รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาพยาธิสรีรวิทยาของโรคทางเดินน้ำดีตีบตันในเด็ก

The study of pathophysiology of biliary atresia in infants

โดย

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
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ABSTRACT

Biliary atresia (BA) remains one of the most intractable liver diseases in pediatric population. In this project, we conducted 3 studies regarding its pathophysiology. In study 1, serum levels of IL-12, IL-18, and IFN-gamma were determined from 46 BA patients and 19 normal controls. We found that serum IL-18 and IFN-gamma levels were increased in BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. In study 2, survivin and caspase-3 expression was determined using immunohistochemistry from liver biopsies of 49 BA patients, and 29 non-BA pediatric patients. We demonstrated that survivin expression of biliary ductular epithelia was related to BA. These suggest that actions of survivin proteins involve in the biliary pathology of BA. In study 3, ninety-four BA patients between 2001 and 2009 were reviewed. Serum total bilirubin (TB) was determined at 7th day after surgery. The data shown by evaluation of serum TB at 7th day after surgery showed a prognostic power to predict a short-term surgical outcome at 6th month.

บทคัดย่อ

โรคทางเดินน้ำดีตีบตันเป็นโรคตับในเด็กที่มีความรุนแรงที่สุดโรคหนึ่ง โครงการวิจัยนี้ประกอบไปด้วยการศึกษาวิจัย ๓ เรื่อง ในการศึกษาที่ ๑ ผู้วิจัยได้ทำการวัดระดับซีรั่มของ IL-12, IL-18, และ IFN-gamma ในผู้ป่วยทางเดินน้ำดีตีบตัน ๔๖ คน เปรียบเทียบกับกลุ่มควบคุม ๑๙ คน พบว่าระดับซีรั่มของ IL-18, และ IFN-gamma สูงกว่ากลุ่มควบคุม และระดับที่สูงขึ้นของซีรั่ม IL-18 มีความสัมพันธ์กับอาการทางคลินิกที่แย่ลง ทั้งหมดนี้บ่งว่า IL-18 และ IFN-gamma มีบทบาทสำคัญใน สรีรพยาธิวิทยาของโรคทางเดินน้ำดีตีบตัน ในการศึกษาที่ ๒ ผู้วิจัยศึกษาการแสดงออกของ survivin และ caspase-3 จากเนื้อเยื่อตับของผู้ป่วยทางเดินน้ำดีตีบตัน ๔๙ คน เปรียบเทียบกับกลุ่มควบคุม ๒๙ คน ผู้วิจัยแสดงให้เห็นว่ามีการแสดงออกของโปรตีน survivin เฉพาะในเซลล์บุทางเดินน้ำดี ซึ่งอาจหมายความว่า การทำงานของ survivin เกี่ยวข้องกับพยาธิสภาพของทางเดินน้ำดีในโรคนี้ ในการศึกษาที่ ๓ ผู้วิจัยได้ทำการเก็บรวบรวมข้อมูลผู้ป่วยทางเดินน้ำดีตีบตัน ๙๔ ราย (พ.ศ. ๒๕๔๔ ถึง ๒๕๕๒) พบว่า สามารถใช้ระดับซีรั่มบิลลิรูบินในวันที่ ๗ หลังการผ่าตัดรักษา เป็นดัชนีบ่งชี้ผลการรักษาที่ ๖ เดือนหลังผ่าตัดได้

Executive Summary

1. Elevated serum IL-18 and interferon-gamma in medium-term survivors of biliary atresia

Biliary atresia (BA) is a fatal disease in children. Its main pathological feature is progressive immune-mediated cholangiopathy. Interleukin (IL)-12, IL-18, and interferon-gamma (IFN-gamma) play important roles in various immunological diseases. The objective was to investigate whether these serum markers were associated with clinical outcome in BA.

Methods: Serum levels of IL-12, IL-18, and IFN-gamma were determined using enzyme-linked immunosorbent assay from 46 BA patients (median age of 9 years) and 19 normal controls. The BA patients were then categorized into three groups according to their outcome: jaundice-free (29 cases), mild to moderate jaundice (10 cases), and marked jaundice (7 cases). The comparisons of serum IL-12, IL-18, and IFN-gamma levels among groups of the patients were performed using one-way analysis of variance with post-hoc tests. Data are expressed as mean + standard deviation.

Results: Serum IL-18 and IFN-gamma in BA patients were higher than the normal controls (IL-18: 113.3 + 82.6 vs. 80.5 + 9.9 pg/mL, $p = 0.011$ and IFN-gamma: 41.7 + 5.1 vs. 38.0 + 1.9 pg/mL, $p < 0.001$). There was no difference in serum IL-12 between BA and controls. Further analysis demonstrated that, in BA patients, only serum IL-18 levels significantly increased with the degree of jaundice (test for trend, $p = 0.004$).

Conclusions: Serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These results suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. Additionally, IL-18 is likely to be involved in the disease progression.

2. Hepatic expression of survivin and caspase-3 in biliary atresia

Biliary epithelia undergo early activation of apoptosis in a mouse model of biliary atresia (BA). Survivin is an inhibitor of apoptosis by impeding caspase activation. Our objective was to investigate hepatic expression of survivin and caspase-3 in BA.

Methods: Survivin and caspase-3 expression was determined using immunohistochemistry from liver biopsies of 49 BA patients (M:F=23:26) during laparotomy, and those of 29 non-BA pediatric patients (M:F=14:15) whose liver tissues were needed in the treatment process. At 12 months after Kasai operation, the BA patients were categorized into good (jaundice-free) and poor outcome (persistent jaundice). Survivin and caspase-3 expression from hepatocyte areas was assessed based on percentage of stained area. Biliary epithelia at portal triad were evaluated as positive or negative staining. Unpaired t-tests and Fisher's exact tests were used. Data are expressed as mean+SD.

Results: There was no difference in survivin or caspase-3 expression of hepatocyte areas between BA and non-BA patients (79.0+14.4 vs. 79.4+16.3%, P=0.92 and 29.9+22.5 vs. 34.5+23.0%, P=0.39 for survivin and caspase-3, respectively). The proportion of BA patients having survivin expression of biliary epithelial cells was significantly higher than that of non-BA patients (40/49 vs. 9/29, P<0.0001). Only 25.0% (2/8) of hepatitis and 33.3% (7/21) of non-cholestatic patients had survivin expression in ductular epithelia.

At 12 months post-Kasai, there was no difference in hepatic survivin or caspase-3 expression at the time of surgery between BA patients with good outcome and those with poor outcome in terms of staining areas or expression in biliary ductular epithelia.

Conclusions: Survivin expression of biliary ductular epithelia was related to BA. These suggest that actions of survivin proteins involve in the biliary pathology of BA, probably via anti-apoptotic process. Its expression may be helpful in the diagnosis but cannot be used as a prognostic marker.

3. Serum bilirubin as a simple marker to predict early outcome in biliary atresia

Biliary atresia (BA) is a seriously progressive inflammation of neonatal liver and ongoing obliterative process of the bile ducts with currently no predictable course nor certain prognosis. The study aimed to investigate the role of post-op serum total bilirubin (TB) as an early predictor for surgical outcome in BA.

Method: BA patients between 2001 and 2009 were reviewed. Serum levels of TB were determined at 7th day after Kasai operation. The patients were categorized into two groups, good outcome (serum TB below 2 mg%) and poor outcome (serum TB above 2 mg%) at 6th month following surgery based on jaundice status. The data of serum TB were then analyzed to evaluate its predictive value for clinical outcome using descriptive study and Fisher's exact tests.

Results: Ninety-four BA patients underwent Kasai operation. Median age at the operation was 80.5 days. Ascending cholangitis occurred in 40 (46%) patients. The outcome at 6th month could be assessed in 86 patients. Nevertheless, data of both serum TB and clinical outcome were able to be analyzed in only 80 patients (good: poor = 48:32). Fifty-four percent (26/48) of jaundice-free patients had a decrease in post-op serum TB levels at 7th day compared with only 25% (8/32) of those who remained jaundice. The drop of more than 20% in serum TB levels at 7th day was significantly associated with good outcome at 6th month ($p = 0.012$) showing predictive value of 76.5%. Further analysis revealed reduction in post-op serum TB at 7th day more than 50%, occurring in 6% (5/80), were all jaundice-free at 6th month.

Conclusions: The data shown by evaluation of serum TB at 7th day after surgery showed a prognostic power to predict a short-term surgical outcome at 6th month. This early prediction using post-op TB concentration may be helpful as an adjunct in planning further management in these patients.

Study 1: Elevated serum IL-18 and interferon-gamma in medium-term survivors of biliary atresia

Introduction

Biliary atresia (BA) remains one of the most intractable liver diseases in pediatric population. It is a disease characterized by an obliterative, inflammatory, and immune-mediated cholangiopathy leading to obstruction of bile flow in neonates and infants [1]. The cause and pathophysiology of this disease are still unclear. Without proper treatment, the consequence is the development of progressive liver fibrosis, indicated by direct hyperbilirubinemia and acholic stools, leading to biliary cirrhosis within a few months and mortality within 2 to 3 years [2,3]. When patients with BA are left without any surgical correction, the majority will die from hepatic decompensation, esophageal variceal bleeding or infection [4,5].

Although there have been a number of studies on serum and tissue markers regarding the pathophysiology of progressive liver fibrosis in BA including various cytokines [6-9], growth factors [10,11], nitric oxide [12,13], and the apoptosis of bile duct cells [14-16], the exact mechanism is still unclear. Our previous studies demonstrated an evidence of the association between serum inflammatory markers, including ICAM-1, IL-8, nitric oxide and selectin [7,9,12,13,17], and clinical outcome in BA. However, the information regarding the association between serum IL-12, IL-18, and IFN-gamma with clinical outcome in BA has not been explored.

Interferon-gamma (IFN-gamma) is a soluble inflammatory cytokine. This interferon was originally called macrophage-activating factor. It has been shown that IFN-gamma can prevent liver fibrosis by inhibiting the activation and proliferation of stellate cells, resulting in reduced expression of procollagen without affecting transforming growth factor-beta1

expression in pig serum-induced rat liver fibrosis in vivo [18]. In addition, interleukin-12 (IL-12) and IL-18 are both critical to the induction of IFN-gamma. Various roles for IL-12 and IL-18 in control of inflammation in an IFN-gamma dependent manner have been demonstrated [19]. Both IL-12 and IL-18 have been characterized as effective IFN-gamma-inducing cytokines. Concomitant treatment with IL-12 and IL-18 has been shown to synergistically induce IFN-gamma synthesis and may be an effective therapy for treating immune-mediated diseases, and infectious diseases [20]. It has been also suggested that IL-18 contributes to infection through the induction of IFN-gamma production by NK cells, but not through the development of Th1 cells, under the condition in which IL-12 synthesis is deficient [21]. Hence, evidences mentioned above indicate that IL-12, IL-18, and IFN-gamma are closely related to each other.

The information of serum levels of IL-12, IL-18, and IFN-gamma, IL-12 in BA patients may improve our understanding of its pathophysiology regarding the inflammatory nature of this disease. Therefore, the objective of this study was to explore possible roles of serum IL-12, IL-18, and IFN-gamma levels in medium-term survivors of BA patients. It is postulated that serum IFN-gamma, IL-12 and IL-18 can be used as prognostic markers for post-operative BA patients.

Materials and Methods

Patients

BA patients and their parents were informed and recruited for the study during the annual follow-up. The age- and gender-comparable control group comprised of healthy children participating in the vaccination program during the same period. Briefly, peripheral venous whole blood was drawn with a sterile syringe, transferred to a centrifuge tube, allowed to clot and then centrifuged at 4^{o}C. The sera were stored at -70^{o}C until they could be assayed.

Measurement of serum IL-12, IL-18, and IFN-gamma levels

Serum levels of IL-12, IL-18, and IFN-gamma were measured using available commercial ELISA kits according to company's protocols (catalogue number D1200 for IL-12, R&D Systems, Minneapolis, USA; code number 7620 for IL-18, MBL International, Illinois, USA; and catalogue number DIF50 for IFN-gamma, R&D Systems, Minneapolis, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibodies specific for IL-12, IL-18 or IFN-gamma were pre-coated onto a microplate. Standards and samples were pipetted into the wells and any IL-12, IL-18, or IFN-gamma present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibodies specific for IL-12, IL-18, or IFN-gamma were added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of IL-12, IL-18, or IFN-gamma bound in the initial step. The color development was then stopped and the intensity of the color was measured.

In addition, liver function tests (LFT) including serum albumin, total bilirubin (TB), direct bilirubin (DB), and alanine aminotransferase (ALT), were performed using an automated chemical analyser (Hitachi 911) at the central laboratory of the hospital.

Categorization of the BA patients

In order to compare different clinical outcome among BA patients, they were divided into 3 groups according to the status of jaundice:

Group A; patients without jaundice (serum total bilirubin or TB <2.0 mg%)

Group B; patients with mild to moderate jaundice (serum TB total =2-10 mg%).

