than in 140 non-BA controls. Among the BA patients, 56.5% showed positivity for at least one type of the autoantibodies (anti-M2-3E, ANA and ANCAs). Among the 80 BA patients, 45% had a perinatal infection with CMV (detected by combined analysis of anti-CMV IgG, IgM and CMV DNA). The percentage of CMV infection in the anti-M2-3E-positive BA patients was similar to that in the anti-M2-3E-negative BA patients (51.9% *vs* 41.5%, *P >* 0.05). The prevalence of CMV infection in ANCAs-positive BA patients was higher than that in the ANCAs-negative BA patients (54.3% *vs* 37.8%, *P >* 0.05). There was no statistical relationship found between the presence of these autoantibodies and CMV infection.

Regardless of the severity of fibrosis, anti-M2-3E, ANA or ANCAs were present in the sera of BA patients. The positivity rate of these autoantibodies showed no difference according to extent of fibrosis (F0-F4) in the 124 BA patients (anti-M2-3E: 25.0%, 27.0%, 16.7%, 32.0% and 22.2% respectively, *P >* 0.05; ANA: 25.0%, 5.4%, 12.5%, 10.0% and 22.2% respectively, *P >* 0.05; ANCA: 50.0%, 30.0%, 41.7%, 32.0% and 44.4% respectively, *P >* 0.05).

Interestingly, as presented in Table 5, the prevalence of ANCAs showed positive correlation to the occurrence of postoperative cholangitis (*r* = 0.61, *P* < 0.05). Thus, the probability of ANCA negativity in postoperative cholangitis is very low. ANCA positivity did not correlate with the persistence of jaundice or acute liver injury, nor did anti-M2-3E positivity or ANA positivity show a statistical association with prognosis for the procedure.

The longitudinal (follow-up) findings for autoantibodies showed that 24 BA patients exhibited slight variation from the first evaluation to re-assessment within an average of 6 mo, but without statistically significant differences between the paired samples.

**DISCUSSION**

In an earlier study, Hadchouel *et al*[11] found IgG deposits on the basement membranes of glandular formations in about one-third of biliary remnants studied. Accumulating evidence suggests that the outset of BA may be triggered by an initial perinatal hepatobiliary viral infection[7,17]. Studies have also demonstrated that CMV infection initiates the autoimmune process in BA by targeting intrahepatic biliary epithelial cells of the host, and that the only reactivity to the α-enolase antibody identified in BA was non-specific in human BA[10,18-20]. To date, serological specificity is too low for the diagnosis of BA, making it impossible to avoid damage from invasive surgery. Thus, the aim of this study was to seek a non-invasive biomarker which can discriminate BA from other causes of neonatal cholestasis or predict prognosis.

In the present study, 56.5% of BA patients were positive for at least one type of the autoantibodies. The presence of autoantibodies in the BA patients was not an epiphenomenon, and high prevalence of autoantibodies in BA provided evidence of an autoimmune-mediated pathogenesis.

For PBC-related autoantibodies, 18.5% of the BA patients showed positivity for one or more autoantibodies. Particularly, anti-BPO was present in 14.8% (12/81) of the BA patients, which was detected at a higher frequency than that in the non-BA controls or healthy subjects but at a lower frequency than that in the PBC patients (84.5%)[21]. To our knowledge, this was the first time anti-BPO has been detected in BA infants, as it is often reported in adults but has been rarely reported in pediatric age patients[22]. Compared with PBC, the sensitivity of both AMA-M2 and anti-BPO was relatively lower in BA patients, and the positivity of anti-BPO was higher than that of AMA-M2.

This finding hinted that the corresponding antigen epitope of AMA in BA serum was different from PBC, whose reaction to E2 subunits of the oxo-glutarate dehydrogenase complex and branched-chain oxo-acid dehydrogenase complex were higher than that to PDC-E2. By enlarging the sample size and adopting ELISA to further confirm the detection of AMA in BA, we determined that 32 (25.8%) of 124 BA patients had a positive reaction for anti-M2-3E. Yet, the markedly increased anti-M2-3E positivity was not associated with CMV infection, severity of liver fibrosis or prognosis of the Kasai procedure in BA.

