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นางสาววันวิสาข์ อุดมสินประเสริฐ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Role of autotaxin for the pathogenesis of liver fibrosis in biliary atresia

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for the Degree of Doctor of Philosophy Program in Medical Biochemistry
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วันวิสาข์ อุดมสินประเสริฐ : บทบาทของโอโต้แทกซินกับการเกิดพยาธิสภาพของการเกิดพังผืดสะสมตับในโรคท่อน้ำดีตีบตัน (Role of autotaxin for the pathogenesis of liver fibrosis in biliary atresia) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร. นพ. สิทธิศักดิ์ หรรษาเวก, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. ยง ภู่วรวรรณ, 168 หน้า.

โรคท่อน้ำดีตีบตันเกิดจากความผิดปกติของท่อน้ำดีทำให้เกิดการคั่งของน้ำดีในทารกแรกคลอด สาเหตุของโรคท่อน้ำดีตีบตันยังไม่เป็นที่ทราบแน่ชัด วัตถุประสงค์ของการศึกษาเพื่อศึกษาหาความสัมพันธ์ระหว่าง ระดับโปรตีนออโต้แทกซิน (autotaxin) การแสดงออกระดับ mRNA ระดับ promoter methylation ของยีนออโต้แทกซิน ความยาวของเทโลเมียร์ ระดับ global methylation (Alu and LINE-1) และภาวะเครียดออกซิเดชัน (oxidative stress) กับอาการทางคลินิก ในผู้ป่วยโรคท่อน้ำดีตีบตันภายหลังด้วยการรักษาด้วยการผ่าตัด จำนวน 114 ราย และกลุ่มควบคุมซึ่งมีสุขภาพดีจำนวน 114 ราย ผลการศึกษาพบ ระดับโปรตีนออโต้แทกซินเพิ่มสูงขึ้นในเลือดของกลุ่มผู้ป่วย และสัมพันธ์กับภาวะตัวเหลือง ภาวะเซลล์ตับถูกทำลาย และค่าพังผืดตับอย่างมีนัยสำคัญทางสถิติ นอกจากนี้พบการแสดงออกระดับ mRNA ของยีนออโต้แทกซินเพิ่มสูงขึ้นในเลือดและในชิ้นเนื้อตับของผู้ป่วยโรคท่อน้ำดีตีบตัน และมีความสัมพันธ์แปรผกผันกับ DNA methylation บริเวณ promoter ของยีนออโต้แทกซินในเลือดอย่างมีนัยสำคัญทางสถิติ เมื่อวิเคราะห์การแสดงออกของโปรตีนออโต้แทกซินในชิ้นเนื้อตับด้วยวิธี immunohistochemistry พบการแสดงออกของโปรตีนออโต้แทกซินบริเวณเซลล์บุผิวน้ำดีในตับ (liver bile duct epithelia) และในเซลล์ตับ (hepatocytes) ของกลุ่มผู้ป่วยโรคท่อน้ำดีตีบตัน ซึ่งสอดคล้องกับระดับโปรตีนออโต้แทกซินในเลือด นอกจากนี้ความยาวของเทโลเมียร์ในเลือดของกลุ่มผู้ป่วยโรคท่อน้ำดีตีบตันสั้นกว่ากลุ่มควบคุม และสัมพันธ์กับระดับความรุนแรงของโรคอย่างมีนัยสำคัญทางสถิติ การศึกษาความสัมพันธ์ของระดับ global methylation ความยาวของเทโลเมียร์ และภาวะเครียดออกซิเดชัน พบระดับ global methylation ในกลุ่มผู้ป่วยโรคท่อน้ำดีตีบตันต่ำกว่าในกลุ่มควบคุม ซึ่งสัมพันธ์กับความยาวของเทโลเมียร์ และภาวะเครียดออกซิเดชัน อย่างมีนัยสำคัญทางสถิติ สรุปได้ว่า ระดับโปรตีนออโต้แทกซิน การแสดงออกระดับ mRNA ของยีนโอโต้แทกซิน ระดับ promoter methylation ของยีนออโต้แทกซิน ความยาวของเทโลเมียร์ ระดับ global methylation และภาวะเครียดออกซิเดชัน มีความสัมพันธ์กับลักษณะอาการทางคลินิกของผู้ป่วยโรคท่อน้ำดีตีบตัน ซึ่งอาจจะนำมาใช้เป็นตัวบ่งชี้ทำนายความรุนแรงของโรคท่อน้ำดีตีบตันโดยเฉพาะในผู้ป่วยที่มีพังผืดตับ และช่วยทำให้ความเข้าใจกลไกและพยาธิกำเนิดของการเกิดพังผืดตับในโรคตับเรื้อรังรวมทั้งโรคท่อน้ำดีตีบตันได้ดียิ่งขึ้น

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีทางการแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5674768530 : MAJOR MEDICAL BIOCHEMISTRY
KEYWORDS: BILIARY ATRESIA / AUTOTAXIN / DNA METHYLATION / TELOMERE SHORTENING / OXIDATIVE DNA DAMAGE

WANVISA UDOMSINPRASERT: Role of autotaxin for the pathogenesis of liver fibrosis in biliary atresia. ADVISOR: PROF. SITTISAK HONSAWEK, M.D., Ph.D., CO-ADVISOR: PROF. YONG POOVORAWAN, M.D., 168 pp.

Biliary atresia (BA) is one of the most common causes of neonatal cholestasia. The etiology of BA remains mysterious. Lack of reliable noninvasive diagnostic biomarkers of BA may leads to delayed diagnosis and worse patient outcome. Hence, the identification of noninvasive biomarkers to assess liver fibrosis is desirable. The main aim of this study was to explore various contributors for hepatic dysfunction including autotaxin (ATX), relative telomere length (RLT), global DNA methylation and oxidative stress whether these biomarkers could be related to the pathogenesis of liver fibrosis in BA. One hundred and fourteen post operative BA patients and 114 age-matched healthy controls were enrolled. We found that BA patients had higher circulating ATX and liver stiffness than controls. Our findings showed that elevated circulating ATX was associated with status of jaundice, hepatic dysfunction, and liver stiffness in postoperative BA. In addition, the current study provides evidences for up-regulation of *ATX* mRNA expression in liver specimens of BA patients compared to those in controls. The up-regulation of *ATX* expression in BA liver samples was performed with immunohischemical detection of ATX antigens within the liver bile duct epithelia and the hepatocytes. *ATX* mRNA expression was also significantly elevated and correlated with a decrease in *ATX* promoter methylation in BA patients compared to the controls. Moreover, this study supports the association between RTL in peripheral blood leukocytes and higher risk of liver fibrosis in BA. RLT in blood leukocytes was also associated with disease severity, showing that BA patients with advanced-stage exhibited excessive telomere shortening. Additionally, this study reported that, independent of risk factors, hypomethylation of retrotransposable DNA elements (Alu and LINE-1) was associated with shorter telomeres, elevated oxidative DNA damage, and a higher risk of liver fibrosis in BA. Based on the aforementioned findings, combinations of circulating ATX levels, hepatic ATX expression, relative telomere length, global DNA methylation, and oxidative DNA damage could serve as possible noninvasive biomarkers reflecting the disease severity and the development of liver fibrosis in the post Kasai BA patients. Autotaxin could play a crucial role in the pathogenesis of liver fibrosis in chornic liver disease including biliary atresia.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Medical Biochemistry	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
8-OHdG	8-hydroxy-2′-deoxyguanosine
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APRI	Aspartate aminotransferase to platelets ratio index
AST	Aspartate aminotransferase
ATX	Autotaxin
AUC	Area under the ROC curve
BA	Biliary atresia
BMI	Body mass index
CC	Choledochal cysts
CpG	Cytosine–guanine dinucleotide
CREB	c-AMP response element-binding
CTGF	Connective tissue growth factor
CVs	Coefficients of variation
DB	Direct bilirubin
DNMTs	DNA methyltransferases
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ENPP-2	Ectonucleotide pyrophosphatases/phosphodiesterase-2
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGT	Gamma-glutamyl transferase
GPCR	G-protein-coupled LPA receptor

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HSCs	Hepatic stellate cells
IFN-γ	Interferon gamma
JNK	Jun N-terminal kinase
KPE	Kasai portoenterostomy
LINE-1	Long interspersed nuclear element-1
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPAR	Lysophosphatidic acid receptor
LPD	Lysophospholipase D
mRNA	messenger RNA
MSK-1	Mitogen- and stress-activated protein kinase-1
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PELD	Pediatric end stage liver disease
PH	Portal hypertension
PI3K	Phosphatidylinositol 3-kinase
PROM-1	PROM-1
qCOBRA	Quantitative combine bisulfite restriction analysis
Q-FISH	Quantitative fluorescent in situ hybridization
ROC	Receiver operating characteristic
ROCK	Rho-kinase
ROS	Reactive oxygen species
RTL	Relative telomere length
SEM	Standard error of the mean
SINE (Alu)	Short interspersed nuclear element

SMB	Somatomedin B-like
TB	Total bilirubin
TGF-β1	Transforming growth factor beta
α-SMA	alpha-smooth muscle actin

CHAPTER I

INTRODUCTION

Background

Biliary atresia (BA) is a devastating cholestatic liver disorder in neonates characterized by inflammatory and fibrotic obliteration of the extrahepatic bile ducts. The obstruction of bile flow presents as a triad of jaundice, acholic stool, and hepatosplenomegaly. If left untreated, the most of BA patients will develop severe hepatic fibrosis, biliary cirrhosis, portal hypertension, hepatic failure, and ultimately die by the age of 2 years. Surgical treatment, which remains the standard of care for first line intervention for infants with BA, is the Kasai portoenterostomy. Failure of the Kasai procedure leaves liver transplantation as the only hope for survival (1). The incidence of BA varies from one in 5,000 to one in 19,000 live births, occurring more commonly in Asian than in European countries (2). The etiology, pathogenesis, and factors modifying the disease progression remain largely elusive. However, more recently, it has been generally recognized that BA is perhaps not a single disease entity. Instead, it is proposed that several distinct pathologic mechanisms can lead to a BA phenotype characterized by provoking a stereotypic response comprised of inflammation, autoimmune-mediated bile duct damage, bile duct proliferation, apoptosis, and progressive portal fibrogenesis (3).

One of the hallmark features of BA is progressive hepatic fibrosis. Hepatic fibrosis of variable degree is an invariable finding. Despite early diagnosis and successful Kasai operation, a significant number of BA children inevitably develop progressive liver fibrosis, cirrhosis with concomitant portal hypertension, and end-stage liver disease. Liver transplantation is an effective treatment modality if the Kasai portoenterostomy fails and serious complications occur such as recurrent cholangitis, persistent jaundice, progressive ascites, and bleeding esophageal varices (4, 5).

Although several possible theories have been proposed for pathogenesis of BA, the precise mechanisms of progressive liver fibrosis in biliary atresia remains a mystery. Understanding the pathogenesis of liver fibrosis during the progression of BA is critical for the development of potential therapies.

Autotaxin (ATX) discovered as an autocrine tumor motility-stimulating factor, which is a secreted member of the nucleotide pyrophosphatase and phosphodiesterase (NPP) family of ectoenzymes (6). ATX has physiological roles in adipogenesis, neurogenesis, and vascular development (7). The biological properties of ATX are explained by its lysophospholipase D (LPD) activity, generating the bioactive mediator lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC) (8). LPA evokes growth factor-like responses in almost all cell types including cell growth, survival, differentiation, and motility. The wide variety of LPA effector functions is attributed to the G-protein-coupled LPA receptors (GPCR) with overlapping specificities and widespread distribution (9).

LPA has been demonstrated as a profibrotic mediator in various organs such as kidney (10), lung (11), and liver (12). Regarding its potential effect on hepatic stellate cell (HSCs), LPA was first shown to stimulate rat HSCs proliferation, suggesting that LPA could be a profibrogenic factor in liver (12). HSCs are a major cell type involved in development of liver fibrosis because they are the major source of extracellular matrix components and can transdifferentiate into hepatic myofibroblasts closely involved in proliferation and collagen synthesis (13). Furthermore, ATX appears to be involved in the regulation of liver fibrosis. Previous investigations have also demonstrated a connection between liver fibrosis and serum or plasma LPA and ATX emerged in patients with chronic hepatitis C virus infection (14, 15). However, the origin and fate of serum ATX must be further elucidated and serum ATX should be evaluated as a possible liver fibrosis marker in not only patients with chronic hepatitis C, but also patients with liver fibrosis in general.

ATX is thought to be play an important role in LPA-mediated liver fibrosis in pathogenesis of BA. The involvement of ATX in hepatic fibrosis of BA has never been

explored. Hence, the primary objective of the present study was to investigate mRNA and protein expression of ATX and hepatic localization of ATX in postoperative BA patients. Serum ATX levels and correlation between *ATX* expression and parameter outcomes such as liver function, liver stiffness, portal hypertension, and hepatic fibrosis were determined in postoperative BA patients. Based on our understanding of these processes, we hypothesize that ATX may play an important role in liver fibrosis by LPA-stimulated HSCs proliferation and could be a promising target for anti-fibrotic therapy in the future.

Although the precise etiology of biliary atresia remains largely elusive, epigenetic regulation, especially DNA methylation has been proposed as a cause of BA. Modifications of DNA methylation can be elicited by viruses and genetic defects, resulting in suppression of gene expression. Recently, a methylation microarray study identified the methylation status of specific genes in human BA livers and highlighted DNA hypomethylation as a potential factor in mediating overexpression of genes associated with BA (16). Based on this knowledge, we hypothesized that DNA hypomethylation might be contributed to the pathogenesis of BA and could upregulate the *ATX* expression in BA patients. Thus, the measurement of promoter methylation status and expression of *ATX* in peripheral blood leukocytes and liver tissues from BA patients might ultimately support the development of effective strategies.

What is more, the molecular mechanisms of cellular senescence have been shown to regulate biliary growth and injury, suggesting cellular senescence might be another contributory factor associated with BA etiology. Telomeres, which are considered as biomarker of cellular senescence, consist of the repetitive DNA sequences of TTAGGG and related proteins of crucial importance for telomere function at the ends of chromosomes (17). Telomere shortening loses capping function at the chromosomal ends, leading to the DNA damage program activation, which contributes to senescence, apoptosis, and neoplastic transformation (18). Telomere shortening has also been evinced to drive progression of liver cirrhosis in both hepatocyte and

senescence associated with fibrotic scaring (18). Hence, telomere shortening is believed to be an important cause in the pathogenesis of chronic liver injury including BA. Although it has been reported to be involved in a number of human liver diseases, the precise molecular mechanisms associated with telomere shortening-induced hepatocellular injury remain uncertain. Importantly, previous investigation has documented the role of global DNA methylation in the variability of telomere length (19).

Methylation of retrotransposable elements has been shown to be associated with global DNA methylation. Alu and LINE-1 are major components of non-long terminal repeat retrotransposons, comprising approximately 11% and 17% of the human genome, respectively (20). Hypomethylation of these elements is hypothesized to facilitate genomic instability by resulting in retrotransposition of transposable elements, dysregulation of DNA repair genes (21, 22), and altered expression of important genes (23). Previous studies have highlighted relationships between global hypomethylation and several human diseases (24-27) and that these regions might be important to epigenetic regulation in telomere maintenance (28, 29), thereby establishing a possible etiologic link between global DNA methylation and telomere length in BA patients.

Taken together, the aforementioned studies show that various contributors for hepatic dysfunction including profibrotic cytokines, epigenetic regulators, telomere length, global DNA methylation and oxidative DNA damage are a worthy study subject for defining the microenvironment in hepatic injury and their involvement in the progression of pathological conditions like biliary atresia. Accordingly, the objective of this study was to investigate whether hepatic *ATX* expression, epigenetic regulation-mediated *ATX* expression, relative telomere length, global DNA methylation, and oxidative DNA damage could be involved in liver impairment related to the pathogenesis of liver fibrosis in biliary atresia. Defining these biomarkers might lead to targeted therapies aimed at slowing the progression of disease and delaying (or negating) the need for transplant.

Research questions

- Is ATX expression in both the circulation and the liver associated with clinical outcomes in postoperative BA patients?

- Does the *ATX* promoter methylation appear to be related with *ATX* expression in postoperative BA patients?

- Is telomere shortening associated with clinical outcomes in BA patients?

- Is global DNA methylation correlated with telomere length, oxidative DNA damage and outcome parameters in BA patients?

Objectives

- To evaluate ATX expression in both the circulation and the liver and to determine the possible correlations of ATX expression and biochemical parameters of postoperative BA patients.

- To compare promoter methylation status and ATX expression in peripheral blood leukocytes and liver tissues from BA patients and controls as well as to identify whether *ATX* promoter methylation and expression were associated with clinical parameters of BA patients.

- To investigate telomere length in peripheral blood leukocytes and liver tissues from both BA patients and age-matched healthy controls and to evaluate the possible association between telomere length and clinical parameters of BA patients.

- To assess methylation levels and patterns of Alu and LINE-1 elements in peripheral blood leukocytes from BA patients and age-matched healthy controls and to explore whether Alu and LINE-1 methylation levels were associated with hepatic dysfunction, oxidative stress, and relative telomere length in BA patients.

Hypothesis

- ATX expression levels in both the circulation and the liver would be elevated and associated with the disease severity and liver stiffness in BA patients.

- Hypomethylation of the *ATX* promoter region could upregulate *ATX* expression in BA patients.

- Telomere length may be shortened and correlated with clinical outcomes in BA patients.

- Epigenetic alterations in the form of global DNA methylation may be associated with outcome parameters, telomere length, and oxidative DNA damage in BA patients.

Key words

Biliary atresia, Autotaxin, DNA methylation, Telomere shortening, Global DNA methylation, Oxidative DNA damage

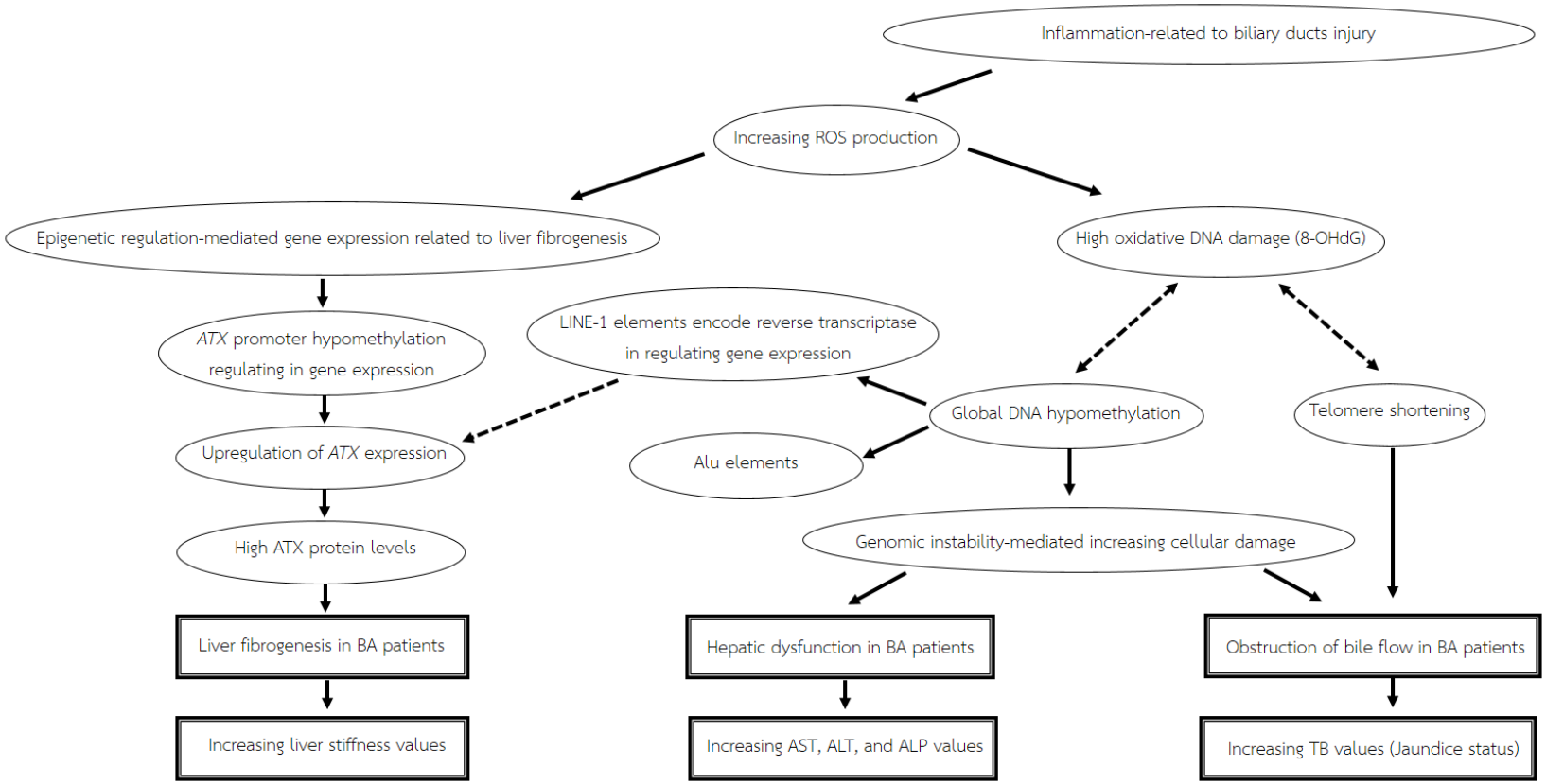

Figure 1. Contributory factors of hepatic dysfunction in biliary atresia. The combination of fibrogenic cytokines including autotaxin, epigenetic regulation especially DNA methylation-mediated autotaxin expression, genomic instability, cellular senescence and aging, cellular damage, as well as inflammatory response might be contributory factors related to poor outcomes including jaundice, portal hypertension, liver fibrosis and cellular damage that could serve as non-invasive biomarkers in postoperative biliary atresia patients.

Experimental design

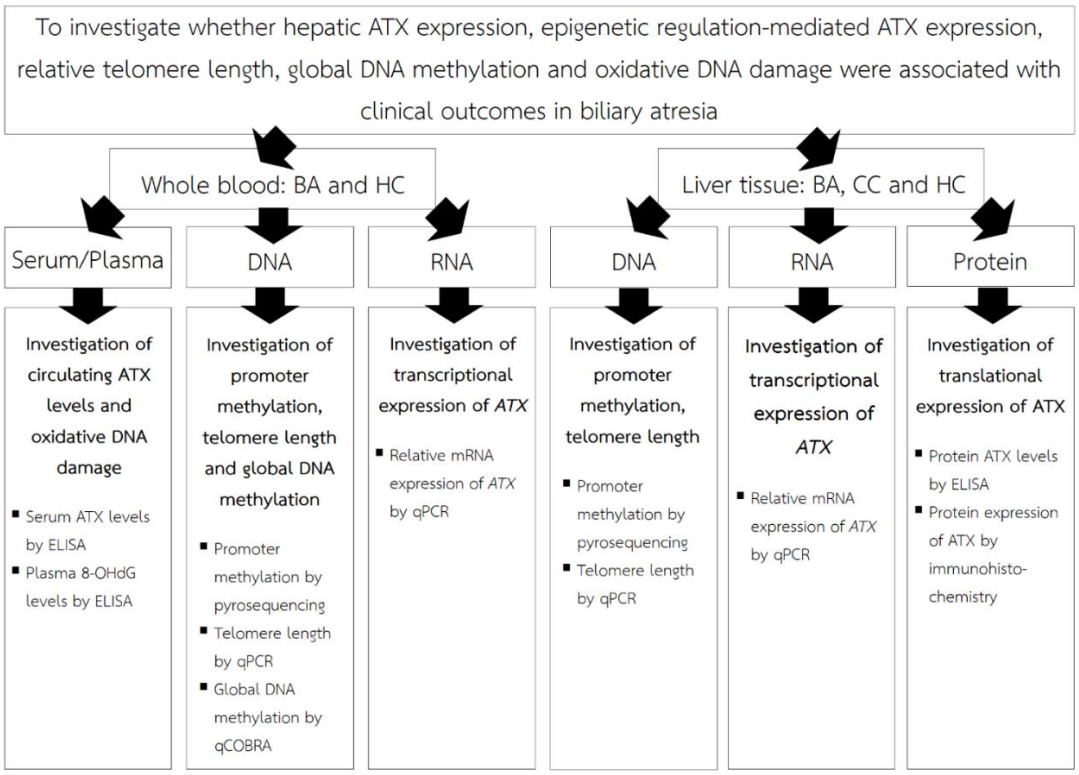

Figure 2. Procedure framework of the present study. The primary aim of this study was to investigate whether numerous biomarkers including ATX expression related to epigenetic regulation, relative telomere length, global DNA methylation, and oxidative DNA damage were associated with the pathogenesis of liver fibrosis in biliary atresia.

