

## Original article

# Serum vascular endothelial growth factor per platelet count in patients with biliary atresia

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**Background:** Biliary atresia (BA) is a progressive, sclerosing, inflammatory process resulting in complete obliteration of the extrahepatic bile ducts. The obstruction of bile flow engenders worsening cholestasis, hepatic fibrosis, and cirrhosis, which lead to portal hypertension and a decline in hepatic synthetic function. Hepatic stellate cells, which play roles in hepatic fibrogenesis, are an important source of various inflammatory mediators including vascular endothelial growth factor (VEGF) in the injured liver.

**Objectives:** Investigate the level of serum VEGF and serum VEGF per platelet count in patients with BA and its relation to clinical characteristics.

**Methods:** Peripheral blood samples were taken from 70 BA patients and 15 healthy control children. Serum VEGF was measured by enzyme-linked immunosorbent assay. We compared serum VEGF and serum VEGF per platelet count in BA patients with the respective results obtained in healthy control children. The relation of serum VEGF per platelet count with clinical variables of BA patients was investigated.

**Results:** Serum VEGF levels and serum VEGF per platelet count in BA patients were not significantly different from those in normal controls ( $289.64 \pm 230.01$  pg/mL vs.  $312.36 \pm 189.05$  pg/mL;  $p=0.72$  and  $1.72 \pm 1.21 \times 10^6$  vs.  $1.57 \pm 0.97 \times 10^6$ ;  $p=0.66$ ). Significant differences were observed among BA patients when VEGF per platelet count was categorized by the presence of esophageal varice ( $p=0.03$ ). Only in BA patients was the serum level of VEGF correlated with the number of platelets ( $r=0.53$ ,  $p<0.001$ ).

**Conclusion:** A high serum VEGF per platelet count is a useful marker for the development of portal hypertension in BA patients, especially for esophageal varice. Serum VEGF per platelet count may be useful for monitoring disease course in BA after hepatic portoenterostomy.

**Keywords:** Biliary atresia, platelet, vascular endothelial growth factor

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Biliary atresia (BA) is a progressive, sclerosing, inflammatory process resulting in complete obliteration of the extrahepatic bile ducts. The obstruction of bile flow engenders worsening cholestasis, hepatic fibrosis, and cirrhosis, which lead to portal hypertension and a decline in hepatic synthetic function. Successful restoration of bile flow by hepatportoenterostomy can be achieved in 60 to 90% of patients, although 75% of them will progress to cirrhosis and eventually end-stage liver disease requiring liver transplantation [1]. The etiology of BA has so far remained unknown.

Several hypotheses refer to infections, congenital factors, toxic agents, and immune-related causes [2]. Various genes involved in cell signaling, transcription regulation, hepatic development, morphogenesis, and fibrogenesis are altered in BA [3]. Previous reports on BA patients who had undergone hepatic portoenterostomy revealed that a number of cytokines, such as endothelin-1, hyaluronan, hepatocyte growth factor, interleukin-8, and tissue inhibitors of metalloproteinase-1, were significantly elevated in patients with persistent jaundice, abnormal transaminases, and/or portal hypertension than in those free from those complications [4-8]. These results suggest that numerous cytokines are involved in hepatic inflammation and fibrogenesis in BA.

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Hepatic stellate cells (HSCs), located in Disse's space, have been strongly implicated in the generation of liver fibrogenesis. Activated HSCs produce transforming growth factor (TGF)- $\beta$  and extracellular matrix (ECM) components, which induce fibrosis [9,10]. Furthermore, increased ECM components impair blood flow and oxygen delivery, resulting in hypoxia [11]. Hepatic vascular cell proliferation, or vascular remodeling, is also closely associated with liver fibrosis [12, 13]. Progression of liver fibrosis has been linked with injuries associated with hypoxia and neovascularization [14]. Neovascularization comprises angiogenesis, the formation of new blood vessels by sprouting of preexisting mature endothelial cells, and vasculogenesis, the formation of blood vessels by differentiation of endothelial progenitor cells [15-17]. Activated HSCs are harmful due to unbalanced expression and secretion of growth factors that enhance fibrogenesis and neovascularization [18].