It has been reported that anti-PML and anti-Sp100 are co-immunogenic in PBC patients, correlating with an unfavorable disease course and a fast progression[23,24]. Our findings showed that the positivity for anti-Sp100, anti-gp210 and anti-PML in BA was rather low, and the co-existence of anti-Sp100 and anti-PML only occurred in a single BA patient, whose liver damage developed rapidly and seriously according to the diagnosis of pathological biopsy. Anti-gp210 is related to severe disease course and poor prognosis of PBC. PBC patients who are positive for anti-gp210 will progress into liver failure easier[25].

In our study, a total of 4 study participants displayed anti-gp210 positivity, including 2 patients with BA, 1 patient with choledochal cysts, and 1 healthy infant. The 2 patients with BA presented with biliary cirrhosis, while the 1 patient with choledochal cysts showed middle-stage liver damage. We were unsure whether the anti-gp210 in the healthy infant appeared earlier than the clinical manifestations, possibly as an alerting signal, similar to AMA-M2 in PBC. The AIH-related antibodies showed low positive rates in both BA and control groups. Among these autoantibodies, anti-LKM was not detected in any of the BA patients, which was consistent with the previous report by A-Kader *et al*[26].

# For ANA, our study detected it at a higher frequency (11.3%) in BA patients than the previous report of 5%[26]. The prevalence was also significantly higher than that in patients with other liver diseases in our study. ANA, presenting mostly with a speckled pattern, is reportedly positive in approximately 6% of healthy children, which is similar to that found in the disease controls and healthy controls of our study[27,28]. As is known, ANA are fundamental for the diagnosis of a variety of autoimmune diseases, and the clinical value of ANA testing for autoimmune liver diseases is beyond doubt[29,30]. In our study, neither ANA nor specific ANA exhibited any correlation to stages of liver fibrosis or prognosis of the KP procedure.

# Both positivity and the titer of ANA are related to age of the autoimmune disease patient (being lower in childhood disease than in adulthood disease)[31,32]. Of note, the average age of BA patients in our study was 2.9 mo, a period in which nearly all of the IgG is maternally-derived. Thus, it is possible that maternally-derived IgG may cause development of the disease. This is in line with the findings of Hannam *et al*[33], who documented cases where placental transfer of maternal AMA was associated with neonatal liver disease. Based on these data, longitudinal analysis of ANA in BA patients was performed (comparison of detection at the first evaluation and at re-assessment within an average 6 mo of follow-up). There were no significant differences between the preoperative and the postoperative findings, suggesting that the autoantibodies that arose in the BA patients may not be transient.

# In addition, the prevalence of ANCA (29.0%) was remarkably higher in sera from BA patients than in that of healthy controls, similar to the positivity rates detected for the special ANCA, which included anti-MPO and anti-PR3. However, the detection of ANCA in BA did not correspond fully with other reports. In a preliminary observation[34], ANCA was detected in 91% of patients with BA, and was suggested as a promising biomarker for an immune-mediated process directed against specific hepatobiliary antigens; however, in another report by A-Kader *et al*[26], the ANCA was positive in only 1 of 20 patients. These differences in findings might reflect the differences in age of the patients studied or in the study methods applied.

# In our study, we found that ANCAs positivity in BA patients appeared more frequently with postoperative cholangitis than did ANCAs negativity. As is known, ANCA is not only a helpful tool for establishing the diagnosis of Wegener’s granulomatosis and microscopic polyangiitis but also appears with non-vascular chronic inflammatory diseases. The presence of ANCA has also been associated with the occurrence of relapses in AIH, with decreased liver synthesis function in PBC and with increased cholestasis in primary sclerosing cholangitis[12,35]. One of the minor antigen targets of antineutrophil cytoplasmic antibodies present in BA has been shown to be α-enolase, which is an enzyme involved in glycolysis and is ubiquitously expressed in a variety of cells, including biliary epithelial cells and hepatocytes[12]. It hinted that neutrophils may be activated in BA. The priming of neutrophils with tumor necrosis factor- induces a marked expression of ANCA antigens on the cell surface[36,37]. The expression of these target antigens facilitates the interaction with ANCA antibodies, resulting in subsequent polymorphonuclear leukocyte activation, which then induces production of reactive oxygen species, release of superoxide and degranulation of neutrophils. ANCA was also reported to be involved in fibrosis of the lung and kidney[38,39]; however, we found that the rates of ANCA positivity were not significantly different in BA patients with fibrosis from stages F0 to F4, indicating that these antibodies might not be associated with the severity of liver fibrosis necessarily.

