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ORIGINAL ARTICLE

Association of circulating osteopontin levels with clinical outcomes in postoperative biliary atresia

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Abstract
Purpose Biliary atresia (BA) is one of the most serious liver disorders in children. The aims of the present study were to investigate circulating levels of osteopontin in BA children compared with healthy controls and to evaluate the relationship between circulating osteopontin and therapeutic outcome of BA patients.
Methods Fifty-nine BA patients post-Kasai operation and 13 healthy children were recruited. The patients were divided into two groups according to their serum total bilirubin levels (TB < 2, jaundice-free vs. TB ≥ 2 mg/dL, persistent jaundice) and alanine aminotransferase (ALT < 45, normal ALT vs. ALT ≥ 45 IU/L, elevated ALT). Plasma osteopontin levels were analyzed using commercial enzyme-linked immunosorbent assay.
Results The circulating osteopontin was higher in BA children compared with that of healthy controls (146.9 ± 19.1 vs. 28.0 ± 8.4 ng/mL, $P = 0.001$). The BA patients with persistent jaundice had more increased plasma osteopontin levels than those without jaundice (157.8 ± 47.9 vs. 27.5 ± 6.4 ng/mL, $P = 0.001$). Furthermore, plasma osteopontin levels in BA patients with elevated ALT were significantly higher than those with normal ALT (103.2 ± 29.2 vs. 24.5 ± 7.9 ng/mL, $P = 0.01$). In addition, circulating osteopontin was positively correlated with serum total bilirubin ($r = 0.526$, $P < 0.001$) and with serum ALT ($r = 0.575$, $P < 0.001$). Subsequent analysis showed that the BA patients with portal hypertension had more elevated plasma osteopontin compared to those without portal hypertension (116.7 ± 31.1 vs. 19.5 ± 9.3 ng/mL, $P = 0.01$).
Conclusion Increased circulating osteopontin was associated with the development of hepatic dysfunction and portal hypertension in BA patients. Circulating osteopontin may serve as a possible marker reflecting disease severity and monitoring the disease progression in postoperative BA patients.

Keywords Biliary atresia · Jaundice · Osteopontin · Portal hypertension

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Introduction

Biliary atresia (BA) is a serious neonatal disorder characterized by progressive, destructive cholangiopathy affecting extra-hepatic ducts of the biliary tree. It may result in obliteration or discontinuity of the biliary tract at any point between the porta hepatis and the duodenum. If BA patients are left untreated, the majority of them will develop severe liver damage, portal hypertension, and ultimately die within the age 2 years [1]. The first line of surgical treatment is early Kasai hepatoportoenterostomy. Despite early diagnosis and successful Kasai operation, more than half of the BA patients inevitably develop hepatic fibrosis, biliary cirrhosis, and end-stage liver disease [2]. Alternatively, liver transplantation is an effective

treatment modality when the Kasai portoenterostomy has failed and serious complications occur as for example, recurrent cholangitis, persistent jaundice, cirrhosis, progressive ascites, and bleeding esophageal varices [3]. Although several etiologies of BA have been postulated, such as neonatal viral infections, genetic disorders, abnormalities in immune response, the precise pathogenesis of BA remains unclear [4]. Previously, it has been reported that a number of cytokines and growth factors including transforming growth factor-beta [5], hepatocyte growth factor [6], and basic fibroblast growth factor [7] play important roles in the pathophysiology of BA. However, little information is available on circulating osteopontin levels in postoperative BA.

Osteopontin is a highly phosphorylated and glycosylated secretory protein that is not only expressed in bone, but also is secreted by a variety of cell types including chondrocytes, immune cells, smooth muscle cells, epithelial cells, and endothelial cells [8, 9]. It serves as a multifunctional factor in both physiologic and pathologic processes such as cell adhesion, migration, inflammatory response, anti-apoptosis, vascular remodeling, and bone calcification [10]. Osteopontin contains multiple functional domains, with a high sialic acid content, aspartate-rich domain, calcium-binding domain, thrombin cleavage site, many residues with consensus for phosphorylation as well as integrin-binding arginine–glycine–aspartate (RGD) motif, which play a role in various inflammatory diseases [11]. The role of osteopontin in liver diseases is not completely elucidated. Carbon tetrachloride administration has been demonstrated to increase osteopontin expression in rat liver where it was localized mostly to activated Kupffer cells, hepatic macrophages, and stellate cells [12]. Recombinant osteopontin also activated rat hepatic macrophage migration in vitro. These results suggest that osteopontin may play a principal role in stimulating inflammation to the liver and promoting liver fibrosis.