Group C; patients with marked jaundice (serum TB >10 mg%)

Further analysis between BA patients based on the status of jaundice and the levels of serum IL-12, IL-18, and IFN-gamma, were carried out.

Statistical analyses

Demographic and clinical data between groups were compared by chi-square tests and unpaired t-tests. The mean and standard deviation were calculated for each variable. The comparisons of these variables between BA patients and controls were performed using parametric tests. The comparisons of serum IL-12, IL-18 and IFN-gamma levels among groups of the patients were performed using one-way ANOVA with post-hoc tests for linear trend. Data are expressed as mean+SD. All statistical analyses were carried out using SPSS Statistics 17.0 and Prism version 5.0.

Results

Serum levels of IL-12, IL-18, and IFN-gamma were determined using ELISA from 46 BA patients (median age of 9 years) and 19 normal controls. The BA patients were then categorized into three groups according to their outcome:

Group A jaundice-free (29 cases)

Group B mild to moderate jaundice (10 cases), and

Group C marked jaundice (7 cases)

The demographic and clinical data are shown in Table 1.

	Group A	Group B	Group C	P-value
Number of cases	29	10	7	-
Age (years)	10.3+5.2	8.3+6.5	5.7+3.1	0.37
Serum albumin (g/dL)	4.4+0.4	3.8+0.5	3.4+0.4	<0.001
Serum total bilirubin (mg%)	0.9+0.5	4.5+2.6	24.8+13.9	<0.001
ALT (IU/L)	125.8+174.3	191.9+120.8	168.3+95.6	0.48
Serum IL-12 (pg/mL)	12.8+2.1	12.7+1.9	13.1+1.5	0.94
Serum IL-18 (pg/mL)	93.1+30.4	118.6+33.6	189.8+190.8	0.017
Serum IFN-gamma (pg/mL)	41.9+5.7	40.7+4.4	42.1+4.0	0.81

Table 1: Demographic and clinical data of BA patients based on the status of jaundice. Data are shown as mean+SD and one-way ANOVA was used.

Serum IL-18 and IFN-gamma in BA patients were higher than the normal controls (IL-18: 113.3+82.6 vs. 80.5+9.9 pg/ml, P=0.011 and IFN-gamma: 41.7+5.1 vs. 38.0+1.9

pg/ml, P<0.001). There was no difference in serum IL-12 between BA and controls (12.8+1.9 vs. 12.9+1.8 pg/ml, P=0.92). Further analysis demonstrated that, in BA patients, only serum IL-18 levels significantly increased with the degree of jaundice (test for linear trend, P=0.004), as shown in Figure 1. In addition, there was no correlation between serum IL-18 and serum IFN-gamma in BA patients.

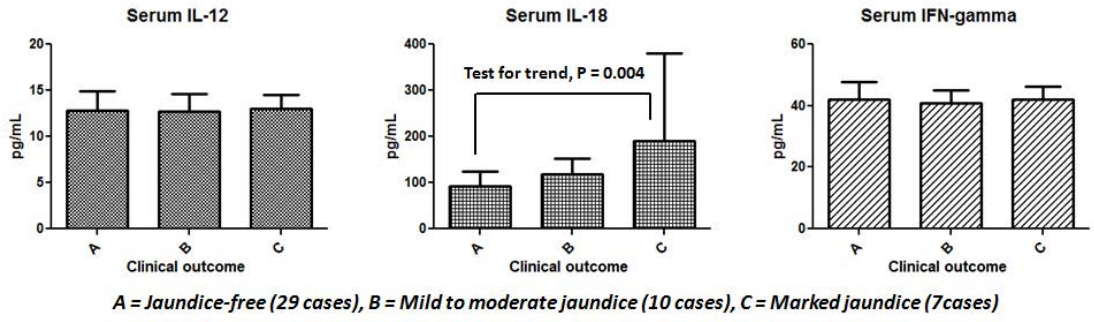

Figure 1: The comparisons of serum IL-12, serum IL-18, and serum IFN-gamma among the three groups of BA patients

Discussion

At present, the knowledge of BA has been better understood and its pathophysiology has been widely investigated since the introduction of Kasai operation [1]. It has been suggested that the role of inflammatory signaling pathways in progressive liver fibrosis is critical to the liver pathology [22]. Although there have been several reports regarding the association between inflammatory markers and BA as mentioned above in the introduction, the study of possible roles of serum levels of IL-12, IL-18, and IFN-gamma in BA receives not much attention.

Based on the role of IL-12 as a key proinflammatory cytokine, it is likely that IL-12 is involved in the pathophysiology of BA. Recent studies showed that loss of IL-12 modified the pro-inflammatory response but did not prevent duct obstruction in experimental BA in animals [23]. However, the role of IL-12 in the pathogenesis of biliary obstruction is not fully understood.

IL-18 levels were demonstrated to correlate with IL-12 and IFN-gamma levels through T helper 1 cytokine production [24]. Mack et al. [25] revealed that there were increases in T cells and Kupffer cells in the portal tracts of BA. Analysis of liver tissue from BA patients showed a Th1-type cytokine profile with significant expression of IFN-gamma and IL-12.

The present study clearly showed that serum levels of IL-18 and IFN-gamma were significantly elevated in medium-term survivors of BA compared to healthy children. The results are consistent with previous studies conducted in adult patients demonstrating that serum IL-18 levels were higher in cirrhotic patients including primary biliary cirrhosis and chronic hepatitis C than those of healthy adults [26,27]. Our findings on the correlation of elevated serum IL-18 levels and the severity of jaundice in BA are also agreeable with Urushihara et al. [28]. Since IL-18 is a potent pro-inflammatory cytokine inducing IFN-

gamma production from activated T cells, elevated serum IL-18 levels found in BA patients may be responsible for the elevation of serum IFN-gamma in BA found in this study. Nonetheless, we found no correlation between serum IL-18 and serum IFN-gamma in BA patients. This may be due to the small sample size or the complex pathways involved in the interaction between IL-18 and IFN-gamma. Investigation of these cytokine levels in the liver tissue will elucidate possible roles of IL-18 and IFN-gamma in BA more precisely.

Interestingly, we demonstrated that there was no change in serum IL-12 levels between BA patients and normal controls. An important biologic activity associated with IL-12 is to promote the differentiation of naive T cells into T-helper cells capable of producing IFN-gamma. Stimulation of IFN-gamma production by IL-12 can be synergistically enhanced by the presence of other pro-inflammatory cytokines such as TNF, IL-1, and IL-2 [19]. Therefore, it is likely that IL-12 is not directly involved in the pathophysiology of BA and the elevation of serum IFN-gamma found in BA patients is not passed through IL-12 signaling pathway but rather via IL-18 pathway in some extent.

As always, this kind of study regarding serum markers does inevitably have some limitations. Firstly, the serum levels of cytokines did not necessarily reflect the action of their signaling pathways within the disease organs. However, with the supported evidence from other studies on the real association between their serum levels and the process of liver fibrosis, it is likely that the elevated serum levels of IL-18 and IFN-gamma found in BA play roles in its pathophysiology. Secondly, there are inflammatory cytokines or markers other than IL-12, IL-18, and IFN-gamma that are involved in the pathophysiology of progressive inflammatory liver injury, for example, IL-8 [7], ICAM-1 [17], selectin [9] and nitric oxide [13]. Therefore, there are other influential factors outside the boundaries of this study needed to be taken into account. What we attempted to do here is to assemble some small pieces from the big picture of complex pathways.

In conclusions, serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These results suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. Additionally, IL-18 is likely to be involved in the disease progression. Serum IL-18 may be used as a prognostic marker in BA.

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Study 2: Hepatic expression of survivin and caspase-3 in biliary atresia

Introduction

Biliary atresia (BA) is one of the most devastating liver diseases in infants. For decades, numerous investigations of its pathophysiology and its cause have been reported. The main pathological features of BA include inflammatory process of the liver and biliary ductular proliferation [1-3]. Our previous studies demonstrated that several inflammatory markers are associated with the pathology of BA [4-7]. Although histopathologic features of BA have been extensively studied from liver biopsies of infants with BA undergoing hepatic portoenterostomy (Kasai operation), the pathogenesis of the disorder remains poorly understood. Furthermore, there are still other aspects regarding its pathophysiology need to be further explored.

Recently, it has been illustrated that cholangiocytes undergo early activation of apoptosis (programmed cell death) in a mouse model of BA. The synergistic role of IFN-gamma in activating caspase-3 in biliary ductular cells was demonstrated. In addition, the inhibition of caspase activity resulted in decreased apoptosis of biliary cells in an animal model of BA [8]. In another study, up-regulation of genes involved in apoptosis of the infective murine model for BA has been demonstrated. Our previous studies also reported an observation of elevated serum IFN-gamma levels in medium-term survivors of BA [6]. This information supports possible roles of apoptosis in the pathogenesis of BA both experimentally and clinically.

Caspase-3 is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes that undergo proteolytic process to form the active enzyme. Immunohistochemical expression of caspase-3 has been used as a marker for

apoptosis in various organs [9,10]. Survivin is a protein of the inhibitor of apoptosis family. Its function is to inhibit caspase activation leading to negative regulation of cellular apoptosis [11,12]. Survivin is dramatically expressed in a cell cycle-dependent manner during liver regeneration and plays a critical role in the regulation of cell proliferation and differentiation [11]. At present, the molecular mechanisms of survivin regulation are still not well understood.

Although there is an evidence of the involvement of apoptotic process in BA, the association between apoptosis and BA patients receives little attention [13]. In addition, no information regarding hepatic survivin and caspase-3 expression in BA patients has been reported. Our objective is this study was to study hepatic expression of survivin and caspase-3, important markers for apoptosis, in BA. We hypothesize that apoptosis plays an active role regarding the liver pathology of BA.

Methods

Liver tissues and patients

The study was approved by the Ethical committee. Liver samples of BA patients undergoing Kasai operation and non-BA patients undergoing liver biopsies between January 2006 and February 2010 were retrospectively studied. All patients were operated by one team of surgeons (PV and SC). The non-BA patients were served as controls. All non-BA patients underwent exploratory laparotomy as the therapeutic means for their diseases. Liver biopsies in this group of patients were an additional procedure and were indicated for medical reasons.

Immunohistochemistry of liver tissues for survivin and caspase-3

Liver samples were fixed in formalin for 24 hours and kept in paraffin embedded blocks using standard procedure. New sections of 4 μm thickness were cut from the formalin-fixed paraffin embedded blocks and mounted on glass slides coated by aminopropyltriethoxysilane (APES; Sigma Chemical Co., St Louise, MO, USA). Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 10-minute incubation in 3% hydrogen peroxide. Antigen retrieval was done by heating the sections in a microwave at approximately 850 W for 5 minutes and 300 W for 10 minutes in 10mM citrate buffer pH 6.0. After washing with 0.1% Tween 20 (MERCK-Schuchardt, Hohanbrunn, Germany) in phosphate buffered saline (PBS), the sections were treated with 5% bovine serum albumin (Sigma Chemical Co.) in PBS for 30 minutes and then treated with primary antibodies for two hours at room temperature. The primary antibodies used in this study were against survivin (R&D Systems, Inc., Minneapolis, USA; AF886) diluted at 1:200 and caspase-3 (R&D Systems, Inc.; AF835) diluted at 1:100. Both antibodies were diluted in PBS. After thorough washing in 0.1% Tween 20 in PBS, labeled polymer (Dako Envision System, Dako Corporation, Carpinteria, CA, USA) was applied to the sections for 30 minutes

and followed by three washes of 0.1% Tween 20 in PBS. Color was developed in freshly made diaminobenzidine (Sigma Chemical Co). Sections were washed briefly in running tap water and lightly stained with Mayer's hematoxylin.

Sections of hepatocellular carcinoma known to have cytoplasmic staining for survivin and caspase-3, were stained at the same run as positive controls. For each specimen, a negative control was done by replacing the primary antibody with non-immune rabbit serum with the same dilution to the relevant primary antibodies. All sections were processed under the same conditions.

Evaluation of hepatic survivin and caspase-3 expression

Immuno-stained sections were evaluated independently by 2 investigators who were unaware of the diagnosis or clinical outcome of the patients. Semi-quantitative methods were used, based on the extent of the antibody stain. Areas of hepatic lobules excluding portal triads and central veins were evaluated, based on Ruifrok and Johnston's method [14,15], using ImageJ software version 1.41o [16].

Areas of immuno-stained liver histology from each patient were pictured and saved as JPEG format. The parameters of the camera set in all cases were identical. The stained areas of survivin and caspase-3 expression was evaluated using the Histogram mode from ImageJ software version 1.41o [16] with color deconvolution plugin [17,18]. By using this option, the blue color of hematoxylin and brown color of diaminobenzidine (DAB) can be separated. With this computerized method, the intensity in DAB channel of a pixel in the selected area is scored into 256 arbitrary levels (from white to dark brown; 255→0).