In summary, we found that prevalence of ANCAs increase prominently in BA, which showed positive correlation to the occurrence of postoperative cholangitis. Although anti-M2-3E was not associated with prognosis of the KP procedure, its high prevalence provided evidence of autoimmune activity in the pathogenesis of BA. Thus, autoantibodies in sera of BA were not an accidental epiphenomenon. Future research in multi-centers focusing on identifying potentially pathogenic, bile duct-specific autoantibodies, the titre and source of autoantibodies in BA should be pursued.

**Article Highlights**

***Research background***

Accumulating evidence supports the biliary atresia (BA) pathogenesis theory of a primary perinatal viral trigger that is followed by an aberrant autoimmune-mediated attack on bile duct epithelia, resulting in inflammatory and fibrosing obstruction of the bile ducts. Similarly, both primary biliary cholangitis and autoimmune hepatitis are characterized by autoimmune-mediated injury to liver cells and biliary ducts, and autoimmune liver disease (ALD)-related autoantibodies play a crucial role in the accurate classification for such. The authors hypothesized that ALD-related autoantibodies would be also associated with BA.

***Research motivation***

No previous study has comprehensively evaluated the prevalence of ALD-related autoantibodies and their roles in prognosis of BA. The authors attempted to explore the ALD-related autoantibodies in BA sera.

***Research objectives***

The authors try to search appropriate non-invasive biomarker for diagnosis and prognosis of BA.

***Research methods***

The authors collected the sera of BA and evaluated the prevalence of ALD-related autoantibodies using multiple methods, including the autoimmune liver diseases profile and specific anti-nuclear antibody (ANA) by line-blot assay; ANA and anti-neutrophil cytoplasmic antibody (ANCA) by indirect immunofluorescence assay; specific ANCA and anti-M2-3E by enzyme linked immunosorbent assay, respectively. Simultaneously, associations of these autoantibodies with the clinical features of followed-up BA were evaluated by Spearman’s correlation coefficient.

***Research results***

The overall positivity of serum ALD-related autoantibodies in BA patients was 56.5%. The prevalence of anti-M2-3E, ANAs and ANCAs were significantly increased in a large cohort of infants with BA. In addition, the authors found that the ANCAs showed positive correlation to the occurrence of postoperative cholangitis.

***Research conclusions***

High prevalence of ALD-related autoantibodies in the BA developmental process strongly reveals the autoimmune-mediated pathogenesis. ANCA positivity may be a useful serum biomarker to predict postoperative cholangitis of BA.

***Research perspectives***

Our future research will focus on identifying potentially pathogenic, bile duct-specific autoantibodies, the titre and source of autoantibodies in BA.

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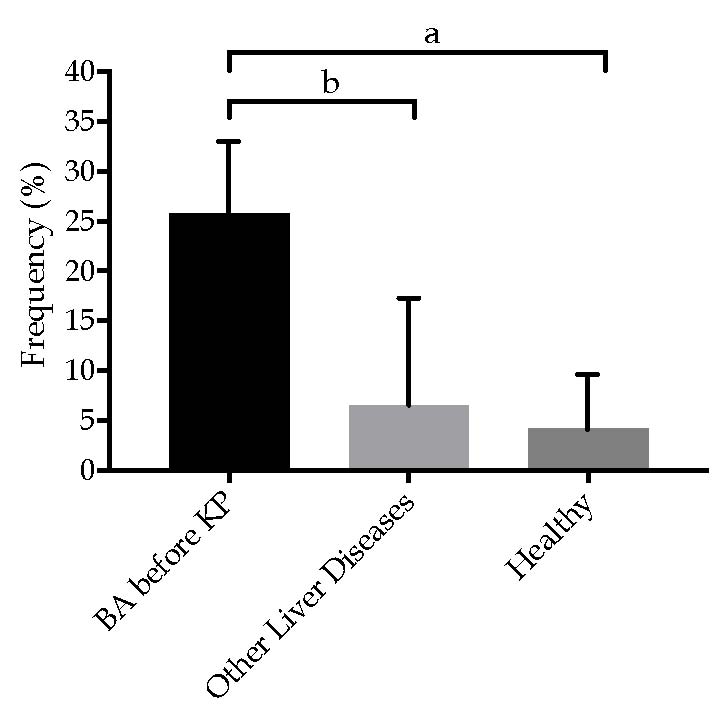
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Figure%201%20updated-11042017

