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ORIGINAL ARTICLE

High serum matrix metalloproteinase-3 and liver stiffness in postoperative biliary atresia

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Abstract
Background Biliary atresia (BA) is a neonatal liver disorder characterized by chronic inflammation and obliteration of extrahepatic bile ducts. The purpose of the study was to investigate serum matrix metalloproteinase-3 (MMP-3) in postoperative BA patients and the association of MMP-3 with clinical outcome and liver stiffness score.
Methods Fifty-eight BA patients post-Kasai operation and 20 controls were enrolled. None of the patients had undergone liver transplantation. BA patients were classified into two groups according to their serum total bilirubin (TB) levels (TB < 2 mg/dL, no jaundice vs. TB ≥ 2 mg/dL, persistent jaundice) and alanine aminotransferase (ALT) levels (ALT < 45 IU/L, normal ALT vs. ALT ≥ 45 IU/L, elevated ALT). Serum MMP-3 levels were determined by enzyme-linked immunosorbent assay. Liver stiffness scores were measured by FibroScan.
Results BA patients had greater MMP-3 levels (10.8 ± 1.0 vs. 7.9 ± 0.8 ng/mL, $P = 0.02$) and higher liver stiffness values than controls (29.7 ± 3.0 vs. 5.1 ± 0.5 kPa, $P < 0.001$). Serum MMP-3 levels were significantly elevated in BA patients with jaundice when compared with those without jaundice (15.3 ± 2.2 vs. 8.5 ± 0.8 ng/mL, $P = 0.004$). In addition, BA patients with elevated ALT had higher levels of serum MMP-3 than those with normal ALT (12.4 ± 1.5 vs. 8.3 ± 0.9 ng/mL, $P = 0.02$). Moreover, BA patients with portal hypertension displayed higher serum MMP-3 than those without portal hypertension (13.5 ± 1.5 vs. 7.4 ± 0.8 ng/mL, $P = 0.001$). There was also a correlation between serum MMP-3 and liver stiffness scores ($r = 0.448$, $P \leq 0.001$).
Conclusion Serum MMP-3 was associated with hepatic dysfunction and liver stiffness in postoperative BA patients. Accordingly, MMP-3 could play a role in the pathophysiology of hepatic fibrosis in BA after Kasai operation.

Keywords Biliary atresia · Jaundice · Liver stiffness · Matrix metalloproteinase-3 · Portal hypertension

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Introduction

Biliary atresia (BA) is a common cause of chronic cholestasis in children and is characterized by progressive fibrosclerotic cholangiopathy resulting in partial or complete obliteration of the extrahepatic biliary tree. The obstruction manifests with a triad of jaundice, acholic stools, and hepatosplenomegaly. Untreated BA patients develop severe liver fibrosis, biliary cirrhosis, and eventually die by 2 years of age [1]. The primary therapeutic option for re-establishing bile flow in BA children is Kasai portoenterostomy. Despite early Kasai procedure, the vast majority of patients still progress to cirrhosis, portal hypertension, and end-stage liver disease [2, 3]. At this stage, liver transplantation is an effective treatment modality when the Kasai operation has failed and serious

complications occur. Potential etiopathogenic mechanisms of BA include perinatal viral infections, genetic defects, and abnormalities in immune response [4].

Matrix metalloproteinases (MMPs), a 28-membered family of zinc-dependent endopeptidases that are the major degrading enzymes of extracellular matrix proteins, play essential roles in the processes of tissue remodeling and repair in physiological and pathological conditions [5, 6]. The MMP family is categorized into four main subtypes, consisting of collagenases, gelatinases, stromelysins, and membrane type-matrix metalloproteinases (MT-MMPs) [7]. Increasing evidence has accumulated for their roles in pathogenicity and progression of fibrosis.