Our preliminary study showed that the average intensity level of the background area (no staining) of DAB channel was 160. Therefore, intensity levels between 160 and 255 were considered as negative staining levels. For the quantification of immuno-staining from

hepatocyte areas, five randomly selected areas of hepatocytes excluding portal triads and central veins were selected as regions of interest. Then, percentage of stained areas was averaged.

In addition, biliary ductular epithelial cells at the portal triad areas were assessed by 2 investigators (PV and SP) as positive or negative staining. Any disagreement between the two investigators would be finalized by discussion. Then, relations between the ratio of patients with positive staining of biliary ductular cells and different diseases were analyzed using Fisher's exact tests.

Categorization of the BA patients

In order to associate the expression of caspase-3 and survivin with clinical outcome at 12 months post-op among BA patients, they were divided into 2 groups according to the status of jaundice (TB <2 mg/dL; good outcome vs. TB ≥2 mg/dL; poor outcome). Subgroup analysis of hepatic expression of either survivin or caspase-3 based on their clinical outcome at 12 months post-op was carried out.

Statistical analyses

Demographic and clinical data between groups were compared by Fisher's exact tests and unpaired t-tests where appropriate. The mean and standard deviation were calculated for each variable. The comparisons of survivin and caspase-3 expression among the two groups (BA and non-BA patients) were performed unpaired t-tests. Association between outcome of BA patients at 12 months post-op and hepatic of survivin and caspase-3 expression was analyzed using univariate analysis.

Significant differences were established at $P<0.05$. For all statistical analyses, either GraphPad Prism version 5.0 (GraphPad Software Inc., California, USA) or SPSS software version 17.0 (SPSS Inc., Chicago, IL) was used. Data are expressed as mean and SD.

Results

During the studied period, there were 49 BA patients (M:F=23:26), and 29 non-BA pediatric patients (M:F=14:15). The non-BA pediatric patients included 21 non-cholestatic patients (11 choledochal cysts, 5 thalassemias, 2 neuroblastomas, 1 adrenal xanthogranuloma, 1 portal vein thrombosis, and 1 hepatoblastoma) and 8 hepatitis patients.

BA vs. hepatitis vs. non-cholestatic patients

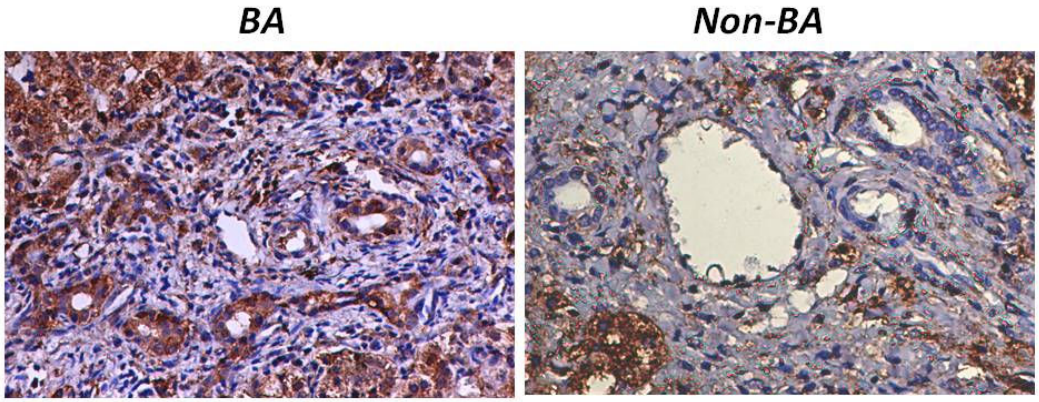
There was no difference in survivin or caspase-3 expression of hepatocyte areas between BA and non-BA patients (79.0+14.4 vs. 79.4+16.3%, P=0.92 and 29.9+22.5 vs. 34.5+23.0%, P=0.39 for survivin and caspase-3, respectively). The proportion of BA patients having survivin expression of biliary epithelial cells was significantly higher than that of non-BA patients (40/49 vs. 9/29, P<0.0001). Only 25.0% (2/8) of hepatitis and 33.3% (7/21) of non-cholestatic patients had survivin expression in ductular epithelia, as shown in Table 1.

Table 1: Expression of survivin and capase-3 of biliary ductular cells at the portal triad. All P-values are related to BA using Fisher's exact tests.

	Survivin				**Caspase-3**		
Diseases	*Positive*	*Negative*	*P-value*		*Positive*	*Negative*	*P-value*
BA	40	9			20	29	
Non-cholestasis	7	14	0.0002		5	16	0.2761
Hepatitis	2	6	0.0028		3	5	1.0

The histological features of portal triad of survivin expression between BA and non-BA are shown in Figure 1.

Figure 1: Hepatic survivin expression of biliary ductular epithelial cells between BA (positive staining) and Non-BA (negative staining)



BA patients with good outcome and BA patients with poor outcome

At 12 months post-Kasai, there were 25 BA patients with good outcome and 19 BA patients with poor outcome. There was no difference in hepatic survivin or caspase-3 expression at the time of surgery between BA patients with good outcome and those with

poor outcome in terms of staining areas (good outcome vs. poor outcome: 78.81+15.91 vs. 78.81+13.70%, P=0.99 and 30.20+22.86 vs. 24.86+17.72%, P=0.40, for survivin and caspase-3 respectively) or ratio of BA patients with positive expression in biliary ductular epithelia (survivin, P=0.71 and caspase-3, P=0.34).

Discussion

At present, the knowledge of BA has been better understood and its pathophysiology has been widely investigated since the introduction of Kasai operation [2]. For decades, it has been suggested that the role of inflammatory signaling pathways in progressive liver fibrosis is critical to the liver pathology [1,4,5,7]. However, roles of apoptotic process in BA have not been explored.

In this study, immunohistochemistry (IHC) was used to investigate the hepatic survivin and caspase-3 expression. The increased use of IHC in both clinical and basic research settings has led to the development of various computerized techniques for acquiring quantitative information from immunostaining levels. Quantitative IHC techniques have often yielded important information regarding patient diagnosis, prognosis, or both. Our previous study demonstrated that immunostaining quantification obtained by a digital computer-assisted method (ImageJ software with color deconvolution plugin) is concordant with a standard semi-quantitative analysis [5]. Moreover, digital measurement is more objective and could resolve either researcher's bias or disagreement between observers.

Our results clearly demonstrated that there is an association between the survivin expression of biliary ductular epithelial cells and BA. This observation suggested that anti-apoptotic process occurred in biliary ductular cells of BA. This may be responsible for biliary ductular proliferation which is a pathognomonic feature for BA in early stage [19]. Since our study showed that there was no biliary survivin expression in cholestatic hepatitis and choledochal cyst, we postulated that biliary survivin expression in BA is not associated with cholestasis or biliary obstruction. In addition, this study demonstrated that caspase-3 expression in biliary cells was not different that of other diseases. It is therefore likely that apoptosis, via caspase-3 pathway activation, is not directly involved in biliary cells of BA but rather anti-apoptotic process via survivin pathways.

Interestingly, survivin and caspase-3 expression found in the areas of hepatocytes in BA was not different from those of other diseases. Hence, apoptosis process seems to not significantly involve in the pathological process of hepatocytes in BA. Subsequent analysis among BA patients revealed that survivin and caspase-3 expression of hepatocytes at the time of Kasai operation was not associated with early clinical outcome in BA at 12 months post-op. Taken all together, hepatic survivin and caspase-3 expression cannot be used as prognostic markers for predicting the early outcome in BA.

Nonetheless, we are aware of some limitations in this study. Firstly, we do not know whether biliary expression of survivin found in BA patients is a primary or secondary effect to the disease pathology. If it is primary, biliary survivin expression will decrease over time in BA patients with good outcome. Secondly, there was no ideal control (normal liver tissues) in this study. However, to obtain normal liver in infants is unethical. In this study, non-cholestatic patients were used as controls. Finally, the sample size of BA patients was probably too small in terms of statistical analysis. However, due to the rarity of BA, the number of 44 BA patients cannot be overlooked.

In conclusions, survivin expression of biliary ductular epithelia was significantly observed in liver tissues of BA. These suggest that actions of survivin proteins involve in the biliary pathology of BA, probably via anti-apoptotic process. Its expression may be helpful in the diagnosis but cannot be used as a prognostic marker. More studies on this aspect are needed to better understand the precise role of survivin towards biliary ductular cells in BA.

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Study 3: Serum bilirubin as a simple marker to predict early outcome in biliary atresia

Introduction

Biliary atresia (BA) is a serious neonatal hepatobiliary disorder of unknown cause with progressive inflammation of liver and ongoing obliterative process of the bile ducts. Untreated patients ultimately die of end-stage liver decompensation. The renown hepatic portojejunostomy procedure by Morio Kasai restoring extrahepatic biliary drainage can rescue 20-30% of BA patients [1, 2]. The remaining or the majority of children will eventually suffer from biliary cirrhosis and being listed for liver transplantation.

The post-op course of the disorder, however, is currently not predictable and uncertain, even after a successful Kasai operation. A number of prognostic markers of post-op success after the operation have been investigated [3-7]. As liver function test, particularly total bilirubin (TB) concentration is routinely used to evaluate post-op icteric status, the implement of this factor as an early predictor is very simple and save. In addition, the use of serum TB to predict a surgical outcome is rarely reported in the literature [8-10]. The present study was to investigate the prognostic value of post-op serum TB at 7^{th} day, focusing on the short-term results at 6^{th} month after operation.

Methods

The ethical approval was granted by the International Review Board of the Faculty of Medicine, Chulalongkorn University. A total of 94 consecutive BA patients who underwent Kasai operation at King Chulalongkorn Memorial Hospital between 2001 and 2009 were retrospectively reviewed. The diagnosis of all BA patients following ultrasonography and liver scintigraphy was confirmed by coeliotomy with intraoperative cholangiography and when the diagnosis was made the original hepatic portoenterostomy was carried out. The operation techniques, roughly including liver mobilization, magnification using a surgical loupe, lateral dissection of the fibrous portal mass, liver biopsy, and Roux-en-Y portojejunostomy as described in the literature previously, was performed by one team of surgeons. Postoperatively, all BA patients received intravenous antibiotics followed by long-term oral cotrimoxazole, ursodeoxycholic acid, vitamin A, D, E, and K, and short-term high-dose prednisolone.

In order to investigate the prognostic role of post-op serum TB, the specimens of peripheral venous blood postoperatively collected at 7^{th} day (± 2 days) were determined for serum TB concentration using an automated chemical analyzer (Hitachi 911) at our central laboratory. The patients were then classified into two groups depending on their icteric status at 6^{th} month after surgery; good (serum TB below 2 mg%) and poor outcome (serum TB above 2 mg%). The correlations between serum TB levels and surgical outcomes at that period of time were analyzed using descriptive study and Fisher's exact test. Demographic data including age, gender, complications, and other hepatic enzymes were also analyzed. The statistical calculation was performed using Statistical Package of the Social Science software version 10.0 (SPSS Inc., Chicago, IL, USA). A significant difference was defined as p value of <0.05.

Results

Male to female ratio was about 1.2:1. The median age of infants at the time of surgery was 80.5 days (range, 21 to 209 days). Approximately 85.1% in the series underwent Kasai operation beyond 60 days of life. The age and gender, however, were not significantly different between those with jaundice and those who were jaundice-free at 6^{th} month. Ascending cholangitis was the most common post-op complication, accounting for 46% of cases in the study. There was no peri- and post-op mortality.

Mean pre-op TB concentration of 11.82 ± 3.44 mg% slightly declined to 10.90 ± 4.46 mg% at post-op day 7. At sixth month, the icteric status could be assessed in 86 patients. Nevertheless, data of both serum TB levels at 7^{th} day and 6^{th} month were able to be analyzed in only 80 patients. Forty-eight patients (60%) were jaundice-free (classified as good outcome), whereas the remaining 32 patients remained jaundice (poor outcome).

Fifty-four percent (26/48) of jaundice-free patients had a decrease in post-op serum TB levels at 7^{th} day compared with only 25% (8/32) of those who were in poor outcome. The 20% or more drop of serum TB levels at 7^{th} day was significantly associated with good outcome at 6^{th} month ($p = 0.012$). And also, the predictive value where the decrease in post-op serum TB at 7^{th} day was used as a predictor for good outcome was 76.5%. Further analysis revealed reduction in post-op serum TB at 7^{th} day more than 50%, which found in 6% (5/80) of all patients, were all jaundice-free at 6^{th} month. Although none in poor outcome obtained half drop in post-op serum TB at 7^{th} day, the difference between the half concentration reduction and outcome was not statistically significant ($p = 0.08$).

Twenty percent drop in post-op serum glutamic pyruvic transaminase (SGPT) and glutamic oxaloacetic transaminase (SGOT) at day 7 accounted for 37.8% and 46.4% of patients in good outcome, respectively. However, both SGPT and SGOT were found no strong correlation between post-op levels and surgical outcome as serum TB.