**Figure 1 Representative strips after color development by line-blot immunoassay.** The line-blot immunoassay strips had been coated with nine autoimmune liver diseases-related antigens, including Ro-52, SLA/LP, LC-1, LKM-1, gp210, PML, Sp100, BPO and AMA-M2 (from left to right). BA group: ALD/93-57 with anti-BPO +, ALD/87-31 with anti-Ro-52 +++, anti-PML +, anti-Sp100 +, anti-BPO ++ and AMA-M2 +, ALD/89-53 with anti-BPO +; Other liver diseases group: Only ALD/198-59 with anti-LC-1 ±; Healthy group: All autoantibodies negative. Positive control (ALD/52-75) showed anti-BPO +++ and AMA-M2 +++. Other liver diseases include choledochal cysts, transient cholestasis of unknown origin and neonatal intrahepatic cholestasis caused by citrin deficiency. ALD: Autoimmune liver disease; BA: Biliary atresia; LC-1: Liver cytosolic antigen type 1.

**Figure** **2 Positivity of anti-M2-3E by enzyme linked immunosorbent assay in biliary atresia patients and controls.** a*P* < 0.05 *vs* healthy controls; b*P* < 0.05 *vs* other liver diseases. Other liver diseases include choledochal cysts, transient cholestasis of unknown origin and neonatal intrahepatic cholestasis caused by citrin deficiency. BA: Biliary atresia; ELISA: Enzyme linked immunosorbent assay.Figure%203b%20cropped

**Figure 3 The main fluorescence patterns of anti-neutrophil cytoplasmic antibodies in biliary atresia patients.** ANCA detection by indirect immunofluorescence assays was performed on ethanol-fixed (upper panel) and formaldehyde-fixed (lower panel) human neutrophils. Depending on whether formaldehyde-fixed human neutrophils reactivities were positive or not, p-ANCA was divided into p-ANCA with formaldehyde resistance and p-ANCA with formaldehyde sensitivity. ANCA: Anti-neutrophil cytoplasmic antibodies; BA: Biliary atresia; (c)-ANCA: Cytoplasmic-ANCA; (p)-ANCA: Perinuclear-ANCA.

**Table 1 Demographic and clinical features, and biochemical parameters1 of biliary atresia patients and non-biliary atresia controls *n* (%)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Variable** | **BA,**  ***n* = 124** | **Non-BA,**  ***n* = 140** | | | |
| **Other liver diseases2,**  ***n* = 92** | | **Healthy,**  ***n* = 48** |
| Age, mo | 2.9 (1.9-3.0) | 2.8 (2.0-3.2) | 3.4 (2.0-4.0) | | |
| Sex, male/female | 60/64 | 50/42 | 25/23 | | |
| METAVIR score |  |  |  | | |
| F0 | 4 (3.2) | NA | NA | | |
| F1 | 37 (29.8) | NA | NA | | |
| F2 | 24 (19.4) | NA | NA | | |
| F3 | 50 (40.3) | NA | NA | | |
| F4 | 9 (7.3) | NA | NA | | |
| ALT, U/L | 162.4 (104.3-204.6)a | 132.0 (70.5-247.5) | 28.1 (22.4-40.8) | | |
| AST, U/L | 190.5 (151.4-257.4)a | 195.9 (117.0-292.5) | 38.0 (26.0-52.3) | | |
| γ-GT, U/L | 716.0 (411.0-1142.5)a | 598.4 (189.5-1043.2) | 32.7 (23.4-46.3) | | |
| ALP, U/L | 406.8 (316.5-522.2)a | 517.0 (409.7-660.3) | 275.8 (189.0-345.6) | | |
| TBIL, μmol/L | 157.0 (126.5-184.0)a | 145.7 (107.1-196.5) | 6.5 (2.8-14.8) | | |
| DBIL, μmol/L | 130.5 (103.5-152.7)a | 110.1 (88.4-137.8) | 3.1 (1.0-4.9) | | |