Recently, the overexpression of osteopontin has been documented by using gene chip array and Northern blot analysis of livers from children with BA [13]. Previous studies have also demonstrated markedly upregulated gene expression of hepatic osteopontin in biliary atresia, which suggests that human bile duct epithelial cells can synthesize osteopontin [14, 15]. These findings prompted us to consider the hypothesis that osteopontin may play a role in the pathogenesis of BA. However, the circulating osteopontin from various clinical stages of BA and its prognostic significance in the BA patients have received little attention. We hypothesize that circulating osteopontin would be more pronounced in BA patients than in healthy controls. Therefore, the purposes of the present study were to investigate circulating concentration of osteopontin in plasma collected from the BA patients after Kasai operation and to determine the relationship between plasma osteopontin and outcome parameters in BA.

Materials and methods

Patients

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, and was conducted in accordance with the Declaration of Helsinki. All parents of children with BA and healthy controls were informed of the research's purpose. Their written informed consent was obtained prior to the children being recruited.

Fifty-nine BA patients (37 girls and 22 boys; mean age 8.2 ± 0.4 years) who came for the follow-up visit in Pediatric Liver Clinic and 13 healthy children (7 girls and 6 boys; mean age 8.3 ± 1.1 years) from the Well Baby Clinic at King Chulalongkorn Memorial Hospital were recruited in this study. All BA children had undergone hepatic portojejunostomy with Roux-en-Y (original Kasai operation) and the children in control group had normal physical findings and had no previous medical problems.

None of the BA patients in this study had any symptoms and signs suspected infection or ascending cholangitis or hemostatic abnormalities at the time of blood sampling. None had obtained liver transplantation. In order to compare the outcomes among BA patients, they were classified into two groups based on serum total bilirubin (TB) and serum alanine aminotransferase (ALT). According to the jaundice status, BA patients were categorized into no jaundice group (TB < 2 mg/dL; good outcome, $n = 38$) and persistent jaundice group (TB ≥ 2 mg/dL; poor outcome, $n = 21$). Based on serum ALT, they were subsequently divided into normal ALT group (ALT < 45 IU/L, $n = 22$) and elevated ALT group (ALT ≥ 45 IU/L, $n = 37$). Portal hypertension (PH) was designated by the presence of esophageal varices demonstrated by endoscopy. Sixteen of 38 BA patients without jaundice and 17 of 21 patients with persistent jaundice had portal hypertension.

Laboratory tests

Plasma samples were obtained from each patient and healthy control and stored at -70°C until tested. Double-blinded quantitative detection of plasma osteopontin was performed using commercial enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions (Immuno-Biological Laboratories Co., Gunma, Japan). Briefly, standards of recombinant human osteopontin and plasma samples were added to 96-well

microtiter plates precoated with rabbit polyclonal antibody against osteopontin and incubated for 1 h at room temperature. The wells were then washed seven times with washing buffer and incubated for 30 min at 4°C with a horseradish peroxidase-labeled mouse monoclonal antibody to human osteopontin. After washing thoroughly with washing buffer nine times, substrate solution was added to each well, and the plate was incubated for 30 min at room temperature in the dark. Eventually, the reaction was stopped with the stop solution, and then absorbance was measured at 450 nm using automated microtiter plate reader. The osteopontin concentration was determined from a standard curve. Twofold serial dilutions of recombinant human osteopontin with a concentration of 5–320 ng/mL were used as standards. Sensitivity level for this assay was 3.3 ng/mL.

The liver function tests, including serum albumin, total bilirubin, direct bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyl transferase, were measured by routine laboratory methods using Hitachi 912 automated machine.

Statistical analysis

Comparison of demographic data and clinical parameters between groups were performed using unpaired *t* test. Correlation among numerical data was obtained using Pearson correlation coefficient (*r*). Sensitivity, specificity, receiver-operating characteristic (ROC) curves and area under the curve (AUC) were also determined. The level of statistical significance was set at $P < 0.05$. Data are expressed as mean and SEM. Statistical analysis was performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Fifty-nine plasma samples from BA patients and 13 from healthy children were obtained for the measurement of plasma osteopontin concentrations. There was no statistically significant difference of age (healthy controls vs. BA patients, 8.3 ± 1.1 vs. 8.2 ± 0.4 years) and gender (healthy controls vs. BA patients, female/male 7/6 vs. 37/22) between healthy controls and the BA patients. The demographic data, liver function tests and plasma osteopontin in BA patients with and without jaundice were displayed in Table 1.