MMP-3 is one of the stromelysin expressed in several cell types including hepatocellular carcinoma cells as well as in hepatic stellate cells [8–10]. In addition, MMP-3 is involved in the shedding of protein ectodomains from the cell surface [11] and can degrade a broad spectrum of extracellular matrix substrates, including types III, IV, and V collagens and non-collagenous extracellular matrix components, such as laminins, fibronectin, osteopontin, and proteoglycans [12]. Therefore, MMP-3 can proteolytically activate other MMPs and pathological conditions may be achieved through the overexpression of MMP genes [13]. Previous study showed that the expression of MMP-3 was associated with liver fibrosis in human [14].

Recently, the expression of MMP-3 has been investigated using semiquantitative reverse transcriptase polymerase chain reaction analysis in liver tissue samples from BA infants [15]. Gene expression of hepatic MMP-3 was significantly upregulated in all samples of BA. In addition, MMP-3 was expressed in the ductal plate of human intrahepatic bile duct development [16]. These observations prompted us to speculate that MMP-3 may be responsible for the pathogenesis of BA. However, serum MMP-3 from various clinical stages of BA and its possible role in BA patients has not received much attention. Therefore, the purpose of this study was to determine serum MMP-3 levels in postoperative BA patients and to evaluate the possible association of serum MMP-3 with clinical outcome.

Methods

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, and complied with the ethical guidelines of the 1975 Declaration of Helsinki. All parents of children were informed of the study purpose and the use of serum samples for biochemical analysis. Written informed consent was obtained from the participants' parents upon informing them about the protocol and procedures involved in the research.

Study population

Fifty-eight BA patients (30 girls and 28 boys with mean age of 9.4 ± 0.7 years) and 20 healthy children (10 girls and 10 boys with mean age of 10.1 ± 0.7 years) were recruited in this study. All patients with type 3 (uncorrectable) isolated BA had undergone hepatic portojejunostomy with Roux-en-Y reconstruction (original Kasai procedure), and they were generally in good health; no signs of suspected infection or bleeding abnormalities at the time of blood sampling. We excluded all patients with BA splenic malformation, severe cardiac anomalies, definite macroscopic cirrhosis, and types 1 or 2 (correctable) BA. None of them had undergone liver transplantation. Healthy controls attending the Well Baby Clinic at King Chulalongkorn Memorial Hospital for vaccination had normal physical findings and no underlying disease. Serum samples were taken during their annual routine follow-up between October 2009 and June 2010. BA patients were classified into two groups according to serum total bilirubin (TB), serum alanine aminotransferase (ALT), and liver stiffness score. Based on their jaundice status, BA children were divided into two groups: patients without visible jaundice (TB < 2 mg/dL) and patients with persistent jaundice (TB ≥ 2 mg/dL). Serum ALT routinely serves as a specific biochemical indicator of liver dysfunction reflecting hepatocellular damage. Further classification was achieved according to their levels of serum ALT status [19] into a normal ALT group (ALT < 45 IU/L, $n = 22$) and a high ALT group (ALT ≥ 45 IU/L, $n = 36$). Subsequently, portal hypertension (PH) was validated by the presence of ascites and/or esophageal varices as diagnosed by endoscopic screening. Fifteen of the 38 BA children without jaundice and 18 of 20 children with persistent jaundice had evidence of portal hypertension.

Laboratory methods

Samples of peripheral venous blood were collected from every participant, and were stored at −70°C for further analysis. Quantitative determination of MMP-3 concentration in serum was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's protocol. Serum samples were first diluted in accordance with manufacturer's recommendation in tenfold dilution. Recombinant human MMP-3 standard and serum samples were pipetted into each well, which had been pre-coated with specific antibody to MMP-3. After incubating for 3 h at room temperature on a microplate shaker at 200 rpm, every well was thoroughly washed six times with wash buffer. After six washes, substrate solution was pipetted into the wells and the

microplate was incubated for 10 min at room temperature protected from light. Lastly, the reaction was stopped by stop solution and the color intensity was measured with an automated microplate reader at 450 nm. The MMP-3 concentration was determined by a standard optical density–concentration curve. Serum hyaluronic acid (HA) level was measured using a competitive inhibition based-ELISA as described previously [17]. The liver function tests, including serum albumin, total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were performed using a Hitachi 912 automated machine at the central laboratory of our hospital. The aspartate aminotransferase to platelets ratio index (APRI) was calculated as follows: (AST/upper limit of normal) × 100/platelet count (10^9/L) [18].

