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There is no association between K469E ICAM-1 gene polymorphism and biliary atresia

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INTRODUCTION

Biliary atresia (BA), the obliteration of extra- and intra-hepatic biliary system, remains one of the most intractable liver diseases in children. When patients with BA are left untreated, the majority of them will die of complications of biliary cirrhosis^[1,2]. At present, it is accepted that hepatic porto-enterostomy or Kasai operation is the first choice of treatment. Surgical correction at an early age is therefore the key to a successful management of infants with BA^[3], especially in countries where liver transplantation program is not widely available. So far, liver transplantation is preserved for BA patients who have gross cirrhosis at diagnosis or who failed Kasai operation^[1]. Since BA is a serious liver disease with poor long-term outcome, extensive research for its exact cause in order to understand the disease has been carried out^[4]. Nevertheless, its etiology is still unclear. A number of hypotheses have been proposed to explain the origin of the disease including peri-natal infection^[5], immune-mediated disorders^[2], and defect of morphogenesis^[6]. It has also been suggested by some research groups that susceptibility to BA is influenced by genetic factors via pro-inflammatory cytokines^[7-9].

Inter-cellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein involved in cell adhesion and acts as a cell surface ligand for members of the leukocyte integrin family. The interaction of leukocyte integrins with endothelial ICAM-1 leads to leukocyte adherence, transendothelial migration and cell activation, which are fundamental to the recruitment of leukocytes to the tissue leading to inflammatory process^[10]. A soluble form of ICAM-1 (sICAM-1) can release into the circulation during the course of an inflammatory reaction^[11,12]. Recently, it has been shown that the elevated serum sICAM-1 is associated with primary biliary cirrhosis. The levels correlate with the disease activity and the degree of cholestasis^[13].

ICAM-1 gene is located on chromosome 19p13. The gene encoding ICAM-1 is affected by a common, functionally important, genetic polymorphism^[14]. The K469E polymorphism of the ICAM-1 gene has already been described^[15,16]. This polymorphism occurs in exon 6 of the ICAM-1 gene and results in a change from lysine to glutamic acid in Ig-like

Abstract

AIM: To determine whether there was an association between inter-cellular adhesion molecule-1 (ICAM-1) gene polymorphism and biliary atresia (BA), and to investigate the relationship between serum soluble ICAM-1 (sICAM-1) and clinical outcome in BA patients after surgical treatment.

METHODS: Eighty-three BA patients and 115 normal controls were genotyped. *K469E ICAM-1* polymorphism was analyzed using PCR assay. Serum sICAM-1 was determined using ELISA method from 72 BA patients. In order to evaluate the association between these variables and their clinical outcome, the patients were categorized into two groups: patients without jaundice and those with persistent jaundice.

RESULTS: There were no significant differences between BA patients and controls in terms of gender, K469E ICAM-1 genotypes, and alleles. The proportion of patients having serum sICAM-1 ≥ 3 500 ng/mL in persistent jaundice group was significantly higher than that in the other group. In addition, there was no association between *K469E ICAM-1* polymorphism and the status of jaundice in BA patients after Kasai operation.

CONCLUSION: ICAM-1 possibly plays an important and active role in the disease progression. However, the process is not associated with genetic variation of *K469E ICAM-1* polymorphism.

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domain 5. The ICAM-1 gene polymorphism can affect the interaction between ICAM-1 and its receptor, namely leukocyte function-associated antigen-1, and influence B-cell activation^[17]. ICAM-1 plays a pivotal role in the migration of leukocytes to inflammatory sites. Therefore, it may be involved in various inflammatory diseases.

As far as we are concerned, there is no investigation regarding ICAM-1 gene polymorphism in BA. Since progressive inflammatory process within the liver is an important feature of BA^[18,19], there may be some links between the pathophysiology of BA and ICAM-1 gene polymorphism. Therefore, the study aimed to evaluate the ICAM-1 gene polymorphism in BA patients and to investigate whether there was an association between serum sICAM-1 and clinical outcome in BA patients after surgical treatment.

MATERIALS AND METHODS

This study was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University, Thailand. All parents of the children with BA and healthy controls had been informed of the purpose of the study. The written informed consents were obtained.

Study of ICAM-1 gene polymorphism in biliary atresia

BA patients were recruited into the study during annual follow-ups between January 2002 and July 2003. All patients were diagnosed as BA by exploratory laparotomy with intra-operative cholangiography during early infancy period. The control group, whose ethnicity is similar to the BA patients (Thai), comprised healthy teenagers among those who participated in an evaluation of hepatitis B vaccination during the same period. Eighty-three BA patients participated in the study. Six out of eighty-three patients received liver transplantation. The healthy control group comprised 115 teenagers.