As demonstrated in Fig. 1 plasma osteopontin levels in control group, overall BA group, no jaundice group, and persistent jaundice group were 28.0 ± 8.4, 146.9 ± 19.1, 27.5 ± 6.4, and 157.8 ± 47.9 ng/mL, respectively. Plasma osteopontin levels were significantly elevated in patients with BA compared with healthy controls ($P = 0.001$). In BA patients, plasma osteopontin levels were significantly lower in the patients without jaundice than those with persistent jaundice ($P = 0.001$). There were no significant differences in plasma osteopontin levels between jaundice-free group and controls. Figure 2 shows the plasma osteopontin in control group in comparison with patients with elevated ALT (ALT ≥ 45 IU/L) and normal ALT (ALT < 45 IU/L). Patients with elevated ALT had significantly higher plasma osteopontin levels than those with normal ALT and control group (103.2 ± 29.2 vs. 24.5 ± 7.9 ng/mL, respectively, $P = 0.01$). Moreover, BA patients with portal hypertension had significantly higher levels of plasma osteopontin compared to those without portal hypertension (116.7 ± 31.1 vs. 19.5 ± 9.3 ng/mL, $P = 0.01$) as shown in Fig. 3. There was a positive correlation between circulating osteopontin and serum total

Table 1 Demographic data, liver function test and plasma osteopontin levels of biliary atresia patients classified based on status of jaundice

	Controls	Overall	No jaundice	Jaundice	*P*
N	13	59	38	21	
Female/male	7/6	37/22	24/14	13/8	0.2
Age (years)	8.3 ± 1.1	8.2 ± 0.4	8.8 ± 0.5	7.0 ± 0.7	0.05
Albumin (g/dL)	–	4.0 ± 0.1	4.3 ± 0.1	3.3 ± 0.2	0.001
Total bilirubin (mg/dL)	–	5.2 ± 1.3	0.8 ± 0.1	13.3 ± 2.8	0.001
Direct bilirubin (mg/dL)	–	3.5 ± 0.9	0.2 ± 0.1	9.5 ± 2.1	0.001
AST (IU/L)	–	136.8 ± 16.3	74.0 ± 10.1	250.3 ± 28.6	0.001
ALT (IU/L)	–	114.8 ± 13.9	80.5 ± 14.4	176.9 ± 23.8	0.001
ALP (IU/L)	–	443.5 ± 39.3	376.2 ± 47.9	565.2 ± 61.2	0.02
GGT (IU/L)	–	200.1 ± 25.9	166.8 ± 31.6	260.3 ± 43.0	0.1
Osteopontin (ng/mL)	28.0 ± 8.4	146.9 ± 19.1	27.5 ± 6.4	157.8 ± 47.9	0.001

The data are expressed as mean ± SEM. *P* values for differences between the no jaundice and persistent jaundice patients

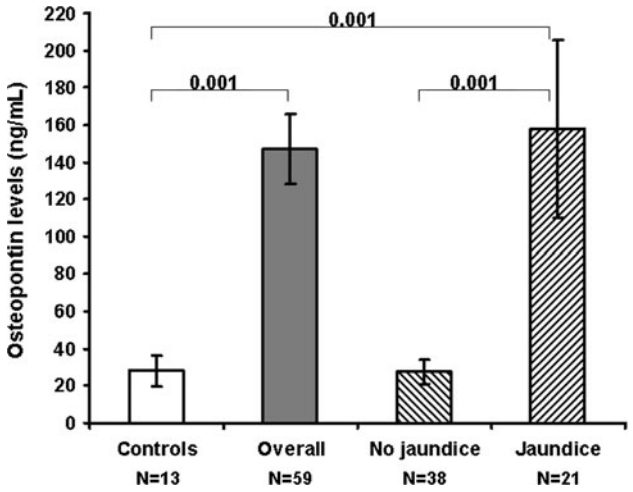

Fig. 1 Plasma osteopontin levels between biliary atresia patients based on total bilirubin and controls. The data are expressed as mean ± SEM

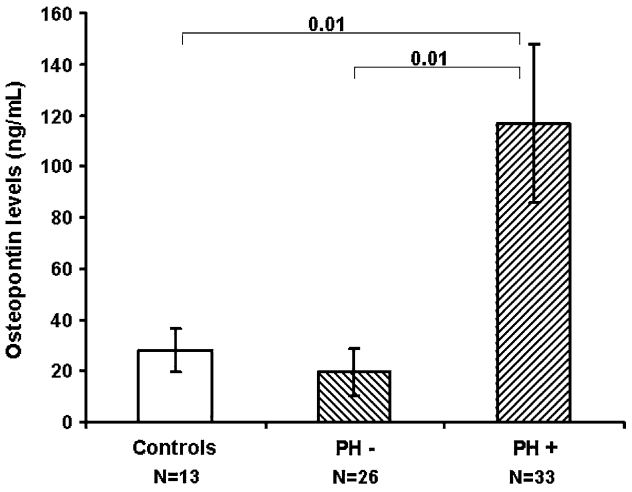

Fig. 3 Plasma osteopontin levels between biliary atresia patients based on the presence of portal hypertension (PH) and controls. The data were expressed as mean ± SEM. *PH* portal hypertension