Discussion

It has been accepted that the standard treatment of BA involves early diagnosis, successful Kasai operation, and prompt aggressive prevention and treatment of post-op ascending cholangitis. With this sequential therapy, approximately half of BA patients at our institution are jaundice-free at one year follow-up [11]. However, the post-op course of this disorder is exactly unpredictable and uncertain, thus providing not all post-op improvement will achieve long-term survival. Apart from the unsuccessful operation, some patients with post-op jaundice-free still insidiously develop biliary cirrhosis and eventually suffer end-stage liver decompensation. As a result, only about one-third of BA patients after surgery can reach the cure [1,2].

Several prognostic features, including histoimmunological findings of the liver and fibrous portal mass, portal pressure index and hepatobiliary imaging, that determined at the time of surgery have been proposed to predict the ultimate outcome after surgery [5,12-15]. Although the authors had found correlation between them and the surgical outcome, all these characteristics are subjective or depend upon the experience and skill of the persons who are responsible for the measurement. Thus, to obtain more objective value, it would rather that the prognostic parameters are measured by means of serum biochemistry.

Since some trace elements such as copper and zinc are involved in several hepatic metabolism and stored in a significant amount in hepatic parenchyma [16,17], they were included in a calculation of BA prognostic index [18]. The interpretation showed that the serum copper to zinc ratio was the most reliable parameter among other liver function variables, including TB, cholinesterase, and gamma-glutamyl transpeptidase, in predicting the severity of liver deterioration in BA. Nevertheless, these trace elements do not directly reflect the status of liver function. Although the serum cholinesterase levels might correspond

more sensitively with the improvement of synthetic liver function, its value in predicting the prognosis after surgery was not demonstrated [19].

A predictive value of the serum TB levels has been proposed and determined as an indication for liver transplantion [10,20,21]. The earliest prediction of ultimate outcome was three months post-surgery [10]. Some authors estimate the post-op prognosis by serum TB clearance or measurement [22] of TB excretion from direct limb of portoenterostomy [23]. All studies were conducted for the purpose that providing unsuccessfully patients an early referral to liver transplantation. Although the present study determine the outcome at 6^{th} month that too early to interpret the success of Kasai operation, the prediction time at 1 week after surgery will serve an early advice to the parents or provision of a great concern to aggressively treat BA patients whose results are suspected to be unsuccessful. As we have shown, the 20% or more decrease in serum TB levels at 7^{th} day was significantly associated with good outcome at 6^{th} month. According to our knowledge, this relationship was found at the earliest time following surgery. The further analysis that more than half TB concentration reductions were all jaundice-free would reflect the satisfying bile drainage from the wide range of the liver. Unfortunately, such patients accounted for a minority of our cases.

We can conclude from our findings that the drop in post-op serum TB at one week showed a strong prognostic power to predict our patients' short-term outcome. Although this early prediction cannot be used as a parameter of long-term survival or, in the long run, an indication for liver transplantation, at least, our findings will help in planning further management in these patients.

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ผลงานที่ได้จากโครงการ

ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

ได้รับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติ ที่มี Impact factor โดยมีหัวหน้าโครงการเป็นผู้วิจัยหลัก 1 เรื่องดังนี้

1. **Vejchapipat P**, Poomsawat S, Chongsrisawat V, Honsawek S, Poovorawan Y. Elevated serum IL-18 and interferon-gamma in medium-term survivors of biliary atresia. Eur J Pediatr Surg. 2012;22(1):29-33. (Impact factor =0.839)

นอกจากนี้ ผู้รับทุนยังเป็นผู้วิจัยร่วม ในงานวิจัยที่ได้รับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติที่มี Impact factor ที่เกี่ยวข้องกับโรคทางเดินน้ำดีตีบตัน ในระหว่างที่ได้รับทุน อีก 6 เรื่อง ดังนี้

1. Honsawek S, **Vejchapipat P**, Payungporn S, Theamboonlers A, Chongsrisawat V, Poovorawan Y. Soluble receptor for advanced glycation end products and liver stiffness in postoperative biliary atresia. Clin Biochem. 2013 Feb;46(3): 214-8. (Impact factor = 2.076)
2. Udomsinprasert W, Tencomnao T, Honsawek S, Anomasiri W, **Vejchapipat P**, Chongsrisawat V, Poovorawan Y. +276 G/T single nucleotide polymorphism of the adiponectin gene is associated with the susceptibility to biliary atresia. World J Pediatr. 2012 Nov;8(4):328-34. (Impact factor = 1.216)
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5. Honsawek S, Praianantathavorn K, Chongsrisawat V, Vejchapipat P, Theamboonlers A, Poovorawan Y. High serum matrix metalloproteinase-3 and liver stiffness in postoperative biliary atresia. Pediatr Surg Int. 2011 Jul;27(7):681-7. (Impact factor = 1.253)
6. Honsawek S, **Vejchapipat P**, Chongsrisawat V, Thawornsuk N, Poovorawan Y. Association of circulating osteopontin levels with clinical outcomes in postoperative biliary atresia. Pediatr Surg Int. 2011 Mar;27(3):283-8. (Impact factor = 1.253)

กิจกรรมอื่นๆที่เกี่ยวข้อง

การไปเสนอผลงาน

ผู้รับทุนได้เดินทางไปเสนอผลงานวิจัยเกี่ยวกับเรื่องโรคทางเดินน้ำดีตีบตันระดับนานาชาติทั้งสิ้น 4 ครั้ง (Oral presentation 3 ครั้ง Poster presentation 1 ครั้ง) ดังนี้

1. การประชุม 12th Congress of Pediatric Surgery เมืองบาเซโลนา ประเทศสเปน วันที่ 15 มิถุนายน 2554 ถึง 18 มิถุนายน 2554

   Vejchapipat P, Poomsawat S, Chongsrisawat V, Honsawek S, Poovorawan Y. Elevated serum IL-18 and interferon-gamma in medium-term survivors of biliary atresia. (Oral presentation)
2. การประชุม 13th Congress of European Pediatric Surgical Association and 59th Congress of British Association of Paediatric Surgeons กรุงโรม สาธารณรัฐ อิตาลี ระหว่างวันที่ 13 มิถุนายน 2555 ถึง 16 มิถุนายน 2555

   Vejchapipat P, Poomsawat S, Chittmittrapap S, Poovorawan Y. Hepatic expression of survivin and caspase-3 in biliary atresia. (Oral presentation)
3. การประชุม 23rd Congress of the Asian Association of Pediatric Surgeons ณ เมือง โซล ประเทศ สาธารณรัฐเกาหลี ระหว่างวันที่ 8 ตุลาคม 2555 ถึง 10 ตุลาคม 2555

   Vejchapipat P. Biliary atresia: experience from one institution. (Oral presentation)
4. การประชุม 14th European Congress of Pediatric Surgery เมือง ไลพ์ซิก สหพันธรัฐ เยอรมนี ระหว่างวันที่ 5 มิถุนายน 2556 ถึง 8 มิถุนายน 2556

Sookpotarom P, **Vejchapipat P**, Tepmalai K, Passakonnirin R, Chittmittrapap S, Poovorawan Y. Serum bilirubin and alanine aminotransferase as simple markers to predict early outcome in biliary atresia. (Poster presentation)

การได้รับเชิญไปบรรยายที่เกี่ยวข้องกับการทำวิจัยโรคทางเดินน้ำดีตีบตัน

1. ผู้รับทุนได้รับเชิญให้เข้าร่วมประชุมในฐานะผู้เชี่ยวชาญ ในรูปแบบของ panel discussion ของโรคทางเดินน้ำดีตัน ในการประชุมวิชาการ 23rd Congress of the Asian Association of Pediatric Surgeons ณ เมือง โซล ประเทศ สาธารณรัฐเกาหลี ระหว่างวันที่ 8 ตุลาคม 2555 ถึง 10 ตุลาคม 2555

การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ

ผู้รับทุนได้สร้างเครือข่ายในการส่งต่อผู้ป่วยโรคทางเดินน้ำดีตีบตันเพื่อการรักษาและการวิจัยกับกุมารศัลยแพทย์ที่อยู่ตามโรงพยาบาลศูนย์และโรงพยาบาลจังหวัดดังนี้

๑. นายแพทย์ ไพบูลย์ สุขโพธารมย์	โรงพยาบาลชลประทาน จังหวัดนนทบุรี
๒. แพทย์หญิง จำเรียง คุ้มจันอัด	โรงพยาบาลบุรีรัมย์ จังหวัดบุรีรัมย์
๓. แพทย์หญิง รจนา ภาสกรนิรินทร์	โรงพยาบาลศูนย์ราชบุรี จังหวัดราชบุรี
๔. แพทย์หญิง ตะวัน อิ่มวิเศษ	โรงพยาบาลธรรมศาสตร์ ศูนย์รังสิต
๕. แพทย์หญิง นัทธ์หทัย กนกนาก	โรงพยาบาลศูนย์สุรินทร์ จังหวัดสุรินทร์
๖. แพทย์หญิง พฐู บุญมหิทธิสุทธิ์	โรงพยาบาลสมุทรปราการ

ภาคผนวก

๑. บทความทางวิชาการ 1 เรื่องโดยมีผู้รับทุนเป็นผู้วิจัยหลัก ที่ได้รับการตีพิมพ์ในวารสารระดับนานาชาติและมี impact factor

๒. เอกสารการไปเสนอผลงานวิจัยในการประชุมวิชาการระดับนานาชาติในต่างประเทศ จำนวน 4 ครั้ง

ภาคผนวก ๑

Elevated Serum IL-18 and Interferon-Gamma in Medium-Term Survivors of Biliary Atresia

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Eur J Pediatr Surg 2012;22:29–33.

Abstract

Introduction Biliary atresia (BA) is a fatal disease in children. Its main pathological feature is progressive immune-mediated cholangiopathy. Interleukin (IL)-12, IL-18, and interferon-gamma (IFN-gamma) play important roles in various immunological diseases. The objective was to investigate whether these serum markers were associated with clinical outcome in BA.

Methods Serum levels of IL-12, IL-18, and IFN-gamma were determined using enzyme-linked immunosorbent assay from 46 BA patients (median age of 9 years) and 19 normal controls. The BA patients were then categorized into three groups according to their outcome: jaundice-free (29 cases), mild to moderate jaundice (10 cases), and marked jaundice (7 cases). The comparisons of serum IL-12, IL-18, and IFN-gamma levels among groups of the patients were performed using one-way analysis of variance with post-hoc tests. Data are expressed as mean + standard deviation.

Results Serum IL-18 and IFN-gamma in BA patients were higher than the normal controls (IL-18: 113.3 + 82.6 vs. 80.5 + 9.9 pg/mL, $p = 0.011$ and IFN-gamma: 41.7 + 5.1 vs. 38.0 + 1.9 pg/mL, $p < 0.001$). There was no difference in serum IL-12 between BA and controls. Further analysis demonstrated that, in BA patients, only serum IL-18 levels significantly increased with the degree of jaundice (test for trend, $p = 0.004$).

Conclusions Serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These results suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. Additionally, IL-18 is likely to be involved in the disease progression.

Keywords
- biliary atresia
- interleukin-12
- interleukin-18
- interferon-gamma

Introduction

Biliary atresia (BA) remains one of the most intractable liver diseases in pediatric population. It is a disease characterized by an obliterative, inflammatory, and immune-mediated cholangiopathy leading to the obstruction of bile flow in neonates and infants.[1] The cause and pathophysiology of this disease are still unsolved. Without proper treatment,

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the consequence is the development of progressive liver fibrosis, indicated by direct hyperbilirubinemia and acholic stools, leading to biliary cirrhosis within a few months and mortality within 2 to 3 years.[2,3] When patients with BA are left without any surgical correction, the majority will die from hepatic decompensation, esophageal variceal bleeding, or infection.[4,5]

Although there were several studies on serum and tissue markers regarding the pathophysiology of progressive liver fibrosis in BA including various cytokines,[6–9] growth factors,[10,11] nitric oxide,[12,13] and the apoptosis of bile duct cells,[14–16] the exact mechanism is still unclear. Our previous studies demonstrated an evidence of the association between serum inflammatory markers, including intercellular adhesion molecule 1 (ICAM-1), interleukin (IL)-8, nitric oxide, and selectin,[7,9,12,13,17] and clinical outcome in BA. However, the information regarding the association between serum IL-12, IL-18, and interferon-gamma (IFN-gamma) with clinical outcome in BA has been lacking.

IFN-gamma is a soluble inflammatory cytokine. This interferon was originally called macrophage-activating factor. It has been shown that IFN-gamma can prevent liver fibrosis by inhibiting the activation and proliferation of stellate cells, resulting in reduced expression of procollagen without affecting transforming growth factor-β1 expression in pig serum-induced rat liver fibrosis in vivo.[18] In addition, IL-12 and IL-18 are both critical to the induction of IFN-gamma. Various roles for IL-12 and IL-18 in control of inflammation in an IFN-gamma-dependent manner have been demonstrated.[19] Both IL-12 and IL-18 have been characterized as effective IFN-gamma-inducing cytokines. Concomitant treatment with IL-12 and IL-18 has been shown to synergistically induce IFN-gamma synthesis. This was postulated to be an effective therapy for treating immune-mediated diseases and infectious diseases.[20] It has also been suggested that IL-18 contributes to infection through the induction of IFN-gamma production by NK cells, but not through the development of T helper 1 (Th1) cells, under the condition in which IL-12 synthesis is deficient.[21] Hence, evidences as mentioned above indicate that IL-12, IL-18, and IFN-gamma are closely related to each other.