ICAM-1 DNA detection

Peripheral whole blood from BA patients and controls was collected in tubes containing EDTA. Genomic DNA was extracted from samples according to the manufacturer's protocol. Single base polymorphism at codon 469 (K469E) in exon 6 of ICAM-1 was determined using a method based on polymerase chain reaction-restriction fragment length polymorphism for the detection of K469E ICAM-1 gene, as previously described^[14,17]. The ICAM-1 F 5'CCCCGAC-TGGACGAGAGG3' is a sense primer and ICAM-1 R 5'GGGGCTGTGGGGGAGGATA3' is an anti-sense primer. We combined 2 mL of DNA sample with a reaction mixture containing 20 mL of 2.5X Eppendorf MasterMix (Hamburg, Germany), 1 mmol/L P1, 1 mmol/L P2, and sterile water, in a final volume of 25 mL. PCR was performed under the following conditions: after an initial 4 min denaturation step at 95 °C, 37 cycles of amplification were performed, each including 30 s denaturation at 95 °C, 30 s annealing at 60 °C and 30 s extension at 72 °C, followed by a final 4 min extension at 72 °C. Each amplified DNA sample (10 mL) was added to loading buffer and run on a 2% agarose gel (FMC Bioproducts, Rockland, ME, USA) at 100 V for 60 min. The 331-bp product stained with ethidium bromide on preparation was visualized on a UV transilluminator.

PCR-RFLP analysis

PCR products were subjected to RFLP analysis, using restriction endonuclease *Bst*UI (New England Biolabs, Beverly, MA, USA) to determine the ICAM-1 polymorphism. Briefly, 10 mL of PCR product was mixed with 1.5 mL of 10× buffer, 3 mL of sterile water and 0.5 mL (5 U) of *Bst*UI incubated at 60 °C for 4 h. After incubation, the samples were run on a composite gel containing 2% NuSieve agarose (FMC BioProducts, Rockland, ME, USA) and 1% standard agarose. The clusters of PCR products were visible under UV light, as a result of prior ethidium bromide staining.

Study of serum sICAM-1 in biliary atresia

Seventy-two serum samples of BA patients were available for the study of sICAM-1. All patients underwent hepatic porto-jejunostomy with Roux-en-Y (original Kasai operation) during infancy period. Patients who received liver transplantation were excluded from the study to avoid confounding effects of different therapeutic modalities. Briefly, peripheral venous whole blood was drawn with a sterile syringe, transferred to a centrifuge tube, allowed to clot and then centrifuged at 4 °C. The sera were stored at -70 °C until they could be assayed. Serum sICAM-1 levels were determined by a commercially available ELISA kit (Quantikine, R&D Systems, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. The serum sICAM-1 levels were expressed as nanogram per milliliter. In addition, liver function tests including serum albumin, total bilirubin (TB), direct bilirubin (DB), alkaline phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ -glutamyl transpeptidase (GGT) were performed using an automated chemical analyzer (Hitachi 911) at the central laboratory of the hospital.

Out of 72 BA patients, none in this study exhibited symptoms and signs of fever or ascending cholangitis at the time of blood sampling. None received liver transplantation. In order to compare the outcome among the BA patients, they were subsequently divided into two groups according to the status of jaundice and levels of TB: patients with no jaundice (TB < 2.0 mg%) and patients with persistent jaundice (TB \geq 2.0 mg%). In addition, subgroup analysis between BA patients as per the presence of jaundice and ICAM-1 gene polymorphism was carried out.

Statistical analyses

Demographic data between groups were compared by Fisher's exact and unpaired *t*-tests. Genotype and allele frequencies were compared by χ^2 test. Significant differences were established at *P* < 0.05. Odds ratios were also calculated. For all statistical analyses, either GraphPad Prism version 3.02 (GraphPad Software Inc., CA, USA) or SPSS software version 10.0 (SPSS Inc., Chicago, IL, USA) was used. Data were expressed as mean \pm SD.

