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Original Article

Calreticulin expression is associated with gamma-glutamyl transferase and intercellular cell adhesion molecule-1 in biliary atresia

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Abstract: Calreticulin plays a critical role in the etiology of multiple autoimmune diseases. It is unknown, however, whether calreticulin levels are of clinical significance for patients with biliary atresia. This study was to confirm possible correlations between serum calreticulin levels and the immunohistochemical features of biliary atresia. Serum was collected from 44 biliary atresia patients, 15 infants with anicteric choledochal cysts, and 12 healthy controls. Serum calreticulin and liver function tests were detected. Western blot and immunohistochemistry were used to examine and localize calreticulin and intercellular cell adhesion molecule-1 expression in the biliary atresia liver tissues. Calreticulin in serum from biliary atresia patients were elevated. Moreover, the levels of calreticulin in biliary atresia patients correlated with gamma-glutamyl transferase. Calreticulin expression levels were elevated in biliary atresia liver specimens as compared to choledochal cysts infants. Protein expression levels of calreticulin and intercellular cell adhesion molecule-1 were positively correlated. Immunohistochemical detection showed augmented positive immunostaining for calreticulin and intercellular cell adhesion molecule-1 in biliary atresia as compared to choledochal cysts liver tissues. These results suggested that calreticulin may play a potential role in the etiology of biliary atresia. Furthermore, the calreticulin and intercellular cell adhesion molecule-1 as biomarkers may be used to monitor the severity of biliary atresia.

Keywords: Biliary atresia, calreticulin, intercellular cell adhesion molecule-1, gamma-glutamyl transferase

Introduction

Biliary atresia (BA) is a devastating disorder of infants that invariably leads to portal hypertension, cirrhosis, and ultimately death within the first two years of life if without effective treatment. Its incidence is approximately in 1/8,000 to 1/18,000 live births, and occurs more frequently in Asia (such as Japan and China) than Western countries [1-4]. Classic clinical manifestations include conjugated jaundice, dark urine, acholic stools, and hepatomegaly [5]. Kasai's portoenterostomy is typically the initial operation offered to infants with BA. Patients need to be operated on within the first 90 days of life to attain a satisfactory long-term outcome [6, 7]. Liver transplantation is another crucial treatment if Kasai surgery fails, there are serious complications, or liver failure occurs [4, 8, 9]. Current research supports a series of possible etiologies for BA but its precise pathogenic mechanisms remain largely unclear. Immunologic abnormalities, particularly autoimmunity, have recently received much focus [10-12]. There is increasing evidence suggesting autoimmunity may play a critical role in pathogenesis of BA.

Calreticulin (CRT) is a calcium-binding protein located in the lumen of endoplasmic reticulum (ER) [13]. CRT acts as a critical innate immune system receptor [14]. A recent study has identified CRT to be involved in a number of autoimmune processes, such as stimulation of inflammatory mediators, complement inactivation, epitope spreading. In addition, CRT has been proved as an autoantigen in rheumatoid arthritis (RA), which can be secreted from cells into the serum. These findings demonstrate that CRT is not only an autoantigen, but also a criti-

Table 1. Distribution of study subjects and serum calreticulin levels

Diagnosis	BA	CC	HC
Disease Type	III[a]	I[b]	N/A
Age (months)[c]	2.51 ± 0.36	36.82 ± 7.63	3.06 ± 1.57
CRT (ng/L)	27.91 ± 5.14	17.91 ± 2.97	16.29 ± 1.92

BA, Biliary Atresia; CC, Choledochal Cysts; HC, Healthy Controls; CRT, Calreticulin. [a]Type III BA is where the most proximal part of the extrahepatic biliary tract within the porta is entirely solid. This type is common and accounts for > 90% of cases. [b]Type I CC is the most common (80-90% of cases) and involves saccular or fusiform dilatation of a portion or the entire common bile duct but the intrahepatic duct is normal. CC with non-icteric and normal liver function served as controls. [c]Age at time of serum collection.

cal mediator in the etiology of multiple autoimmune diseases [15-18]. Current studies reveal that CRT expression is augmented in fibrotic tissues, demonstrating that CRT function associated with fibrogenesis [19]. Furthermore, CRT can directly or indirectly induce production of matrix proteins such as collagen and fibronectin [20, 21].