ATX, Autotaxin; BA, Biliary atresia; CC, Choledochal cyst; HC, Healthy control; qPCR, Quantitative Polymerase Chain Reaction; 8-OHdG, 8-hydroxy-2-deoxyguanosine; ELISA, Enzyme-linked immunosorbent assay; qCOBRA, Quantitative combine bisulfite restriction analysis

Expected benefits of the study

The benefit of this work is to identify novel biological markers for monitoring the disease severity and detection of liver fibrosis in biliary atresia. Further understanding of the pathogenesis of BA will provide new therapeutic approaches for this disorder.

Limitations

The current study was limited by liver biopsy tissue collection; the major problem would be the collection of liver biopsy tissue from age-matched normal participants that serve as control groups.

Ethical considerations

This study have been approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University. The Belmont Report identifies three fundamental ethical principles for any human subject research:

- Respect for persons: All parents or legal guardians of the recruited children with BA, and CC, and of healthy controls were informed of the study's purpose. Written informed consent were obtained from the parents prior to the children entering the study.

- Beneficence: There is no benefit to the participants. The specimens of participants were used only for laboratory research. There were a small risk of bruising, a rare risk of infection.

- Justice: The subjects were selected fairly and the risks and benefits of research were distributed equitably. The participants were recruited following to the inclusion and exclusion of this proposal.

CHAPTER II

REVIEW OF RELATED LITERATURES

Pathogenesis of biliary atresia

Biliary atresia is the most common cause of chronic liver disease in infancy, characterized by progressive fibrosclerotic cholangiopathy. It stems from an inflammatory and fibrosing obstruction of extrahepatic bile ducts leading to severe hepatic fibrosis, biliary cirrhosis, portal hypertension, hepatic failure, and end-stage liver disease. Damage to the small and medium sized intrahepatic bile ducts is a characteristic feature of BA and generally evokes a compensatory repair response that involves in expansion of portal tracts with proliferating bile ductules even at end-stage. With time, the fibroproliferative response bridge adjacent portal areas and culminate in biliary cirrhosis. It results from unsuccessful repair of biliary injury leading to cholestatic liver damage. As in most chronic liver disease, fibrogenesis in BA incorporates evolution and proliferation of hepatic stellate cells to myofibroblasts capable of producing extracellular matrix (ECM) eventually leading to progressive scarring at the expense of hepatic parenchyma (30). This rapid progression of liver fibrosis has not been elucidated; however, it probably reflects a simultaneous activation of multiple fibrogenic pathways in the background of ongoing cholestasis, a distinguishing pathological consequence of BA. Epithelial mesenchymal transition (EMT) is a well-recognized mechanism for forming myofibroblasts in injured tissues (31).

Many attempts to characterize the process of liver fibrogenesis have shown in biliary atresia. Malizia and colleagues described increased expression of type I procollagen mRNA by mesenchymal cells and perisinusoidal cells at sites of inflammation and scarring, where transforming growth factor beta (TGF-β1) immunoreactivity was often found. They also reported the expression of platelet-derived growth factor (PDGF) messenger RNA (mRNA) in proliferating ductules (32).

Michael and colleagues evaluated hepatic connective tissue growth factor (CTGF) mRNA expression and its relationship to hepatic histology in children with BA. Their findings found the expression of *CTGF* mRNA in biliary epithelial cells, endothelial cells, and myofibroblast-like cells (presumably hepatic stellate cells) were high and correlated with the severity of fibrosis. These data are consistent with an important regulatory role for biliary epithelial cells in the signaling cascades that stimulate fibrogenesis in obstructive biliary disorders (3). In 2010, Farrington et al. found significantly higher TGF-β1 levels in the livers of patients with advanced stages of BA than healthy controls. Furthermore, it was demonstrated that the expression of TGF-β1 was significantly higher in centrolobular compared with portal regions. In contrast, α-SMA expression was found to be significantly elevated in portal regions in the liver parenchyma. They suggest a paracrine mechanism of TGF-β-driven fibrogenesis in advanced BA (33). Further investigation has also demonstrated the overexpression of α-SMA appears to be associated with early hepatic fibrosis (34). Recently, Mavila et al. demonstrated that a potential functional linkage between the expansion of prominin-1 (PROM-1)-expressing cell population and the newly evolving fibrosis associated with BA mediated by fibroblast growth factor (FGF) and TGF-β1 signaling (35).

Previous studies have also documented the relationship between high serum cytokines and clinical outcome-related liver fibrosis in children with BA. Over decade ago Honsawek et al. reported elevated serum levels of many cytokines including tissue inhibitors of metalloproteinase (TIMP)-1 (36), osteopontin (37), galectin-3 (38), matrix metalloproteinase (MMP)-3 (39), adiponectin (40), soluble receptor for advanced glycation end products (sRAGE) (41), and CTGF (42). These cytokines were associated with liver dysfunction and degree of fibrosis in postoperative BA patients. These findings indicated that serum levels of cytokines might serve as biochemical parameters for progression of hepatic fibrosis in BA. Additionally, Vejchapipat et al. investigated serum TGF-β1 and epidermal growth factor (EGF) levels and their association with therapeutic outcomes in postoperative BA patients. They found that both serum TGF-β1 and EGF levels were associated with a good outcome in BA

patients. There was a positive correlation between serum TGF-β1 and EGF concentrations. The authors concluded that serum TGF-β1 and EGF levels may be used as prognostic markers in long-term postoperative BA patients (43).

Several cytokines are thought to play an important role in the process of fibrogenesis, which have become topics of research interest in an attempt to evaluate the possibility of serum cytokine as a biochemical marker of liver fibrosis. Understanding the cellular and molecular mechanisms associated with cholestasis-induced hepatocellular injury and fibrogenesis may provide novel markers to aid in better prognosis BA disease, detection of liver fibrosis, and prediction of the outcome in biliary atresia.

Contributors of hepatic dysfunction in biliary atresia

Role of ATX on fibrotic process

Autotaxin (ATX), also known as ectonucleotide pyrophosphatase phosphodiesterase (ENPP) 2 family, comprising seven members with structurally related catalytic domains. The ENPPs hydrolyze phosphodiester or pyrophosphate bonds in various substrates, including nucleoside triphosphates, lysophospholipids and choline phosphate esters (44). ATX is a secreted glycoprotein with lysophospholipase D (LPD) that converts lysophosphatidylcholine (LPC) into the lipid mediator lysophosphatidic acid (LPA) (8). ATX is made of two somatomedin B-like (SMB) domains, followed by a catalytic domain and a nuclease-like domain (45). ATX deficiency in mouse leads to embryonic lethality, while ATX heterozygous mice appear healthy but show half-normal plasma LPA levels, indicating that ATX is the major LPA-producing enzyme in vivo and required for normal development (14). Recently, emerging data have indicated an important role of ATX in regulating immune responses. For instance, ATX is highly expressed in the high endothelial venules of lymphoid organs to facilitate the entry of lymphocytes from the blood into secondary lymphoid organs (46). Most, if not all of, biological functions of ATX are mediated via the LPA signaling through the specific LPA receptors on cell membrane (7). The broad range of LPA cellular function

is accomplished by several LPA receptors (LPAR1-6) differentially coupled to distinct G proteins, through which various downstream signaling molecules, including phospholipase C, phosphatidylinositol 3-kinase (PI3K), Ras-MAPK and Rho, are activated. LPA is an important lipid mediator with a wide variety of biological actions including cell proliferation, migration, and survival in many cell types (Figure 3) (9).

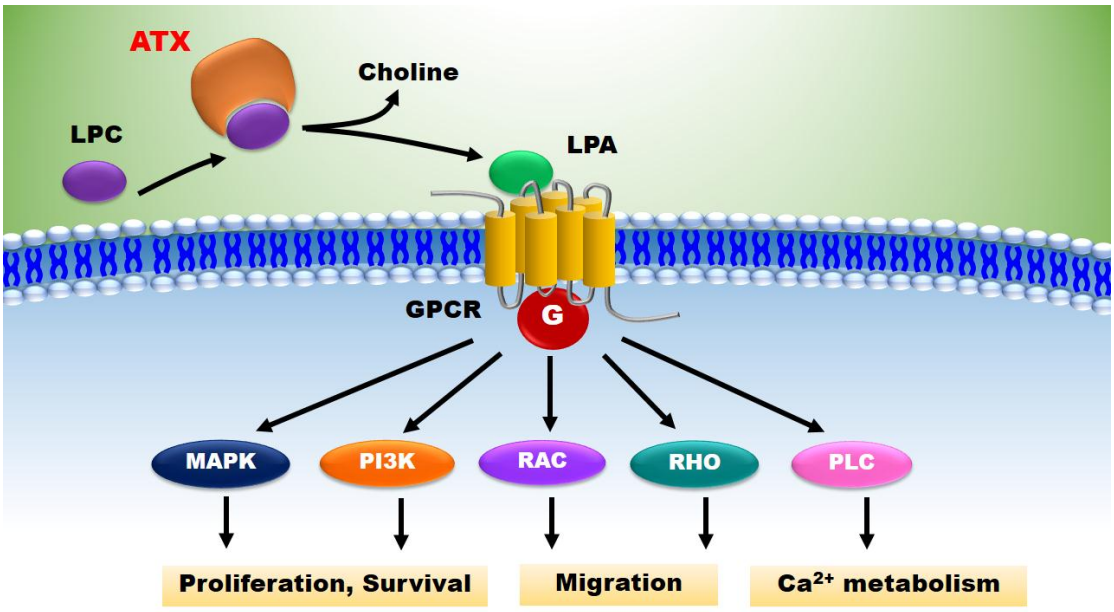

Figure 3. The molecular machanisms of autotaxin in regulating biological functions of human cells via its enzymatic production of lysophosphatidic acid. Adapted from Tabchy et al, 2011 (47).

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ATX and LPA have been shown to be involved in both pathological and physiological states including tumor progression and metastasis (48, 49), atherosclerosis (50), chronic inflammatory disorders (51), lung fibrosis (52), and liver fibrosis (3, 53). Previous investigations have also demonstrated the effect of ATX on LPA-mediated fibrosis.

Evidence for the effect of ATX on LPA-mediated liver fibrosis

Liver fibrosis is one of the main characteristics of liver cirrhosis, a disease most commonly caused by alcoholism, hepatitis B and C, fatty liver disease, and cholestasis. One of the pivotal cell types of liver fibrosis is the HSCs, which normally store vitamin A, but can also be activated into contractile myofibroblasts that obstruct blood flow and secrete the profibrotic mediator TGF-β1 and ECM (54).

Growing evidence from *in vitro* studies has shown that LPA can activate hepatic myofibroblasts (55) and increase the proliferation and survival of HSCs (12, 56). Correlations have been established between plasma LPA concentration and the histological stages of liver fibrosis markers in patients with chronic hepatitis C (57), as well as in rats treated with a chemically induced fibrosis (58). Interestingly, overexpression of the LPA-producing enzyme ATX and proteins involved in LPA signaling, especially the Rho-GTPase binding proteins (Cdc42s) has been reported in liver biopsies from patients with cancer compared with hepatitis patients or healthy controls (59). The above reports indicate a probable involvement of LPA in liver fibrosis with possible consequences for the development of hepatocellular carcinoma. In 2012, Ikeda and Yatomi reviewed the relationship between ATX or LPA and liver fibrosis, the usefulness of serum ATX levels for predicting the stages of liver fibrosis, and the potential roles of increased serum ATX and plasma LPA levels in liver fibrosis (Figure 4) (53).

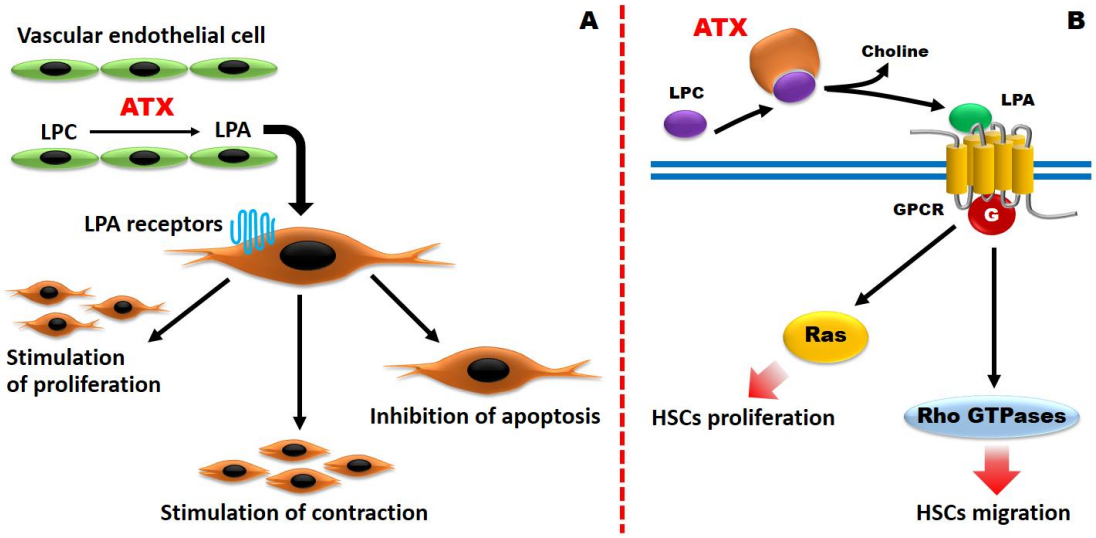

Figure 4. The effect of autotaxin-mediated lysophosphatidic acid on liver fibrosis. (A) Autotaxin has been shown to simulate hepatic stellate cells proliferation and contraction and also inhibit apoptosis through the altered activation of lysophosphatidic acid signal transductions; (B) lysophosphatidic acid has been reported to activate hepatic stellate cells proliferation and migration via Ras and Rho GTPases signaling pathways, respectively. Adapted from Ikeda H and Yatomi, 2012 (53) and Tania et al, 2012 (60), respectively.

The possible pathway involved in the profibrotic action of LPA is its capacity to increase the expression of CTGF (61). This was observed in mesengial cells (62), endothelial cells (63), epithelial cells (64), skeletal muscle cells (65), and keloid fibroblasts (66). This involves the Rho-kinase (ROCK) pathway (67) the integrin-linked kinase (68), and may require transactivation of TGF-β-receptors as well as c-Jun N-terminal kinase (JNK), pathway activation (69). Finally, the profibrotic action of LPA is reinforced by its capacity to activate cell contraction and fibronectin assembly, which are two important events involved in the wound-healing process (70, 71). However, more recently, LPA appears to be involved in the induction Rat-2 fibroblast cell proliferation via cooperation between ERK/MAPK signaling and c-AMP response element-binding (CREB) activation. It was shown that LPA strongly stimulated CREB

through mitogen- and stress-activated protein kinase-1 (MSK-1) and that MSK-1-dependent CREB phosphorylation is mediated by ERK1/2 and p38 MAPK pathways in Rat-2 fibroblast cells (72).

Several lines of evidence indicate an important role for LPA in the etiology of fibrosis. The profibrotic action of LPA has been established in kidney, lung, and liver. Therefore, the effect of ATX on LPA-mediated liver fibrosis is thought to be involved in the pathogenesis of BA. However, the mechanism of ATX-mediated LPA that induces hepatic fibrosis in BA has, to date, not been fully understood.

DNA hypomethylation: a potential mechanism propagating hepatic injury in biliary atresia

As mentioned above, the etiology of BA is unknown and theories of pathogenesis include viral infection, chronic inflammatory or autoimmune-mediated bile duct injury and abnormalities in bile duct development. Importantly, it has been demonstrated a possible link between epigenetic regulation and the etiologic mechanism of intrahepatic bile duct defects in BA (73). Epigenetic mechanisms are thought to play a critical role in gene regulation during development and differentiation of disease. Methylation of CpG residues within promoter and enhancer regions leads to suppression of gene expression (74) and DNA methylation alterations can be elicited by viruses and genetic defects (75, 76). DNA hypomethylation has been reported to be involved in the development of several autoimmune disorders, such as systemic lupus erythematosus and rheumatoid arthritis (77, 78). Importantly, recent evidences indicate that alterations to epigenetic DNA methylation patterns could indirectly play a part in the pathogenesis of BA. The hypermethylation of promoter regulatory elements contributes to the lower CD11a expression in T lymphocytes of BA patients (79). However, the hypomethylation of interferon gamma (IFN-γ) gene promoter may be responsible for increased IFN-γ expression in BA infants (80), providing further significance to the potential role of DNA hypomethylation in BA. Based on previous reports suggesting the significance of aberrant DNA methylation in the development of biliary atresia, the present study addressed the question of

whether or not the ATX expression-mediated liver injury was altered due to DNA hypomethylation.

The relationship of telomere shortening in the pathogenesis of biliary atresia

Cellular senescence is a state of irreversible cell cycle arrest that has been involved in several gastrointestinal diseases, including human cholestatic liver disorders. Senescence might play a prominent role in the progression of biliary atresia. Telomere shortening, double stranded DNA damage, inflammation, and other forms of cell stress all function as stimuli for cell senescence. Telomeres, which are located at the ends of chromosomes, consist of repetitive DNA sequences of TTAGGG and related proteins of crucial importance for telomere function. Telomeres help maintain genomic integrity and stability by shielding chromosome ends from deterioration, fusion, and atypical recombination (17). Telomere length shortens each time cells divide, because DNA polymerases are not capable of completely replicating chromosomes during cell division. This is commonly referred to as the end-replication problem. This alteration in telomere length precipitates capping function loses at the chromosomal ends, leading to DNA damage program activation, which contributes to senescence, apoptosis, and neoplastic transformation (18). As such, telomere length is an indicator of the biological age of a cell.

There is also emerging evidence that describes an association between attrition of telomere length and several human pathologies (81, 82). This also includes a variety of cancers and chronic liver disorders such as liver hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (83-85). These findings strongly suggest telomere shortening in the development of liver cirrhosis. Accordingly, evaluation of telomere size may serve as a feasible and reliable constitutive indicator in determining the risk prognosis for BA. In support of this proposed causal relationship, a study by Sanada et al. (86) measured hepatocyte telomeres using quantitative fluorescent in situ hybridization (Q-FISH) and reported normalized telomere/centromere ratios, being markedly reduced when compared to healthy controls. Although the role of telomere

dysfunction in BA remains poorly understood, the aforementioned studies suggest that hepatocyte telomere length might be used for an adjunct biomarker to clinical scoring models of cirrhosis and biliary injury.

Global DNA hypomethylation, oxidative DNA damage, and telomere shortening in biliary atresia

It is well known that the etiologies of BA have not been well established. However, several theories have been proposed to explain the pathogenesis of BA, including viral infections, toxins, and immunologic insults; notably, the interplay between environmental and genetic factors (87). Growing evidence suggests that epigenetic variation can be elicited by viruses, toxins, and genetic defects (88), which may have relevance in the development of BA. DNA methylation, one type of epigenetic change, is a reversible modification of cytosine residues in the genome through the addition of a methyl group to cytosine nucleotides. This variation is an important mechanism in regulating expression of human genes, maintenance of genomic stability, and telomere length (89). A substantial portion of methylation sites throughout the human genome are found in repetitive sequences and transposable elements, such as Alu or short interspersed nuclear element (SINE) and long interspersed nuclear element-1 (LINE-1). Alu and LINE-1 are major components of non-long terminal repeat retrotransposons, comprising approximately 11% and 17% of the human genome, respectively (20). Because repetitive DNA sequences account for over 40% of methylation in the genome, DNA methylation measured in retrotransposon elements has served as a useful proxy for global DNA methylation (90). Alu and LINE-1 elements are usually heavily methylated in normal cells, thus maintaining transcriptional inactivation and inhibiting retrotransposition. Hypomethylation of these elements is hypothesized to facilitate genomic instability resulting in retrotransposition of transposable elements, dysregulation of DNA repair genes (21, 22), and altered expression of important genes (23). Previous studies have highlighted relationships between global hypomethylation and several human diseases (24-27). Methylation of these elements also makes them susceptible to oxidative stress (91), which may be a possible factor associated with biliary atresia.

Oxidative stress constitutes the majority of DNA damage in human cells, which is due mainly to excess production of reactive oxygen species (ROS) (92). Generation of ROS can lead to a wide range of DNA lesions, including base deletions, mutations, DNA strand breakage, chromosomal rearrangements, and cross-linking with proteins (93). Oxidative DNA damage can modify epigenetic alterations by multiple mechanisms. One form of DNA damage induced by oxidative stress is the change in genomic base to species like 8-hydroxy-2-deoxyguanosine (8-OHdG). 8-OHdG is able to interfere with the ability of DNA to function as a substrate for the DNA methyltransferases (DNMTs), leading to global DNA hypomethylation and subsequent genomic instability (94). Alu and LINE-1 may be critical elements in chromosome and genomic stability and may be induced by an increase in oxidative stress, leading to genomic instability and DNA damage. As such, these elements may contribute to the pathophysiology of BA. To date, there has been no evidence regarding the possible association between global methylation and oxidative DNA damage in BA patients. This information could improve our understanding of the relationship between epigenetic alteration-mediated DNA damage and BA etiology. Interestingly, epigenetic mechanism appears to be an important component of telomere regulation. Several studies have reported that hypomethylation of subtelomeric regions was related to telomere length and that these regions might be important to epigenetic regulation in telomere maintenance (28, 29), thereby establishing a possible etiologic link between global DNA methylation and telomere length in BA patients. Albeit methylation of retrotranposon elements has been investigated in relation to a variety of disorders, little is known about the association of global DNA methylation and the exact patho-etiology of BA. Further understanding of global DNA methylation, oxidative damage, and telomere length would shed light on the role of epigenetic aberrations play in the etiology of BA and might ultimately support the development of effective strategies.

CHAPTER III

SERUM AUTOTAXIN LEVELS CORRELATE WITH HEPATIC DYSFUNCTION AND SEVERITY IN POSTOPERATIVE BILIARY ATRESIA

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Abstract

Objective: To investigate correlation of serum autotaxin (ATX) and disease severity in biliary atresia (BA).

Methods: Eighty postoperative BA patients and 15 controls were recruited. Serum ATX levels were determined by enzyme-linked immunosorbent assay.