Vascular endothelial growth factor (VEGF) is a well-known, potent angiogenic factor which enhances vascular permeability, promoting the extravasation of protein to form a stromal matrix and tumor invasion [19]. VEGF has proven a potent, diffusable and specific endothelial growth factor. In recent years, several members of the VEGF family have been described, namely, placenta growth factor (PlGF), VEGF-A, VEGF-B, VEGF-C and VEGF-D [20]. Serum VEGF can be influenced by VEGF released from platelets during clotting [21, 22]. In a recent study, serum VEGF per platelet count has been applied to correct variations of serum VEGF levels in patients with different platelet counts [23]. It has been shown that serum VEGF per platelet count correlates with advanced stage colorectal cancer, suggesting its role as a standard measure of circulating VEGF. DNA array analysis conducted on livers of patients with chronic hepatitis C, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and nonalcoholic steatohepatitis has demonstrated overexpression of genes essential for fibrogenesis and neovascularization including those encoding growth factors such as hepatocyte growth factor (HGF) and VEGF, their receptors, adhesion molecules, and matrix remodeling molecules [24, 25]. Nonetheless, data on the role of VEGF in patients with BA is still scarce. The aim of this study has been to determine the relationship of serum VEGF and serum VEGF per platelet count, calculated by dividing serum VEGF

concentration by the platelet count, with clinical parameters in patients with BA.

### **Materials and methods**

The study protocol has been approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Likewise, the parents of all participating children have provided their consent to the study.

### **Subjects**

The study included 70 patients with BA who attended the liver clinic at King Chulalongkorn Memorial Hospital. Fifteen healthy children served as controls.

### **Clinical data**

Physical examination, liver function test, and complete blood count were performed on each patient. Splenomegaly was diagnosed based on physical examination whereas esophageal varice was recognized by endoscopy. Patients with total bilirubin  $\geq 2$  mg/dL were classified as jaundiced.

### **Blood sampling and enzyme immunoassay of serum VEGF concentration**

Serum samples were stored at  $-70^{\circ}\text{C}$  until tested. Serum VEGF was measured in duplicate by commercially available ELISA kits (Quantikine human VEGF, R&D System, Minneapolis, USA). According to the manufacturer's specifications, the minimal detectable dose of VEGF was  $<5.0$  pg/mL, the intra-assay and the inter-assay variables were 6.7-5.1% and 8.8-6.2%, respectively. Absorbance was determined using a microplate reader 3550 (Bio-Rad, Hercules, USA) at 450 nm. Serum VEGF per platelet count was calculated by dividing serum VEGF concentration by the platelet count.

### **Statistical analysis**

The results were expressed as the mean  $\pm$  standard deviation (SD). To investigate the relationships between serum levels of VEGF, VEGF per platelet count and other variables, "t"-test and correlation coefficient ( $r$ ) were used when appropriate. A value of  $p < 0.05$  based on a two-tailed test was considered statistically significant. All the statistical analyses were performed using a statistical software package (SPSS, Version 13, Chicago, USA).

## Results

### *Serum VEGF in patients with biliary atresia and healthy controls*

The baseline characteristics of the patients with BA and healthy controls are shown in **Table 1**. There was no significant difference in serum VEGF levels between the patients with BA and healthy controls.

### *Correlation of serum VEGF level and platelet count*

In the patients with BA, there was a statistically significant correlation between serum VEGF level and platelet count ( $r=0.53$ ,  $p<0.001$ ) (**Fig.1**). Increase in serum VEGF level was directly related to the increase in platelet count. On the other hand, there was no correlation between serum VEGF levels and platelet

count in healthy controls ( $p=0.06$ ).

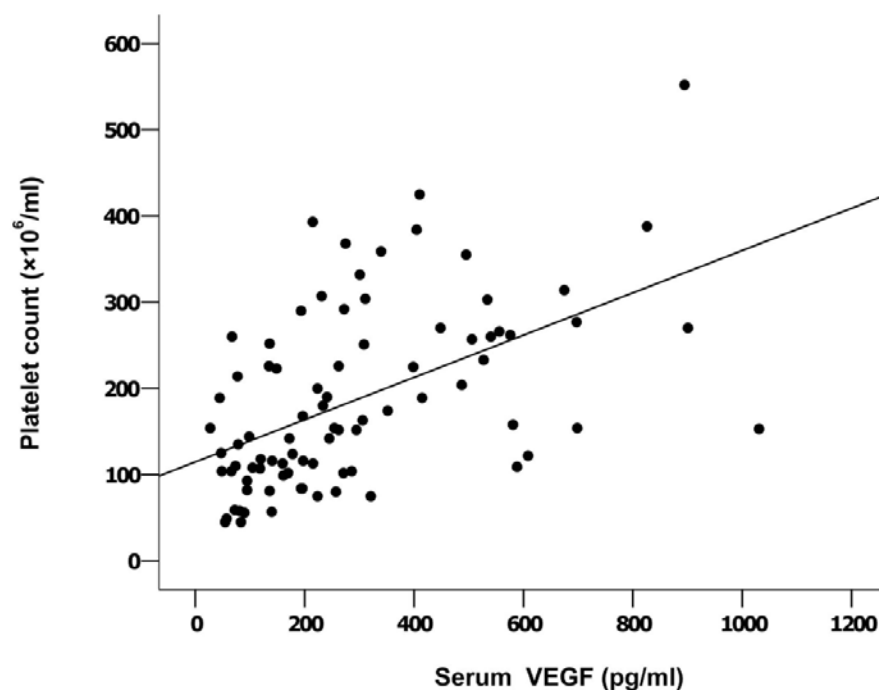
### *Correlation between serum VEGF and serum VEGF per platelet count and clinical features in patients with biliary atresia*