Intercellular cell adhesion molecule-1 (ICAM-1) is a cytokine-inducible glycoprotein. ICAM-1 plays important roles during lymphocyte adhesion to target cells or antigen-presenting cells [22]. Expression of certain adhesion molecules is of great importance for proper immune reactions and useful for monitoring the activity of certain diseases. ICAM-1 has been reported to be significantly expressed in the bile ducts and hepatic parenchyma of livers from BA patients [23]. One pathologic marker of BA includes an immunological reaction targeted against the biliary epithelial cell characterized by ductal expression of ICAM-1. A soluble form of ICAM-1 (sICAM-1) has been detected in sera. Elevated levels of sICAM-1 have been associated with chronic hepatitis and immune-mediated liver diseases such as primary biliary cirrhosis [24, 25].

Diagnosing BA is challenging, as there is a high degree of overlap in clinical manifestations; and biochemical, imaging, and histological characteristics between BA and other causes of neonatal cholestasis (NC) [26]. Elevated CRT expression has been reported to be associated with autoimmune processes and fibrotic diseases. For example, the sera concentration level of CRT is raised in patients with RA and a correlation exists between sera levels of CRT and RA disease activity [15]. It is unknown, however, whether sera levels of CRT are augmented in BA patients. Our study, therefore focused on assessing serum levels of CRT in infants with BA, and investigated possible correlations between CRT levels and various clinical features, laboratory parameters, and ICAM-1 expression. The aim of our study was to evaluate potentials of CRT and ICAM-1 that may play a key role in the pathogenesis of BA, and to identify any additional molecular markers to be used for biomarkers and predisposition.

Materials and methods

Study population

The study population comprised of 44 infants with BA, 15 infants with anicteric choledochal cysts (CC) and 12 healthy controls (HCs). Diagnosis of BA or CC was based on clinical patterns and pathological examination of resected biliary remnants and liver biopsy. BA patients were not accompanied by CMV antibody positive, any syndromic and cystic variants. HCs were selected from hospital patients who had undergone elective surgery (e.g. inguinal hernia or adhesion of labia minora operation). Both CC and HC subjects had normal liver function and were without infection, inflammation, or immune-mediated disease. Study participants were prospectively recruited from the Children's Hospital of Fudan University. Demographic data of each group is shown in **Table 1**. Clinical data for BA patients were obtained from hospital databases. The protocol of this experiment was approved by the Ethics Committee at the Children's Hospital of Fudan University ([2013] 044; Feb, 2013). Written informed consent was obtained from each participating patient before enrollment in the study.

Sample collection and preparation

All serum samples were collected from peripheral blood and kept at 4°C for 1 h to allow for clotting before the surgery, and were then centrifuged at 4,000 RPM/min for 10 min and stored immediately at -80°C until further analysis. Liver samples of BA and CC patients from surgery used to assay CRT and ICAM-1 were immediately snap-frozen in liquid nitrogen in the operating room, and stored at -80°C until further analyses.

In vitro liver function tests

In this study, liver function tests were performed to assess the condition of a patient's liver. The following parameters were measured: alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AKP), gamma-glutamyl transpeptidase (GGT), direct bilirubin (DB), total bilirubin (TB), total bile acid (TBA), and albumin (ALB). These parameters were examined by a Reagent Kit (Pointe Scientific, Inc. MI, USA).