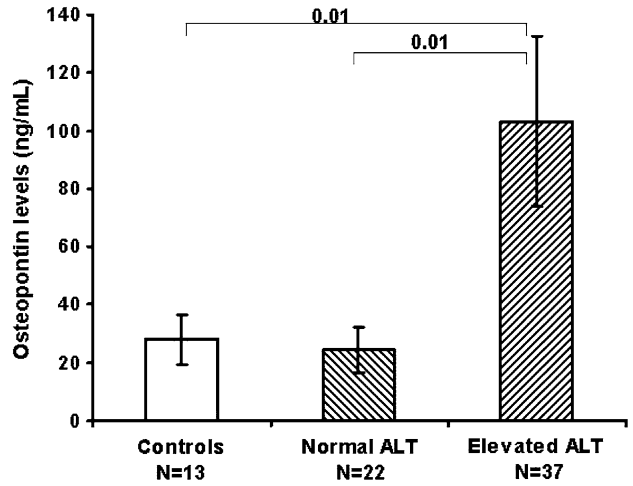

Fig. 2 Plasma osteopontin levels between biliary atresia patients based on serum ALT and controls. The data were expressed as mean ± SEM. *ALT* alanine aminotransferase

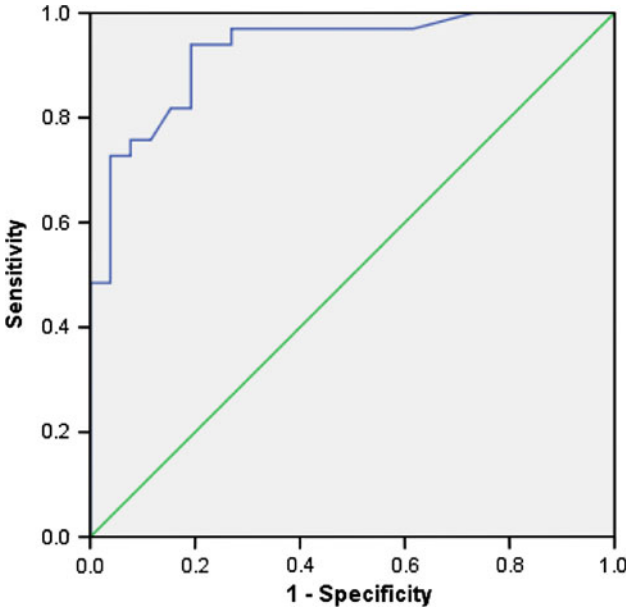

Fig. 4 ROC curves testing the ability of osteopontin to predict esophageal varices in BA patients, with an AUC of 0.928. The best cut-off value to detect varices was 21.4 ng/mL, with sensitivity of 73% and specificity of 96%

bilirubin levels ($r = 0.526$, $P < 0.001$). Furthermore, circulating osteopontin was positively correlated with serum ALT ($r = 0.575$, $P < 0.001$). ROC curve analysis illustrated a good discriminating power of circulating osteopontin for portal hypertension/esophageal varices in postoperative BA patients, with a cut-off value of 21.4 ng/mL and an AUC of 0.928 (95% CI 0.864–0.993) (Fig. 4).

Discussion

Biliary atresia is a rare and intractable liver disease of unknown cause that affects hepatic bile ducts resulting in chronic cholestasis, hepatic fibrosis and biliary cirrhosis. BA infants are generally presented with a triad of obstructive jaundice, acholic stools, and hepatosplenomegaly. It is the most common indication for liver transplantation in infants and children [1]. Even after effective bile flow has been established by the Kasai procedure, the disease still progresses to the end-stage liver disease which patients will suffer from the complication of portal hypertension and hepatic dysfunction [2, 3]. Nevertheless, the precise pathophysiology of liver fibrosis or cirrhosis in BA patients is not completely defined.