Liver stiffness measurement

Transient elastography measured the liver stiffness between 25 and 65 mm from the skin surface, which is approximately equivalent to the volume of a cylinder of 1-cm diameter and 4-cm length. The measurements were performed by placing a transducer probe of FibroScan on the intercostal space at the area of the right lobe of the liver with patients lying in a dorsal decubitus position with maximum abduction of the right arm. The target location for measurement was a liver portion that was at least 6-cm thick, and devoid of major vascular structures. The measurements were performed until 10 validated results had been obtained with a success rate of at least 80%. The median value of 10 validated scores was considered the elastic modulus of the liver, and it was expressed in kilopascals (kPa).

Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 for Windows. Comparisons of demographic and clinical parameters between groups were performed using χ^2 and Student's unpaired t test. Correlations between numerical data were acquired using the Pearson correlation coefficient (r). Data were expressed as mean ± SEM. $P < 0.05$ were considered statistically significant.

Results

Comparison between BA patients and healthy controls

A total of 58 BA patients and 20 healthy controls were enrolled in this study. The characteristics of participants in both groups are shown in Table 1. Mean age and gender ratio in controls and BA patients were not different, while serum MMP-3 levels were significantly elevated in BA patients when compared with healthy controls (10.8 ± 1.0 vs. 7.9 ± 0.8 ng/mL, $P = 0.02$) (Fig. 1). BA patients had significantly greater serum hyaluronic acid levels than controls (50.1 ± 7.2 vs. 23.9 ± 1.5 ng/mL, $P = 0.001$). In addition, liver stiffness scores in BA patients were remarkably higher than those in controls (29.7 ± 3.0 vs. 5.1 ± 0.5 kPa, $P < 0.001$).

Comparison between BA patients with and without persistent jaundice

We further categorized BA patients into a jaundice ($n = 20$) and non-jaundice group ($n = 38$). As presented in

Table 1 Demographic data, biochemical characteristics, and liver stiffness scores of controls and biliary atresia patients

Variables	Controls ($n = 20$)	BA patients ($n = 58$)	P
Age (years)	10.1 ± 0.7	9.4 ± 0.7	0.1
Gender (female:male)	10:10	30:28	0.5
Albumin (g/dL)	–	4.3 ± 0.1	NA
Total bilirubin (mg/dL)	–	2.6 ± 0.5	NA
Direct bilirubin (mg/dL)	–	2.1 ± 0.5	NA
AST (IU/L)	–	128.4 ± 11.3	NA
ALT (IU/L)	–	111.8 ± 10.9	NA
ALP (IU/L)	–	437.3 ± 28.3	NA
Platelet count (10^3/mm^3)	–	164.7 ± 13.2	NA
APRI	–	3.0 ± 0.4	NA
MMP-3 (ng/mL)	7.9 ± 0.8	10.8 ± 1.0	0.02
Hyaluronic acid (ng/mL)	23.9 ± 1.5	50.1 ± 7.2	0.001
Liver stiffness (kPa)	5.1 ± 0.5	29.7 ± 3.0	<0.001

The data are expressed as mean ± SEM

BA biliary atresia, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *APRI* aspartate aminotransferase to platelets ratio index, *MMP-3* matrix metalloproteinase-3, *NA* not applicable