The information of serum levels of IL-12, IL-18, and IFN-gamma in BA patients may improve our understanding of its pathophysiology regarding the inflammatory nature of this disease. Therefore, the objective of this study was to explore possible roles of serum IL-12, IL-18, and IFN-gamma levels in medium-term survivors of BA patients. We hypothesized that serum IFN-gamma, IL-12, and IL-18 can be used as prognostic markers for postoperative BA patients.

Materials and Methods

Patients

The study was approved by the ethical committee. BA patients and their parents were informed and recruited for the study during the annual follow-up. The age- and gender-comparable control group comprised healthy children participating in the vaccination program during the same period. 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.

Measurement of Serum IL-12, IL-18, and IFN-Gamma Levels

Serum levels of IL-12, IL-18, and IFN-gamma were measured using available commercial enzyme-linked immunosorbent assay (ELISA) kits according to company's protocols (catalog number D1200 for IL-12, R&D Systems, Minneapolis, MN, USA; code number 7620 for IL-18, MBL International, Chicago, IL, USA; and catalog number DIF50 for IFN-gamma, R&D Systems). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibodies specific for IL-12, IL-18, or IFN-gamma were precoated onto a microplate. Standards and samples were pipetted into the wells and any IL-12, IL-18, or IFN-gamma present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibodies specific for IL-12, IL-18, or IFN-gamma were added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of IL-12, IL-18, or IFN-gamma bound in the initial step. The color development was then stopped and the intensity of the color was measured.

In addition, liver function tests, including serum albumin, total bilirubin (TB), direct bilirubin, and alanine aminotransferase, were performed using an automated chemical analyser (Hitachi 911, Hitachi, Tokyo, Japan) at the central laboratory of the hospital.

Sample Size Calculation

Comparisons of serum IL-18 between BA and normal controls were selected as the primary outcome. From our pilot data, standard deviation (SD) of serum IL-18 from normal controls was 20 pg/mL. The true difference of means between two groups was proposed at 15 pg/mL. By using StatMate 2.00 (GraphPad Software Inc., San Diego, CA, USA), it revealed that sample size of 25 in each group has a 80% power to detect a difference between means of 15 with a significance level (α) of 0.05 (two-tailed). Further adjustment calculation of sample size in ratio of 1:2.5, with the same power, suggested that sample size of BA group is 44 and sample size of control group is 18 (http://www.stattools.net/SSizunequal_Pgm.php).

Categorization of the BA Patients

To compare different clinical outcome among BA patients, they were divided into three groups according to the status of jaundice:

Group A; patients without jaundice (serum TB <2.0 mg%).
Group B; patients with mild to moderate jaundice (serum TB total = 2 to 10 mg%).
Group C; patients with marked jaundice (serum TB > 10 mg%).

Table 1 Demographic and Clinical Data of BA Patients Based on the Status of Jaundice. Data are Shown as Mean + SD and One-Way Analysis of Variance was Used

	Group A	Group B	Group C	p Value
Number of cases	29	10	7	–
Age (years)	10.3 + 5.2	8.3 + 6.5	5.7 + 3.1	0.37
Serum albumin (g/dL)	4.4 + 0.4	3.8 + 0.5	3.4 + 0.4	<0.001
Serum TB (mg%)	0.9 + 0.5	4.5 + 2.6	24.8 + 13.9	<0.001
ALT (IU/L)	125.8 + 174.3	191.9 + 120.8	168.3 + 95.6	0.48
Serum IL-12 (pg/mL)	12.8 + 2.1	12.7 + 1.9	13.1 + 1.5	0.94
Serum IL-18 (pg/mL)	93.1 + 30.4	118.6 + 33.6	189.8 + 190.8	0.017
Serum IFN-gamma (pg/mL)	41.9 + 5.7	40.7 + 4.4	42.1 + 4.0	0.81

BA, biliary atresia; SD, standard deviation; TB, total bilirubin; ALT, alanine aminotransferase; IL, interleukin; IFN-gamma, interferon-gamma.

Further analysis between BA patients based on the status of jaundice and the levels of serum IL-12, IL-18, and IFN-gamma were performed.

Statistical Analyses

Demographic and clinical data between groups were compared by chi-square tests and unpaired *t* tests. The mean and SD were calculated for each variable. The comparisons of these variables between BA patients and controls were performed using parametric tests. The comparisons of serum IL-12, IL-18, and IFN-gamma levels among groups of the patients were performed using one-way analysis of variance with post-hoc tests for linear trend.

All data are expressed as mean + SD. All statistical analyses were performed using SPSS Statistics 17.0 (SPS, Inc., Chicago, IL, USA) and Prism version 5.0 (Graphpad software, Inc., San Diego, CA, USA).

Results

Serum levels of IL-12, IL-18, and IFN-gamma were determined using ELISA from 46 BA patients (median age of 9 years) and 19 normal controls. Serum IL-18 and IFN-gamma in BA patients were higher than normal controls (IL-18: 113.3 + 82.6 vs. 80.5 + 9.9 pg/mL, $p = 0.011$ and IFN-gamma: 41.7 + 5.1 vs. 38.0 + 1.9 pg/mL, $p < 0.001$). However, there was no difference in serum IL-12 between BA and controls (12.8 + 1.9 vs. 12.9 + 1.8 pg/mL, $p = 0.92$).

The BA patients were then categorized into three groups according to their outcome: Group A—jaundice-free (29 cases), Group B—mild to moderate jaundice (10 cases), and Group C—marked jaundice (7 cases). The demographic and clinical data of BA patients are shown in ►Table 1.

Further analysis demonstrated that, in BA patients, only serum IL-18 levels significantly increased with the degree of jaundice (test for linear trend, $p = 0.004$), as shown in ►Fig. 1. In addition, there was no correlation between serum IL-18 and serum IFN-gamma in BA patients.

Discussion

At present, the knowledge of BA has been better understood and its pathophysiology has been widely investigated.[1] It has been suggested that the role of inflammatory signaling pathways in progressive liver fibrosis is critical to the liver pathology.[22] Although there were several reports regarding the association between inflammatory markers and BA, the study of possible roles of serum levels of IL-12, IL-18, and IFN-gamma in BA receives not much attention.

Based on the role of IL-12 as a key proinflammatory cytokine, it is likely that IL-12 is involved in the pathophysiology of BA. Recent studies showed that the loss of IL-12

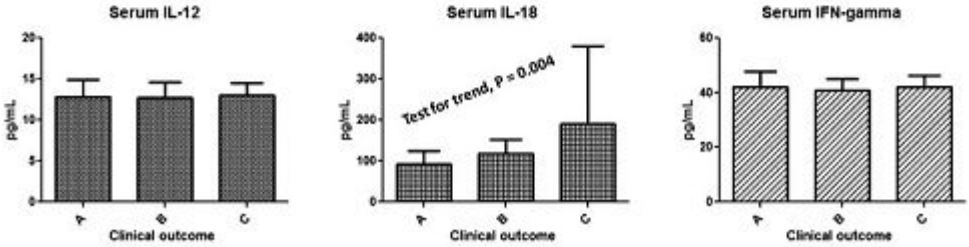

Figure 1 The comparisons of serum IL-12, serum IL-18, and serum IFN-gamma among the three groups of BA patients.

modified the proinflammatory response but did not prevent duct obstruction in experimental BA in animals.[23] Furthermore, IL-18 levels were demonstrated to correlate with IL-12 and IFN-gamma levels through Th1 cytokine production.[24] Mack et al[25] revealed that there were increases in T cells and Kupffer cells in the portal tracts of BA. Analysis of liver tissue from BA patients showed a Th1-type cytokine profile with significant expression of IFN-gamma and IL-12.

The present study clearly showed that serum levels of IL-18 and IFN-gamma were significantly elevated in medium-term survivors of BA compared with healthy children. The results are consistent with previous studies conducted in adult patients demonstrating that serum IL-18 levels were higher in cirrhotic patients including primary biliary cirrhosis and chronic hepatitis C than those of healthy adults.[26,27] Our findings on the correlation of elevated serum IL-18 levels and the severity of jaundice in BA are also agreeable with Urushihara et al.[28] Since IL-18 is a potent proinflammatory cytokine-inducing IFN-gamma production from activated T cells, elevated serum IL-18 levels found in BA patients may be responsible for the elevation of serum IFN-gamma in BA found in this study. Nonetheless, we found no correlation between serum IL-18 and serum IFN-gamma in BA patients. This may be due to the small sample size or the complex pathways involved in the interaction between IL-18 and IFN-gamma. Investigation of these cytokine levels in the liver tissue will elucidate possible roles of IL-18 and IFN-gamma in BA more precisely.

Interestingly, we demonstrated that there was no difference in serum IL-12 levels between BA patients and normal controls. An important biologic activity associated with IL-12 is to promote the differentiation of naive T cells into T-helper cells capable of producing IFN-gamma. Stimulation of IFN-gamma production by IL-12 can be synergistically enhanced by the presence of other proinflammatory cytokines such as tumor necrosis factor, IL-1, and IL-2.[19] Therefore, it is likely that IL-12 is not directly involved in the pathophysiology of BA and the elevation of serum IFN-gamma found in BA patients is not passed through IL-12 signaling pathway but rather via IL-18 pathway in some extent. Additionally, since there is no difference in serum IL-12 among BA patients with different jaundice status, it is likely that the association between serum IL-18 and jaundice status found in this study is not due to poor liver function.

As always, this kind of study regarding serum markers does inevitably have some limitations. First, the serum levels of cytokines did not necessarily reflect the action of their signaling pathways within the disease organs. However, with the supported evidence from other studies on the real association between their serum levels and the process of liver fibrosis, it is likely that the elevated serum levels of IL-18 and IFN-gamma found in BA play roles in its pathophysiology. Second, there are inflammatory cytokines or markers other than IL-12, IL-18, and IFN-gamma that are involved in the pathophysiology of progressive inflammatory liver injury, for example, IL-8,[7] ICAM-1,[17] selectin,[9] and nitric oxide.[13] Third, we would like to emphasize that the results are weak due to very small patient numbers and more data are needed to confirm our findings. Therefore, there are other influential factors outside the boundaries of this study needed to be taken into account. What we attempted to do here is to assemble some small pieces from the big picture of complex pathways.

In conclusions, serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These results suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. Additionally, IL-18 is likely to be involved in the disease progression. Serum IL-18 may be used as a prognostic marker in BA.

Conflict of Interest

None

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--
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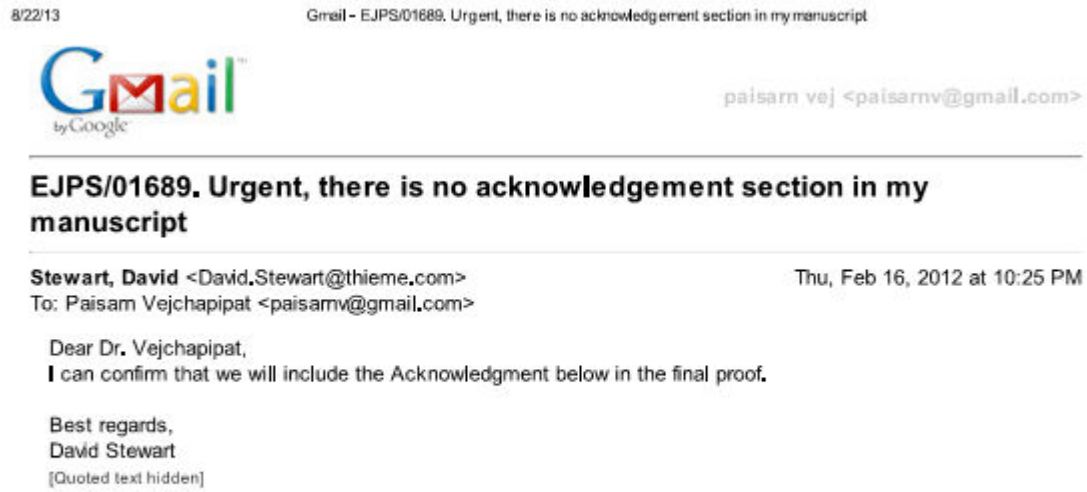

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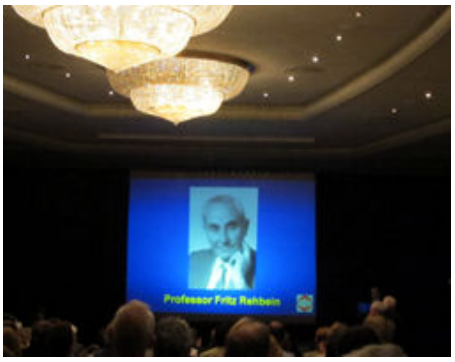