Results: BA patients had greater serum ATX and liver stiffness than controls. Serum ATX and liver stiffness were markedly elevated in BA patients with jaundice compared to those without jaundice. Furthermore, serum ATX was correlated with liver stiffness and biochemical parameters in BA.

Conclusions: Elevated serum ATX was correlated with hepatic dysfunction in BA. Accordingly, serum ATX is a promising biomarker reflecting the severity in BA.

Introduction

Biliary atresia (BA) is a devastating cholestatic liver disorder in neonates characterized by progressive inflammatory cholangiopathy. It results from the fibrosclerotic destruction of the extrahepatic bile duct, leading to complete obliteration of the biliary tract at any point between the porta hepatic and duodenum. The obstruction of bile flow presents as a triad of jaundice, acholic stool, and hepatosplenomegaly. If left untreated, the majority of BA patients will develop severe hepatic fibrosis, biliary cirrhosis, portal hyper tension, hepatic failure, and ultimately die by the age of 2 years (1). Surgical treatment which remains the standard of care for first line intervention for infants with BA is the Kasai portoenterostomy (95). Failure of the Kasai procedure leaves liver transplantation as the only hope for survival (1). The precise etiology of biliary atresia remains a mystery; however, several possible theories have been proposed for pathogenesis of BA, including genetic defect, perinatal viral infection, abnormality of bile duct morphogenesis, and immune-mediated bile duct injury (96).

Autotaxin (ATX), also known as ENPP-2 (ectonucleotide pyrophosphatases/ phosphodiesterase-2), is a 125 kDa secreted glycoprotein that belongs to the ENPP family (6). It was originally characterized as an autocrine motility stimulating factor from the conditioned medium of A2058 melanoma cells (97). Since then, the increased expression of ATX has been shown in various malignant tumor growth and metastasis (97). ATX uniquely exhibits a lysophospholipase D (LPD) activity through which it hydrolyzes lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA) (8). ATX is widely expressed in tissues such as brain, placenta or high endothelial venules (98-100). In heterozygous ATX-null mice, both the lysoPLD activity and the LPA concentrations were about half of those observed in wild-type mice, whereas complete knock-out of ATX is embryonic lethal due to blood vessel abnormalities, showing that ATX is responsible for the bulk of LPA production in blood (7, 14).

Regarding its potential effect on hepatic stellate cells (HSCs), LPA was first shown to stimulate rat hepatic stellate cell proliferation, suggesting that LPA could be

a profibrogenic factor in liver (12). Development of liver fibrosis is coordinated by various cell types, including HSCs. In continuously injured livers hepatic stellate cells are activated and transdifferentiated into myofibroblasts, resulting in the production of abundant extracellular matrices (13). Previous investigations have also suggested a connection between liver fibrosis and serum or plasma LPA and ATX was elevated in patients with chronic hepatitis C virus (HCV) infection (15, 57). However, the origin and fate of serum ATX must be further studied and serum ATX should be investigated as a plausible liver fibrosis marker in not only patients with chronic hepatitis C, but also patients with liver fibrosis in general.

According to our knowledge, serum ATX in various clinical stages of BA and its potential role in BA patients have not yet been demonstrated. The present study is the first to evaluate the correlation of serum ATX, liver stiffness, and biochemical parameters in postoperative BA. We postulated that serum ATX would be elevated and associated with the disease severity and liver stiffness in BA patients, and to prove this hypothesis, we examined serum ATX and liver stiffness in BA patients compared with healthy controls. Therefore, the objective of this study was to analyze serum ATX levels collected from BA patients and to determine the possible correlations of serum ATX and biochemical parameters of postoperative BA patients.

Materials and Methods

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All parents of children were informed of the purpose of the study and of any interventions involved in this study. Written informed consents were obtained from the participants' parents upon informing them about the protocol and procedures involved in the research

Study population

Eighty BA patients (42 girls and 38 boys with mean age of 9.6±0.7 years) who came for the follow-up visit to the Pediatric Liver Clinic and 15 healthy children (8 girls and 7 boys with mean age of 9.5±0.7 years) were enrolled in this prospective study. All patients with type 3 (uncorrectable) isolated BA had undergone hepatic portojejunostomy with Roux-en-Y reconstruction (original Kasai procedure), and they were generally in good health; no signs of suspected infection or bleeding abnormalities at the time of blood sampling. None of the participants had histories of liver transplantation or adjuvant steroid therapy, but the patients with serum total bilirubin exceeding 2 mg/dl had been treated with ursodeoxycholic acid. Healthy controls attending the Well Baby Clinic at King Chulalongkorn Memorial hospital for vaccination had normal physical findings and no underlying disease. Serum samples were taken during their routine follow-up between January 2011 and December 2013. The duration of follow-up after the Kasai operation was 8.8±0.9 years. BA patients were categorized into two groups according to serum total bilirubin (TB). Based on their jaundice status, BA children were divided into a non-jaundice group (TB<2 mg/dL) and a persistent jaundice group (TB≥2 mg/dL). Subsequently, portal hypertension (PH) was validated by the presence of ascites and/or esophageal varices as diagnosed by endoscopic screening. Twenty-eight patients had no evidence of PH whereas the rest of the 52 patients suffered from PH.

Laboratory methods

Samples of peripheral venous blood were collected from every participant, and were stored at -80 °C for further measurement. Quantitative determination of ATX concentration in serum was performed using a commercially available enzyme-linked immunosorbent assay (ELISA) development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Serum samples were first diluted in accordance with manufacturer's recommendation. Recombinant human ATX standards and serum samples were added into each well, which was pre-coated with a monoclonal antibody against ATX. After incubating for 2 hours at room temperature,

every well was washed thoroughly 4 times with wash buffer. Then, a horseradish peroxidase-conjugated polyclonal antibody specific for ATX was pipetted into each well and incubated for a further 2 hours at room temperature. After 4 washes, substrate solution was pipetted into the wells and then the microplate was incubated for 30 minutes at room temperature with protection from light. Finally, the reaction was stopped by the stop solution and the optical density was measured with an automated microplate reader at 450 nm. The amount of colour generated is directly proportional to the amount of ATX in the sample. Autotaxin concentration was determined by a standard optical density-concentration curve. Twofold serial dilutions of recombinant human ATX with a concentration of 0.781–50 ng/mL were used as standards. The intra- and inter-assay coefficients of variation (CVs) were 2.6–3.7% and 2.9–4.7%, respectively. The sensitivity of this assay was 0.157 ng/mL. The liver function tests including serum albumin, total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) were performed using a Hitachi 912 automated machine at the central laboratory of our hospital. The aspartate aminotransferase to platelets ratio index (APRI) was calculated as follows: (AST/upper limit of normal) × 100/platelet count (10^9/L) (101).

Liver stiffness measurement

Liver stiffness measurement was performed on the same day as blood collection. Transient elastography measured the liver stiffness between 25 and 65 mm from the skin surface, which is approximately equivalent to the volume of a cylinder of 1 cm diameter and 4 cm length. The measurements were performed by placing a transducer probe of FibroScan (Echosens, Paris, France) on the intercostal space at the area of the right lobe of the liver with patients lying in a dorsal decubitus position with maximum abduction of the right arm. The target location for measurement was a liver portion that was at least 6 cm thick, and devoid of major vascular structures. The measurements were performed until 10 validated results had been obtained with a

success rate of at least 80%. The median value of 10 validated scores was considered the elastic modulus of the liver, and it was expressed in kilopascals (kPa).

Statistical analysis

Statistical analysis was performed using the SPSS version 16.0 statistical software package (SPSS Inc., Chicago, IL). Comparisons of demographic and clinical parameters between groups were performed using Chi-square and Student's unpaired *t*-test when appropriate. Correlation between numerical data was acquired using Pearson's correlation coefficient (*r*). Data were expressed as mean±standard error of the mean. All the *P* values<0.05 based on a two tailed test were considered statistically significant.

Results

Comparison between BA patients and healthy controls

A total of 80 BA patients and 15 healthy controls were prospectively recruited in the present study. The characteristics of participants in both groups are summarized in Table 1. Mean age, gender ratio, and body mass index (BMI) in BA patients and controls were not different, while liver stiffness scores in BA patients were considerably higher than those in controls (28.3±2.6 versus 5.2±0.7 kPa, *P*<0.001).

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

Variables	**BA** (n=80)	**Controls** (n=15)	***P*-value**
Age (years)	9.6±0.7	9.5±0.7	0.9
Gender (Female: Male)	42:38	8:7	0.5
BMI (kg/m^2)	18.0±0.6	18.0±0.5	0.8
Albumin (g/dL)	4.3±0.1	-	NA
Total bilirubin (mg/dL)	2.5±0.5	-	NA
Direct bilirubin (mg/dL)	2.0±0.5	-	NA
AST (IU/L)	128.1±11.2	-	NA
ALT (IU/L)	117.8±12.3	-	NA
ALP (IU/L)	430.0±28.9	-	NA
Platelet count (10^3/mm^3)	162.2±12.8	-	NA
APRI	3.0±0.4	-	NA
Liver stiffness (kPa)	28.3±2.6	5.2±0.7	<0.001
Autotaxin (ng/mL)	905.9±53.6	290.0±37.1	<0.001

The data are expressed as mean±SEM. BA, biliary atresia; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; APRI, aspartate aminotransferase to platelets ratio index; NA, not applicable

In addition, BA patients had significantly higher serum ATX levels than healthy controls (905.9±53.6 versus 290.0±37.1 ng/mL, *P*<0.001), as shown in Figure 5.

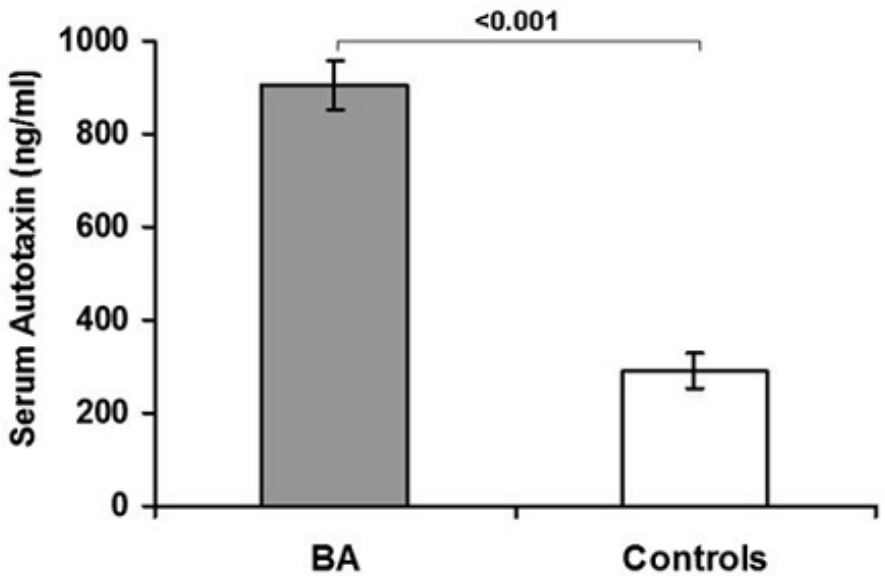

Figure 5. Comparison of serum ATX levels in postoperative biliary atresia patients and healthy controls. The data are expressed as mean±SEM.

Comparison between BA patients with and without persistent jaundice

We further classified BA patients into a persistent jaundice (n=42) and non-jaundice group (n=38). The demographic data and biochemical parameters including liver function tests, serum ATX, and liver stiffness values based on jaundice status are demonstrated in Table 2. BA patients with persistent jaundice had significantly lower albumin levels than those patients without jaundice. In contrast, serum bilirubin, AST, ALT, ALP, GGT, and APRI were markedly elevated in BA patients with jaundice compared to those without jaundice. Moreover, the mean liver stiffness values of patients with persistent jaundice were remarkably higher than those of patients without jaundice (40.8±3.7 versus 15.1±2.3 kPa, P<0.001).

Table 2. Comparison between biliary atresia patients with and without jaundice.

Variables	**BA patients with jaundice** (n=42)	**BA patients without Jaundice** (n=38)	***P*-value**
Age (years)	9.6±1.0	9.5±0.9	0.9
Gender (Female: Male)	21:21	21:17	0.5
BMI (kg/m^2)	17.0±0.7	18.8±0.9	0.1
Albumin (g/dl)	3.9±0.1	4.6±0.1	<0.001
Total bilirubin (mg/dL)	5.1±1.0	0.5±0.1	<0.001
Direct bilirubin (mg/dL)	4.3±1.0	0.2±0.1	<0.001
AST (IU/L)	187.7±15.1	82.5±10.8	<0.001
ALT (IU/L)	156.4±21.2	88.2±12.4	0.005
ALP (IU/L)	568.5±34.0	317.5±33.3	<0.001
Platelet count (10^3/mm^3)	123.3±17.2	192.0±16.8	0.007
APRI	5.2±0.6	1.5±0.2	<0.001
Liver stiffness (kPa)	40.8±3.7	15.1±2.3	<0.001
Autotaxin (ng/mL)	1,144.4±72.2	642.3±54.2	<0.001

The data are expressed as mean ± SEM. BA, biliary atresia; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; APRI, aspartate aminotransferase to platelets ratio index

As presented in Figure 6, serum ATX levels in BA patients with jaundice were substantially greater than those in BA patients without jaundice (1,144.4±72.2 versus 642.3±54.2 ng/mL, *P*<0.001).

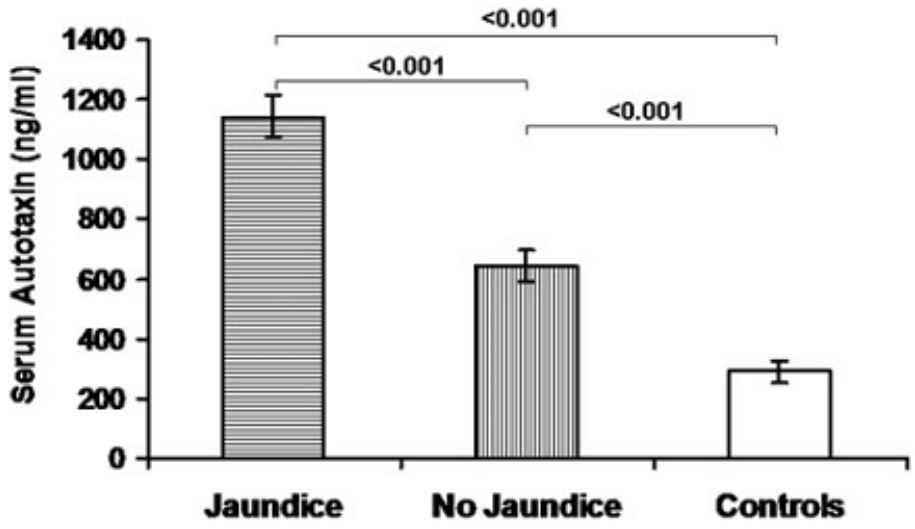

Figure 6. Comparison of serum autotaxin levels in biliary atresia patients with jaundice, biliary atresia patients without jaundice, and controls. The data are expressed as mean±SEM.

Further analysis showed that serum ATX levels were markedly elevated in BA patients with PH than those without PH (1,078.5±62.8 versus 585.4±65.6 ng/mL, *P*<0.001) (Figure 7).

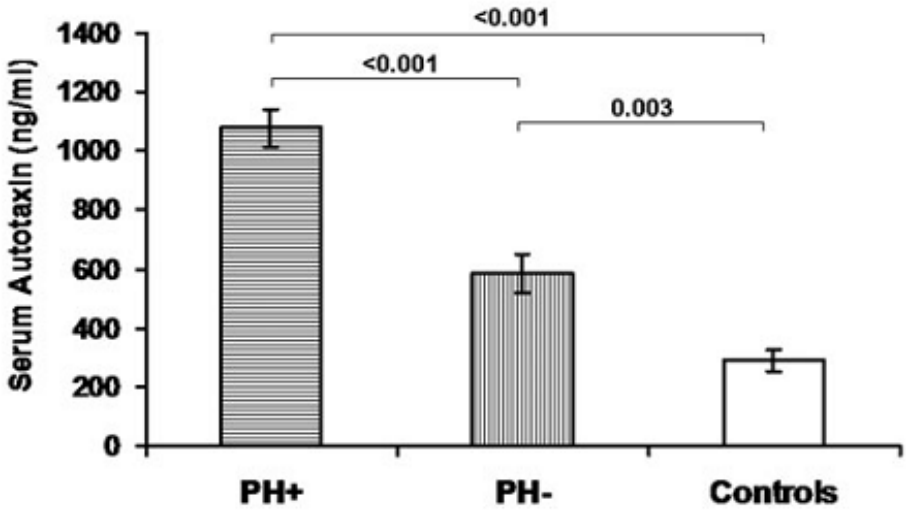

Figure 7. Comparison of serum autotaxin levels in biliary atresia patients with portal hypertension, biliary atresia patients without portal hypertension, and controls. The data are expressed as mean±SEM.

Additionally, serum ATX levels were positively correlated with serum TB (*r*=0.46, *P*<0.001), ALP (r=0.66, *p*<0.001), AST (*r*=0.67, *P*<0.001), ALT (*r*=0.35, *P*=0.006), and liver stiffness values (*r*=0.66, *P*<0.001). Conversely, serum levels of ATX were inversely correlated with serum albumin (*r*=0.57, *P*<0.001). Correlations between serum ATX, ALP, AST, ALT, liver stiffness, and serum albumin are illustrated in Figure 8.

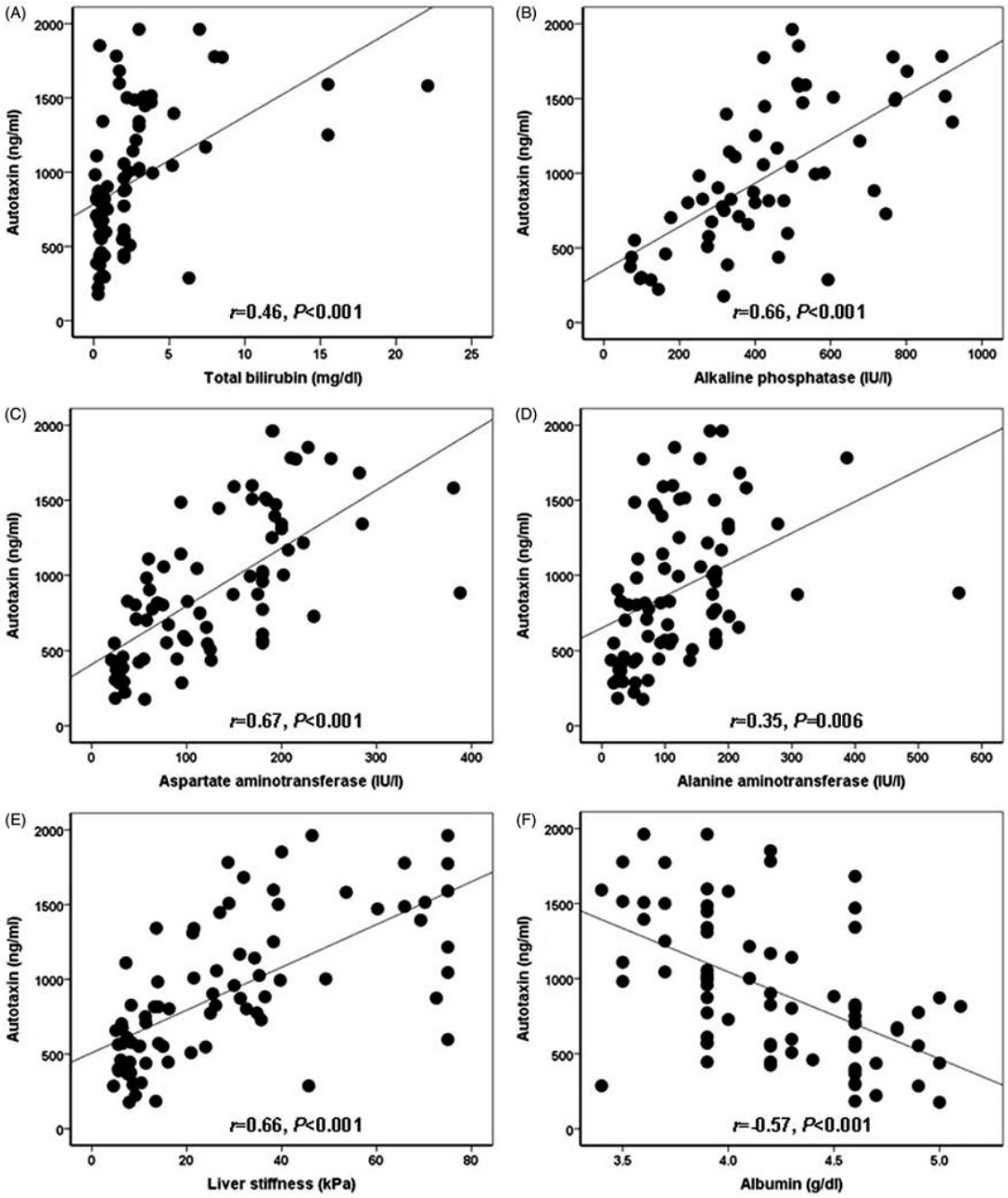

Figure 8. Scatter diagram and correlation analysis in biliary atresia patients. Serum autotoxin levels are correlated with total bilirubin (A), alkaline phosphatase (B), aspartate aminotransferase (C), alanine aminotransferase (D), liver stiffness (E), and albumin (F).

Discussion

BA is an inflammatory obstructive cholangiopathy of unknown etiology, and therapeutic options are unsatisfactory. Despite early diagnosis and successful Kasai operation, a significant number of BA children inevitably develop progressive liver fibrosis, cirrhosis with concomitant portal hypertension, and end-stage liver disease. Liver transplantation is an effective treatment modality if the Kasai portoenterostomy fails and serious complications occur such as recurrent cholangitis, persistent jaundice, progressive ascites, and bleeding esophageal varices. Therefore, there remains a critical need for the assessment of fibrogenic progression in BA patients. The functional basis for liver fibrosis and cirrhosis is activation of non-parenchymal cells, such as hepatic stellate cells. After HSCs are stimulated, these key effecter cells in hepatic fibrogenesis are transformed into extracellular matrix producing myofibroblasts. This process results in the production and the accumulation of collagen and other extracellular matrices in liver parenchyma, thus initiating and perpetuating the fibrosis (68). Progression of liver fibrosis is associated with an increased number of HSCs (102). LPA, which appears to be the major biological effector of ATX, inhibits apoptosis, stimulates, and contracts rat HSCs (12). Therefore, one can speculate that elevated serum ATX levels could be a biochemical indicator for activation of HSCs during the development of liver fibrosis. This study has been aimed to evaluate the association between serum ATX, liver stiffness measurements, and biochemical parameters in BA patients after Kasai procedure.

In the present study, we demonstrated that serum ATX levels were significantly higher in BA patients compared with healthy controls. Furthermore, serum autotoxin levels were substantially higher in BA patients with persistent jaundice than those without jaundice. Subsequent analysis revealed that serum ATX was positively correlated with serum total bilirubin, suggesting that serum ATX was associated with jaundice status in BA patients. We further found that elevated serum ATX was positively correlated with serum TB, AST, ALT, and ALP in postoperative BA patients. Serum AST and ALT routinely serves as biochemical parameters of liver dysfunction

reflecting hepatocellular damage. In addition, serum ALP is likely to be an indicator for the severity of biliary obstruction. Further analysis also showed a negative correlation between serum ATX and serum albumin. Thus, these findings indicate that ATX could be a useful biochemical marker in determining hepatic dysfunction and biliary obstruction in postoperative BA patients.