RESULTS

Study of ICAM-1 gene polymorphism in biliary atresia

The distribution of ICAM-1 genotypes and alleles in 83 BA patients and 115 controls is shown in Table 1. There

Table 1 ICAM-1 genotypic and allele distribution in BA patients and controls

	BA patients, <i>n</i> (%) <i>n</i> = 83	Controls, <i>n</i> (%) <i>n</i> = 115	Odds ratio (95% CI)	<i>P</i>
Age (yr)	6.09±4.67	13.07±2.13		<0.0005
Male	37:46	57:58		0.49
Genotype				
E/E	28 (33.74)	41 (35.65)	0.92 (0.51–1.66)	0.78
E/K	7 (8.43)	11 (9.57)	0.87 (0.32–2.35)	0.78
K/K	48 (57.83)	63 (54.78)	1.13 (0.64–2.00)	0.67
Alleles				
E	63 (37.95)	93 (40.43)	0.90 (0.60–1.36)	0.62
K	103 (62.05)	137 (59.57)	1.11 (0.74–1.67)	
E/K ratio	0.61	0.68		

were no significant differences between two groups in terms of gender, K469E ICAM-1 genotypes, and K469E ICAM-1 alleles.

Study of serum sICAM-1 in biliary atresia

Serum sICAM-1 levels in 72 BA patients were determined. The demographic data between patients with no jaundice and patients with persistent jaundice are shown in Table 2. There was no difference in age and gender between the two groups. Patients with persistent jaundice had lower levels of albumin and higher levels of AST, ALT, AP, and GGT compared to patients without jaundice.

We also found that 24 out of 72 patients had sICAM-1 levels above the upper limit detected by the ELISA kit (3 500 ng/mL), therefore, the cut point of 3 500 ng/mL was selected to subdivide the patients. By using χ^2 test, the proportion of patients having serum sICAM-1 levels ≥ 3 500 ng/mL in persistent jaundice group was significantly higher than that of patients having serum sICAM-1 levels

≥ 3 500 ng/mL in the other group ($P < 0.0005$), as shown in Table 2.

Association between K469E ICAM-1 gene polymorphism and status of jaundice

After excluding the BA patients who had undergone liver transplantation, 63 out of 83 BA patients had both data of genotype and total bilirubin levels. Therefore, an analysis of the association between ICAM-1 gene polymorphism and the status of jaundice after Kasai operation was conducted in 63 patients. The analysis showed that there was no difference in terms of age, gender, K469E ICAM-1 genotypes, and alleles between patients with no jaundice and with persistent jaundice (Table 3).

DISCUSSION

BA remains one of the major hepatic causes of death in early childhood. Although a number of hypotheses have

Table 2 Demographic data, liver function test, and serum sICAM-1 levels between BA patients without jaundice and with persistent jaundice (mean±SD)

BA patients	No jaundice (<i>n</i> = 41)	Persistent jaundice (<i>n</i> = 31)	<i>P</i>
Age (yr)	6.41±4.05	6.00±5.91	0.73
Male:female	22:19	11:20	0.12
Albumin (g/dL)	4.41±0.56	3.71±0.83	<0.0005
Total bilirubin (mg%)	0.79±0.44	12.04±10.53	<0.0005
DB (mg%)	0.24±0.26	9.36±7.85	<0.0005
AST (IU/L)	84.12±67.28	228.26±130.45	<0.0005
ALT (IU/L)	96.93±93.83	161.84±97.93	0.006
AP (IU/L)	380.90±249.80	593.20±293.49	0.002
GGT (IU/L)	163.16±175.49	384.65±404.72	0.004
sICAM-1 (<3 500: ≥ 3 500 ng/mL)	37:4	11:20	<0.0005

Table 3 ICAM-1 genotypic and allele distribution in BA patients without jaundice and with persistent jaundice

	No jaundice, <i>n</i> (%) <i>n</i> = 34	Jaundice, <i>n</i> (%) <i>n</i> = 29	Odds ratio (95%CI)	<i>P</i>
Age (yr)	5.91±3.92	5.66±5.17		0.84
Male	18:16	11:18		0.23
Genotype				
E/E	9 (26.47)	10 (34.48)	0.68 (0.23–2.01)	0.49
E/K	3 (8.82)	3 (10.34)	0.83 (0.15–4.52)	0.84
K/K	22 (64.71)	16 (55.18)	1.49 (0.52–4.11)	0.44
Alleles				
E	21 (30.88)	23 (39.66)	0.68 (0.32–1.42)	0.30
K	47 (69.12)	35 (60.34)	1.47 (0.70–3.07)	
E/K ratio	0.45	0.66		