Measurement of CRT protein levels in sera by ELISA

Serum CRT levels were measured using ELISA kits for human CRT (R&D Systems, Minneapolis, MN, USA). ELISAs were performed according to the manufacturer's protocol. Briefly, add sample dilution 40 ul to testing sample well, then add testing sample 10 ul (sample final dilution is 5-fold), add sample to wells, don't touch the well as far as possible, and gently mix. After closing plate with closure plate membrane, incubate for 30 min at 37°C. Wells were then washed for five times for 30 s for each rinse, discard liquid, dry by pat. Add HRP-Conjugate reagent 50 ul to each well, except blank well. Then incubate for 30 min at 37°C and wash like previously. The chromogenic agent was applied and the reaction was developed for 15 minutes at 37°C in the dark. Add stop solution 50 ul to each well, stop the reaction, read absorbance at 450 nm after adding the stop solution and within 15 min. Pretreatment with protein A/G PLUS-agarose (Santa Cruz Biotechnology, Dallas, TX, USA) was used.

Western blot analysis

Liver tissue samples were homogenized with ELB lysis buffer (50 mM Tris, 140 mM NaCl, 0.5% NP-40, and 100 mM NaF [pH = 7.6]) containing 1 mM phenylmethlsulfonyl fluoride (PMSF). Protein concentrations were measured by the Lowry method, and absorbance was examined spectrophotometrically. Protein specimen were electrophoresed on 10% denatured sodium dodecyl sulfate gels, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking in 5% fat-free milk, the membrane was incubated overnight at 4°C with primary antibodies against CRT and ICAM-1 (R&D Systems, Minneapolis, MN, USA), followed by washing and incubation with an HRP-goat anti-rabbit antibody (Sigma-Aldrich, St. Louis, MO, USA). The membrane was stripped and incubated with a mouse anti-GADPH antibody (Sigma-Aldrich, St. Louis, MO, USA) followed by incubation with an HRP-goat anti-mouse antibody (Kangcheng Co., Ltd., Shanghai, China). The stained membranes were visualized by using enhanced chemiluminescence (ECL) detection (Amersham Pharmacia Biotech Co., Ltd., UK) and the quantification of Western Blot by Imagine J software (NIH, USA). GAPDH protein was used as the internal control. Western blotting experiments were repeated at least three times per sample.

Immunohistochemistry

Liver samples were fixed with 4% paraformaldehyde in phosphate buffered saline for 24 h. After alcohol dehydration, livers were embedded in paraffin and sectioned. Immunohistochemistry (IHC) was performed using the two-step EnVision/HRP technique (Dako Cytomation, Denmark) according to the manufacturer's instructions. CRT and ICAM-1 expression were examined using a monoclonal anti-human CRT or ICAM-1 antibody (R&D Systems, Minneapolis, MN, USA) diluted to 1:100 in phosphate buffered saline (PBS). Brown stained cells were scored as positive. The expression of CRT and ICAM-1 were quantitatively evaluated using an Olympus BH2 microscope with a computer-aided image analysis system (Qiu Wei Inc., Shanghai, China). Images were obtained with a digital camera (Nikon 4500, Tokyo, Japan). The positive areas and optical density (OD) of CRT and ICAM-1 positive cells were determined by measuring three randomly selected microscopic fields for each section. The expression of CRT and ICAM-1 in BA liver tissue was semi-quantitatively analyzed for the percentage of positive cells and the degree of staining. Less than 1% positive cells was scored as 0; 2-25% positive cells was scored as 1, 26-50% positive cells was scored as 2, 51-75% positive cells was scored as 3, and more than 75% positive cells was scored as 4. Degree of staining was scored as follows: no staining scored as 0, straw yellow (weak) scored as 1, palm yellow (moderate) scored as 2, and puce (intense) scored as 3. The overall IHC total score was the percent positive rating multiplied by the staining degree [27].

Statistics

Data passed the normality test and were presented as the mean ± standard deviation (SD).