There was no significant association between serum VEGF levels and various clinical variables, including esophageal varice and jaundice except for splenomegaly (**Table 2**). Serum VEGF levels in BA patients with jaundice, splenomegaly, or esophageal varice were not different from those in the control group. Patients without splenomegaly had higher platelet counts than those with splenomegaly ( $268.17\pm98.19\times10^6/\text{mL}$  vs.  $138.43\pm80.04\times10^6/\text{mL}$ ,  $p<0.001$ ).

**Table 1.** The characteristics and serum vascular endothelial growth factor (VEGF) level of the patients with biliary atresia and healthy controls.

	Healthy controls	Biliary atresia
Number	15	70
Gender (Male/Female)	11/4	35/35
Age (year)*	8.13 $\pm$ 5.34	6.08 $\pm$ 4.51
Serum VEGF (pg/mL)**	352.29 $\pm$ 190.13	289.64 $\pm$ 230.01

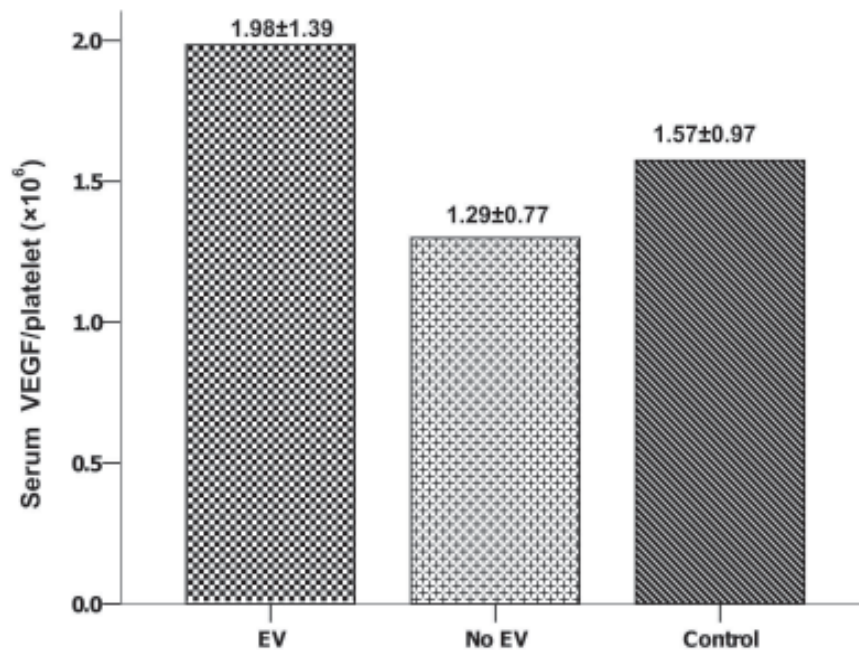
Data are expressed as mean $\pm$ SD. \* $p=0.13$ ; \*\* $p=0.33$



**Fig. 1** The correlation between serum vascular endothelial growth factor (VEGF) level and platelet count in the patients with biliary atresia ( $r=0.53$ ,  $p<0.001$ ).

Serum VEGF per platelet count in patients with BA was not significantly different from that in healthy controls ( $1.72 \pm 1.21$  vs  $1.57 \pm 0.97 \times 10^6$ ,  $p=0.66$ ). In patients with BA, the serum VEGF per platelet count was higher in the group with esophageal varice than

in those without esophageal varice ( $1.98 \pm 1.39 \times 10^6$  vs.  $1.29 \pm 0.77 \times 10^6$ ,  $p=0.03$ ) (**Fig. 2**). However, there was no correlation between serum VEGF per platelet count and other clinical features including jaundice and splenomegaly (**Table 2**).



**Fig. 2** Mean serum vascular endothelial growth factor (VEGF) levels in patients with biliary atresia with and without esophageal varice (EV). Data represent mean $\pm$ SD.