Data are described as median (interquartile range: 25th-75th percentile). 1Reference intervals: ALT, 3-35 U/L; AST, 5-60 U/L; γ-GT, 13-57 U/L; ALP, 118-390 U/L; TBIL, 2-17 μmol/L; DBIL, 0-7 μmol/L. 2Choledochal cysts, transient cholestasis of unknown origin and neonatal intrahepatic cholestasis caused by citrin deficiency, included as disease controls. a*P* < 0.05 *vs* healthy controls. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BA: Biliary atresia; DBIL: Direct bilirubin; FO-F4: Fibrosis scores 0-4; γ-GT: Gamma-glutamyl transpeptidase; NA: Not applicable; TBIL: Total bilirubin.

**Table 2 Prevalence profile of autoimmune liver disease in biliary atresia patients and non-biliary atresia controls *n* (%)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variable** | **BA,**  ***n* = 81** | **Non-BA,**  ***n* = 88** | | |
| **Other liver diseases1,**  ***n* = 44** | **Healthy, *n* = 40** | **Total** |
| PBC-related antibodies | 15 (18.5)c | 4 (9.1) | 1 (2.5) | 5 (5.7) |
| AMA-M2 | 1 (1.2) | 0 (0) | 0 (0) | 0 (0) |
| Anti- BPO | 12 (14.8)a,c | 2 (4.5) | 0 (0) | 2 (2.2) |
| Anti-Sp100 | 1 (1.2) | 0 (0) | 0 (0) | 0 (0) |
| Anti-gp210 | 2 (2.5) | 1 (2.3) | 1 (2.5) | 2 (2.2) |
| Anti-PML | 3 (3.7) | 1 (2.3) | 0 (0) | 1 (1.1) |
| AMA-M2 + anti-BPO | 1 (1.2) | 0 (0) | 0 (0) | 0 (0) |
| AMA-M2 + anti-BPO + anti-Sp100 + anti-PML | 1 (1.2) | 0 (0) | 0 (0) | 0 (0) |
| AIH-related antibodies | 6 (7.4) | 3 (6.8) | 3 (7.5) | 6 (6.8) |
| Anti-LKM-1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Anti-LC-1 | 5 (6.2) | 3 (6.8) | 3 (7.5) | 6 (6.8) |
| Anti-SLA/LP | 1 (1.2) | 0 (0) | 0 (0) | 0 (0) |
| Anti-Ro-52 | 5 (6.2) | 2 (4.5) | 2 (5) | 4 (4.5) |

1Choledochal cysts, transient cholestasis of unknown origin and neonatal intrahepatic cholestasis caused by citrin deficiency, included as disease controls. AMA-M2 + M2-3E: combined the positivity to AMA-M2 and BPO; AMA-M2 + BPO + Sp100 + PML: combined the positivity to AMA-M2, BPO, Sp100 and PML. c*P* < 0.05 *vs* non-BA; a*P* < 0.05 *vs* healthy controls. AIH: Autoimmune hepatitis; BA: Biliary atresia; PBC: Primary biliary cholangitis; LKM-1: Liver-kidney microsomes type 1; LC-1: Liver cytosolic antigen type 1.

**Table 3 Prevalence of anti-nuclear antibody in biliary atresia patients and non-biliary atresia controls *n* (*%*)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Variable** | **BA,**  ***n* = 124** | **Other liver diseases1,**  ***n* = 92** | **Healthy,**  ***n* = 48** |
| ANA, by IIF | 14 (11.3)c | 3 (3.3) | 2 (4.2) |
| Fluorescence patterns |  |  |  |
| Homogeneous | 3 (2.4) | 0 (0) | 0 (0) |
| Speckled | 3 (2.4) | 0 (0) | 1 (2.1) |
| Nucleolar | 3 (2.4) | 0 (0) | 1 (2.1) |
| Nuclear dots | 1 (0.8) | 0 (0) | 0 (0) |
| Centrosome | 1 (0.8) | 0 (0) | 0 (0) |
| Cytoplasm | 1 (0.8) | 0 (0) | 0 (0) |
| Ring or rod Golgi | 1 (0.8) | 2 (2.2) | 0 (0) |
| Centromere | 1 (0.8) | 0 (0) | 0 (0) |
| Spindle apparatus | 0 (0) | 1 (1.1) | 0 (0) |
| Specific ANA2, by line-blot |  |  |  |
| SSA | 1 (0.8) | 0 (0) | 0 (0) |
| Ro-52 | 5 (4.0) | 2 (2.2) | 2 (4.2) |
| CENP B | 4 (3.2) | 1 (1.1) | 0 (0) |
| dsDNA | 3 (2.4) | 0 (0) | 0 (0) |