Table 2. Correlations of serum calreticulin levels with the liver function tests in patients with biliary atresia

	BA	CRT (ng/L)	*r*	*p*
ALT (IU/L)	99.36 ± 73.42	27.91 ± 5.14	-0.185	0.229
AST (IU/L)	14.82 ± 73.96		-0.181	0.240
AKP (IU/L)	624.2 ± 175.7		-0.035	0.819
GGT (IU/L)	950.5 ± 662.5		0.509	0.0004
DBIL (μmol/L)	110.5 ± 34.51		-0.077	0.620
TBIL (μmol/L)	161.6 ± 51.78		-0.069	0.657
TBA (μmol/L)	148.0 ± 45.16		0.045	0.772
ALB (g/L)	39.06 ± 3.058		0.071	0.648

ALT, alanine transaminase; AST, aspartate transaminase; AKP, alkaline phosphatase; GGT, gamma glutamyl transpeptidase; DBIL, direct bilirubin; TBIL, total bilirubin; TBA, total bile acid; ALB, albumin; BA, biliary atresia; CRT: calreticulin.

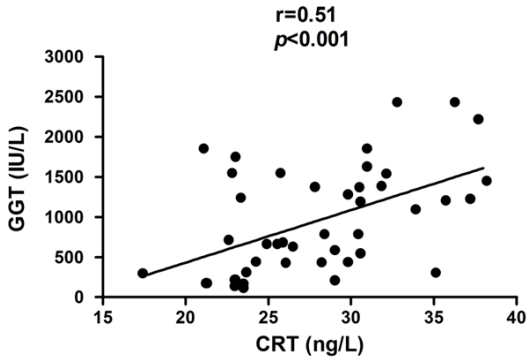

Figure 1. Serum calreticulin levels positively correlated with gamma-glutamyl transferase (GGT) levels in infants with biliary atresia (r = 0.51, *P* < 0.001).

Statistical significance between groups was determined using the Student's *t*-test. Correlation between two groups was calculated by Pearson's correlation. A *p*-value less than 0.05 was considered to be statistically significant. All statistical analyses were performed using the GraphPad Prism statistics software.

Results

Clinical characteristics and serum CRT levels

In this study, a total of 71 participants were involved, including of 44 BA, 15 CC, and 12 HC. The detailed clinical status of these participants is shown in **Table 1**. There was no statistical difference in gender between infants with BA and control groups.

Serum CRT levels were measured by ELISA in all participants. The mean concentration of sera CRT in the BA, CC and HC infants were 27.91 ± 5.14, 17.91 ± 2.97 and 16.29 ± 1.92 ng/L, respectively. Statistical analysis revealed that levels of serum CRT were significantly higher in the BA group compared to the CC (*P* < 0.01) and HC (*P* < 0.01) groups. There was no statistical difference between CC and HC groups (*P* > 0.05). The relationships between sera CRT levels and liver function tests (ALT, AST, AKP, GGT, DBIL, TBIL, TBA, ALB) are shown in **Table 2**. A strong positive correlation exists between CRT and GGT levels in the BA patients (r = 0.51, *P* < 0.001, **Figure 1**).

Protein expression levels of CRT and ICAM-1 are elevated in infants with BA

Using Western blot analysis, CRT and ICAM-1 protein expression levels were investigated in liver biopsy tissues of BA patients and CC control patients. The CRT and ICAM-1 expression levels were significantly higher in BA infants as compared to CC controls (*P* < 0.05). Moreover, a strong positive correlation existed between CRT and ICAM-1 expression in BA patients (r = 0.55, *P* < 0.001, **Figure 2**).

IHC analysis of CRT and ICAM-1 expression in BA tissue samples

Immunohistochemical analysis was performed on BA and CC liver samples with antibodies against CRT and ICAM-1 (**Figure 3**). There was enhanced expression of CRT and ICAM-1 in the livers of BA patients as compared to livers of CC patients. Sections from infants with BA revealed a mild inflammatory cell infiltrate that was sparse in cases with an abundant fibrotic matrix where the liver was morphologically severely disordered. Additionally, extensive ductular proliferation was observed and was characterized by angulated and elongated ductules. The total IHC scores of CRT and ICAM-1 positive cells were higher in BA patients than in CC infants. This was confirmed using the Fisher definite probability method (*P* < 0.01). CRT and ICAM-1 expression positively correlated in infants with BA (r = 0.60, *P* < 0.001, **Figure 4**).