**Table 2.** Serum vascular endothelial growth factor (VEGF) level of the patients with biliary atresia.

	Serum VEGF level (pg/mL)	P-value	VEGF/platelet ( $\times 10^6$ )	P-value
<b>Jaundice</b>		0.50		0.18
Yes (n=28)	266.90 $\pm$ 248.60		1.97 $\pm$ 1.37	
No (n=42)	304.80 $\pm$ 218.52		1.56 $\pm$ 1.09	
<b>Splenomegaly</b>		0.04		0.17
Yes (n=47)	251.30 $\pm$ 214.91		1.86 $\pm$ 1.26	
No (n= 23)	367.99 $\pm$ 244.55		1.43 $\pm$ 1.08	
<b>Esophageal varice</b>		0.27		0.03
Yes (n=42)	258.49 $\pm$ 234.28		1.98 $\pm$ 1.39	
No (n=22)	323.60 $\pm$ 193.52		1.29 $\pm$ 0.77	

Data are expressed as mean $\pm$ SD.

## Discussion

Hepatic stellate cells (HSCs) are an important source of inflammatory mediators in the injured liver, assuring autocrine and paracrine regulation of proliferation and activation of neighboring cells and HSCs. Under a hypoxic atmosphere mimicking a microenvironment of injured liver, HSCs were activated to produce VEGF protein to participate in hepatic angiogenesis and fibrogenesis [26-28]. VEGF is up-regulated by hypoxia and a number of cytokines including epidermal growth factor (EGF), TGF- $\beta$ , IL-1, and IL-6 [29]. Cellular hypoxia is known to occur in alcoholic liver injury, hepatic fibrosis, and in tumors, three conditions in which HSCs play an important role. Activated HSCs produce cyclooxygenase-2 (COX-2) protein to induce VEGF production that is believed to play a key role in angiogenesis and fibrogenesis in the injured liver [26]. Previous reports have shown that VEGF protein derived from HSCs is involved in the proliferation of sinusoidal endothelial cells [30, 31]. Hepatocellular hypoxia and angiogenesis have been associated with fibrogenesis in experimental cirrhosis [27, 32]. Hypoxic tissue injury is frequently due to microvascular disruption, which causes cessation of blood flow while hypoxia simultaneously enhances the production of angiogenic factors such as VEGF protein.

Portal hypertension is a frequent and severe complication of chronic liver diseases including biliary atresia. It is characterized by a pathological increase in portal venous pressure, by the subsequent formation of an extensive network of portosystemic collateral vessels (PSC), including gastroesophageal varices, splenomegaly, and by the development of hyperdynamic circulatory syndrome [33, 34]. It has been recently demonstrated that the expression of VEGF is increased in experimental models of portal hypertension, and that the formation of PSC is, in part, a VEGF-dependent angiogenic process [35, 36]. Both circulating and tissue bound VEGF tend to increase in acute and chronic hepatitis and to decrease in cirrhosis [37]. However, in this study there has been no significant difference between serum VEGF levels of BA patients with portal hypertension (either splenomegaly or esophageal varice) and normal controls. According to this study, serum VEGF levels in BA patients did not correlate with the jaundice status, consistent with the study by Aw et al. [38] showing no correlation between serum VEGF levels and aspartate aminotransferase or bilirubin in children with acute liver failure.

Principally, serum VEGF is a combination of both VEGF released from platelets on coagulation and the circulating plasma VEGF. Platelets may normally contain a certain amount of VEGF in their granules. Therefore, serum VEGF level can be influenced by platelet count. A direct correlation between platelet counts and serum VEGF has also been reported [39-41]. In addition, platelets have been reported to engulf and store various angiogenic factors such as VEGF and platelet-derived growth factor (PDGF) in their granules, and these molecules are secreted immediately after platelet activation [42]. In this study, serum VEGF levels significantly correlated with platelet counts in BA patients ( $r=0.53$ ,  $p<0.001$ ), but there was no such correlation in healthy controls. These facts would imply increased amounts of VEGF in platelets of the BA patients.

Serum VEGF per platelet count is an indirect theoretical estimate of VEGF in platelets. In this study, serum VEGF levels and platelet counts were higher in BA patients without splenomegaly than in those with splenomegaly, but serum VEGF per platelet count was not different in those with or without splenomegaly. In contrast, BA patients with esophageal varice showed higher serum VEGF per platelet count than those without esophageal varice. Accordingly, serum VEGF per platelet count can predict the development of serious complications such as esophageal varice in patients with BA who undergo portoenterostomy.

## Conclusion

This study demonstrates the possible role of serum VEGF per platelet count as an indicator of the development of esophageal varice in patients with BA. Therefore, serum VEGF per platelet count could be a useful clinical parameter for BA patients' follow-up. Further studies conducted on a larger patient population will be required in order to elucidate its value in clinical application.

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