1Choledochal cysts, transient cholestasis of unknown origin and neonatal intrahepatic cholestasis caused by citrin deficiency, included as disease controls. 2Specific ANA include 12 different antibodies, with only SSA, Ro-52, CENP B and dsDNA positive in the study. c*P* < 0.05 *vs* other liver diseases. ANA: Anti-nuclear antibody; BA: Biliary atresia; IIF: Indirect immunofluorescence.

**Table 4 Prevalence of anti-neutrophil cytoplasmic antibodies in biliary atresia patients and non-biliary atresia controls *n* (*%*)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Variable** | **BA,**  ***n* = 124** | **Other liver diseases1,**  ***n* = 92** | **Healthy,**  ***n* = 48** |
| ANCA, by IIF | 36 (29.0)a | 23 (25.0) | 2 (4.2) |
| c-ANCA | 10 (8.1) | 1 (1.1) | 0 (0) |
| p-ANCA | 25 (20.2) | 21 (22.8) | 2 (4.2) |
| a-ANCA | 1 (0.8) | 1 (1.1) | 0 (0) |
| Specific ANCA, by ELISA |  |  |  |
| Anti-MPO | 11 (8.9) | 6 (6.5) | 2 (4.2) |
| Anti-PR3 | 19 (15.3) | 13 (14.2) | 0 (0) |
| Anti-MPO and/or anti-PR3 | 23 (18.0)a | 13 (14.2) | 2 (4.2) |
| ANCAs | 47 (37.9)a | 31 (33.7) | 3 (6.3) |

1Choledochal cysts, transient cholestasis of unknown origin and neonatal intrahepatic cholestasis caused by citrin deficiency, included as disease controls. a*P* < 0.05 *vs* healthy controls. Anti-MPO and/or anti-PR3: Any positivity of specific ANCA; ANCAs: Any positivity of ANCA by IIF or specific ANCA by ELISA. ANCA: Anti-neutrophil cytoplasmic antibody;BA: Biliary atresia;ELISA: Enzyme linked immunosorbent assay; IIF: Indirect immunofluorescence.

**Table 5 Association of autoantibodies and prognosis of Kasai procedure with follow-up of 52 biliary atresia patients *n* (*%*)**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Parameter** | **Anti-M2-3E** | |  | **ANA** | |  | **ANCAs** | |
| **Positive,**  ***n* = 17** | **Negative,**  ***n* = 35** |  | **Positive,**  ***n* = 8** | **Negative,**  ***n* = 44** |  | **Positive,**  ***n* = 29** | **Negative,**  ***n* = 23** |
| TB, μmol/L | 105.5 ± 80.3 | 109.4 ± 106.8 |  | 41.6 ± 49.5 | 127.9 ± 135.0 |  | 133.0 ± 120.7 | 85.3 ± 66.2 |
| ALT, U/L | 184.1 ± 119.8 | 171.7 ± 121.4 |  | 127.9 ± 135.0 | 185.8 ± 115.7 |  | 171.46 ± 95.5 | 179.6 ± 140.3 |
| Occurrence of cholangitis | 9 (52.9) | 20 (57.1) |  | 5 (62.5) | 24 (54.5) |  | 24 (82.8)a | 5 (21.7)a |

Data are described as mean ± SD. a*P* < 0.05, *r* = 0.61; ANCAs showed positive correlation to the occurrence of postoperative cholangitis. TB showed as parameter of persistence of jaundice (TB > 34 μmol/L); ALT showed as parameter of acute liver injury (ALT > 35 U/L). ANCAs: Any positivity of ANCA or specific ANCA. ALT: Alanine aminotransferase; ANA: Anti-nuclear antibody; ANCA: Anti-neutrophil cytoplasmic antibody; BA: Biliary atresia; TB: Total bilirubin.