Discussion

BA is a progressive, inflammatory, and fibrotic cholangiopathy of infants, affecting both the intrahepatic and extrahepatic bile ducts that results in ruin and obstruction of the biliary tree

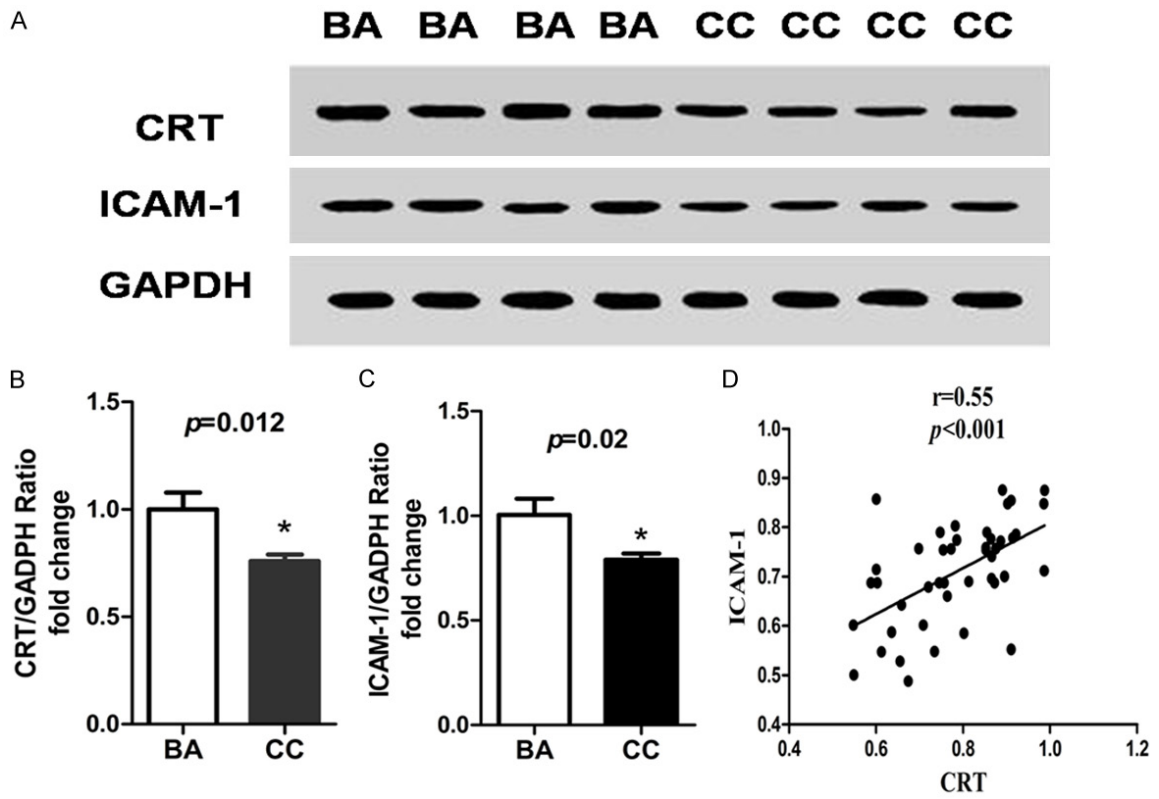

Figure 2. Western blot analysis of liver tissue calreticulin and intercellular cell adhesion molecule-1 protein expression. A. Western blot of calreticulin and Intercellular cell adhesion molecule-1 protein expression in the livers of bilisry atresia (n = 44) and choledochal cysts (n = 15) patients. B. The level of liver calreticulin protein expression (CRT/GADPH ratio) was higher in biliary atresia patients (0.797 ± 0.115) than choledochal cysts controls (0.482 ± 0.089) (P = 0.012). C. The level of liver Intercellular cell adhesion molecule-1 protein expression (ICAM-1/GADPH ratio) was higher in biliary atresia patients (0.646 ± 0.073) than that of choledochal cysts controls (0.391 ± 0.067) (P = 0.02). D. The calreticulin expression positively correlated with intercellular cell adhesion molecule-1 expression in the livers of biliary atresia patients (r = 0.55, P < 0.001).

[12, 28]. Its poor clinical outcome underscores a compelling need for new, efficient measures of early diagnosis, prognosis prediction, and therapy. In our study results, CRT was significantly upregulated in BA patients. Additionally, CRT and GGT expression were found to be positively correlated in serum of BA