Osteopontin, also known as early T cell activation gene-1 (Eta-1), is a secreted phosphoprotein 1 (SPP1) that has been implicated in the pathogenesis of various inflammatory and fibrotic disorders. It stimulates T cell proliferation and induces T cells and macrophages to express other Th1 cytokines during inflammation [16]. Elevation of circulating osteopontin levels has been reported in patients with rheumatic mitral stenosis [17], multiple sclerosis [18], osteoarthritis [19], as well as gastric and liver cancer [20]. However, there has been no published article investigating the relationship between circulating osteopontin levels and the outcome parameters of biliary atresia.

From this study, we showed that plasma osteopontin levels in BA patients were significantly higher than those in healthy controls. In BA patients, the patients with elevated ALT had increased levels of plasma osteopontin compared to those with normal ALT. Subsequent analysis showed that plasma osteopontin levels were higher in BA patients with persistent jaundice than those without jaundice. These findings indicate that circulating osteopontin is associated with serum ALT and status of jaundice in postoperative BA patients. The elevated circulating osteopontin was positively correlated with the serum ALT and serum total bilirubin in postoperative BA patients. ALT is routinely utilized as a specific biochemical marker of liver disorders reflecting hepatocellular injury. Additionally, jaundice status in BA patients is likely to be a parameter for intrahepatic biliary obstruction. Hence, these results suggest that osteopontin play a plausible role in the pathogenesis of hepatocellular injury in BA, and that seem to be involved in the degree of biliary obliteration.

According to our knowledge, the present study is the first to illustrate that circulating osteopontin was elevated in BA patients compared with healthy controls, and that osteopontin concentration was associated with therapeutic outcome (status of jaundice, hepatic dysfunction, and portal hypertension) in BA. Elevated circulating osteopontin has been reported in a number of liver diseases, including acute hepatic dysfunction, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and primary biliary cirrhosis [21–25]. In agreement with the findings of this study, Zhao et al. [22] revealed that plasma osteopontin increased significantly in cirrhosis patients, which was correlated with the severity of hepatic damage. Kim et al. [24] also demonstrated an elevation of plasma osteopontin with advancing degree of hepatocellular carcinoma. These findings suggest that high plasma osteopontin is associated with hepatic injury and hence reflect liver dysfunction. In this regard, we observed that a circulating osteopontin level >21.4 ng/mL had a sensitivity of 73% and a specificity of 96% to predict portal hypertension/esophageal varices in postoperative BA patients. These findings indicate that circulating osteopontin could be used as a predictive marker for ongoing deterioration of liver function and perpetuation of esophageal varices in these patients and, hence, become a prognostic factor for better timing of liver transplantation.

Several possible mechanisms may be responsible for the elevation of plasma osteopontin in BA patients. Firstly, the production of osteopontin in the damaged liver might contribute to high circulating osteopontin. Recent studies have shown that osteopontin mRNA and protein expression were significantly increased in the livers of BA patients and correlated with the degree of hepatic fibrosis in BA livers [14, 15], suggesting that osteopontin production by the liver in BA is elevated. Secondly, the increased osteopontin levels could be attributed to the imbalance between osteopontin production and osteopontin clearance. In advanced stage of BA, reduced osteopontin clearance may possibly result in enhance circulating osteopontin levels. Furthermore, since other organs apart from liver can synthesize osteopontin, the main sources of increased plasma osteopontin in this study could be extrahepatic organs. Additional investigation will be necessary to clarify this observation.

It should be pointed out, however, that a limitation of the present study is that the sample size was not large enough to make strong conclusions. Our results should be confirmed in a larger number of subjects. In addition, incomplete assessment of potential confounders including age, gender, medical comorbidities need to be taken into account. Another limitation of this study is that this research was designed as a cross-sectional study, definite cause and effect relationship may not be concluded. Finally, overexpression of plasma osteopontin might be just the non-specific finding of cholestasis. Additional studies on non-BA children with cholestatic liver diseases will solve this concern. However, with the supporting evidence from other studies regarding the association between osteopontin expression and the degree of systemic inflammatory response [13–15], it is likely that the elevated plasma osteopontin levels found in postoperative BA patients may be involved in the pathophysiology of hepatocellular injury and perpetuation of portal hypertension.

Taken together, this study revealed that BA patients had significantly increased circulating levels of osteopontin compared with healthy controls. Plasma osteopontin was more pronounced in the BA children with persistent jaundice than in those without jaundice. In addition, patients with PH had markedly higher osteopontin levels than those without PH. Osteopontin measurement may be a valuable index to reflect the disease severity and the development of PH in the post-Kasai BA patients. More studies should address the relationships between plasma and hepatic osteopontin levels among BA children at the time of surgery and include the different groups of age-matched

patients with other cholestatic liver diseases for comparison. Whether osteopontin will prove to be a real prognostic marker of postoperative BA will require further investigation.

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