According to our knowledge, the present study is the first to show that serum ATX is elevated in BA patients compared with healthy controls. We also found that serum ATX was positively correlated with AST, ALP, TB, and liver stiffness, but negatively correlated with serum albumin. These results support that serum ATX is associated with jaundice status, hepatic dysfunction, and liver fibrosis in BA patients. Previous investigation has also indicated that ATX is a key enzyme for converting LPC to LPA and plasma LPA levels are correlated with the serum ATX activity in patients with chronic liver disease (57). In agreement with our findings, Watanabe and colleagues demonstrated that serum ATX levels were elevated in patients with chronic hepatitis C (57, 58). A recent study using hepatectomized rats suggested that elevated ATX activity in rats with liver injury was caused by a decrease in ATX clearance (58). In addition, Wu and coworkers further reported that the increased ATX expression was detected mainly in hepatocellular carcinoma (HCC) tissues compared to normal liver tissues and that ATX overexpression in HCC was specifically correlated with inflammation and liver cirrhosis (103).

It is notable that the more elevated serum ATX was observed in BA children with PH. PH is a consequence of advanced hepatic fibrosis that obstructs sinusoidal blood flow leading to the perpetuation of multiple varices. In this regards, portal-systemic shunting could affect the clearance of ATX. A reduced first-pass effect in the liver may be responsible for the greater serum ATX in the patients with PH. In line with our findings, Pleli et al. have documented that serum ATX was associated with the stage of liver cirrhosis, the prevalence of esophageal varices, and portal hypertensive gastropathy, suggesting that serum ATX could be an indicator for the severity of liver disease and the prognosis of cirrhosis patients (104).

Several possible mechanisms may contribute to the significant elevation of serum ATX in BA patients, especially in those with a poor outcome. The elevated serum ATX is likely attributed to an increase in ATX production, a reduction in ATX clearance from the circulation or a combination of both. In the advanced BA patients with jaundice and/or PH, the decreased clearance could be caused by reduced uptake of ATX by liver sinusoidal endothelial cells (LSEC) (105). Lack of LSEC fenestration and formation of an organized basement membrane resulting in the capillarization of liver sinusoids, not only precedes fibrosis, but is also permissive for HSC activation and fibrosis (106). Thus, dysregulation of the LSEC phenotype is a critical step in liver fibrosis. This process may lead to a reduction in ATX clearance thereby increasing circulating ATX levels. Furthermore, other organs apart from the liver can produce and secrete ATX in systemic circulation. In recent years, ATX expression has been evident in brain, lung, heart, liver, duodenum, adrenals, and skeletal muscle, indicating that ATX could be expressed in various tissues or organs (44). The major sources of high serum autotoxin in this study may be extrahepatic organs. The higher ATX levels could be regarded as indicating hepatic damage and cholestatis in BA children.

A number of caveats need to be emphasized regarding the current study. First, the study is cross-sectional in design with relatively small numbers of patients and controls. Accordingly, cause-and-effect relationships cannot be concluded and require prospective longitudinal studies to elucidate any relationships. However, with a small sample size, caution must be applied, as the findings might not be transferable to other populations. Secondly, incomplete evaluation of possible confounding factors including medical comorbidities needs to be taken into account. Other limitations would be the lack of serum creatinine and pediatric end stage liver disease (PELD) values. Future studies could evaluate whether serum creatinine correlates with serum ATX and further determine the PELD score for assessing the severity of chronic liver disease. Moreover, this study was limited to those patients who attended our hospital. As a result, the findings might not be directly applicable to subjects from other ethnic groups. Ultimately, tissue expression of ATX has not been determined. Additional

immunohistochemical analysis of ATX hepatic expression could render more valuable information on the pathophysiologic role of ATX in BA.

In summary, the current evidence revealed that BA patients had significantly elevated serum ATX and liver stiffness values compared with healthy controls. Serum ATX and liver stiffness values were markedly higher in BA patients with persistent jaundice than in those without jaundice. Subsequent analysis showed that BA patients with PH had substantially greater serum ATX than those without PH. Furthermore, serum ATX was associated with status of jaundice, hepatic dysfunction, and liver stiffness in postoperative BA. Based on these findings, serum ATX and liver stiffness measurements could serve as possible noninvasive biomarkers reflecting the disease severity and the development of liver fibrosis in the post Kasai BA patients. Further studies will be needed to determine the exact mechanisms resulting in increased serum ATX in BA. Although underlining mechanisms of the cause-and-effect relationships are not entirely elucidated, there is abundant room for further research regarding the potential role of ATX in the pathogenesis of biliary atresia.

CHAPTER IV

AUTOTAXIN OVEREXPRESSION IN THE LIVERS OF INFANTS WITH BILIARY ATRESIA

(Under revised before submission)

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Abstract

Background/Purpose: Progressive liver fibrosis, which is a clinical hallmark of biliary atresia (BA) and the primary indication for liver transplantation, remains incomprehensible. Autotaxin (ATX) has an effect on hepatic fibrogenesis via the enzymatic production of lysophosphatidic acid. We therefore aimed to investigate mRNA and protein expression of ATX in BA livers.

Methods: Liver specimens from BA infants (n=20) were compared with samples from those who underwent liver biopsy for choledochal cysts (CC, n=7) and liver transplantation for healthy controls (n=5). Transcriptional and translational expression of ATX were quantified using real-time PCR and immunohistochemistry.

Results: Our finding demonstrated overexpression of *ATX* mRNA in BA livers. In immunohistochemical evaluation, ATX was positively stained on the hepatic parenchyma and the biliary epithelium within BA patients compared to CC controls. Immunostaining score of ATX in BA livers was also significantly higher than that in CC livers ($P<0.0001$). Despite no relationship of ATX expression with BA outcomes, there were associations between age and clinical indicators (TB; $r=-0.52$, $P=0.009$, DB; $r=-0.46$, $P=0.024$, ALP; $r=-0.43$, $P=0.046$, and GGT; $r=-0.89$, $P=0.019$) in BA patients.

Conclusions: Our data show that mRNA and protein expression of ATX are increased in BA livers. These findings support the mounting evidence that ATX could be responsible for hepatic injury in post-operative BA patients; however, the precise mechanisms for the effect of ATX in disease progression will be addressed in future studies.

Introduction

Biliary atresia (BA) is neonatal cholestasis disease characterized by fibrosclerosing and inflammatory obliteration of the biliary tracts leading to progressive liver damage (1). The primary treatment for BA, Kasai portoenterostomy (KPE), establishes good bile flow and long-term survival. However, many infants are at risk of a new biliary reconstruction even after timely KPE resulting in chronic cholestasis, increased fibrosis, cirrhosis, and eventually to end-stage liver disease, thus making this entity the leading cause of liver transplantation in patients. Although the precise pathogenesis of BA remains elusive, theories of pathogenesis include viral infections, toxins, and chronic inflammatory or immunology-mediated bile duct injury and abnormalities in bile duct development (107). Some experts believe that the main cause of adverse prognosis in congenital BA patients is progressive liver fibrosis. An increased understanding of what causes the inflammatory cholangiopathy of BA could lead to therapies aimed at protecting the intrahepatic biliary system from inflammatory-mediated fibrosis. Nevertheless, the molecular mechanisms involved in the pathogenesis of liver fibrosis in BA are still mysterious. Indeed, hepatic fibrosis is a reversible physiologic and pathologic events, and the role of cytokines-mediated pathogenesis in the disorder elicits a great deal of interest.

Autotaxin (ATX; ectonucleotide pyrophosphatase/phosphodiesterase family member 2 [ENPP2]) is a secreted lysophospholipase D that generates the lipid mediator lysophosphatidic acid (LPA) from extracellular lysophospholipids, predominantly lysophosphatidylcholine (8). The ATX-LPA signaling has been implicated in multiple biological and pathophysiological processes consisting of vasculogenesis, cholestatic pruritus, tumor progression, and fibrosis through six distinct G-protein coupled LPA-receptors (LPAR1-6) (108). A link between the ATX-LPA axis and liver fibrosis came when Ikeda et al. found that intradermal LPA induces hepatic stellate cells (HSCs) proliferation, stimulates their contraction, and inhibits their apoptosis. HSCs are known as prototypic profirogenic cells in the liver (56). After transformation into myofibroblasts in response to a liver injury, HSCs start to produce abundant

extracellular matrices and profibrogenic cytokines like ATX-derived LPA. In addition, both LPA and ATX concentrations were increased in chronic hepatitis C patients related to liver fibrosis (57). The ATX-LPA axis has also been reported to be up-regulated in human hepatocellular carcinoma (103, 109), thereby establishing the possible effect of ATX in inflammation-related hepatic fibrosis diseases such as biliary atresia.

Although, until now, ATX concentrations have been shown to be associated with liver fibrosis, no previous study concerning transcriptional or translational expression of ATX in liver tissue and its relationship to BA outcomes have been identified. Accordingly, the purposes of this study were to investigate mRNA and protein expression of ATX in liver tissues from BA patients, choledochal cysts (CC), as well as healthy controls, and additionally to determine the plausible correlations between ATX expression and outcome parameters of both pre- and post- operative BA patients.

Materials and Methods

Patients and liver specimens

The study protocol conformed to the ethical standards outlined in the Declaration of Helsinki and was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. All participants, parents, or legal guardians were fully informed regarding the study protocol and procedures prior to participating in the study. Written informed consent was obtained from all patients and from parents or legal guardians of patients younger than 18 years of age.

All available perioperative liver biopsies were obtained from 20 infants with BA at the time of KPE, 7 infants with choledochal cysts (CC), and 5 healthy controls at the Department of Surgery, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. Infants diagnosed with BA or CC were included in the study based on clinical, cholangiogram, and histological findings. The control group was comprised of CC patients with no history of immune-mediated diseases. Healthy controls were selected

from liver donors who had not been diagnosed with any other disease. Liver biopsies in control group of patients were an additional procedure and were required for medical reasons.

Collected clinical data at the time of KPE and during follow-up including age, albumin, total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) were performed on a Roche Hitachi 912 chemistry analyzer (Roche Diagnostics, Basel, Switzerland).

RNA extraction and quantitative real time-PCR for mRNA expression of *ATX*

Total RNA was isolated from liver biopsies using RNeasy Mini Kit (Qiagen, Hilden, Germany), with cDNA reverse transcribed by TaqMan Reverse Transcription Reagents (Applied Biosystems, Branchburg, NJ, USA). Real-time PCR was performed using QPCR Green Mastermix HRox (biotechrabbit GmbH, Hennigsdorf, Germany) on StepOnePlus Real Time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). Primers used for *ATX* and *glyceraldehyde 3-phosphate dehydrogenase (GADPH)* amplification were, as follows: *ATX* forward primer 5'-CGTGGCTGGGAGTGTACTAA-3'; *ATX* reverse primer 5'AGAGTGTGTGCCACAAGACC-3', as previously described by Kondo et al. (110); *GADPH* forward primer 5'-GTGAAGGTCGGAGTCAACGG-3', and *GADPH* reverse primer 5'-TCAATGAAGGGGTCATTGATGG-3'. Real-time PCR condition was performed, as follows: (initial step) 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and then 60°C for 1 min. Relative mRNA expression of *ATX* was normalized to *GADPH* as an internal control and was determined using $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical analysis for protein expression of ATX

Liver specimens were originally paraffin embedded and then sectioned according to standard protocols. Routine staining with hematoxylin and eosin and immunohistochemical staining with antibodies was performed to detect protein expression of ATX (Merck Millipore, Darmstadt, Germany). For ATX staining, cells with brown stained cytoplasm were scored as positive. All of tissue sections were analyzed

by pathologist, who was blinded to the clinical status and the diagnosis of the patients. Immunoreactivity of ATX in the biliary epithelium and the parenchyma was semi-quantitatively analyzed for the percentage of positive cells and the intensity of staining. The percent of positive cells less than 1% was scored as 0, 2–25% as 1, >25–50% as 2, and >50% as 3. The intensity of ATX immunostaining was determined by using the following staining scores: 0=none staining, 1=mild staining, 2=moderate staining, and 3=strong staining. Based on these staining scores, the positive areas of ATX positive cells were determined by measuring five randomly selected microscopic fields (400×) for each slide.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences version 22.0 (SPSS, Inc., Chicago, IL, USA). One way ANOVA was applied to determine statistical significance between demographic data of patients and among controls. Mann-Whitney U-test was used for comparing mRNA expression of ATX between groups. In addition, results of immunohistochemistry were analyzed with Chi-square test for trend or Fisher's test when applicable. Finally, correlations between expression levels of ATX and clinical parameters in BA infants were calculated with Spearman's coefficient analysis. Values are presented as mean±standard error of the mean (SEM). A *P*-value of <0.05 was considered statistically significant for differences and correlations.

Results

Clinical characteristic of study participants

The baseline characteristic from a total of 20 infants with BA and 7 choledochal cysts (CC) controls are summarized in Table 3. There was no statistically significant difference in sex ratio between BA patients and CC controls, while outcome parameters including TB, DB, AST, and ALP in BA patients were remarkably higher than CC controls.

Table 3. Clinical characteristics of biliary atresia patients and choledochal cysts controls.

	BA patients (n=20)		CC controls (n=7)	*P*-value
	Pre-operation	Post-operation		
Age (years)	0.26±0.019	0.68±0.018	2.40±0.98	0.001
Sex (male:female)	8 (40%):12 (60%)	8 (40%):12 (60%)	2 (40%):5 (60%)	1.00
Albumin (g/dL)	4.04±0.081	3.82±0.12	4.30±0.20	0.248
TB (mg/dL)	12.15±0.75	5.05±1.16	3.12±1.72	<0.0001
DB (mg/dL)	9.04±0.56	2.24±0.77	2.26±1.59	<0.0001
AST (IU/L)	238.50±26.40	117.11±14.13	114.00±30.04	0.046
ALT (IU/L)	191.78±25.77	116.00±18.04	138.76±48.32	0.39
ALP (IU/L)	580.61±37.23	485.32±44.21	295.75±33.91	0.002
GGT (IU/L)	1,443.75±599.66	302.50±285.50	244.00±175.00	0.26

The data are expressed as mean±SEM

P-value<0.05 indicates statistically significant difference of clinical data at time of KPE between BA patients and CC patients

BA, biliary atresia; CC, choledochal cysts; TB, total bilirubin; DB, direct bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase

ATX mRNA expression

In an endeavor to identify transcriptional expression of ATX in infants with BA, relative *ATX* mRNA expression was quantified by real-time polymerase chain reaction (PCR) in liver biopsies from 15 BA patients and 5 healthy controls. Real-time PCR analysis showed that relative *ATX* mRNA expression was higher in BA livers than that of controls (Figure 9).

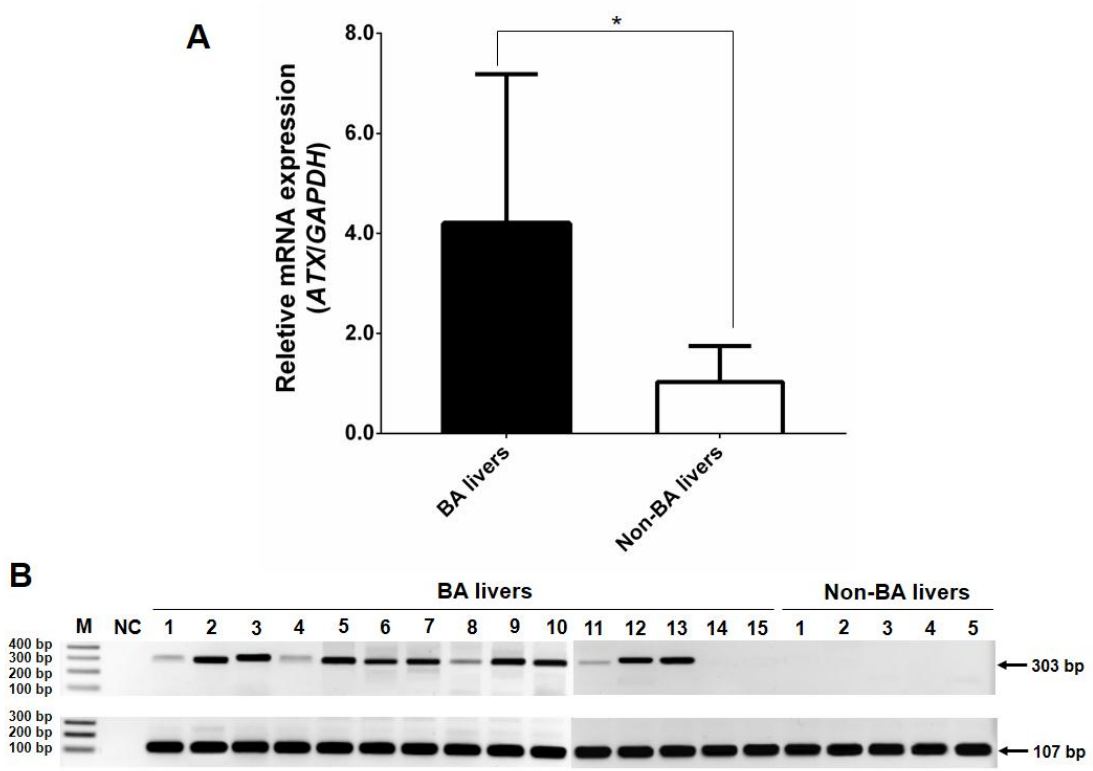

Figure 9. Relative *ATX* mRNA expression between biliary atresia livers and healthy livers. (A) Up-regulated mRNA expression of *ATX* normalized by *GAPDH* in livers from BA infants; (B) Representative gel of *ATX* and *GAPDH* products from real-time PCR products. *$P<0.05$ vs control group

Immunohistochemistry analysis of ATX expression

Immunohistochemical evaluation for ATX was performed in both BA and CC liver tissues. Representative immunohistochemical findings of ATX are illustrated in Figure 10. In congenital BA liver specimens, overexpression of ATX was detectable in the hepatic parenchyma, more in the center of the liver nodules, the inflammatory cells, the biliary epithelial cells, and cells of the surrounding connective tissue. On the other hand, ATX expression was scarcely evident as a faint cytoplasm staining in the livers used as control (Figure 10A). Whereas, the distribution of ATX in positive cells was classified as cytoplasm-localized pattern.

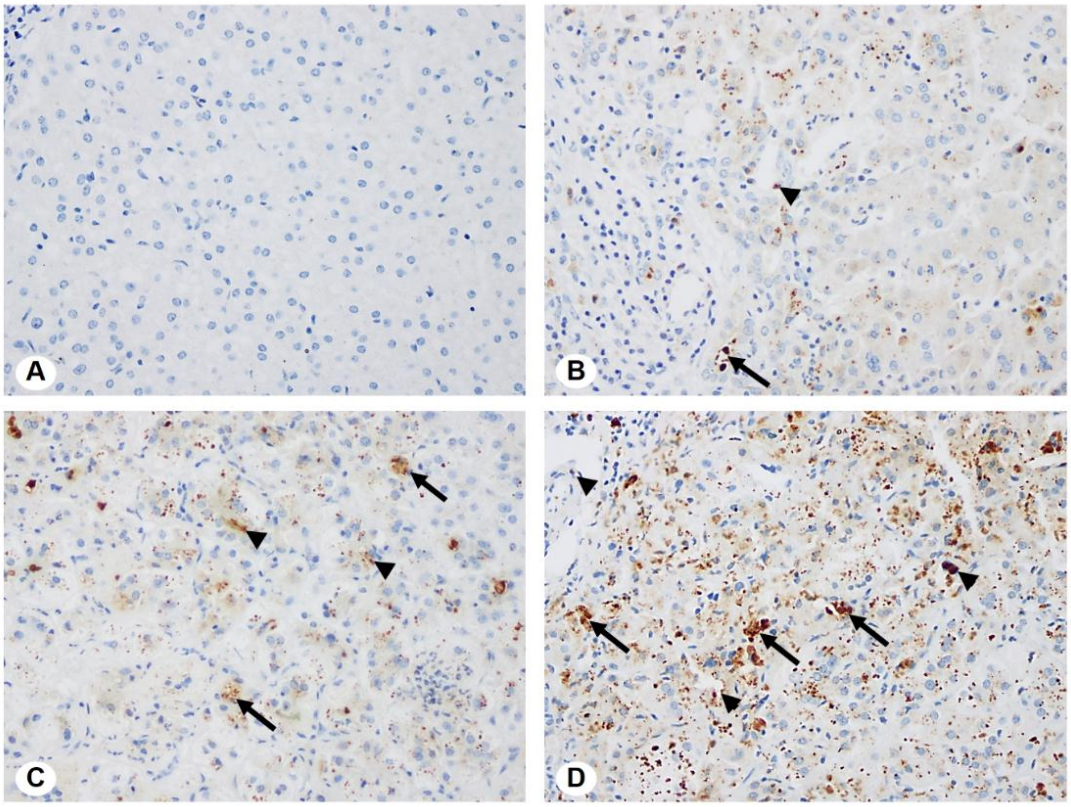

Figure 10. Immunohistochemical staining for ATX protein expression. Specific staining of ATX protein is represented by brown coloration. The expression of ATX in BA livers is mostly in the hepatic parenchyma (arrows) and the biliary epithelium (head arrows). ATX staining scores were defined, as follows; (A) 0=none expression of ATX in a liver used as control; (B) 1=mild expression of ATX in BA liver; (C) 2=moderate expression of ATX in BA liver; and (D) 3=strong expression of ATX in BA liver. (Original magnifications 400x).

In order to compare expression levels of ATX protein between BA patients and CC controls, the intensity staining and percentage of ATX positive cells were assessed by visual scoring method. The ATX immunoreactive scores in both BA patients and CC controls are depicted in Table 4. In BA livers at the time of KPE, the degree of ATX staining was markedly increased in BA patients compared with CC controls when measured as an immunointensity staining on histological liver sections (P<0.0001).

Likewise, the percentage of ATX positive cells in both the hepatic parenchyma cells and the biliary epithelial cells was significantly higher in BA patients than those in controls (*P*<0.0001).

Table 4. Autotaxin expression levels in biliary atresia patients and choledochal cysts controls.

Immunohistochemistry staining score	Autotaxin		*P*-value
	BA patients	CC controls	
The intensity staining score			
None (-)	0 (0.00%)	7 (100.00%)	<0.0001
Mild (+)	10 (50.00%)	0 (0.00%)	
Moderate (++)	4 (20.00%)	0 (0.00%)	
Strong (+++)	6 (30.00%)	0 (0.00%)	
The percentage of positive cells staining score			
<1% (-)	0 (0.00%)	7 (100.00%)	<0.0001
1-25% (+)	10 (50.00%)	0 (0.00%)	
>25-50% (++)	7 (20.00%)	0 (0.00%)	
>50% (+++)	3 (30.00%)	0 (0.00%)	

BA, biliary atresia; CC, choledochal cyst

Associations of hepatic ATX expression with clinical outcomes in BA patients

To obtain a clue as to how ATX expression affects the pathogenesis of BA, we analyzed correlation between ATX immunohistochemistry staining score and blood chemistry data in BA patients. Subsequent analysis revealed no association between ATX expression and outcome parameters in pre- and post-operative BA children (Table 5).

Table 5. Correlations between autotaxin immunohistochemistry staining score and clinical parameters in biliary atresia patients.