patients. GGT is generally distributed in many human tissues involved in secretory and absorptive processes, especially the bile canaliculus. It is a microsomal enzyme expressed mostly by biliary epithelial cells in the liver that catalyzes the transfer of the γ-glutamyl moiety of glutathione and participates in the detoxification of xenobiotics [29]. Sera GGT levels are typically elevated when there are liver disorders affecting the biliary tree, e.g. progressive familial intrahepatic cholestasis, sclerosing cholangitis, and BA, among others [30, 31]. GGT levels higher than 300 IU/L has been demonstrated to have a sensitivity of 98.1% in clinical diagnosis of BA [32]. Particularly, GGT level is generally regarded as an indicator of biliary obstruction rather than hepatocyte lesion [33]. Although the preferable liver function test for the assessment of intrahepatic cholestasis is alkaline phosphatase (AKP) and GGT [32], there might be a useful indication for pediatric patients, because AKP activity varies in infants. In our study, the positive correlation between CRT and GGT demonstrates that CRT might play an important role in the pathogenesis of BA. CRT might be a helpful molecular marker, in addition to GGT, for the diagnosis of BA.

Previous studies have identified CRT be to function in neuroblastoma differentiation and autoimmune diseases [34]. CRT is distributed in a wide array of tissues and is expressed at high