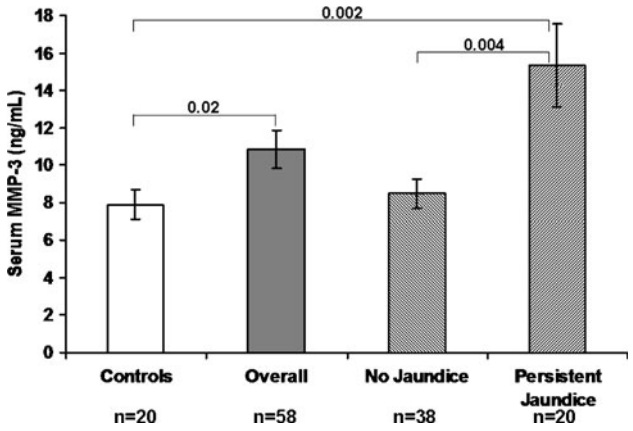

Fig. 1 Comparison of serum MMP-3 levels in biliary atresia patients based on total bilirubin and controls. The data are expressed as mean ± SEM

Table 2, BA patients with jaundice had significantly higher aspartate aminotransferase to platelets ratio index (APRI), hyaluronic acid, and liver stiffness values when compared with those without jaundice. In BA patients, serum MMP-3 levels of patients with persistent jaundice were markedly elevated when compared with those of patients without jaundice (15.3 ± 2.2 vs. 8.5 ± 0.8 ng/mL, $P = 0.004$) (Fig. 1). We also found that BA patients with elevated ALT ($n = 36$) had higher levels of serum MMP-3 than those with normal ALT ($n = 22$) (12.4 ± 1.5 vs. 8.3 ± 0.9 ng/mL, $P = 0.02$) (Fig. 2). Moreover, BA patients with portal hypertension ($n = 33$) displayed higher serum MMP-3 than those without portal hypertension ($n = 25$) as shown in Fig. 3 (13.5 ± 1.5 vs. 7.4 ± 0.8 ng/mL, $P = 0.001$). There was also a positive correlation between serum MMP-3 levels and liver stiffness scores ($r = 0.448$, $P < 0.001$) (Fig. 4).

Discussion

Biliary atresia remains one of the most common causes of pathologic jaundice in neonates. The etiopathogenic mechanisms of BA are still not entirely clear, and therapeutic options are unsatisfactory. Irrespective of the initiating disorder, all children with BA share a unique progressive inflammatory and fibrosis obstruction of the biliary system [1–3]. The progression of BA results in liver fibrosis and portal hypertension with severe complications such as gastroesophageal varices, splenomegaly, and progressive ascites. Although a number of cytokines and growth factors have been previously documented in the pathophysiological role of BA [19–22], published data on serum MMP-3 from different clinical stages of BA have received little attention. This study aimed to evaluate serum MMP-3 concentrations, liver stiffness scores, and clinical parameters in BA patients post-Kasai procedure.

The current study showed that serum MMP-3 levels in BA patients were markedly higher than those in healthy controls. In the BA patients, serum MMP-3 levels were substantially greater in BA patients with persistent jaundice than those without jaundice. Subsequent analysis revealed that the BA patients with high ALT had more increased concentrations of serum MMP-3 as compared to those with normal ALT. It has been known that high serum ALT is a specific indicator for liver injury. Jaundice status in BA patients is believed to be an indicator for intrahepatic biliary obstruction. In addition, serum MMP-3 levels positively correlated with liver stiffness scores. Furthermore, this study showed more elevated MMP-3 levels in BA patients with portal hypertension than those without portal hypertension. Therefore, these findings indicate that the elevation of MMP-3 may reflect the degree of hepatic

Table 2 Comparison of biliary atresia patients without and with jaundice

Variables	BA patients without jaundice ($n = 38$)	BA patients with Jaundice ($n = 20$)	P
Age (years)	8.9 ± 0.8	10.1 ± 1.2	0.4
Gender (female:male)	20:18	10:10	0.5
Albumin (g/dL)	4.5 ± 0.1	3.9 ± 0.1	<0.001
Total bilirubin (mg/dL)	0.7 ± 0.1	6.2 ± 1.2	<0.001
Direct bilirubin (mg/dL)	0.4 ± 0.1	5.4 ± 1.2	<0.001
AST (IU/L)	107.8 ± 14.2	167.6 ± 15.6	0.01
ALT (IU/L)	105.6 ± 15.7	123.7 ± 10.0	0.4
ALP (IU/L)	387.8 ± 36.7	531.5 ± 35.5	0.01
Platelet count (10^3/mm^3)	190.3 ± 16.2	116.2 ± 18.7	0.01
APRI	2.1 ± 0.4	4.8 ± 0.6	<0.001
MMP-3 (ng/mL)	8.5 ± 0.8	15.3 ± 2.2	0.004
Hyaluronic acid (ng/mL)	29.3 ± 3.5	89.6 ± 16.9	<0.001
Liver stiffness (kPa)	18.9 ± 2.5	50.1 ± 4.5	<0.001