Clinical characteristics	Spearman's rho correlation	ATX immunohistochemistry staining score			
		The intensity staining		The positive cells staining	
		Pre-OP	Post-OP	Pre-OP	Post-OP
Age (years)	Coefficient (*r*)	-0.37	-0.36	-0.30	-0.30
	P-value	0.12	0.12	0.20	0.20
Sex (male:female)	Coefficient (*r*)	0.085	0.085	0.009	0.009
	P-value	0.72	0.72	0.97	0.97
Albumin (g/dL)	Coefficient (*r*)	-0.18	-0.054	-0.090	0.12
	P-value	0.51	0.84	0.74	0.65
Total bilirubin (mg/dL)	Coefficient (*r*)	-0.15	-0.24	-0.27	-0.19
	P-value	0.55	0.35	0.28	0.46
Direct bilirubin (mg/dL)	Coefficient (*r*)	-0.076	-0.11	-0.15	-0.071
	P-value	0.76	0.66	0.54	0.78
AST (IU/L)	Coefficient (*r*)	-0.008	0.22	-0.15	-0.23
	P-value	0.98	0.37	0.58	0.37
ALT (IU/L)	Coefficient (*r*)	-0.14	-0.13	-0.34	-0.055
	P-value	0.60	0.60	0.18	0.83
ALP (IU/L)	Coefficient (*r*)	0.075	0.34	0.18	-0.22
	P-value	0.77	0.17	0.50	0.38
GGT (IU/L)	Coefficient (*r*)	-0.21	-0.25	0.26	0.28
	P-value	0.79	-0.63	0.74	0.65

ATX, autotaxin; OP, operation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase

Regarding clinical outcome at 6 months post-operative BA patients, there were 12 BA patients with good outcome (TB<2.0 mg/dL) and 8 BA patients with poor outcome (TB≥2.0 mg/dL). Interestingly, we observed strongly negative associations between age at the time of KPE and clinical parameters consisting of TB (*r*=-0.52,

P=0.009), DB (*r*=-0.46, *P*=0.024), ALP (*r*=-0.43, *P*=0.046), and GGT (*r*=-0.89, *P*=0.019) (Table 6).

Table 6. Correlations between age and clinical parameters in biliary atresia patients.

Clinical characteristics	**Spearman's rho correlation**	**Age (years)**	
		Pre-operation	**Post-operation**
Sex (male:female)	Coefficient (*r*)	-0.18	-0.18
	P-value	0.43	0.43
Albumin (g/dL)	Coefficient (*r*)	0.30	-0.40
	P-value	0.20	0.10
Total bilirubin (mg/dL)	Coefficient (*r*)	-0.52	0.25
	P-value	0.009	0.30
Direct bilirubin (mg/dL)	Coefficient (*r*)	-0.46	0.26
	P-value	0.024	0.28
AST (IU/L)	Coefficient (*r*)	-0.30	-0.15
	P-value	0.19	0.54
ALT (IU/L)	Coefficient (*r*)	0.010	-0.29
	P-value	0.97	0.22
ALP (IU/L)	Coefficient (*r*)	-0.43	-0.36
	P-value	0.046	0.14
GGT (IU/L)	Coefficient (*r*)	-0.89	-0.73
	P-value	0.019	0.025

ATX, autotaxin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase

Discussion

Despite extensive research efforts, understanding of mechanisms that regulates biliary atresia (BA) progression following Kasai portoenterostomy (KPE) is scarce. Biliary

atresia causes rapidly progressive liver fibrosis and cirrhosis in neonates and is the most common indication for liver transplantation in children. Although the precise cause of liver fibrosis in BA remains obscure, several cytokines have been proposed to act an important role in the regulation of hepatic fibrogenesis (33, 111-115). Our group has previously reported associations between elevation of circulating autotaxin (ATX) and poor outcomes in BA patients, especially severity of fibrosis (116). This is important evidence supporting the hypothesis that ATX could be responsible for hepatic pathology in BA. In our present experiment, we investigated mRNA and protein expression of ATX in liver biopsies from BA children, choledochal cysts (CC), and healthy controls, showing overexpression of ATX mRNA in BA patients. We further performed immunohistological analysis to determine protein expression of ATX and unveiled an intense increase in ATX staining of BA infants, predominantly in the hepatic parenchyma and the biliary epithelium at the time of KPE. To our knowledge, the present study describes for the first time mRNA and protein expression of ATX in BA livers.

The biological outcome of ATX has been documented to induce a variety of inflammatory phenomena via LPA activity, and its role in disease pathophysiology has been verified in several human diseases (108), making the ATX-derived LPA signaling an attractive therapeutic. Indeed, emerging evidence suggests that the liver is the main source of ATX metabolism in both human and animal models (53). Additionally, ATX expression has been detected on all types of liver cells, including the biliary epithelium (117). This suggests the possibility a regulative role of ATX in the liver. A lack of differential mRNA and protein expression in BA livers was confirmed in the current experiment. Our result demonstrated that ATX mRNA was up-regulated in liver of infants with BA when compared with CC controls. This finding is in line with previous investigation demonstrating overexpression of ATX in liver tissues of patients with hepatocellular carcinoma (HCC) (59), suggesting that up-regulated ATX mRNA is associated with hepatic damage and hence, reflects liver fibrosis. Besides up-regulation of ATX mRNA in livers of infants with BA, it has shown an increase of hepatic protein expression of ATX. Recently published report also confirmed that overexpression of

ATX protein was specifically associated with inflammation and cirrhosis in HCC patients (103), agreed with our finding, implying that ATX could have a potential effect in the inflammation related to progressive BA. Although our immunohistochemistry data demonstrated positive cytoplasmic ATX expression in the inflammatory cells and the biliary epithelial cells, its expression did not correlate with biochemical abnormalities in BA patients; thereby, its diagnostic or prognostic applications remain limited at the moment. In accordance with our finding, Vejchapipat et al. demonstrated no association between hepatic expression of inducible nitric oxide synthase and clinical outcomes in BA children (118). Whereas, Honsawek et al. reported that cyclooxygenase-2 expression was associated with serum total bilirubin, AST, and ALT in BA infants (119). The reason for this discrepancy may be attributed to the difference in their effects on the disease pathophysiology. Rather, ATX might be associated with the nature of BA disease itself. Thus, it is reasonable to postulate that increased expression of ATX in BA livers might be a defensive response of the body to fight against hepatic impairment, or simply a compensatory response to ATX sensitivity resistance which leads to its compensatory up-regulation.

The physiological significance of elevated ATX expression in BA remains imprecise. The aberrant production of ATX may results in the altered activation of LPA signaling pathways through G-protein coupled LPA-receptors, but no limited to activation of their signaling-associated cell proliferation, migration and apoptosis. Hepatic stellate cells (HSCs) are known to play a major role in the fibrotic process in the liver that could contribute to the etiology of BA. For this reason, many factors with potentially fibrogenic activities in the liver have been evaluated in light of their effects on HSCs activation and apoptosis. Regarding the potential effect of ATX-mediated LPA on HSCs, LPA has been shown to stimulate the contractility of HSCs and to inhibit their apoptosis through Rho/Rho kinase activation (56, 120). Although ATX may not play a primary role in the pathogenesis of liver fibrosis, it could accelerate fibrogenesis by stimulating the proliferation of HSCs in patients with liver fibrosis via its ability to produce LPA. Collectively, the aberrant expression of ATX along with the consequently abnormal production of LPA in the liver microenvironment may fuel the development

of biliary atresia. Further experiments isolating the biliary epithelial cells or HSCs from BA livers would be required to determine the precise biological or pathological significance of the observations presented in this report.

Interestingly, the present study further revealed negative associations between age at time of KPE and clinical factors including TB, DB, ALP, and GGT in BA children, despite our finding of no association between hepatic ATX expression and BA outcomes. Age at the time of KPE is believed to have a detrimental effect on post-operative prognosis of BA patients (121, 122). This finding suggests that a successful portoenterostomy might be involved in age at the moment of operation; however, more work is needed before reaching this conclusion.

There are inherent limitations to our study that must be recognized. The most important limitation is the lack of normal liver tissue from an age-matched cohort available for study due to ethical considerations in obtaining liver biopsy samples from healthy infants. Given that BA is a rare disorder, the sample size of study population is relatively small. A further a large scale and multicenter studies should be conducted to make a more definite conclusion. Our other major limitation is that the causal association between hepatic ATX expression and BA was not fully addressed in the study. Additional research is required to evaluate whether increased hepatic ATX expression is causally related to progressive BA or is just a compensatory response to the disease.

To sum up, the current study provides evidence for up-regulation of *ATX* mRNA in liver specimens of BA patients compared to those in controls. ATX was expressed not only in the hepatic parenchyma but also in the biliary epithelial cells from infants with BA at the time of KPE. These findings suggest that ATX might be related to the progression of liver injury in biliary atresia. Further investigations examining the possible effect of selective ATX inhibitors on inflammation and progression of liver fibrosis in BA are needed for the development of non-transplant therapeutic strategies to prevent the progression of this devastating disease in affected infants.

CHAPTER V

ASSOCIATION BETWEEN PROMOTER HYPOMETHYLATION AND OVEREXPRESSION OF AUTOTAXIN WITH OUTCOME PARAMETERS IN BILIARY ATRESIA

(Under revised process in Plos One)

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Abstract

Objective: Biliary atresia (BA) is a progressive fibroinflammatory liver disease. Autotaxin (ATX) has a profibrotic effect resulting from lysophosphatidic acid activity. The purpose of this study was to examine *ATX* expression and *ATX* promoter methylation in peripheral blood leukocytes and liver tissues from BA patients and controls and investigate their associations with outcome parameters in BA patients.

Methods: A total of 130 subjects (65 BA patients and 65 age-matched controls) were enrolled. DNA was extracted from circulating leukocytes and liver tissues of BA patients and from and age-matched controls. *ATX* promoter methylation status was determined by bisulfite pyrosequencing. *ATX* expression was analyzed using quantitative real-time PCR and enzyme-linked immunosorbent assay.

Results: Decreased methylation of specific CpGs were observed at the *ATX* promoter in BA patients. Subsequent analysis revealed that BA patients with advanced stage had lower methylation levels of *ATX* promoter than those with early stage. *ATX* promoter methylation levels were found to be associated with hepatic dysfunction in BA. In addition, *ATX* expression was significantly elevated and correlated with a decrease in *ATX* promoter methylation in BA patients compared to the controls. Furthermore, promoter hypomethylation and overexpression of *ATX* were inversely associated with jaundice status, hepatic dysfunction, and liver stiffness in BA patients.

Conclusion: Accordingly, it has been hypothesized that *ATX* promoter methylation and *ATX* expression in peripheral blood may serve as possible biomarkers reflecting the progression of liver fibrosis in postoperative BA. These findings suggest that the promoter hypomethylation and overexpression of *ATX* might play a contributory role in the pathogenesis of liver fibrosis in BA.

Introduction

Biliary atresia (BA) is a devastating cholestatic liver disorder of uncertain etiology in neonates and manifests as impaired liver function and fibroinflammatory obliterative cholangiopathy of both the intrahepatic and extrahepatic bile ducts (87). BA patients initially develop neonatal jaundice due to hepatic cholestasis and progress to liver fibrosis, which leads to cirrhosis (1). Since no medical therapies exist, sequential treatment by surgical hepatoportoenterostomy or Kasai procedure and liver transplantation is the only option for therapy in most affected children due to complications of cirrhosis (123). Although the precise pathogenesis of BA has yet to be determined, multiple theories exist regarding the etiology of BA, including viral infection, inflammation, bile duct proliferation, and fibrogenesis (124).

Autotaxin (ATX), a secreted glycoprotein, belongs to the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) enzyme family. ATX exhibits a unique lysophospholipase D (LPD) activity, converting lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) (8). LPA acts via activation of at least six different G-protein-coupled receptors to influence a number of biological processes (108). Both ATX and LPA are considered to be involved in the development of liver fibrosis and elevated serum ATX was associated with liver fibrosis in cirrhotic patients and hepatocellular carcinoma (HCC) patients (104, 110). LPA can induce hepatic stellate cell (HSC) proliferation, stimulate their contraction, and inhibit their apoptosis (56). Liver fibrosis is the excessive accumulation of extracellular matrix (ECM) proteins that occurs in most types of chronic liver diseases. HSCs were recognized as the main matrix-producing cells in the liver. In continuously injured livers, HSCs are activated and transdifferentiate into myofibroblasts, resulting in the production of abundant extracellular matrices and profibrogenic cytokines including ATX-mediated LPA. Serum ATX has been proposed as a marker for liver fibrosis (53). Recent studies have also suggested a connection between liver fibrosis and circulating LPA, and serum ATX was elevated in chronic hepatitis C patients (15, 57). Importantly, the ATX-LPA axis has been shown to be up-regulated in human HCC, suggesting that ATX possibly plays an important role in

inflammation-related liver fibrogenesis (103, 109). Given that elevated ATX levels contribute to the pathogenesis of liver fibrosis in BA, we hypothesized that the hypomethylation of the *ATX* promoter region could upregulate *ATX* expression in BA patients.

It has been demonstrated that there is a possible link between epigenetic regulation and the etiologic mechanism of intrahepatic bile duct defects in BA (73). Methylation of cytosine–guanine dinucleotide (CpG) residues within the promoter and enhancer regions leads to suppression of gene expression and DNA methylation alterations can be elicited by viruses and genetic defects (74-76). DNA hypomethylation may result in the development of several autoimmune disorders, such as systemic lupus erythematosus and rheumatoid arthritis (77, 78). Recent evidence indicates that alterations to epigenetic DNA methylation patterns contribute to the pathogenesis of BA. The hypermethylation of promoter regulatory elements contributes to the lower CD11a expression in T lymphocytes of BA patients (79). However, the hypomethylation of the *interferon gamma (IFN-γ)* gene promoter may be responsible for increased *IFN-γ* expression in BA infants (80). These findings suggest the significance of aberrant DNA methylation in the development of BA.

Until now, there have been no reports regarding the role of *ATX* promoter methylation and expression in BA patients. We recently reported that elevated serum ATX was significantly associated with hepatic dysfunction and severity in BA patients (116). The purpose of this study was to investigate promoter methylation status and expression of *ATX* in peripheral blood leukocytes and liver tissues from BA patients compared with controls. We also explored whether *ATX* promoter methylation and expression were associated with the clinical parameters of BA patients.

Materials and Methods

Study population

The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB number 279/57). This study was conducted in accordance with the ethical standards outlined in the 1975 Declaration of Helsinki. All participants, parents, or legal guardians were fully informed regarding the study protocol and procedures prior to participating in the study. Written informed consent was obtained from all patients and from parents or legal guardians of patients younger than 18 years of age.

This cross-sectional study evaluated 65 BA patients, 65 age-matched unaffected volunteers. BA patients were diagnosed by intra-operational cholangiography and were surgically treated with Kasai portoenterostomy at King Chulalongkorn Memorial Hospital, Bangkok, Thailand. Patients who had undergone liver transplantation were excluded. Unaffected volunteers who attended the Well Baby Clinic at King Chulalongkorn Hospital for vaccination who had normal physical findings and no underlying diseases were included. We stratified BA patients according to liver stiffness values into two groups: non-fibrotic BA (<9.7 kPa, n=22) and fibrotic BA (>9.7 kPa, n=43). We also classified BA patients according to serum total bilirubin (TB) into either the non-jaundice group (TB<2 mg/dL, n=41) or the persistent jaundice group (TB≥2 mg/dL, n=24). Based on the severity of hepatic injury (aspartate aminotransferase, AST value), BA patients were divided into either the early-stage group (AST<100 IU/L, n=36) or late-stage group (AST≥100 IU/L, n=29).

Liver tissue samples

Liver tissue samples of 15 BA patients (8 females and 7 males; age range 1–4 months; mean 66 days) who underwent Kasai operations and 5 non-BA patients who are liver donors with no signs of fibrosis were obtained at the Department of Surgery, King Chulalongkorn Memorial Hospital, between 2001 and 2006. The non-BA patients who had no clinical jaundice, served as controls. All non-BA patients underwent

exploratory laparotomy as the therapeutic treatment for their diseases. Liver biopsies in this group of patients were an additional procedure and were required for medical reasons. All samples were obtained with the families' consent.

Assessment of clinical outcome

Venous blood samples were drawn from each subject in ethylenediaminetetraacetic acid and clot blood tubes for routine laboratory tests, including TB, AST, alanine aminotransferase (ALT), alkaline phosphatase (ALP), and albumin. All of the immediately aforementioned tests were performed on a Roche Hitachi 912 Chemistry Analyzer (Roche Diagnostics, Basel, Switzerland). Assessment of liver stiffness by transient elastography was performed using a Fibroscan (EchoSens, Paris, France). Briefly, measurements were performed by placing the Fibroscan transducer probe on the intercostal space at the area of the right lobe of the liver. Measurements were then performed until 10 validated results were obtained with a success rate of at least 80%. The median value from 10 validated scores represented the elastic modulus measurement of the liver, which was expressed in kilopascals (kPa).

Bisulfite Pyrosequencing of *ATX* Promoter

Genomic DNA was isolated from peripheral blood leukocytes and liver tissue samples using DNA isolation kits (GE Healthcare, Buckinghamshire, UK and Vivantis, Selangor Darul Ehsan, Malaysia, respectively). Our assay was designed to examine methylation levels at four CpG sites within the ATX promoter. Quantitative DNA methylation analysis of each CpG was measured on bisulfite-treated DNA using highly quantitative analysis based on PCR pyrosequencing. Briefly, extracted DNA (50 ng; concentration: 2.5 ng/μL) was bisulfite converted using an EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Orange, CA, USA). Each 30 μL PCR reaction contained 10X PCR buffer, 200 mM dNTPs, 0.2 mM primers, 0.5 U HotStar Taq DNA polymerase (Qiagen, Inc., San Diego, CA, USA), and 5 ng of bisulfite-treated DNA. The polymerase was activated by incubation at 95°C for 15 min, followed by 40 cycles of 95°C for 1

min, 56°C for 1 min, and 72°C for 1 min. The reaction was then allowed to develop for 7 min at 72°C. The primers used to measure *ATX* promoter methylation were as follows: forward primer 5'-TAGGTATTGTAGGGGGTGGGAA-3'; reverse primer biotinylated-5'-ACCTTTAACAAAACACACACATAACC-3'; and, sequencing primer 5'-GGGTGGGAATGTGGA-3'. PCR products (20 µl) were purified and analyzed in the PyroMark MA System (Pyrosequencing, Inc., Westborough, MA, USA) and methylation data from the amplified regions were analyzed by Pyro Q-CpG software 1.0.6. The degree of methylation was expressed for each CpG site as a percent of methylated cytosine.

Quantitative real-time polymerase chain reaction (QPCR)

To evaluate mRNA expression of *ATX* in BA patients and controls using real-time PCR, total RNA from peripheral blood leukocytes and liver tissue samples was extracted by Trizol (Invitrogen, Carlsbad, CA, USA), with cDNA reverse transcribed by a Roche Transcriptor cDNA synthesis kit (Roche, Branchburg, NJ, USA). Real-time PCR was performed using QPCR Green Mastermix HRox (biotechrabbit GmbH, Hennigsdorf, Germany) on a StepOnePlus Real Time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). The primers used for *ATX*, *DNA methyltransferase 1 (DNMT1),* and *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* amplification were as follows: *ATX* forward primer 5'-CGTGGCTGGGAGTGTACTAA-3'; *ATX* reverse primer 5'AGAGTGTGTGCCACAAGACC-3', as previously described (110); *DNMT1* forward primer 5'- CAGGCCCAATGAGACTGACA-3', and *DNMT1* reverse primer 5'-GTGGGTGTTCTCAGGCCTGTAG-3'; *GAPDH* forward primer 5'-GTGAAGGTCGGAGTCAACGG-3', and *GAPDH* reverse primer 5'-TCAATGAAGGGGTCATTGATGG-3'. The real-time PCR conditions were performed as follows: (initial step) 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and then 60°C for 1 min. The relative mRNA expression of *ATX* was normalized to GAPDH as an internal control and was determined using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Total protein from liver tissue was extracted by a RIPA assay (Cell Signaling Technology, Inc., Danvers, MA, USA), which contained a protease inhibitor (Merck Millipore, Darmstadt, Germany). Serum and protein lysate ATX concentrations were determined using a commercially available sandwich enzyme-linked immunosorbent assay development kit (R&D Systems, Inc., Minneapolis, MN, USA).

Statistical analysis

All statistical analyses were performed using SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). The statistical significance between demographic data of patients and controls was compared by unpaired Student's *t*-test. Given that *ATX* promoter methylation data were not normally distributed, nonparametric tests were applied as follows: Mann-Whitney *U* test for comparing DNA methylation levels between groups and Kruskal-Wallis *H* test for continuous variables. The association of methylation changes as well as *ATX* expression and clinical parameters were detected using multivariate linear regression analysis. Correlations between DNA methylation, mRNA expression, protein concentration, and clinical parameters were evaluated using Spearman's correlation coefficient (*r*). Receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were calculated to assess the feasibility of using *ATX* methylation status as a diagnostic tool in discriminating BA patients from controls. Data were shown as a mean ± standard error of the mean. *P*-values less than 0.05 were considered statistically significant for differences and correlations.

Results

Characteristics of study participants

The baseline clinical characteristics of study participants are summarized in Table 7. A total of 130 subjects (65 BA patients and 65 age-matched healthy controls) were recruited in the current study. There were no significant differences in age or gender between BA patients and healthy controls. As expected, clinical parameters,

including liver stiffness values, AST, and ALT were significantly higher in BA patients than those in controls (*P*<0.0001).

Table 7. Clinical characteristics of study participants.

Clinical characteristics	**BA patients (n=65)**	**Healthy controls (n=65)**	***P*-value**
Age (years)	8.55±0.52	8.27±0.69	0.92
Gender (female:male)	35:30	32:33	0.87
Liver stiffness (kPa)	25.30±2.71	4.01±0.19	<0.0001*
TB (mg/dL)	2.10±0.41	-	NA
AST (IU/L)	105.07±8.94	26.66±0.82	<0.0001*
ALT (IU/L)	93.97±9.19	9.24±0.65	<0.0001*
ALP (IU/L)	368.17±27.61	-	NA
Albumin (mg/dL)	4.24±0.10	-	NA

Data presented as mean ± standard error of the mean, unless otherwise specified

*Differences in descriptive data are considered significant at P-value less than 0.05 (two-tailed)

Abbreviations: BA=biliary atresia; TB=total bilirubin; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=Alkaline phosphatase; NA=not available

ATX promoter hypomethylation in peripheral blood leukocytes

To explore promoter methylation of *ATX* in BA, we first verified promoter methylation in peripheral blood leukocytes in BA patients and unaffected volunteers. Figure 11 illustrates a schematic representation of four CpG islands within the *ATX* gene promoter region. Overall, methylation levels at the *ATX* promoter were significantly lower in the BA group than healthy controls (*P*<0.0001).