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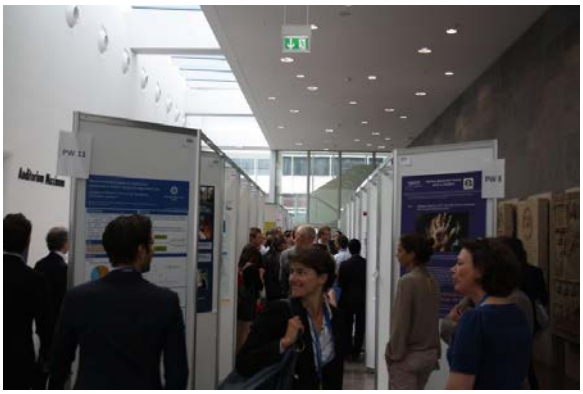

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Program and abstracts book

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Aleksandar Sretenovic, Zoran Krstic, Dragana Vujovic, Polina Pavicevic, Dusica Simic. *University Children's Hospital, Belgrade, Serbia*

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Arnoud Potgieser, Willemien de Vries, Yuk Kueng Sze, Ger Sieders, Henkjan Verkade, Josette Hoekstra-Weebers, Robert Porte, Jan Hulscher. *Beatrix Children's Hospital, University Medical Centre Groningen, Groningen, Netherlands*

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Hanna Lampela, Silja Kosola, Mikko Pakarinen. *Hospital for Children and Adolescents, Helsinki University, Helsinki, Finland*

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A Grieve, E Makin, M Davenport. *Kings College Hospital, London, United Kingdom*

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12.50-12.55 **ELEVATED SERUM IL-18 AND INTERFERON-GAMMA IN MEDIUM-TERM SURVIVORS OF BILIARY ATRESIA**

Paisarn Vejchapipat[1], Voranush Chongsrisawat[2], Sopee Poomsawat[3], Sittisak Honsawek[4], Yong Poovorawan[2]. *1, Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; 2, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; 3, Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand; 4, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

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12 55-13.00 **POST- KASAI CHOLANGITIS IN CHILDREN WITH BILIARY ATRESIA**

Augusto Zani, Mark Davenport. *Kings College Hospital, London, United Kingdom*

KP. Three measures of outcome were used: clearance of jaundice (to <20 umol/L); actuarial native liver survival and the development of varices requiring endoscopic intervention. Non-parametric statistical comparison was used. Survival curves were compared using log-rank test. Data are quoted as median (range). P ≤ 0.05 was regarded as significant.
Results: 263 infants underwent KP at a median age of 58 days (14-209). Median APRi was 0.69 (0.11 – 10.8). There was a significant difference between those who became anicteric (n=134 (52%)) and those with persistent jaundice (p=0.0004). There was a significant difference in native liver survival (P=0.009) when the group was divided into four groups on the basis of initial APRi. 42/249 (17%) children developed varices requiring endoscopic intervention. There was no difference between their initial APRi and controls (0.83 (0.1 – 3) vs 0.68 (0.11 – 10) (P=0.6).
Conclusion: APRi is a simple variable and the first which appears to have reasonable prognostic value in determining some elements of outcome in BA post-Kasai portoenterostomy.

O-25

ELEVATED SERUM IL-18 AND INTERFERON-GAMMA IN MEDIUM-TERM SURVIVORS OF BILIARY ATRESIA

Paisarn Vejchapipat[1], Voranush Chongsrisawat[2], Sopee Poomsawat[3], Sittisak Honsawek[4], Yong Poovorawan[2].
1, Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; 2, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; 3, Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand; 4, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
Aims: Biliary atresia (BA) is a fatal disease in children. Its main pathological feature is progressive immune-mediated cholangiopathy. Interleukin-12 (IL-12), IL-18, and interferon-gamma (IFN-gamma) plays important roles in various immunological diseases. The objective was to investigate whether these serum markers were associated with clinical outcome in BA.
Methods: Serum levels of IL-12, IL-18 and IFN-gamma were determined using ELISA from 46 BA patients (median age of 9 years) and 19 normal controls. The BA patients were then categorized into three groups according to their outcome: jaundice-free (29 cases), mild jaundice (10 cases), and severe jaundice (7 cases). The comparisons of serum IL-12, IL-18 and IFN-gamma levels among groups of the patients were performed using one-way ANOVA with post-hoc tests. Data are expressed as mean+SD.
Results: Serum IL-18 and IFN-gamma in BA patients were higher than the normal controls (IL-18: 113.3+82.6 vs. 80.5+9.9 pg/ml, P=0.011 and IFN-gamma: 41.7+5.1 vs. 38.0+1.9 pg/ml, P<0.001). There was no difference in serum IL-12 between BA and controls. Further analysis demonstrated that, in BA patients, only serum IL-18 levels significantly increased with the degree of jaundice (test for trend, P=0.004).
Conclusion: Serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA. The elevated serum IL-18 in BA patients was associated with worse clinical outcome. These results suggest that IL-18 and IFN-gamma play roles in the pathophysiology of BA. Additionally, IL-18 is likely to be involved in the disease progression.

O-26

POST- KASAI CHOLANGITIS IN CHILDREN WITH BILIARY ATRESIA

Augusto Zani, Mark Davenport. *Kings College Hospital, London, United Kingdom*
Aims: The incidence of cholangitis in patients with biliary atresia (BA) treated with Kasai portoenterostomy (KP) is controversial and had ranged from 30-60%. The aim of the present study was to establish the risk of developing cholangitis after KP in a current series.
Methods: Retrospective review of all infants with BA who underwent a KP over a recent 5 year period (Jan 2006 – Dec 2010). Prophylactic antibiotics were used for 1 month post-KP. Definition: suspected cholangitis (SC)=pyrexia (>38°C) requiring antibiotics with no increase in serum bilirubin; true cholangitis (TC)=pyrexia (>38°C-excluding other sources of infection) and increase in bilirubin level. Time period analysed was between the KP and either the date of liver transplant or the last clinic or date of death. Data is quoted as median (IQ range) and compared using non-parametric tests. P <0.05 was regarded as significant.
Results: Of 121 patients; 25 (21%) developed TC, 29 (24%) had SC, with 67 (55%) having no evidence of sepsis. TC

Elevated serum IL-18 and interferon-gamma in medium-term survivors of biliary atresia

Paisarn Vejchapipat, Sopee Poomsawat, Voranush Chongviriyawat
Sittisak Honsawek, Yong Poovorawan

Department of Surgery and Department of Pediatrics
Faculty of Medicine, Chulalongkorn University
& King Chulalongkorn Memorial Hospital, Bangkok, Thailand

Introduction

- Biliary atresia (BA) is a devastating liver disease in pediatric population
- Its pathophysiology involves immune-mediated inflammatory dysregulation
- Recent evidence suggested that interferon (IFN)-gamma play a key role in animal model of BA

Introduction

- Generally, IL-12 and IL-18 are both strong inducers of IFN-gamma production
- Objective: To investigate serum levels of IL-12, IL-18, and IFN-gamma in medium-term survivors of BA

Methods

- Serum IL-12, IL-18 and IFN-gamma were determined using ELISA
- 46 BA patients (median age =9 years) and 19 normal controls
- Comparisons of these 3 serum cytokines between BA and controls were carried out

Methods

- The BA patients were divided into three groups according to their jaundice status

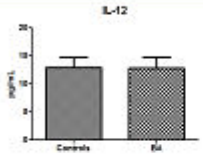
Group	Status	Serum total bilirubin	No. of cases
A	Jaundice-free	<2 mg/dL	29
B	Mild to Moderate	2-10 mg/dL	10
C	Marked jaundice	>10 mg/dL	7

Methods

- The comparisons of these serum cytokines among three groups of patients were performed using one-way ANOVA with post-hoc tests
- Data are expressed as mean+SD

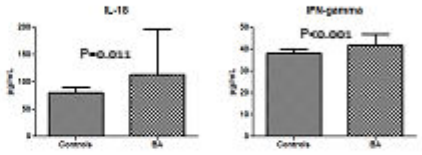
Results
Serum IL-12 (Control vs. BA)

- There was no difference in serum IL-12 levels between controls and BA



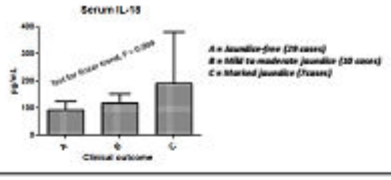
Results
IL-18 & IFN-gamma (Control vs. BA)

- Serum IL-18 and IFN-gamma in BA patients were higher than the controls



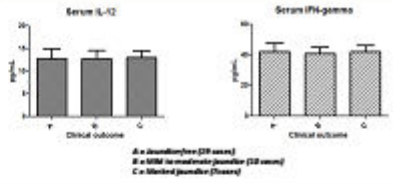
Results: IL-18

- In BA patients, only serum IL-18 levels significantly increased with the degree of jaundice



Results: IL-12 & IFN-gamma

- There was no difference in serum IL-12 and IFN-gamma among 3 groups of BA patients



Conclusions

- Serum IL-18 and IFN-gamma levels were increased in medium-term survivors of BA
- The elevated serum IL-18 in BA patients was associated with worse clinical outcome.
- IL-18 and IFN-gamma are likely to play roles in the pathophysiology of BA

Acknowledgements

- The Thailand Research Fund
- Travelling grant, Chulalongkorn University

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11.25-11.30 **VITAMIN D LEVELS IN INFANTS WITH BILIARY ATRESIA: PRE AND POST KASAI PORTOENTEROSTOMY.**
A Paul[1], NJ Wright[1], N Hadzic[1], M Davenport[1], *[1]Kings College Hospital, London, UK*

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P Vejchapipat[1], S Poomsawat[2], S Chittmittrapap[1], Y Poovorawan[3], *[1]Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, [2]Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand, [3]Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

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C O v. Sochaczweski[1], I Pintelon[2], I Brouns[2], J-P Timmermanns[2], J Kuebler[1], C Petersen[1], *[1]Department of Pediatric Surgery, Medical University, Hannover, Hannover, Germany, [2]Department of Veterinary Science, University of Antwerp, Antwerp, Belgium*

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A Shalaby[1], R Hajhosseiny[1], A Quaglia[1], A Knisely[1], Y Zen[1], M Davenport[1], *[1]Kings College Hospital, London, UK*

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D Chauhan[1], I Zamora[1], D Lazar[1], S Hassan[1], O Olutoye[1], *[1]Texas Children's Hospital, Houston, TX, USA*

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P Laje[1], LJ States[1], SA Becker[1], AA Palladino[1], CA Stanley[1], NS Adzick[1], *[1]The Children's Hospital of Philadelphia, Philadelphia, PA, USA*

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E Maillard[1], SE Cross[1], PA Bateman[1], P Tuiedor[2], D Brandhorst[1], A Clark[2], DWR Gray[1], SJ Hughes[1], PRV Johnson[1], *[1]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, UK, [2]Oxford Centre for Diabetes, Endocrinology, and Metabolism, Churchill Hospital, Oxford, UK*

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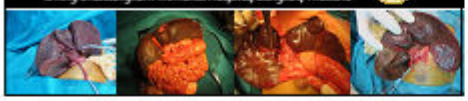
PA Bateman[1], SE Cross[1], D Brandhorst[1], K Jones[1], DWR Gray[1], SJ Hughes[1], PRV Johnson[1], *[1]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, UK*

Hepatic Expression of Caspase-3 and Survivin in Biliary Atresia

Paisarn Vejchapipat, Sopee Poomsawat, Soottiporn Chittmittrapap, Yong Poovorawan

Department of Surgery and Department of Pediatrics
Faculty of Medicine, Chulalongkorn University
& King Chulalongkorn Memorial Hospital, Bangkok, Thailand



Disclosures

NONE

Introduction

- Biliary atresia (BA) is a devastating liver disease in pediatric population
- Its pathophysiology involves immune-mediated inflammatory process

Introduction

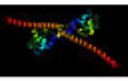
- Recent evidence suggested that apoptosis also plays roles in animal model of BA
- Biliary epithelia undergo early activation of apoptosis in a mouse model of BA

Erickson N, et al. Hepatology 2008;47:1567-77

Caspase-3

- Apoptosis is a complex process
- Caspase-3 is one of the key executioners of apoptosis
- Expression of active caspase-3 is associated with apoptosis process

Survivin



- Survivin is an inhibitor of apoptosis
- Survivin is highly expressed during fetal development and cancer cell malignancy
- By binding and inhibiting caspase-3, survivin controls the cell cycle by inhibiting apoptosis and promoting cell division

Objective

- To investigate hepatic expression of caspase-3 and survivin in BA

Methods

- Caspase-3 and Survivin expression was determined using immunohistochemistry from liver biopsies
- 49 BA patients vs. 29 non-BA pediatric patients
- Comparisons of these 2 proteins between BA and non-BA were carried out