been proposed to account for the disease, its etio-pathogenesis is poorly understood. One possibility is that BA is an immune-mediated disease, which occurs following either a toxic or an infectious insult on a genetically susceptible host^[20]. Therefore, a number of studies have investigated whether bile duct epithelial cells are susceptible to immune or inflammatory attack because of abnormal expression of human leukocyte antigens (HLA) or ICAM-1 on their cell surface^[18]. It has been demonstrated that the expression of ICAM-1 and HLA-DR antigen in BA patients increases compared to that in controls^[21]. In addition to immune-mediated theory, a role of inflammation in the pathophysiology of BA has long been proposed. Histopathologic studies of the hepatic bile duct demonstrate various stages of inflammation causing progressive destruction of biliary system^[3]. This process might be caused by an overexpression of ICAM-1^[22]. The ICAM-1 gene is located on chromosome 19p13 and two non-synonymous single nucleotide polymorphisms are known as 12959G>A (G241R) and 13848A>G (K469E)^[23].

Several investigators have demonstrated the genetic association between ICAM-1 gene polymorphism and chronic inflammation diseases including inflammatory bowel disease^[14], multiple sclerosis^[16], Behcet's disease^[24], and endometriosis^[25]. Although there is a lot of information supporting the role of ICAM-1 gene polymorphism as a risk factor for diseases characterized by inflammatory process, the study of genetic susceptibility to BA, however, receives little attention. Donaldson *et al.*^[8], suggested that BA is not a HLA-associated disease, and that IL-1 and IL-10 gene polymorphisms are not risk factors for BA. Since inflammatory process and obliterative cholangiopathy within the liver are important features of BA, together with the fact that ICAM-1 molecule plays a major role in initiating inflammation, it is of our interest to evaluate whether ICAM-1 gene polymorphism is associated with BA. According to our knowledge, such a study has not been carried out.

The present study revealed that there was no association between K469E ICAM-1 gene polymorphism and BA in Thai population. Our results suggest that K469E ICAM-1 gene polymorphism is not a risk factor for both the etio-pathogenesis and the prediction of success after Kasai operation. However, another study of serum sICAM-1 in BA patients showed that high levels of serum sICAM-1 are associated with the presence of jaundice and poor liver function, as illustrated by lower levels of serum albumin as well as higher levels of serum ALT, AST, and GGT^[26]. Our findings support the results from other research groups. Kobayashi *et al.*^[27], demonstrated that serum sICAM-1 levels can be used as a marker of end-stage liver disease in BA. Serum sICAM-1 also increases in BA patients but does not correlate with liver function^[28]. Davenport *et al.*^[2], reported, that a reduction in ICAM-1 expression on infiltrating cells in the biliary remnants is associated with a better post-operative prognosis. It might be possible that elevated sICAM-1 levels in BA patients with persistent jaundice found in this study reflect the impaired clearance of ICAM-1 from significant liver damage or, alternatively that the elevated sICAM-1 levels are caused by the increased ICAM-1 expression or by either mechanisms. Thus, the regulatory mechanisms of serum sICAM-1 seem to be complex. It is not easy to explain

the mechanisms of our observation from the study of serum sICAM-1 levels alone. At least, however, our findings show that the association between serum sICAM-1 and liver function in BA patients is significant enough to bring about a serious consideration.

According to our findings that high serum sICAM-1 levels are associated with poor outcome in BA patients following Kasai operation and the fact that ICAM-1 signaling is responsible for the initiation of inflammation, sICAM-1 levels in BA patients might have a prognostic role. In the study of serum sICAM-1, we recruited only patients who did not receive liver transplantation, in order to avoid the confounding effects from different therapeutic modalities.

It has been demonstrated that serum sICAM-1 levels in normal controls are low (less than 500 ng/mL)^[27,28]. Although there was no normal control group in our study, at least we found that the proportion of BA patients having serum sICAM-1 ≥ 3 500 ng/mL was significantly higher than that of patients without jaundice. Together with evidence supporting the role of ICAM-1 signaling pathway in inflammation, the manipulation of ICAM-1 expression might benefit BA patients. Therefore, apart from the prognostic role of serum sICAM-1 levels in BA, the therapeutic potential for BA using the immunomodulation of BA deserves attention. However, the specific mechanism requires further investigation.

In conclusion, the absence of association between K469E polymorphism of ICAM-1 and BA in Thai population has been demonstrated. BA patients with persistent jaundice following Kasai operation have higher serum sICAM-1 levels than those without jaundice. These findings suggest that ICAM-1 signaling is involved in the immunopathology of BA. Although ICAM-1 plays an important and active role in the disease progression, the process is not influenced by genetic susceptibility of K469E ICAM-1 gene polymorphism.

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