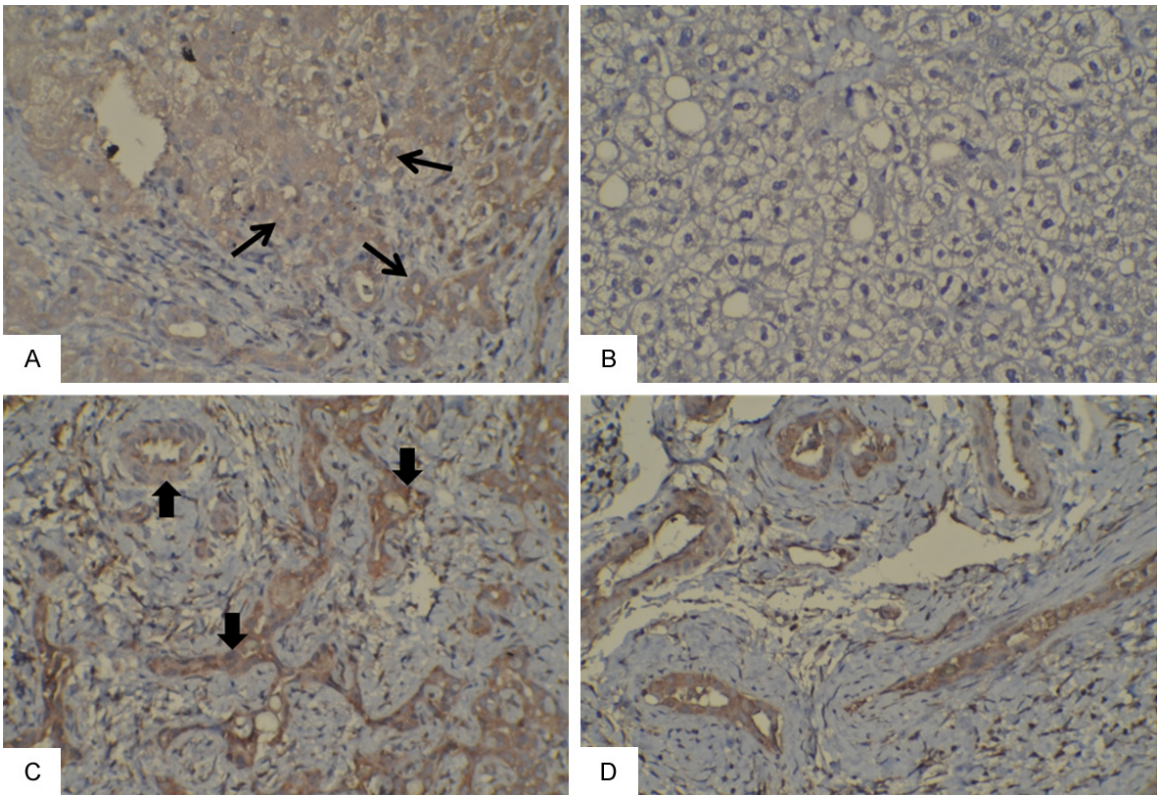

Figure 3. Liver immunohistochemical analysis of calreticulin and intercellular cell adhesion molecule-1. (A) Representative immunostaining patterns of calreticulin. Calreticulin had strong expression in biliary atresia livers (A, 200×), as compared to choledochal cysts livers (B, 200×) ($P < 0.01$). Strongly stained brown cells were considered calreticulin positive. Arrows indicate positive cells. (B) The choledochal cysts group had normal hepatic lobule structure. (C) Representative immunostaining patterns of intercellular cell adhesion molecule-1. Intercellular cell adhesion molecule-1 had strong expression in biliary atresia livers (C, 200×), as compared to choledochal cysts livers (D, 200×) ($P < 0.01$). The strongly stained brown cells were considered intercellular cell adhesion molecule-1 positive. Arrows indicate positive cells. (D) The choledochal cysts group had normal hepatic lobule structure. The biliary atresia group is characterized by abnormal lobular structure, and the extensive ductular proliferation is shown. The livers were morphologically severely disordered with abundant fibrotic matrix (A, C. 200×).

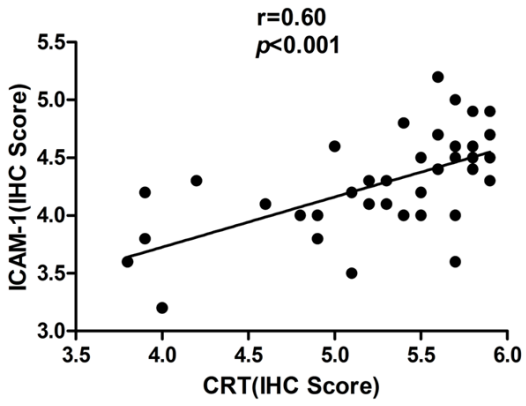

Figure 4. Expression of calreticulin positively correlated with expression intercellular cell adhesion molecule-1 in the liver of biliary atresia patients with immunohistochemical ($r = 0.60$, $P < 0.001$).

levels in the liver, lungs, spleen, kidneys, and heart [35]. In our study, we observed elevated serum levels of CRT in infants with BA as compared with controls. Moreover, a significant correlation was observed between CRT and GGT levels in BA patients. Other recent studies also showed that CRT levels are elevated in patients with RA as compared with other autoimmune disorders [15, 34, 36]. These accumulated data together indicate that CRT plays a pathophysiological role in autoimmune diseases. Interestingly, BA has been recently considered as an autoimmune disease. In our study, Western blot analysis was used to confirm ELISA results measuring CRT protein levels. These data suggest that CRT may serve as a helpful indicator of BA.