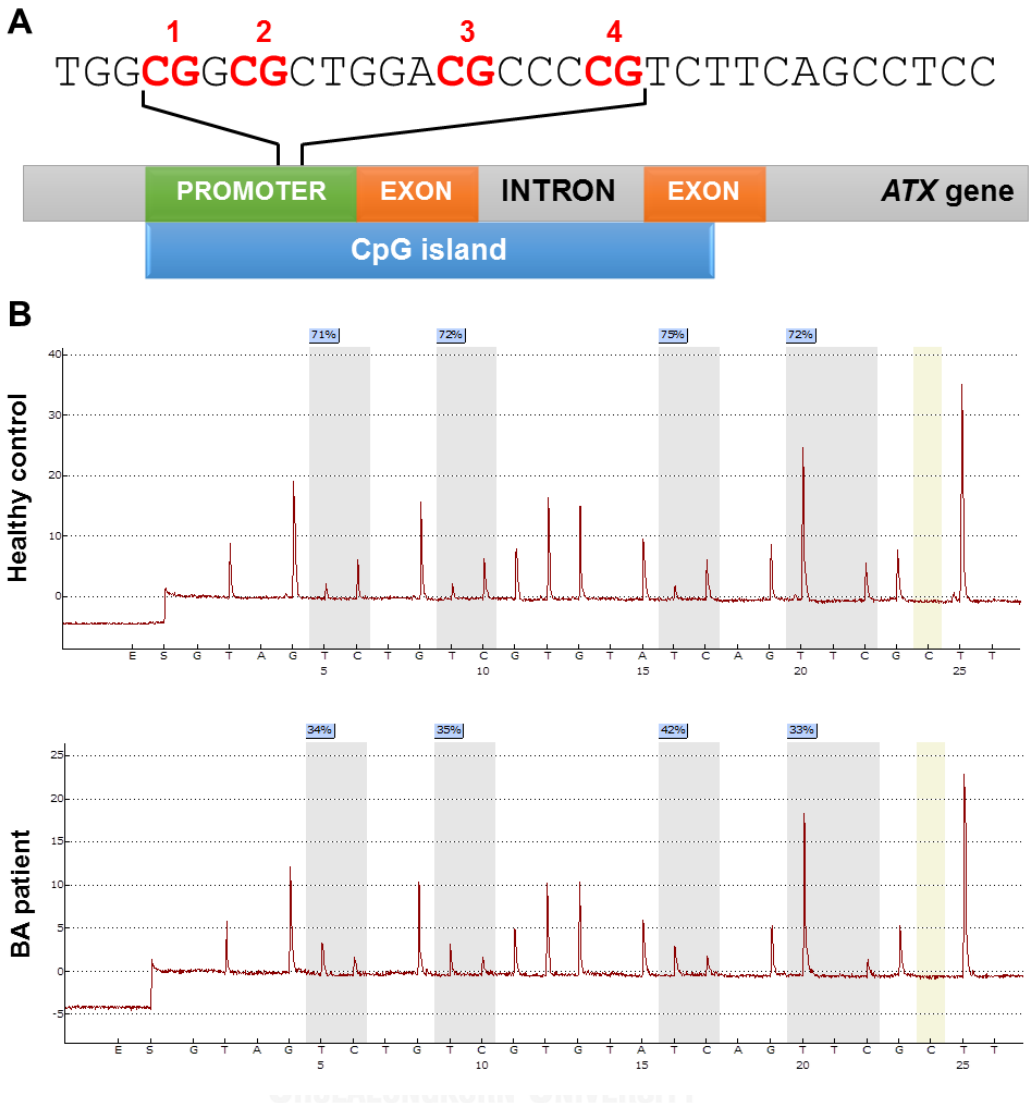

Figure 11. DNA methylation at four CpG islands within *ATX* gene promoter. (A) Schematic diagram representing CpG islands at the *ATX* promoter showing the four CpG sites. (B) Pyrosequencing results in healthy controls and BA patients.

Similarly, BA patients demonstrated significantly reduced methylation levels when compared to healthy controls across four CpG sites, as follows: CpG 1: P=0.0029, CpG 2: P=0.0005, CpG 3: P=0.0057, and CpG 4: P<0.0001 (Figure 12A). In analyses stratified by disease severity, we classified BA patients according to fibrosis, jaundice status, and hepatic dysfunction marker (AST value). Overall, decreased methylation

levels of the *ATX* promoter were detected in advanced BA patients with fibrosis, persistent jaundice, and late stage hepatic dysfunction as compared with those in early stage (P=0.0003, P=0.0077, and P=0.023, respectively) (Figures 12B-D).

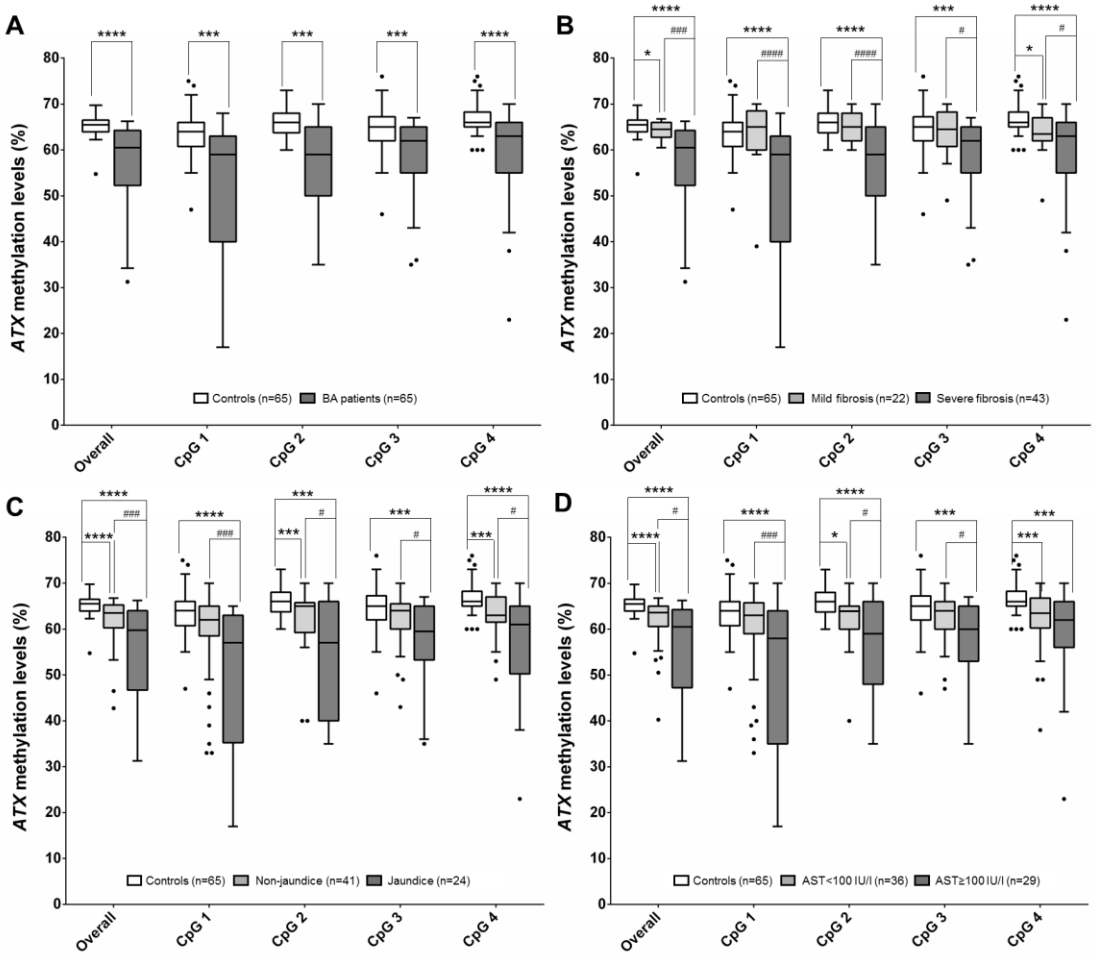

Figure 12. Box-plot illustrating methylation levels of the *ATX* promoter in peripheral blood leukocytes of subjects among different groups. (A) Methylation levels of the *ATX* promoter at four CpG sites in BA patients and healthy controls. (B) Methylation levels of *ATX* promoter at four CpG sites in BA subgroups, including mild fibrosis (F0-F2) and severe fibrosis (F3-F4). (C) Methylation levels of the *ATX* promoter at four CpG sites in patients with and without jaundice. (D) Methylation levels of *ATX* promoter at four CpG sites in BA patients according to severity of hepatic damage (AST values). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control group and $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, $^{\#\#\#\#}P$<0.0001 for comparisons between BA subgroups.

Additionally, we observed a negative correlation between *ATX* promoter methylation levels and liver stiffness, TB, AST, and ALP (*r*=-0.43, *P*=0.001; *r*=-0.31, *P*=0.015; *r*=-0.41, *P*=0.001; and, *r*=-0.35, *P*=0.006, respectively). There was a positive association between *ATX* promoter methylation and serum albumin (*r*=0.37, *P*=0.009) (Table 8). For each of the four CpG sites, methylation of the *ATX* promoter across the three CpG sites (CpG 1, CpG 2, and CpG 3) was inversely correlated with liver stiffness, TB, AST, ALT, and ALP. *ATX* promoter methylation levels were found to be positively associated with serum albumin. No relationship between *ATX* methylation at the CpG 4 residue and clinical outcome was observed in BA patients (Table 8).

Table 8. Correlations between methylation levels of CpG islands at the *ATX* promoter and clinical parameters in BA patients.

Clinical characteristics	**Spearman's rho correlation**	**CpG islands within the *ATX* promoter**				
		Overall	**CpG 1**	**CpG 2**	**CpG 3**	**CpG 4**
Age (years)	Coefficient (*r*)	0.083	0.11	0.13	-0.084	-0.024
	P-value	0.52	0.38	0.30	0.52	0.85
Liver stiffness (kPa)	Coefficient (*r*)	-0.43	-0.50	-0.40	-0.35	-0.24
	P-value	0.001*	<0.0001*	0.001*	0.005*	0.060
TB (mg/dL)	Coefficient (*r*)	-0.31	-0.36	-0.30	-0.26	-0.18
	P-value	0.015*	0.004*	0.020*	0.042*	0.16
AST (IU/L)	Coefficient (*r*)	-0.41	-0.48	-0.40	-0.35	-0.25
	P-value	0.001*	<0.0001*	0.002*	0.006*	0.056
ALT (IU/L)	Coefficient (*r*)	-0.14	-0.22	-0.14	-0.14	0.020
	P-value	0.29	0.085	0.30	0.30	0.88
ALP (IU/L)	Coefficient (*r*)	-0.35	-0.41	-0.33	-0.26	-0.24
	P-value	0.006*	0.001*	0.011*	0.049*	0.066
Albumin (mg/dL)	Coefficient (*r*)	0.37	0.38	0.30	0.29	0.38
	P-value	0.009*	0.007*	0.038*	0.040*	0.007*

*Correlation is considered statistically significant at *P*-value less than 0.05 (two-tailed)

Overexpression of *ATX* mRNA in peripheral blood leukocytes

ATX mRNA expression in peripheral blood leukocytes was significantly elevated in BA patients, as compared with healthy controls (*P*=0.0096) (Figure 13A). When disease severity was measured, advanced BA patients (severe fibrosis, jaundice, and a high AST value) had significantly higher relative *ATX* mRNA expression than early stage BA patients with mild fibrosis, non-jaundice, and a low AST value (*P*=0.0056, *P*=0.015, and *P*=0.022, respectively) and also healthy controls (*P*=0.0003, *P*=0.0003, and *P*=0.0001, respectively) (Figures 13B-D).

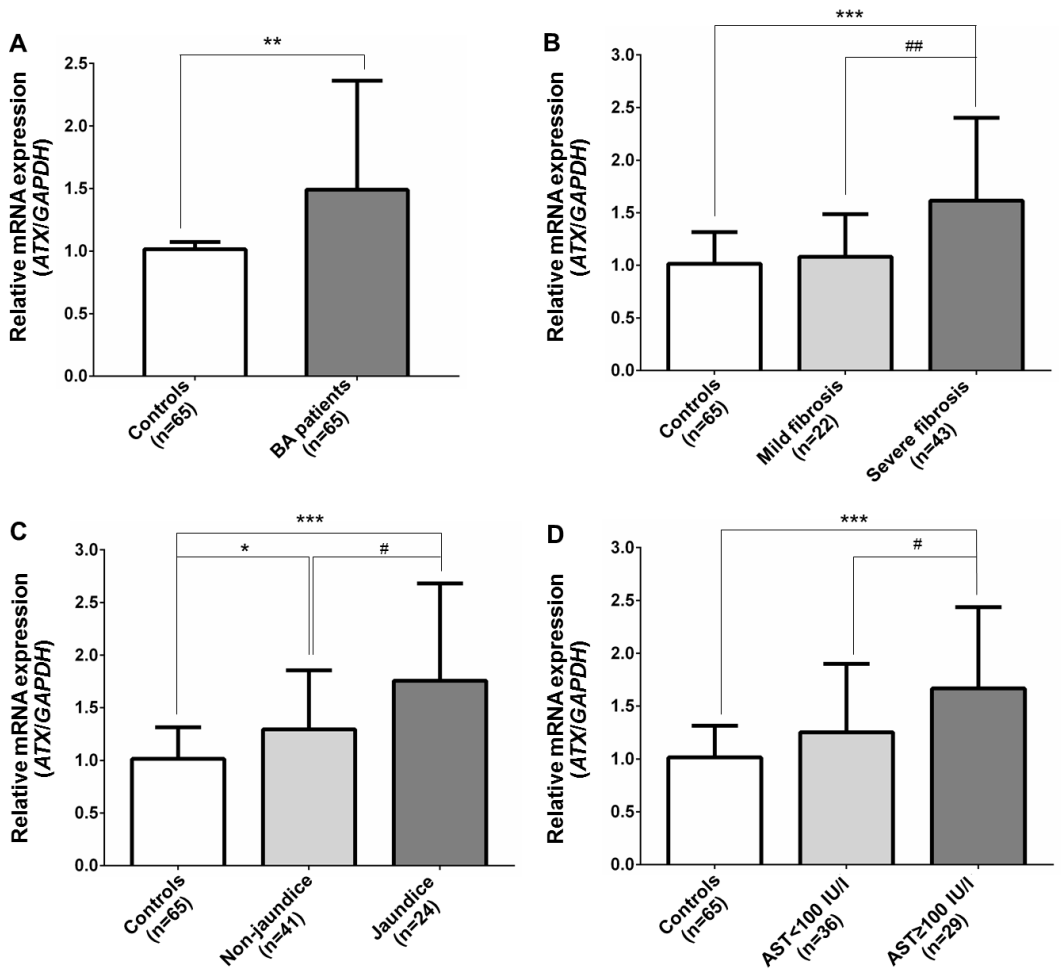

Figure 13. *ATX* mRNA expression in peripheral blood leukocytes of subjects among different groups. (A) Relative *ATX* mRNA expression in BA patients and healthy controls. (B) Relative *ATX* mRNA expression in BA patients with mild and severe fibrosis. (C) Relative *ATX* mRNA expression in BA patients with and without jaundice. (D) Relative *ATX* mRNA expression in early and late stage of hepatic dysfunction in BA patients. Expression was normalized using *GAPDH* as an internal control. Data are expressed as mean and standard deviation. **P*<0.05, ***P*<0.01, ****P*<0.001 vs control group and $^{\#}P$<0.05, $^{\#\#}P$<0.01 for comparisons between BA subgroups.

Subsequent analysis demonstrated positive relationships between the level of *ATX* mRNA expression and clinical parameters including liver stiffness (*r*=0.43, *P*=0.001),

TB (*r*=0.49, *P*<0.0001), AST (*r*=0.36, *P*=0.005), ALT (*r*=0.35, *P*=0.006), and ALP (*r*=0.47, *P*<0.0001) of BA patients. However, there was no significant correlation between relative *ATX* expression and albumin. We used linear regression to adjust these associations for confounding factors and revealed that upregulation of *ATX* expression was found to be associated with the severity of liver stiffness (β coefficient: 0.012, 95% CI: 0.004 to 0.021, *P*=0.006), AST values (β coefficient: 0.04, 95% CI: 0.002 to 0.007, *P*=0.002), and ALP values (β coefficient: 0.001, 95% CI: 0.000 to 0.002, *P*=0.006) (Table 9).

Table 9. Spearman's correlation and multivariate linear regression analysis of *ATX* relative expression estimates.

Variables	Relative mRNA expression (*ATX/GAPDH*)			
	Spearman's rho correlation		Linear regression[a]	
	Coefficient (*r*)	*P*-value	β coefficient (95% CI)	*P*-value
Age (years)	0.067	0.61	0.013 (-0.032 to 0.058)	0.57
Liver stiffness (kPa)	0.43	0.001*	0.012 (0.004 to 0.021)	0.006*
TB (mg/dL)	0.49	<0.0001*	0.15 (-0.025 to 0.094)	0.25
AST (IU/L)	0.36	0.005*	0.04 (0.002 to 0.007)	0.002*
ALT (IU/L)	0.35	0.006*	0.02 (-0.001 to 0.005)	0.13
ALP (IU/L)	0.47	<0.0001*	0.001 (0.000 to 0.002)	0.006*
Albumin (g/dL)	-0.19	0.20	-0.033 (-0.33 to 0.26)	0.83
ATX methylation levels (%)				
- Overall	-0.47	<0.0001*	-0.053 (-0.072 to -0.035)	<0.0001*
- CpG 1	-0.48	<0.0001*	-0.032 (-0.044 to -0.020)	<0.0001*
- CpG 2	-0.52	<0.0001*	-0.050 (-0.067 to -0.033)	<0.0001*
- CpG 3	-0.32	0.011*	-0.049 (-0.069 to -0.028)	<0.0001*
- CpG 4	-0.27	0.030*	-0.043 (-0.066 to -0.019)	0.001*

*Correlation is considered statistically significant at *P*-value less than 0.05 (two-tailed)

[a]The coefficient is adjusted for age and gender

Abbreviations: TB=total bilirubin; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=Alkaline phosphatase

Elevated serum ATX levels

The mean ATX concentration in BA patients was significantly higher than that in healthy controls (*P*=0.012), consistent with evidence from our recent study that serum ATX concentrations were elevated in BA patients (116). Furthermore, a positive association between relative mRNA expression and serum ATX was observed in BA individuals (*r*=0.44, *P*<0.0001).

Correlation between *ATX* methylation, its expression, and protein levels

We investigated associations between changes in DNA methylation, mRNA expression, and circulating protein levels of ATX in BA. The relative mRNA expression and serum ATX were both inversely correlated with overall *ATX* methylation levels (*r*=-0.47, *P*<0.0001 and *r*=-0.55, *P*<0.0001, respectively). Using linear regression model, we observed negative associations between *ATX* expression and overall *ATX* methylation level (β coefficient: -0.053, 95% CI: -0.072 to -0.035, *P*<0.0001) and also each of the four CpG sites, as follows: CpG 1 (β coefficient: -0.032, 95% CI: -0.044 to -0.020, *P*<0.0001), CpG 2 (β coefficient: -0.050, 95% CI: -0.067 to -0.033, *P*<0.0001), CpG 3 (β coefficient: -0.049, 95% CI: -0.069 to -0.028, *P*<0.0001), and CpG 4 (β coefficient: -0.043, 95% CI: -0.066 to -0.019, *P*=0.001) (Table 10). Subsequent analysis revealed that serum ATX levels were correlated with biochemical parameters in BA patients (Table 10).

Table 10. Spearman's correlation and multivariate linear regression analysis of serum ATX level estimates.

Variables	Serum ATX levels (ng/mL)			
	Spearman's rho correlation		Linear regression[a]	
	Coefficient (*r*)	*P*-value	β coefficient (95% CI)	*P*-value
Age (years)	-0.038	0.77	-6.43 (-36.85 to 23.99)	0.67
Liver stiffness (kPa)	0.71	<0.0001*	15.77 (11.13 to 20.42)	<0.0001*
TB (mg/dL)	0.63	<0.0001*	83.18 (49.06 to 117.30)	<0.0001*
AST (IU/L)	0.77	<0.0001*	5.15 (3.90 to 6.39)	<0.0001*
ALT (IU/L)	0.53	<0.0001*	3.15 (1.54 to 4.75)	<0.0001*
ALP (IU/L)	0.68	<0.0001*	1.46 (1.00 to 1.93)	<0.0001*
Albumin (g/dL)	-0.68	<0.0001*	-285.32 (-470.00 to -99.93)	0.003*
ATX methylation levels (%)				
- Overall	-0.55	<0.0001*	-33.80 (-46.42 to -21.18)	<0.0001*
- CpG 1	-0.61	<0.0001*	-21.11 (-29.17 to -12.95)	<0.0001*
- CpG 2	-0.46	<0.0001*	-29.86 (-41.63 to -18.09)	<0.0001*
- CpG 3	-0.34	0.006*	-28.66 (-43.14 to -14.18)	<0.0001*
- CpG 4	-0.42	0.001*	-28.64 (-44.38 to -12.91)	0.0001*
Relative mRNA expression (*ATX/GAPDH*)	0.44	<0.0001*	288.60 (129.36 to 447.85)	0.001*

*Correlation is considered statistically significant at *P*-value less than 0.05 (two-tailed)

[a]The coefficient was adjusted for age and gender

Abbreviations: TB=total bilirubin; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=Alkaline phosphatase

ATX promoter hypomethylation increased its expression in liver tissue samples

To determine whether *ATX* promoter methylation in genomic DNA reflects epigenetic alterations in liver tissue, we examined methylation levels of the *ATX* promoter in the liver tissues of 15 BA patients, compared with those of 5 non-BA controls. The total methylation status was significantly lower in BA livers than in control livers (*P*=0.033), consistent with methylation levels in peripheral blood leukocytes. The three CpG sites at the *ATX* promoter showed less methylation in BA livers, when compared to control livers (CpG 1: *P*=0.033, CpG 2: *P*=0.045, and CpG 3: *P*=0.047,

respectively), but there were no significant differences in methylation levels of the *ATX* promoter at CpG 4 in either group (Figure 14A).

Further analysis showed that *ATX* mRNA expression levels in BA livers were markedly higher than those in non-BA control livers. In quantitative real-time PCR, we also observed a significantly higher *ATX* expression in the BA livers than in the control livers ($P<0.05$) (Figure 14B). Moreover, ATX protein levels were significantly higher in the BA livers, when compared to the non-BA control livers ($P<0.05$) (Figure. 14C).