Non-BA patients

- The non-BA pediatric patients include
  - **8 hepatitis**
  - **21 non-cholestatic patients**
    - *Choledochal cysts 11*
    - *Thalassemias 5*
    - *Neuroblastomas 2*
    - *Adrenal xanthogranuloma 1*
    - *Portal vein thrombosis 1*
    - *Hepatoblastoma 1*

Methods

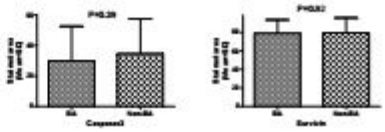
- Caspase-3 and survivin expression from hepatocyte areas was assessed based on percentage of immuno-stained areas
- Caspase-3 and survivin expression of biliary epithelia at portal triad were evaluated as positive or negative staining

Methods

- The comparisons of the 2 proteins between 2 groups of patients were performed using unpaired t-tests or Fisher's exact tests
- Data are expressed as mean+SD

Results: Caspase-3 & Survivin Hepatocyte areas (BA vs. non-BA)

- There was no difference in caspase-3 or survivin expression of hepatocyte areas between BA and non-BA patients



Results: Caspase-3 expression Biliary cells (BA & non-BA)

- There was no difference in caspase-3 expression of biliary epithelial cells between BA and non-BA patients

Biliary epithelial cells	BA	Non-BA
Caspase-3 positive	20	8
Caspase-3 negative	29	21

P= 0.3295

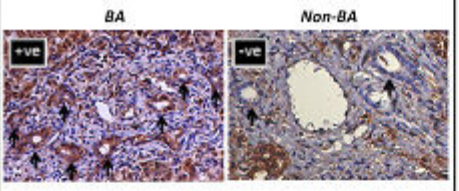
Results: Survivin expression Biliary cells (BA & non-BA)

- The proportion of BA patients having survivin expression of biliary epithelial cells was significantly higher than that of non-BA patients
- 81.6% (40/49) of BA patients had +ve staining of survivin in biliary ductular epithelial cells

Biliary epithelial cells	BA	Non-BA
Survivin positive	40	9
Survivin negative	9	20

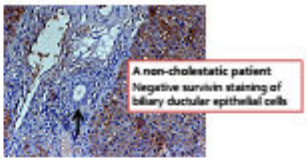
P<0.0001

Survivin expression of biliary ductular epithelial cells in BA vs non-BA



Results: Survivin expression Biliary cells (BA & non-BA)

- Only 25.0% (2/8) of hepatitis and 33.3% (7/21) of non-cholestatic patients showed +ve survivin expression in biliary ductular epithelia



Limitations of the study

- Small sample size
- Ages between BA & non-BA are unequal
- No normal controls
- More studies are needed

Conclusions

- Survivin expression of biliary ductular epithelia was related to BA
- Actions of survivin (via anti-apoptosis) may involve in biliary pathology of BA
- Its expression may be helpful in the diagnosis of BA

Acknowledgements

- The Thailand Research Fund
- Travelling grant, Chulalongkorn University

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AAPS
Seoul 2012

23rd Congress of
the Asian Association of Pediatric Surgeons

AAPS 2012

"Current Pediatric Surgery in Asia"

October 8(Mon)-10(Wed), 2012

Eun Myeong Auditorium, Severance Hospital
Yonsei University College of Medicine
Seoul, Korea

Program & Abstracts

Hosted by

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23rd Congress of
the Asian Association of Pediatric Surgeons
AAPS 2012

Invitation

March 19, 2012

Dr. Paisarn Vejchapipat

Associate Professor of Surgery/ Head of Pediatric Surgery Unit
Chulalongkorn University & King Chulalongkorn Memorial Hospital
Thailand

Dear Dr. Vejchapipat,

On behalf of the Organizing Committee of AAPS 2012, it is our great honor to invite you as an invited speaker in the 23rd Congress of the Asian Association of Pediatric Surgeons (AAPS 2012), which will be held on October 8-10, 2012 at the EunMyeong Auditorium, Severance Hospital, Seoul, Korea.

The congress is aiming at 'Current Pediatric Surgery in Asia' by presenting in-depth discussions for medical professionals in the field of pediatric surgery. The congress covers 6 panel discussion categories – Urology, Congenital biliary tract anomaly, Hirschsprung's disease, Anorectal anomalies, Managements of diaphragmatic hernia and Minimal invasive surgery.

We respectfully ask you be a panel speaker of 'Congenital Biliary Tract Anomaly'.

It is expected that there will be approximately 300 attendees at the congress – surgeons and trainees. This congress will be a cheerful one to share personal and institutional experience, knowledge, ideas and controversies in the management of pediatric surgeries through the scientific programs.

For details of AAPS 2012, please refer to the speaker manual, brochure and the congress website, www.asian-aaps2012.org.

The AAPS 2012 looks forward to seeing you in Seoul, Korea!

Cordially yours,

Seung Hoon Choi, M.D.
President
Organizing Committee
23rd Congress of the Asian Association of Pediatric Surgeons

Secretariat
Address: Euihak Bldg. 3rd Floor, 448-13 Seogyo-dong, Mapo-gu, Seoul, Korea
Tel : +82-2-6711-4762 Fax: +82-2-325-2095 Email: secretariat@asian-aaps2012.org

Biliary atresia: an experience at one institution

Paisarn Vejchapipat, Soottiporn Chittmittrapap, Bidya Chandrakamol, Yong Poovorawan, Voranush Chongsrisawat, Prapapan Rajatapiti, Katawaetee Decharun, Somboon Reukviboonsri, Dusit Viravaidya*

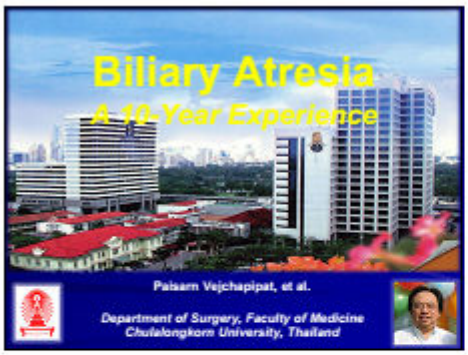
Pediatric Surgery Unit, Department of Surgery, Faculty of Medicine, Chulalongkorn University & King Chulalongkorn Memorial Hospital, Bangkok, Thailand. E-mail: paisarnv@gmail.com

Biliary atresia (BA) is one of the most fatal liver diseases in children. Its main pathological feature is progressive inflammatory cholangiopathy. We describe our experience in the management of BA at King Chulalongkorn Memorial Hospital, Bangkok, Thailand.
Over 26 years, between 1986 and 2011, there were 320 BA patients treated with Kasai operation. We can divide our patients into 4 periods based on our published articles; 1986-1995 (115 cases), 1996-2001 (92 cases), 2002-2005 (53 cases), and 2006-2011 (60 cases). Our surgical outcome improved over times. The good outcome (clearance of jaundice within 6 months following surgery) was achieved from 38% of cases during 1986-1995 period to 58.3% of cases during 2006-2011 period.
At present, the pre-operative diagnosis of BA at our institution is based on clinical presentation, liver function tests, ultrasonography and, occasionally, hepatobiliary scintigraphy. Post-operative high-dose steroids were implemented in our treatment protocol since 2003. Serum total bilirubin (TB) at 7^{th} day post-op is helpful in predicting the outcome only when the drop of serum TB is beyond 50%. One of our problems in the management of BA is that the majority of the patients were referred to our hospital quite late. Median age at Kasai operation over the last 8 years was 80.5 days.

Conclusions: BA still remains a significant problem in Thailand. Our short-term result of Kasai operation is acceptable according to international standard. At least 40% of our patients eventually need liver transplantation. A solution for the enhancement of earlier referral and treatment is urgently required in Thailand.

Acknowledgement: Thailand Research Fund (RMU-5380028), Ratchadapisek Sompoth Fund, and Faculty of Medicine, Chulalongkorn University.

Biliary Atresia
A 10-Year Experience



Paisarn Vejchapipat, et al.

Department of Surgery, Faculty of Medicine
Chulalongkorn University, Thailand

Topics

- Description of our experience
- Our practice guidelines
- Our research

Thailand

- Statistics in 2011
- Population: 64 million
- Pediatric: 12.8 million
- Births: 800,000 /year

National Statistical Office of Thailand

Incidence of BA = 1:10,000 Live Births
→ 80 New Cases of BA per Year

Introduction

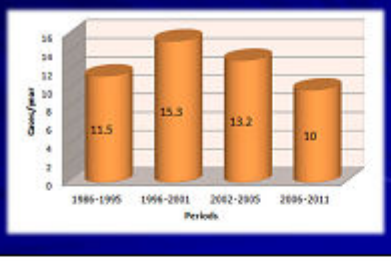
- Biliary atresia (BA) has been our interest since 1990s
- Between 1986 and 2011, there were 320 BA patients undergoing Kasai operation

Chulalongkorn's Experience

- 1986-1995:
  - 115 cases
  - Chula Med J 1996
- 1996-2001:
  - 92 cases
  - Asian J Surg 2005
- 2002-2005:
  - 53 cases
  - J Pediatr Surg 2007
- 2006-2011:
  - 60 cases
  - RCST meeting 2012

Chulalongkorn University

Cases/year



Clearance of jaundice after Kasai op. in various countries

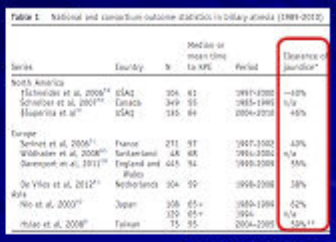
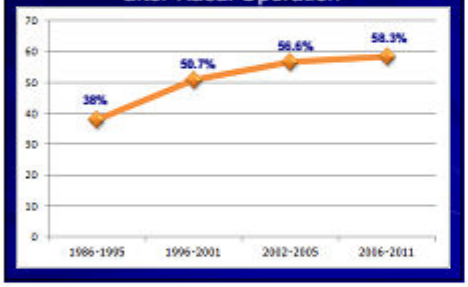


Table 1 National and consortium outcome statistics in biliary atresia (1981-2012)

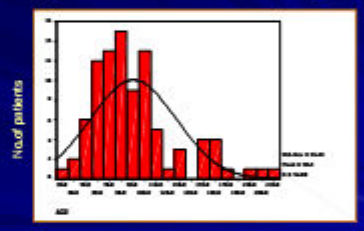
Series	Country	N	Median or mean time to KPE	Period	Clearance of jaundice*
North America					
Schneider et al. 2006[14]	USA2	304	61	1997-2002	~60%
Schreiber et al. 2007[15]	Canada	349	55	1985-1995	n/a
Duperrois et al.[16]	USA3	185	64	2004-2010	46%
Europe					
Sarnacki et al. 2006[17]	France	271	57	1997-2002	43%
Wildhaber et al. 2008[18]	Switzerland	68	68	1996-2006	n/a
Davenport et al. 2011[19]	England and Wales	445	54	1999-2008	55%
De Vries et al. 2012[11]	Netherlands	104	59	1998-2008	38%
Nio et al. 2003[20]	Japan	108	65+	1989-1998	62%
Hsiao et al. 2008[21]	Taiwan	129	65+	1994-	n/a
		75	55	2004-2005	43%[11]

Davenport M. Semin Pediatr Surg 2012

Clearance of jaundice within 6 months after Kasai Operation



Age at The Time of Surgery



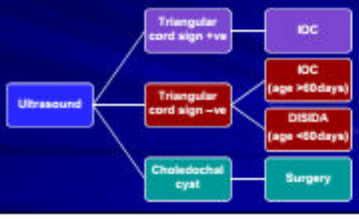
- Only 23% had Kasai operation before 60 days of age
- 63% operated before 90 days of age

Asian J Surg 2005

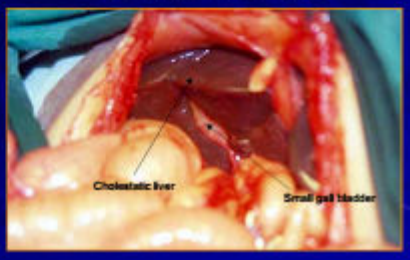
The diagnosis of BA

Current guidelines at our hospital

- Babies (0-4 months old) with cholestatic jaundice and persistent acholic stools



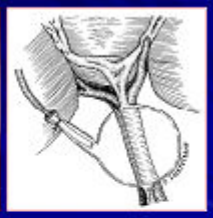
Operative Findings



IOC of BA

- Most of the BA patients (95%) had atresia at porta hepatis (type III)
- BA type III with patent cystic duct and common bile duct were found in 23% of our series

Report of Cystic Biliary Atresia



- An unusual type of Biliary Atresia

Eur J Pediatr Surg 2007

Operation

- Original Kasai operation
- Mobilization of the liver
- Free the portal vein from the fibrous mass
- No frozen section

The Effects of Post-op Steroids

- The use of high-dose post-op steroids is controversial
- 53 BA patients over 4 years
- Steroids did not statistically improve early good outcome in BA
  - Jaundice-free: 61% vs. 50% (steroid vs. non-steroid)
- The results may be due to small sample size