CRT expression on the surface of many cells provides evidence that this intracellular chaperone protein functions outside the ER [37]. It has been reported that exogenous CRT stimulates fibroblast migration and upregulates integrin expression in fibroblasts [38]. CRT in the extracellular matrix can modulate its structure and act as a molecular link between the matrix and cardiac myocytes [39]. We verify that BA patients have higher serum levels of CRT, and upregulated levels in liver tissue using IHC, we also found that CRT is expressed in the hepatocytes and extracellular matrix within the BA liver. These results therefore infer that bile duct damage and evidence of abnormal liver function tests, as well as increased levels of GGT and CRT, can stimulate fibroblast migration, thereby increasing collagen production and thus resulting in liver fibrosis. As liver fibrosis proceeds, liver function worsens, and the liver becomes more fibrotic, thereby creating a vicious cycle.

Cell adhesion molecules play an important role in the cell's response to inflammation and are integral to the processes of leukocyte attachment, adherence, and migration into the matrix [40]. ICAM-1 is been expressed on the surface of many human cells, such as dermal fibroblasts, liver sinusoidal endothelial cells, and leukocytes [41]. Previous IHC studies have revealed that ICAM-1 is significantly expressed in regions of necrosis during hepatitis and in the remnant bile duct tissue [42]. IHC analysis has also determined there is a close relationship between expression of ICAM-1 and disease activity in primary biliary cirrhosis and other liver disorders. It has been shown that ICAM-1 expression in liver diseases may be indicative of hepatocyte damage, and may be a useful biomarker for evaluating disease activity and prognosis [42, 43]. In our study, there is significant expression of ICAM-1 in the BA liver. The distribution of hepatocyte staining for ICAM-1 reveals a potential relationship with the fibrogenesis of cirrhosis, since fibroblasts can be stimulated to express ICAM-1 in vitro. In vivo, activated fibroblasts secrete a lot of collagen in the extracellular matrix.

It has been reported that CRT treatment distinctly upregulates the expression of adhesion molecules such as ICAM-1 in neoplasm endothelial cells [44]. In this study, we also showed that the expression of CRT and ICAM-1 is elevated in liver tissue from BA patients, and that there is a positive correlation between CRT and ICAM-1. Our outcome is consistent with a recent report [44]. In particular, CRT overexpression by autoimmune disorders subsequently enhances ICAM-1 expression in bile duct endothelial cells. ICAM-1 overexpression can result in augmented leukocyte-endothelial cell interaction and enhanced lymphocyte infiltration into the liver, thus proposing a mechanism for lymphocyte infiltration into BA liver tissue and hence suggesting that BA is identical to an inflammatory disease. Furthermore, the fibroblasts that were stimulated and activated by abundant CRT and ICAM-1 deposited a significant amount of collagen into the extracellular matrix, thereby causing hepatic lobule incision and formation of liver cirrhosis.

Overall our results demonstrated elevated levels of CRT in the serum and liver tissue of BA patients. In addition, increased serum CRT levels correlated with GGT levels. This is the first study to suggest such a connection. Our study also reveals that expression of ICAM-1 is elevated in livers of BA patients. In particular, we identified a positive correlation between expressions of CRT and ICAM-1 in livers of BA patients. We therefore propose CRT may play a potential role in the etiology of BA. CRT and ICAM-1 as possible biomarkers may be used to monitor the severity of BA, which may provide new view of the mechanisms regulating the biliary damage. Future studies will explore the mechanistic role of CRT and the relationship between CRT and ICAM-1 in BA, and test the serum CRT and ICAM-1 in normal infants. Finally, complementary research studies are required to elucidate the mechanistic relationship between CRT and liver fibrosis to prevent liver cirrhosis progression.

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Disclosure of conflict of interest

None.

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