The data are expressed as mean ± SEM

BA biliary atresia, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *APRI* aspartate aminotransferase to platelets ratio index, *MMP-3* matrix metalloproteinase-3

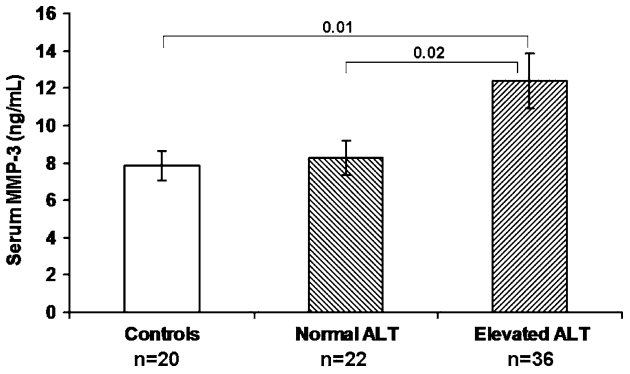

Fig. 2 Comparison of serum MMP-3 levels in biliary atresia patients based on serum ALT and controls. The data are expressed as mean ± SEM. *ALT* alanine aminotransferase

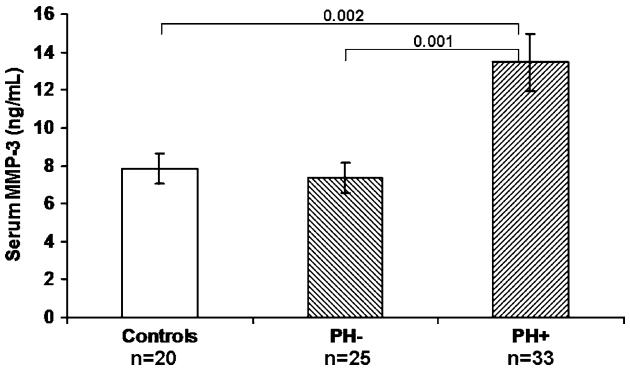

Fig. 3 Comparison of serum MMP-3 levels in biliary atresia patients based on the presence or absence of portal hypertension and controls. The data are expressed as mean ± SEM. *PH* portal hypertension

fibrosis and development of portal hypertension in postoperative BA.

According to our knowledge, the present study is the first to demonstrate that serum MMP-3 was elevated in BA patients when compared with healthy controls, and that serum MMP-3 was associated with clinical outcome (status of jaundice, hepatic dysfunction, and portal hypertension) in BA. Recently, MMP-3 has been reported to play potential roles in a number of liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [23–25]. In accordance with our findings, Baba et al. demonstrated that MMP-3 was overexpressed in all liver tissues from BA patients and was associated with liver fibrosis [15]. In addition, hepatic expression of MMP-3 has been demonstrated in carbon tetrachloride-induced liver injury in rats as described by Herbst et al. [26]. In contrast, Murawaki et al. [23] demonstrated that serum MMP-3 levels were significantly lower in patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma when compared with healthy controls, which were not related to the severity of hepatic damage or liver fibrosis.