Vejchapipat et al. J Pediatr Surg 2007

The use of serum TB at 7th day post-op in predicting short-term outcome after Kasai op

- Observational study
- 80 patients underwent Kasai op (2002-2009)
- Median age at Kasai operation was 80.5 days
- The patients were categorized into 2 groups
  - Good outcome (jaundice-free within 6 months post-op)
  - Poor outcome (persistent jaundice)

Results

- The drop of serum TB at 7th day post-Kasai >20% compared to pre-op can predict good outcome in 76.5%

Serum TB at 7th day post-op	Good outcome	Poor outcome	Total
The drop >20%	26	8	34
The drop <20% or no drop	22	24	46
Total	48	32	80

P=0.012

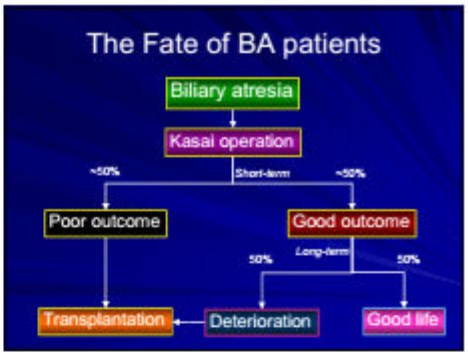
Results

- If the drop of serum TB at 7th day post-Kasai is >50%, all patients will have good outcome

Serum TB at 7th day post-op	Good outcome	Poor outcome	Total
The drop >50%	5	0	5
The drop < 50% or no drop	43	32	75
Total	48	32	80

P=0.08

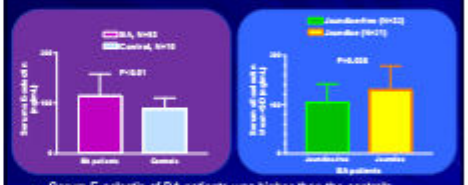
The Fate of BA patients



Other Aspects of Our Research in Biliary Atresia

- Inflammatory markers
  - ICAM-1
  - Selectin
  - Nitric oxide
  - Interleukins
  - IFN-gamma
- Growth factors
  - TGF-beta1
  - EGF
  - HGF
  - bFGF
  - CTGF
- Fibrotic markers
  - TIMP-1
  - Hyaluronan
  - Stem cell factor

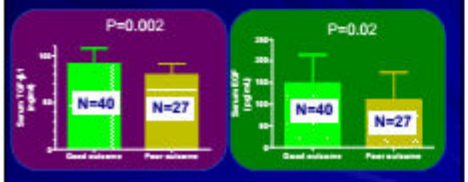
Circulating E-Selectin in Biliary Atresia



- Serum E-selectin of BA patients was higher than the controls
- There was an increase in serum E-selectin in BA patients with jaundice compared to those without jaundice

Eur J Pediatr Surg 2008

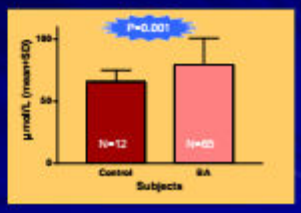
Serum TGF-β1 and EGF in BA



- BA patients with good outcome had serum TGF-β1 and EGF higher than those with poor outcome

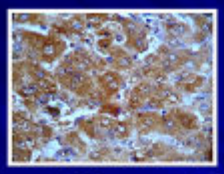
Eur J Pediatr Surg 2008

Systemic nitric oxide production was elevated in BA



Pediatr Surg Int 2006

Hepatic iNOS Expression in BA



- Strong hepatic iNOS expression in BA patients
- In BA, hepatocyte is likely to be the major source of nitric oxide production

Hepatol Res 2008

Conclusions (1)

- BA still remains a significant problem in Thailand
- Our short-term result of Kasai operation is comparable to other published articles
- In short-term, at least 40% of our patients eventually need liver transplantation

Conclusions (2)

- Earlier referral and treatment is urgently required in Thailand
- Studies suggested that inflammatory process and growth factors play important roles in its pathophysiology
- More research is needed to improve outcome

The Team

Acknowledgement

- The Thailand Research Fund (RMU5380028)
- The Royal College of Surgeons of Thailand
- Chulalongkorn University

Thank you very much

14TH CONGRESS OF THE EUROPEAN PAEDIATRIC SURGEONS' ASSOCIATION

LEIPZIG, 5 – 8 JUNE 2013

EUPSA

		Gamez[1], Gemma Bartolo[2], Esteban Frauca[2], Paloma Jara[2], Manuel Lopez-Santamaria[1], Juan A. Tovar[1], *[1]Pediatric Surgery Department, Hospital Universitario La Paz, Madrid, Spain, [2]Pediatric Hepatology Department, Hospital La Paz, Madrid, Spain*
17	12.00-12.10	**CONGENITAL PORTOSYSTEMIC SHUNTS IN CHILDREN: PROPOSITION FOR A PRACTICAL SURGICAL CLASSIFICATION BASED ON 15 YEARS' EXPERIENCE** Florent Guerin[1], Thomas Blanc[2], Stéphanie Franchi-Abella[1], Olivier Bernard[1], Frédéric Gauthier[1], Sophie Branchereau[1], *[1]Bicêtre Hospital. Univeristy Paris XI, LE KREMLIN BICETRE, France, [2]Necker Hospital. University Paris V., PARIS, France*
18	12.10-12.15	**SURGICAL SUCCESS IN CHRONIC PANCREATITIS: COMBINED USE OF ERCP AND LONGITUDINAL PANCREATO-JEJUNOSTOMY (PUESTOW PROCEDURE)** Anu Paul[1], Kat Ford[1], Maria Sellars[1], Shailesh Patel[1], Philip Harrison[2], Mark Davenport[1], *[1]Kings College Hospital, London, UK, [2]Institute of Liver Studies, London, UK*
19	12.15-12.25	**EFFECT OF TACROLIMUS AND INSULIN IN LIVER REGENERATION MODEL IN GROWING ANIMALS WITH PORTAL VEIN STENOSIS: IMUNOHISTOCHEMICAL AND MOLECULAR STUDIES** Ana Tannuri[0], Ariane Backes[0], Uenis Tannuri[0], Maria Mendonça[0], Josiane Gonçalves[0], Evandro Melo[0], Amadeu Queiroz[0], Elenice Lima[0], *[1]University of São Paulo Medical School, São Paulo, Brazil*
20	12.25-12.35	**HEALTH RELATED QUALITY OF LIFE IN PEDIATRIC INTESTINAL TRANSPLANTATION PATIENTS** Eva Dominquez-Amillo[1], Ane M. Andres-Moreno[1], Alicia Alameda-Angulo[2], Olga Mayoral-Pulido[3], Sara Hernandez-Martin[1], Francisco Hernandez-Oliveros[1], Esther Ramos-Boluda[2], Eva Martinez-Ojinaga[2], Gerardo Prieto-Bonzano[2], Manuel Lopez-Santamaria[1], Juan A. Tovar[1], *[1]Pediatric Surgery Department, Hospital La Paz, Madrid, Spain, [2]Pediatric Gastroenterology Department, Hospital La Paz, Madrid, Spain, [3]Psychology Department, Universidad Autonoma de Madrid, Madrid, Spain*
21	12.35-12.45	**IMMUNOHISTOCHEMICAL ANALYSIS OF COLLAGEN TYPE 1, α-SMA AND CD34 AT THE TIME OF KASAI PORTOENTEROSTOMY IN CHILDREN WITH BILIARY ATRESIA** Janne Suominen[1], Hanna Lampela[1], Hannu Jalanko[2], Mikko Pakarinen[1], *[1]Department of Paediatric Surgery, Children's Hospital, University of Helsinki, Helsinki, Finland, [2]Departments of Paediatrics and Paediatric Transplantation, Children's Hospital, University of Helsinki, Helsinki, Finland*
22	12.45-12.50	**CHOLEDOCHAL MALFORMATION, MIB-1 INDEX AND CA19-9 IN BILE: SEARCH FOR A BIOMARKER OF EPITHELIAL INSTABILITY** Mark Davenport[1], Yo Zen[2], *[1]Kings College Hospital, London, UK, [2]Institute of Liver Studies, London, UK*
23	12.50-12.55	**LONG-TERM RESULTS of INTRAPORTAL TRANSPLANTATION of AUTOLOGOUS STEM CELLS in CHILDREN with PORTAL HYPERTENSION SYNDROME** Vladislav Grin[1], Ivan Zhurylo[0,2], Andrew Popandopulo[1], Konstantin Latyshov[0,2], Peter Lepikhov[0,2], Oksana Selezneva[1], *[1]International Center of Biotechnology "Biostem" Institute of Emergency and Restorative Surgery of the Academy of Medical Sciences, Donetsk, Ukraine, [2]Donetsk Gorky Donetsk National Medical University, Donetsk, Ukraine*
24	12.55-13.00	**AGE AT OPERATION IS AN INDEPENDENT FACTOR FOR PREDICTION OF LIVER CIRRHOSIS OF PATIENTS WITH BILIARY ATRESIA** Chen Yajun[1,2], Xing Guodong[1], Wang Zengmeng[1,2], Pang Wenbo[1,2], Zhang Dang[1,2], *[1]Beijing Children's Hospital, Beijing, China, [2]Capital Medical University, Beijing, China*

POSTER WALK 1 - GASTROINTESTINAL I (13.30 - 14.30)

186		**SERUM BILIRUBIN AND ALANINE AMINOTRANSFERASE AS SIMPLE MARKERS TO PREDICT EARLY OUTCOME IN BILIARY ATRESIA** Paiboon Sookpotarom[1,2], Paisarn Vejchapipat[1], Kanokan Tepmalai[1], Rojjana Passakonnirin[1], Soottiporn Chittmittrapap[1], Yong Poovorawan[3], *[1]Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand,*

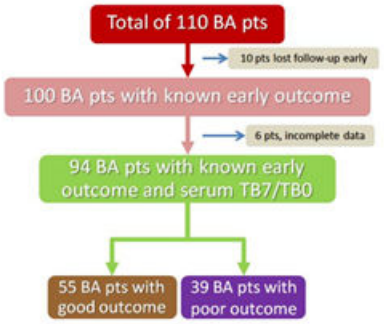
SERUM TOTAL BILIRUBIN AND ALANINE AMINOTRANSFERASE AS SIMPLE MARKERS TO PREDICT EARLY OUTCOME IN BILIARY ATRESIA

Paiboon Sookpotarom, Paisarn Vejchapipat*, Kanokan Tepmalai
Rojjana Passakonnirin, Soottiporn Chittmittrapap, Yong Poovorawan
Division of Pediatric Surgery, Department of Surgery,
Faculty of Medicine, Chulalongkorn University,
*Bangkok, Thailand 10330. *E-mail: paisarnv@gmail.com*

Biliary atresia (BA) is a serious liver disease with uncertain prognosis. We investigate whether serum total bilirubin (TB) at 7th day post-Kasai to pre-op TB ratio (TB7/TB0) and serum alanine aminotransferase (ALT) at 7th day post-Kasai to pre-op ALT ratio (ALT7/ALT0) are associated with early outcome.

Methods: BA patients undergoing Kasai operation between 2001 and 2011 were reviewed. Serum TB7/TB0 and ALT7/ALT0 were calculated for each patient. The patients were categorized into two groups at 6th month post-op based on jaundice status; good outcome (serum TB <2 mg%) and poor outcome (TB >2 mg%). Fisher's exact tests were performed. Binary logistic regression was used to identify possible prognostic markers.

Results: 110 BA patients (M:F=58:52) underwent Kasai operation. Median age at Kasai operation was 82 days. Clinical outcome at 6th month could be assessed in 100 patients (good: poor = 57:43). However, data of both serum TB and clinical outcome were available for analysis in 94 patients (good: poor = 55:39). TB7/TB0 of <0.8 and ALT7/ALT0 of <0.8 can predict good outcome in 72.5% (29/40, p=0.021), and 51.3% (19/37, p=0.275), respectively. Further analysis revealed that TB7/TB0 of <0.5 occurred in only 7.4% (7/94) and they all had good outcome.



p=0.021	Serum TB7/TB0 ratio		
BA patients	≤0.8	>0.8	Total
Good outcome	29	26	55
Poor outcome	11	28	39
Total	40	54	94

p=0.275	Serum ALT7/ALT0 ratio		
BA patients	≤0.8	>0.8	Total
Good outcome	19	32	51
Poor outcome	18	18	36
Total	37	50	87

Logistic regression analysis showed that gender, age at operation, cholangitis, and ALT7/ALT0 ratio were not significant factors impacting on early outcome (p>0.05). Only TB7/TB0 ratio of <0.8 was an independent factor for good outcome (odds ratio =2.8, p=0.02).

Conclusions: TB7/TB0 ratio of <0.8 may be used as a good prognostic marker for BA. However, ALT7/ALT0 ratio of <0.8 is not a good marker in predicting good outcome.

Acknowledgement:
The study was supported by Thailand Research Fund (Grant no. RMU5380028) and Faculty of Medicine, Chulalongkorn University, Thailand.