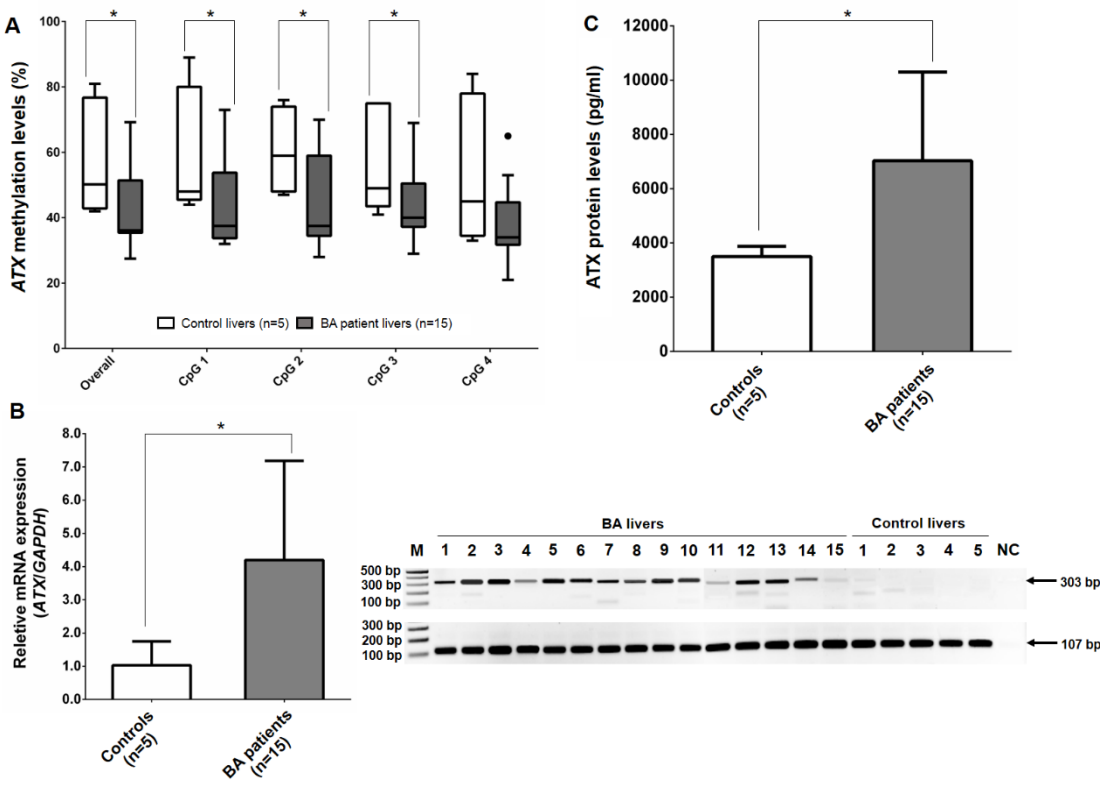

Figure 14. Distribution of the *ATX* promoter methylation, relative mRNA expression, and protein levels in liver tissue of BA patients and controls. (A) Decreased methylation levels of the *ATX* promoter in BA liver tissue samples. (B) Higher mRNA expression of *ATX* in BA cases and representative gel of ATX and GAPDH products from real-time PCR analysis. (C) Elevated *ATX* levels in liver tissue of BA patients. M, molecular weight marker, and NC, negative control. *$P<0.05$ vs control group.

Upregulated *DNMT1* expression in biliary atresia

To determine *DNMT1* expression that may be responsible for *ATX* promoter methylation, we conducted quantitative real-time PCR for *DNMT1* in peripheral blood leukocytes and liver tissue samples. Quantitative real-time PCR showed that relative *DNMT1* mRNA expression in peripheral blood leukocytes was significantly higher in BA patients than healthy controls ($P<0.05$) (Figure 15A). There were no significant differences in relative *DNMT1* expression between early stage BA patients (non-jaundice, mild fibrosis, and low AST value) and advanced BA patients (jaundice, severe fibrosis, and high AST value) ($P>0.05$). Furthermore, relative *DNMT1* mRNA expression in BA livers was significantly increased when compared with that in control livers as shown in Figure 15B ($P<0.01$). No correlation between relative *DNMT1* expression and *ATX* methylation status was found in BA livers. Subsequent analysis illustrated that *DNMT1* mRNA expression was inversely correlated with *ATX* methylation ($r=-0.37$, $P<0.0001$) (Figure. 15C) but *DNMT1* mRNA expression was positively correlated with *ATX* mRNA expression in peripheral blood leukocytes of BA patients ($r=0.51$, $P<0.0001$) (Figure. 15D).

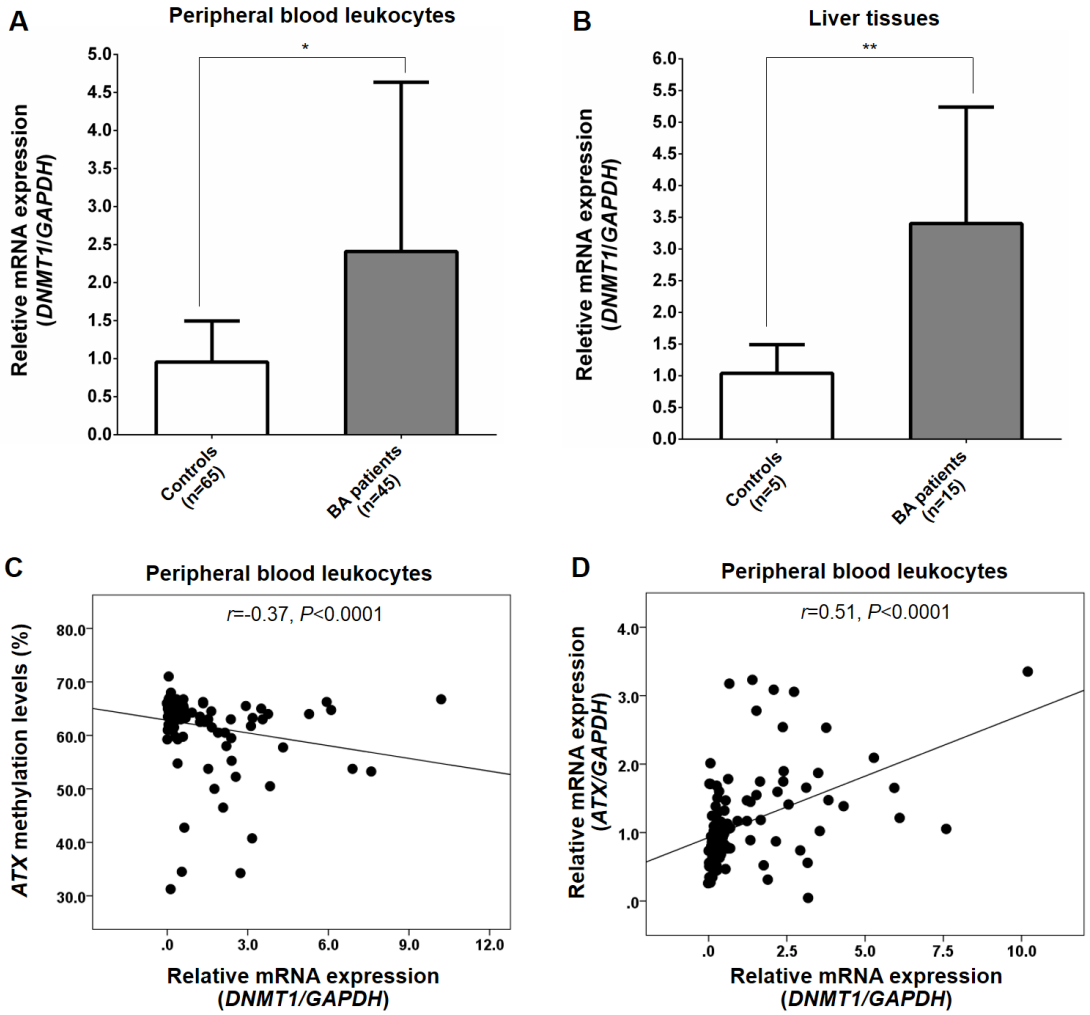

Figure 15. *DNMT1* mRNA expression in peripheral blood leukocytes and livers of BA patients and controls. (A) Relative *DNMT1* mRNA expression in peripheral blood leukocytes from BA patients and healthy controls. (B) Relative *DNMT1* mRNA expression in liver tissue samples from BA patients and healthy controls. (C) A negative correlation between relative *DNMT1* mRNA expression and *ATX* methylation in peripheral blood leukocytes from BA patients. (D) A positive correlation between relative *DNMT1* mRNA expression and relative *ATX* mRNA expression in peripheral blood leukocytes from BA patients. Data are expressed as mean and standard deviation. **P*<0.05, ***P*<0.01 vs control group.

ATX promoter hypomethylation as a possible biomarker

Additionally, we calculated the area under curve of the ROC curve, which was constructed using *ATX* methylation values. Based on the ROC curve, the optimal cutoff values of *ATX* methylation for overall, CpG 1, CpG 2, CpG 3, and CpG 4 as a possible marker for discriminating BA patients were projected to be 63.63, 63.50, 62.50, 63.50, and 63.50, respectively, which yielded the sensitivity of 81.60%, 63.20%, 81.60%, 65.80%, and 84.20% and the specificity of 60.00%, 63.10%, 51.60%, 50.80%, and 53.80%, respectively. The AUC of *ATX* methylation for overall, CpG 1, CpG 2, CpG 3, and CpG 4 were 0.79 (95% CI: 0.70 to 0.87, *P*<0.0001), 0.68 (95% CI: 0.57 to 0.78, P=0.003), 0.71 (95% CI: 0.61 to 0.81, *P*<0.0001), 0.68 (95% CI: 0.57 to 0.78, P=0.003), as well as 0.72 (95% CI: 0.62 to 0.82, *P*<0.0001), respectively (Figure 16A-E).

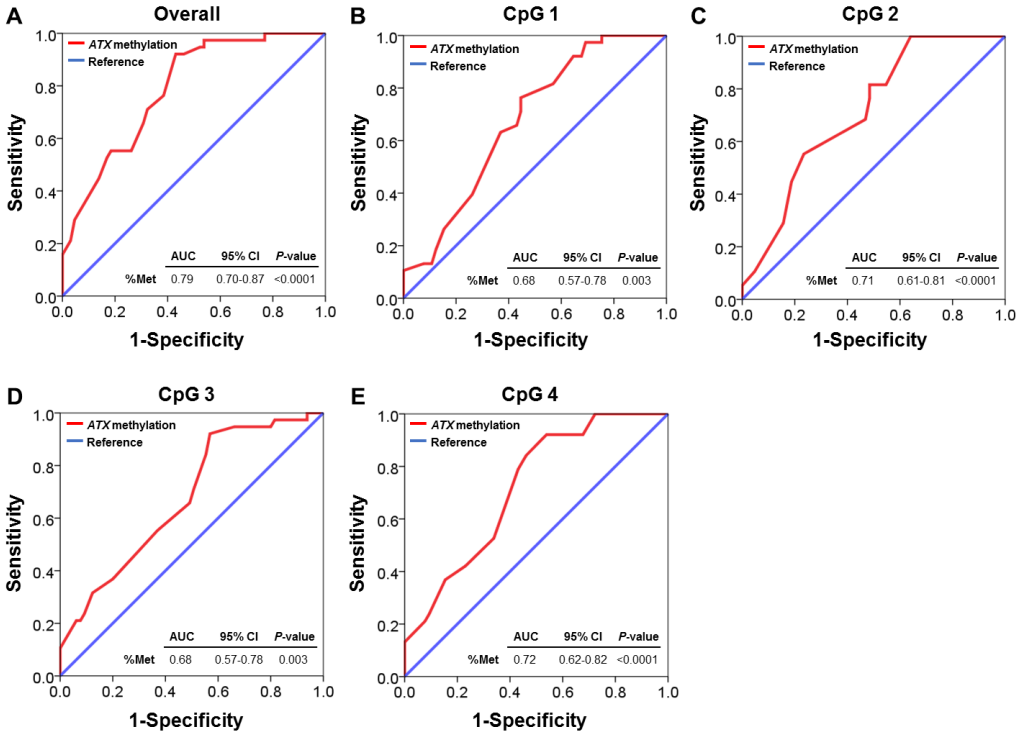

Figure 16. Receiver operating characteristic (ROC) curve representing diagnostic value of *ATX* promoter methylation in BA patients. (A) Overall *ATX* promoter methylation. (B) *ATX* promoter methylation at CpG 1 site. (C) *ATX* promoter methylation at CpG 2 site. (D) *ATX* promoter methylation at CpG 3 site. (E) *ATX* promoter methylation at CpG 4 site.

Discussion

The findings of this study add to an emerging body of literature that has investigated and reported on the molecular processes in peripheral blood leukocytes and liver tissues of individuals affected by biliary atresia. In this study, we demonstrated the presence of reduced DNA methylation at four CpGs within the promoter region of *ATX* in peripheral blood leukocytes and liver tissues of BA individuals. We also found that DNA hypomethylation of the *ATX* promoter might be responsible for elevated *ATX* mRNA expression. Notably, *ATX* expression was significantly more abundant in BA patients than in controls. In addition, upregulated *ATX* expression was observed in BA patients with fibrosis, as compared to BA patients without fibrosis. Data from our previous study provided evidence that increased serum ATX levels in BA patients were significantly correlated with the severity of BA (116). We demonstrated the presence of higher *ATX* expression and protein levels in liver tissues of BA patients. Taken together, we found that higher *ATX* mRNA expression and protein levels were inversely correlated with hypomethylation of the gene promoter. The present study provides further evidence of the primary role that epigenetic modifications assume in influencing level of gene expression in biliary atresia.

To the best of our knowledge, this is the first study to report data regarding the potential epigenetic regulation of the *ATX* gene. We showed that specific CpGs within the *ATX* promoter were hypomethylated in BA patients, which was supported by significantly elevated *ATX* expression and a corresponding increase in ATX protein levels. To validate these findings, we also compared DNA methylation in BA livers with control livers and found CpGs within the *ATX* promoter to have lower methylated DNA in BA livers, which is consistent with the findings observed in peripheral blood leukocytes. The *ATX* promoter hypomethylation correlated negatively with the severity of clinical parameters and hepatic fibrosis (TB, AST, ALP, and liver stiffness) but positively with hepatic protein synthesis (albumin), suggesting that epigenetic mechanisms could play a possible role in the regulation of *ATX* expression regarding hepatic dysfunction and/or hepatic fibrosis. It seems plausible that the selected

promoter regions of *ATX* contain the coding sequence, thus affecting the transcription of the *ATX* gene. However, there was no relationship between *ATX* methylation at the CpG 4 residue and clinical outcome in BA. We speculate that the CpG 4 residue might not contain the coding sequence related to the transcription of the *ATX* gene, leading to no association of *ATX* promoter methylation at the CpG 4 site with outcome parameters in postoperative BA patients.

The current study also revealed that *DNMT1* mRNA expression in peripheral blood leukocytes and liver tissues was significantly elevated in BA patients compared with that in the controls. Further analysis showed that *DNMT1* mRNA expression was negatively associated with *ATX* methylation status but *DNMT1* mRNA expression was positively associated with *ATX* mRNA expression in peripheral blood leukocytes. The explanation for increased *DNMT1* mRNA expression in BA remains obscure. It might be related to the spectrum of the tested DNMTs and the detection method. The enzymatic activity of DNMT1 is controlled by both posttranscriptional and posttranslational mechanism (125). In the present study, we investigate the association between *ATX* methylation status and *DNMT1* expression, but not with the enzymatic activities. Therefore, it is likely that the activity (and not the amount) of the enzyme is more important in BA. Additional research will be needed to determine the association of enzymatic activity of *DNMT1* with the *ATX* methylation status.

Subsequent analysis demonstrated that BA patients had a significantly lower *ATX* methylation status and higher expression of *DNMT1* mRNA. The elevated *DNMT1* mRNA expression involved in *ATX* hypomethylation in BA patients is still unclear. DNA hypomethylation and higher expressions of *DNMTs* have been observed in chronic hepatitis, cirrhosis, and hepatocellular carcinoma (126). This suggested a feedback mechanism of DNMTs on the methylation status. The increased *DNMT1* mRNA expression could be attributed to an indirect response to *ATX* hypomethylation in BA patients. It is postulated that the DNMT activity is itself regulated partially by DNA methylation status which represent a feedback mechanism. Accordingly, we suggest that *ATX* promoter methylation might depend upon several factors, including *DNMT*

mRNA expression, DNMT activity, DNMT protein expression, and transcript levels of other enzymes involved in the DNA methylation. Furthermore, the promoter methylation and expression of *ATX* could depend on multiple pathways and molecules except the DNMT1 function only.

Our findings support prior evidence that DNA methylation is an important determinant of BA (73), as well as a stimulator of stellate cells and progressive hepatic fibrosis in animal models (127). Nevertheless, no direct investigation of gene-specific methylation involving liver fibrosis in BA has been demonstrated. ATX has been shown to affect hepatic fibrogenesis, which has been implicated in the pathogenesis of liver fibrosis in BA, especially the stimulation of hepatic stellate cell proliferation via its enzymatic product, LPA (12). Since serum levels of ATX and LPA have been correlated with the development of liver fibrosis (57), upregulated *ATX* expression might be associated with the severity of BA. In addition, our study showed significantly higher liver *ATX* mRNA expression in BA patients than in controls. This is consistent with previously reported observations demonstrating that *ATX* was more highly expressed in liver tissue of patients with HCC (59). Wu et al. reported extensive evidence of *ATX* overexpression associated with progression of inflammation and liver cirrhosis in HCC patients (103), which supports our findings of upregulated *ATX* expression in BA patients with severe fibrosis. In recent years, elevated circulating ATX has been documented in patients with other liver diseases including chronic hepatitis C virus (HCV) infection, HCV-associated fibrosis, and cirrhosis (15, 57, 104). Moreover, serum ATX was increased in hepatocellular carcinoma with liver fibrosis, nonalcoholic fatty liver disease, and cholestatic disorders (110, 117, 128). *ATX* expression was also shown to be augmented in chronic cholestatic diseases such as primary biliary cholangitis and primary sclerosing cholangitis (129). These findings lead us to hypothesize that ATX might serve as a possible parameter reflecting the severity of liver diseases including biliary atresia.

Currently, BA is accepted as a heterogeneous disease, with various forms of clinical presentation. The clinical manifestation of postoperative BA patients may

reveal striking heterogeneity with a spectrum ranging from mild cases with early stages of liver fibrosis to severe cases with advanced stages of liver fibrosis. In the present study, promoter hypomethylation and overexpression of *ATX* varied between BA patients and, perhaps, between different stage of liver fibrosis. The possible explanation for this observation could be that *ATX* mRNA expression might be lower in the early stage liver fibrosis, and *ATX* expression may continuously increase during the disease progression. Additionally, the variation of hepatic *ATX* expression could be ascribed to the heterogeneity of liver fibrosis, with different stages being present in different areas of the BA livers. The etiology of BA remains unknown and theories of pathogenesis include viral infection, defects in bile duct development, genetics, and toxic factors [2-4]. It is also tempting to speculate that epigenetic factors may modulate the phenotypic manifestations of BA. Moreover, a number of growth factors and cytokines involved in the liver fibrosis remain largely unexplored. Autotaxin may act in concert with many other pro-inflammatory and profibrogenic cytokines and growth factors, which contribute to the liver fibrogenesis in BA.

Although the precise origin and fate of elevated circulating ATX levels remains unknown, both human and animal studies suggest that ATX is metabolized by the liver (53). High serum ATX might result from decreased ATX clearance, increased expression, or a combination of both. A reduction in ATX clearance may result from diminished uptake by liver sinusoidal endothelial cells (105). Yet ATX activity in liver disease is closely related with liver function, as ATX clearance is impaired when liver function failed in the case of BA, and other liver diseases (53, 110). This mechanism may result in the high expression levels of ATX in BA. In the present study, we also found a positive association between mRNA expression and circulating protein levels of ATX in BA patients. Thus, it is speculated that a factor capable of increasing ATX expression (or reducing its clearance) in BA may be caused or created by phenotypic changes in liver sinusoidal endothelial cells during liver fibrosis.

This study suggests the possible involvement of ATX on liver fibrosis in BA. The aberrant production of ATX may lead to the altered activation of LPA signal

transductions through G-protein coupled LPA receptors including, but not limited to, activation of Rho, Ras, phosphoinositide 3-kinase (PI3K) signaling pathways. LPA was shown to stimulate the proliferation and contraction of hepatic stellate cells and inhibit the apoptosis of these cells in vitro through Rho/Rho kinase activation, suggesting that LPA could be profibrogenic in liver (56). LPA acts on its own G-protein coupled receptors and thereby elicit multiple cellular responses, such as Rho/Rac-regulated cell migration, Ras-mediated cell proliferation, and PI3K-mediated cell survival (60). Accordingly, the aberrant expression of ATX along with the consequently abnormal production of LPA in the liver microenvironment may fuel the progression of liver fibrosis in biliary atresia.

It should be noted, however, that we are aware of some inherent limitations. First, the cross-sectional design prevents determination of causal relationships, and the potential for confounding variables cannot be dismissed. To further address this question of cause or consequence, prospective cohort or experimental studies will help to elucidate the effect of epigenetic changes on ATX expression and the risk of BA. Secondly, the number of cases and controls in our study was relatively small, especially regarding the effect of epigenetic changes in ATX produced in liver tissues. This factor diminishes the statistical power and generalizability of our results. It is recommended that future research should be conducted on a large scale using multicenter studies to further address how increased *ATX* expression may influence the pathogenesis in BA patients. Lastly, incomplete assessment of possible confounding variables including medical comorbidities needs to be taken into consideration.

Conclusion

This study revealed that hypomethylation of CpG islands within the promoter region of the *ATX* gene was associated with overexpression of *ATX* mRNA and protein levels, which might play a plausible role in the pathogenesis of liver fibrosis in BA. Our results demonstrate a possible correlation between *ATX* methylation and levels of

ATX transcription and translation. These findings imply that the epigenetic aberrance of *ATX* promoter hypomethylation status might contribute to liver fibrosis and has been hypothesized to be a possible biomarker for monitoring the progression of liver fibrosis in postoperative biliary atresia. Further validation with prospective studies is necessary to determine the utility of ATX as a biomarker for the risk of liver fibrosis in BA. Given the established role of ATX in promoting liver fibrosis, additional studies of potential underlying mechanisms related to the effect of ATX on the pathogenesis of liver fibrosis in BA are warranted.

CHAPTER VI

TELOMERE LENGTH IN PERIPHERAL BLOOD LEUKOCYTES IS ASSOCIATED WITH SEVERITY OF BILIARY ATRESIA

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Abstract

Objective: The purpose of this study was to investigate the association of telomere length in peripheral blood leukocytes with the severity of biliary atresia (BA).

Methods: One hundred and fourteen BA patients and 114 age-matched healthy controls were enrolled. Relative telomere length (RTL) was assessed using a quantitative real-time polymerase chain reaction. Multivariate regression analysis was used to estimate RTL as an independent risk factor of BA. Receiver operating characteristic curve analysis was used to calculate the accuracy of biomarkers in the prediction of liver cirrhosis.

Results: BA patients had significantly shorter telomeres than healthy controls ($P<0.0001$). The RTL in BA patients with jaundice was considerably lower than that of patients without jaundice ($P=0.005$). Moreover, RTL was markedly shorter in patients with cirrhosis (F4), as compared to patients with mild fibrosis (F2) and non-fibrosis (F0-F1, $P<0.0001$). Logistic regression analysis indicated that short RTL was associated with a higher risk of liver cirrhosis in BA. Tertile analysis showed a dose-response effect for this association (P trend<0.0001). Additionally, RTL in BA children revealed a negative correlation with age ($r=-0.50$, $P<0.001$). We noted an association between reduction of RTL and liver stiffness scores, adjusted for age and gender ($b=-0.01$, $P<0.0001$). Short RTL can be employed to distinguish cirrhosis patients from non-cirrhosis patients (area under curve=0.78). Further analysis showed a linear correlation between leukocyte RTL and liver RTL in BA patients ($r=0.83$, $P<0.001$).

Conclusion: The findings of this study provide evidence that telomere shortening is associated with an elevated risk of liver cirrhosis in BA.

Introduction

Biliary atresia (BA), the most common cause of cholestatic liver disorder in infants, is characterized by progressive fibrosclerosing cholangiopathy affecting the extra- and intrahepatic biliary ducts. BA patients who experience obstruction of bile flow suffer persistent jaundice, acholic stools, hepatomegaly, and/or splenomegaly. If left untreated, the majority of BA children will develop chronic liver disease (severe hepatic fibrosis, biliary cirrhosis, and liver failure) and most likely die by the age of 2 years (1). Kasai portoenterostomy, the first-line intervention for infants with BA, reestablishes bile flow to the gastrointestinal tract. Liver transplantation is another treatment option in cases where Kasai portoenterostomy fails or is not practical (130). The precise etiology and pathophysiology of BA remains elusive. Environmental factors may be a cause of BA in a genetically susceptible individual during early infancy. If this is the case, variants of genes playing a role in hepatobiliary development or immunological tolerance tend to be candidates for mediating susceptibility. Moreover, evidence supporting the role of genetic factors as a cause of BA has been accumulating for a number of years (131, 132). In addition to results from epidemiological studies, polymorphism studies, and data on twins, the concept of shortened telomere length as a genetic risk factor for liver fibrosis and BA has been proposed.