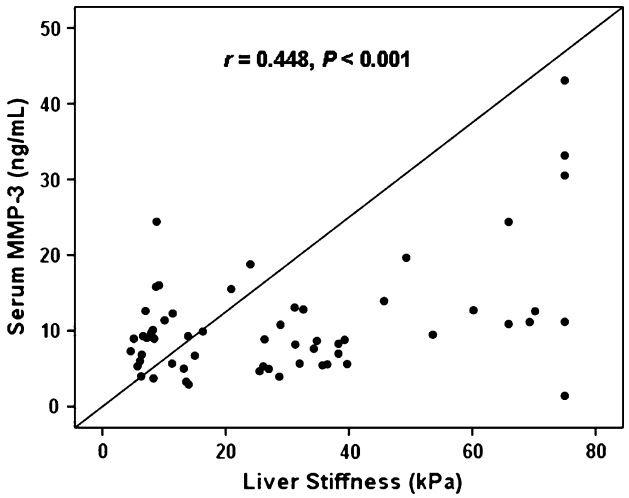

Fig. 4 Correlation between serum MMP-3 levels and liver stiffness scores in postoperative BA patients ($r = 0.448$, $P < 0.001$)

More prospective studies on hepatic MMP-3 expression are needed to elucidate the mechanisms of MMP-3 in BA.

In light of these considerations, several potential mechanisms may be responsible for the significant elevation of serum MMP-3 in BA patients, especially in those with a poor outcome. First, the production of MMP-3 in the damaged liver may result in high serum MMP-3. Secondly, the elevated MMP-3 concentrations could be attributable to the imbalance between MMP-3 production and MMP-3 clearance. In advance stage of BA, reduced biliary clearance of MMP-3 may likely contribute to elevated serum MMP-3 levels. Furthermore, extrahepatic organs can synthesize and secrete MMP-3 in systemic circulation. The higher MMP-3 levels might be regarded as indicating hepatic damage and cholestasis in BA patients. Additional investigations will render more valuable information on the pathophysiological roles of MMP-3 in BA.

It is interesting to note that there was no significant difference in serum MMP-3 levels between BA patients without jaundice and healthy controls. No difference in serum concentrations was observed between BA patients without PH and controls. These findings suggest that the high MMP-3 levels may be an epiphenomenon of cholestasis and/or hepatocellular injury rather than BA by itself, as it is known that even BA patients who are no jaundice have advancing disease process as evidenced by their ongoing abnormal liver enzymes. It should be mentioned that MMP-3 is elevated in BA patients with jaundice and progressive liver disease due to unsuccessful or partially successful Kasai procedure and that patients who achieved adequate biliary drainage after surgical operation or were not jaundiced had serum MMP-3 levels similar to healthy controls. The exact reason for this observation is unclear, but it could be attributed to the wide variation within the

group and small sample size in this study. The mechanism that underlies serum MMP-3 elevation in BA requires additional investigation.

It should be pointed out, however, that there are some limitations to our study. The sample size of patients recruited in this study was relatively small. A further study with a random sample of a larger population will be essential to make a more definite conclusion. In addition, the study was limited to those patients who attended our hospital. Consequently, the results may not be directly applicable to subjects from other ethnic groups. Moreover, this study was cross-sectional in its design and, therefore, the conclusion regarding a causal relationship may not be drawn. However, with the supporting evidence from other studies based on the role of MMP-3 in liver cirrhosis and hepatic fibrosis [14, 15, 24–26], it is plausible that the elevated serum MMP-3 observed in postoperative BA patients could be involved in the pathophysiology of hepatocellular injury and perpetuation of portal hypertension.

To summarize, the present study showed that BA patients had significantly elevated serum MMP-3 levels compared with healthy controls. Serum MMP-3 was more pronounced in the BA patients with persistent jaundice as compared to those without jaundice. In addition, serum MMP-3 in BA children with high ALT was significantly elevated as compared to those with normal ALT. Subsequent analysis demonstrated that BA patients with PH had remarkably higher serum MMP-3 levels than those without PH. High serum MMP-3 is associated with jaundice status, hepatocellular injury, portal hypertension, and liver stiffness in postoperative BA. These findings suggest that MMP-3 could play a potential role in the pathogenesis of hepatic fibrosis in postoperative BA patients. Further studies on serum and hepatic MMP-3 expression of BA patients at the time of surgery are needed to better understand the precise role of MMP-3 in BA.

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