Telomeres, which are located at the ends of chromosomes, consist of repetitive DNA sequences of TTAGGG and related proteins of crucial importance for telomere function. Telomeres help maintain genomic integrity and stability by shielding chromosome ends from deterioration, fusion, and atypical recombination (17). The telomere length shortens each time cells divide, because DNA polymerases are not capable of completely replicating chromosomes during cell division. This is commonly referred to as the end-replication problem. This alteration in telomere length precipitates capping function losses at the chromosomal ends, leading to DNA damage program activation, which contributes to senescence, apoptosis, and neoplastic transformation (18). As such, telomere length is an indicator of the biological age of a cell.

There is also emerging evidence that describes an association between attrition of telomere length and several human pathologies (81, 82), including a variety of cancers and chronic liver disorders, such as liver hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (83-85). These findings strongly suggest telomere shortening in the development of liver cirrhosis. Accordingly, evaluation of telomere length may serve as a feasible and reliable non-invasive indicator for determining the risk and prognosis of BA. In support of this proposed causal relationship, a previous study demonstrated telomere shortening in liver tissues of BA patients at the time of liver transplantation (86). Until now, no report has specifically examined the relationship between telomere length in peripheral blood leukocytes and biochemical parameters in BA patients, particularly by considering DNA from leukocytes as a non-invasive biomarker. This proposed method would provide a cost-effective and time-saving alternative, as peripheral blood leukocytes are easier to collect and evaluate than liver tissue.

In this study, quantitative real-time polymerase chain reaction (PCR) was used to compare and evaluate telomere length in patients with BA and age-matched healthy controls. We hypothesized that shortened telomere length could be positively correlated with increased severity of BA. To prove this hypothesis, we investigated telomere length in peripheral blood leukocytes from both BA patients and age-matched healthy controls and evaluated the association between telomere length and clinical parameters of BA patients.

Materials and Methods

Study population

This cross-sectional analytical study was composed of 114 patients with BA (66 females and 48 males) and 114 healthy age-matched controls (64 females and 50 males). BA patients who came for the follow-up visit to the Pediatric Liver Clinic were enrolled. All BA patients were diagnosed using intra-operative cholangiography and were surgically treated with original Kasai portoenterostomy. BA children who had undergone liver transplantation were excluded from this study. Age-matched

unaffected volunteers who had normal physical findings and no underlying disease were included as the controls. In addition, two pairs of monozygotic twins with BA discordance (one set of whom suffered from BA) were recruited for this investigation. The BA children were stratified in terms of bile flow establishment according to serum total bilirubin (TB) into a non-jaundice group (TB<2 mg/dL) and a persistent jaundice group (TB≥2 mg/dL). Based on the severity of liver fibrosis (liver stiffness values), BA patients were also divided into four groups: non-fibrosis (F0-F1: 0–7.2 kPa), mild fibrosis (F2: 7.3–9.7 kPa), severe fibrosis (F3: 9.8–17.2 kPa), and cirrhosis (F4: ≥17.3 kPa), as previously described by Corpechot et al. (133).

The liver function tests including serum albumin, total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were performed by a Hitachi 912 Chemistry Analyzer at the central laboratory of our hospital. Blood samples from every participant were drawn using ethylenediaminetetraacetic acid for anticoagulation. After centrifugation at 4,000 rpm for 10 minutes, the blood was separated into plasma and leukocytes. The plasma and leukocytes were stored at -80°C until further analysis. Due to the availability to collect liver tissue of BA patients in some cases, we obtained only 6 liver tissue samples and matched DNA samples from peripheral blood leukocytes of BA patients. All liver tissue samples were immediately frozen and stored at -80°C for further measurement.

The protocol for this study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB number 279/57). This study was conducted in accordance with the ethical standards outlined in the 1975 Declaration of Helsinki. All participants, parents, or legal guardians were fully informed regarding the study protocol and procedures prior to participating in the study. Written informed consent was obtained from the participants' parents upon informing them about the protocol and procedures involved in the research.

Measurement of telomere length

Telomere length in genomic DNA extracted directly from peripheral blood leukocytes and liver tissue according to the instruction of DNA extraction kit (GE Healthcare, Buckinghamshire, UK) was measured by applying a quantitative real-time PCR method, as previously described by Cawthon et al. (134). Telomere length was measured according to the ratio of the telomere repeat copy number (T) to the single-copy gene copy number (S) in each given sample. The single-copy gene refers to the 36B4 gene, which encodes the acid ribosomal phosphoprotein (PO). The ratio (T/S) is proportional to the average telomere length. DNA samples were amplified in 10 µl PCR reactions with StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used for *the telomere repeat copy number* and *the single-copy gene copy number* amplification were as follows: telomere forward 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'; telomere reverse 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3,; single-copy gene forward 5'-CAGCAAGTGGGAAGGTGTAATCC-3'; and, single-copy gene reverse 5'-CCCATTCTATCATCAACGGGTACAA-3'. Both PCRs were activated in a final volume of 10 µL that contained SYBRGreen Master Mix none-ROX (2x) (RBC Bioscience, Taipei, Taiwan), 3.12 ng of DNA template, and 0.5 nM of telomere primers or 0.5 nM of single-copy gene primers. The thermal cycling profile for both telomeres and single copy genes started with 95°C incubation for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 54°C. All amplification specificity was regulated by employing melting curve analysis. In each sample, the quantity of telomere repeats and the quantity of single-copy genes were normalized to a reference DNA. The same reference DNA sample (from a single individual) was included in each measurement to control inter-assay variability.

Liver stiffness assessment

The assessment of liver stiffness was performed on the same day as blood sampling. Transient elastography determined the liver stiffness between 25 and 65 mm from the skin surface. The measurements were performed by placing a transducer

probe of Fibroscan (EchoSens, Paris, France) on the intercostal space at the area of the right lobe of the liver. Measurements were then performed until 10 validated results were obtained with a success rate of at least 80%. The median value of 10 validated scores represented the elastic modulus measurement of the liver and it was expressed in kilopascals (kPa).

Statistical analysis

Statistical analyses were performed with the SPSS statistical package, version 20.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test and quantile-quantile plot were used to assess whether relative telomere length (RTL) was normally distributed. Comparisons between means were evaluated by Student's *t*-test, while the Mann-Whitney *U* test and Kruskal-Wallis H test were employed for comparison of abnormally distributed continuous variables. Spearman's rank correlation coefficient test was used to define the relationship between telomere length and age. The associations of RTL with the risk of BA were measured by applying univariate and multivariate logistic regression analyses to determine the roles of confounding factors. Receiver operating characteristic (ROC) curves were constructed to evaluate the specificity and sensitivity of predicting cirrhosis using RTL values, and the area under curve (AUC) was calculated. Data are presented as mean±standard error of the mean. For all statistics, a *P*-value less than 0.05 (based on a two-tailed test) was considered statistically significant.

Results

Characteristics of the study participants

The baseline characteristics of the 114 BA patients and 114 unaffected volunteers are summarized in Table 11. Participants were age-matched between BA patients and healthy controls. Although the number of females was higher than males in both controls and BA patients, there was no significant difference. As expected, liver stiffness values in BA patients were substantially higher than those in controls

(*P*<0.0001). In addition, there were significantly higher serum AST and ALT levels in BA patients than in controls (*P*<0.0001).

Table 11. Clinicopathologic characteristics of biliary atresia patients and age-matched healthy controls.

	BA patients (n=114)	**Controls (n=114)**	***P*-value**
Age (years)	8.95±0.45	8.95±0.45	NS
Gender (female:male)	66:48	64:50	NS
Albumin (g/dL)	4.04±0.09	-	NA
Total bilirubin (mg/dL)	2.72±0.37	-	NA
AST (IU/L)	117.92±9.27	26.66±0.82	<0.0001
ALT (IU/L)	97.23±8.38	9.24±0.65	<0.0001
ALP (IU/L)	421.00±29.74	-	NA
Liver stiffness (kPa)	32.78±2.38	4.01±0.19	<0.0001

Abbreviations: BA=biliary atresia; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=alkaline phosphatase; NS=not significant; NA=not available

Relative telomere length distribution in the study subjects

We investigated telomere length in leukocytes from the BA group and the unaffected controls. Overall, the RTL in leukocytes was significantly lower in BA children compared to healthy controls (*P*<0.0001), as shown in Figure 17A. Given that telomere length is age-related, we classified the subjects of both groups into 3 age categories (3 to 8 years, n=60; 9 to 14 years, n=40; and, 15 to 21 years, n=14). A significantly shortened telomere length could be found in BA patients within each of the 3 age categories, as compared to the control group (*P*=0.020, *P*<0.0001, and *P*<0.0001, respectively), as presented in Figure 17B.

In stratified analysis according to jaundice status, BA patients were divided into persistent jaundice and non-jaundice groups (Table 12). Interestingly, the RTL in BA patients with persistent jaundice was markedly shorter than that in BA patients without jaundice (*P*=0.005). Furthermore, there was a significant difference in RTL between BA patients with jaundice and healthy controls (*P*<0.0001). We also found that BA patients without jaundice had markedly shorter telomere length than unaffected volunteers (*P*<0.0001) (Figure 17C).

Table 12. Clinicopathologic characteristics of biliary atresia patients with and without jaundice.

	BA patients (n=114)		***P*-value**
	Non-jaundice (n=77)	**Jaundice (n=37)**	
Age (years)	8.38±4.32	9.31±0.94	NS
Gender (female:male)	46:31	20:17	NS
Albumin (g/dL)	4.14±0.11	3.78±0.12	0.032
Total bilirubin (mg/dL)	0.77±0.13	7.05±0.74	<0.0001
AST (IU/L)	90.87±19.24	186.13±19.24	<0.0001
ALT (IU/L)	94.76±10.04	131.97±12.59	0.024
ALP (IU/L)	358.64±31.62	586.68±58.17	0.001
Liver stiffness (kPa)	26.93±2.78	45.44±3.83	<0.0001

Abbreviations: BA=biliary atresia; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=alkaline phosphatase; NS = not significant

We further explored telomere length in leukocytes from a subgroup of BA patients according to liver stiffness value (F0-F1: 0–7.2 kPa, n=15; F2: 7.3–9.7 kPa, n=17; F3: 9.8–17.2 kPa, n=18; and, F4: >17.2 kPa, n=69). Table 13 illustrates the clinical characteristics of the BA subgroups based on the severity of liver fibrosis. BA children with cirrhosis (F4) had significantly greater telomere shortening than both patients with mild fibrosis (F2, *P*<0.0001) and patients without liver fibrosis (F0-F1, *P*<0.0001).

However, RTL did not differ among BA children with severe fibrosis (F3) and other stages (Figure 17D).

Table 13. Clinicopathologic characteristics of biliary atresia patients with non-fibrosis, mild fibrosis, severe fibrosis, and liver cirrhosis.

	BA patients (n=114)				***P*-value**
	F0-F1 (n=15)	**F2 (n=17)**	**F3 (n=18)**	**F4 (n=64)**	
Age (years)	7.33±0.74	8.52±0.88	8.17±1.13	9.62±0.67	NS
Gender (female:male)	12:3	6:11	10:8	38:26	NS
Albumin (g/dL)	3.77±0.35	3.90±0.29	4.16±0.24	4.13±0.08	NS
TB (mg/dL)	1.39±0.86	1.34±0.65	1.46±0.5	3.77±0.55	0.015
AST (IU/L)	68.53±16.75	78.76±16.91	100.94±16.93	147.67±13.97	0.002
ALT (IU/L)	59.26±14.38	74.52±15.03	91.06±13.67	128.46±12.5	0.006
ALP (IU/L)	300.26±42.75	292.47±53.97	386.23±77.22	501.49±43.92	0.01
Liver stiffness (kPa)	5.56±0.19	8.75±0.17	14.17±0.54	50.57±2.53	<0.0001

Abbreviations: BA=biliary atresia; TB=total bilirubin; AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=alkaline phosphatase; NS=not significant.

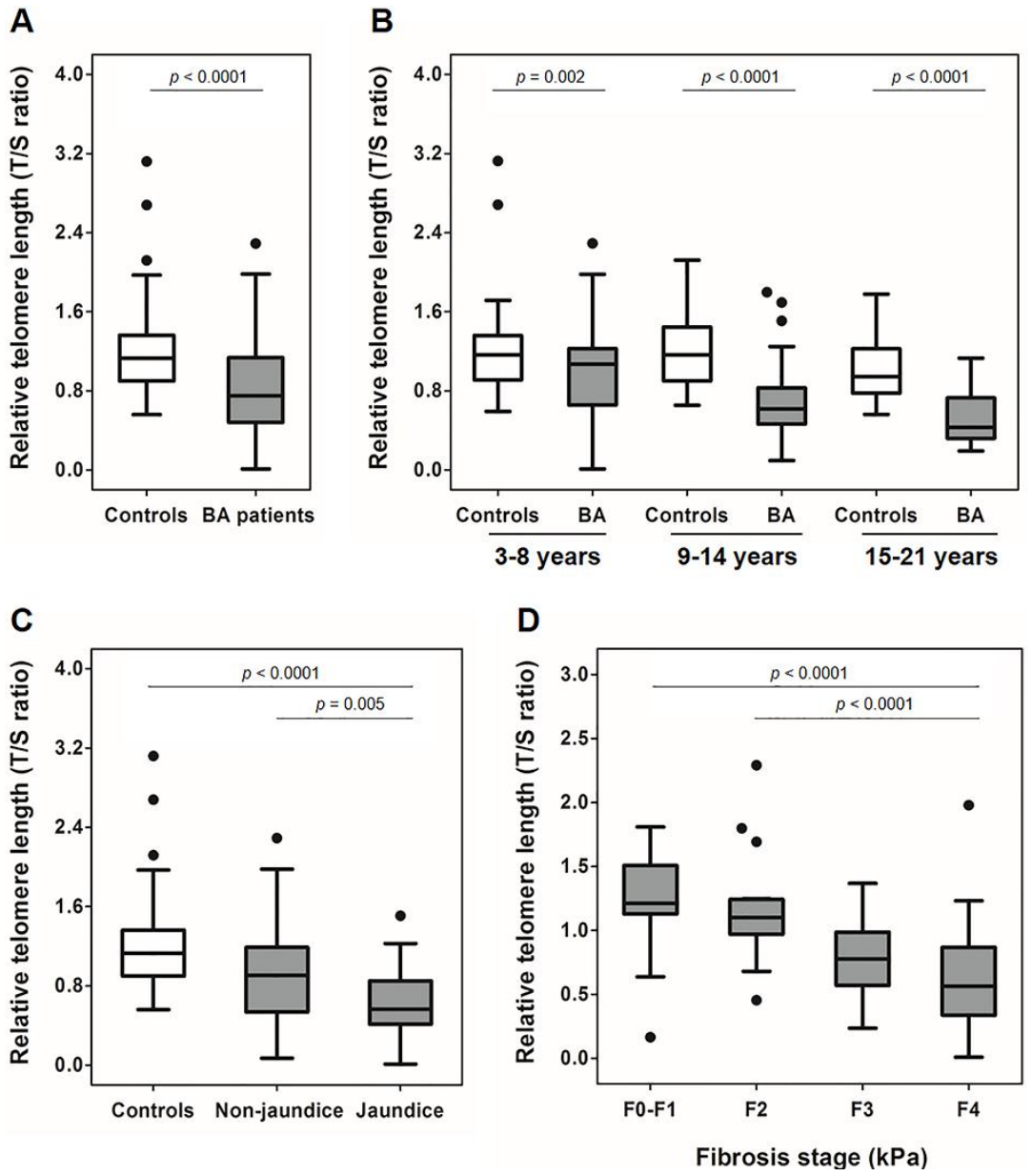

Figure 17. Box-plot illustrating telomere length distribution in subjects among different groups. The line through the middle of the boxes represents the median of T/S value and the top and bottom of each box represents the first and third quartiles. The lower and upper error bars are computed as the lower and upper quartiles, respectively. (A) Relative telomere length in BA patients and healthy controls; (B) Relative telomere length in BA patients and controls, according to age group; (C) Relative telomere length in patients with and without jaundice; (D) Relative telomere length in BA subgroups, including non-fibrosis (F0-F1), mild fibrosis (F2), severe fibrosis (F3), and liver cirrhosis (F4).

Relative telomere length in twins discordant for biliary atresia

Subsequently, we examined telomere length in two sets of twins with discordant pathology in BA. Set 1: the patient is a nine-year-old girl who was diagnosed to have BA, with her twin sister being born healthy and remaining so to date. The RTL was found to be shorter in the BA twin, as compared to her healthy sister that served as the control group (T/S ratio: 0.80 vs. 1.82, respectively). Set 2: a case of 19-year-old twin women, one of whom suffers from BA. Her twin sister has remained healthy with normal liver function tests. We also observed that the BA twin had a shorter telomere length than her twin sister without BA (T/S ratio: 0.13 vs. 0.26, respectively), as demonstrated in Figure 18.

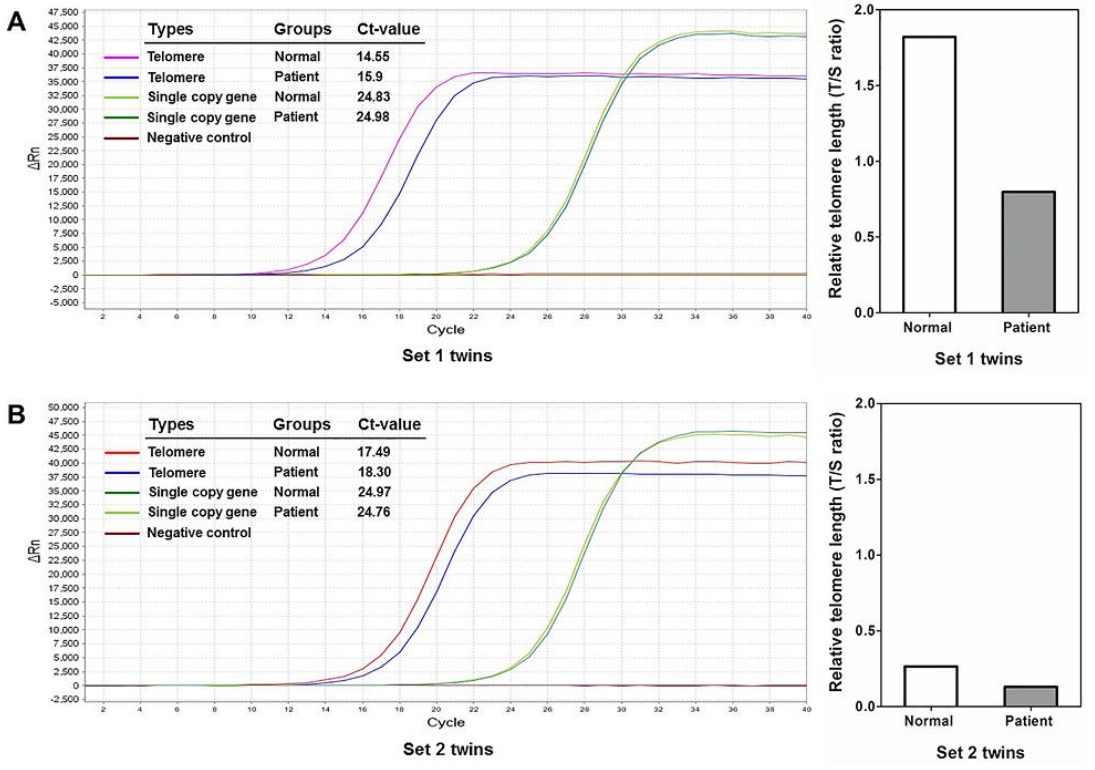

Figure 18. Telomere length assessment finding in two sets of twins by BA discordance. (A) Amplification plot and relative telomere length analysis in nine-year-old twin girls who were discordant for BA (set 1); (B) Amplification plot and relative telomere length analysis in nineteen-year-old twin women affected by BA discordance (set 2).

Short relative telomere length and increased risk of BA

Since telomere length is also influenced by age and gender, we employed logistic regression analysis to control the role of confounding variables. After adjusting for age and gender, RTL in childhood BA was substantially shorter than that of the controls by an average of 0.089 units (95% CI: 0.038 to 0.21, $P<0.0001$). The RTL of participants were separated into short RTL and long RTL groups, based on the median distribution of RTL in healthy controls. As shown in Table 14, patients with short RTL had a significantly elevated risk of BA, as compared to patients with long RTL in both univariate (unadjusted OR: 3.07, 95% CI: 1.75 to 5.39, $P<0.0001$) and multivariate analysis (adjusted OR: 3.25, 95% CI: 1.82 to 5.81, $P<0.0001$). We further categorized study subjects into three groups according to the tertile of RTL values in controls and investigated a significant dose-response association between short RTL and higher risk of BA. Specifically, using the third tertile (longest) as the reference group, the odds ratios (OR) for the first and second tertiles were 5.68 (95% CI: 2.65 to 12.16, $P<0.001$) and 2.53 (95% CI: 1.14 to 5.64, $P=0.023$), respectively, in unadjusted univariate analysis and 6.15 (95% CI: 2.82 to 13.42, $P<0.0001$) and 2.54 (95% CI: 1.14 to 5.67, $P=0.023$), respectively, in multivariate analysis. The *P* trend was less than 0.0001 in both analyses, suggesting quite strong evidence for a dose-response effect of short RTL-related higher risk of BA.

Table 14. Logistic regression analysis of association between relative telomere length and risk of biliary atresia.

RTL	BA	Controls	Unadjusted	*P*-value	Adjusted[a]	*P*-value
			OR (95% CI)		OR (95% CI)	
Overall	114	114	0.11 (0.048-0.24)	<0.0001	0.089 (0.038-0.21)	<0.0001
By median						
Short	86	57	3.07 (1.75-5.39)	<0.0001	3.25 (1.82-5.81)	<0.0001
Long	28	57	1 (reference)		1 (reference)	
By tertile						
1st tertile	70	38	5.68 (2.65-12.16)	<0.0001	6.15 (2.82-13.42)	<0.0001
2nd tertile	33	38	2.53 (1.14-5.64)	0.023	2.54 (1.14-5.67)	0.023
3rd tertile	11	38	1 (reference)		1 (reference)	
P trend				<0.0001		<0.0001

Abbreviations: BA = biliary atresia; RTL = relative telomere length

[a]Adjusted for age and gender

Association between telomere length and clinical characteristics

The association between RTL and age in BA patients and healthy controls is shown in Figure 19. As expected, no association between age and RTL was observed in healthy controls (r=-0.12, P=0.20), while the RTL in BA patients showed an inverse association with age. There was a significant relationship between RTL and age, with RTL being longer in younger patients (r=-0.50, P<0.001). We then performed multiple linear regression analysis, adjusting for age and gender to estimate the interaction between RTL and biochemical variables (Table 15). Interestingly, liver stiffness was found to be associated with a reduction in relative telomere length after adjusting for age and gender (b=-0.01, P<0.0001).

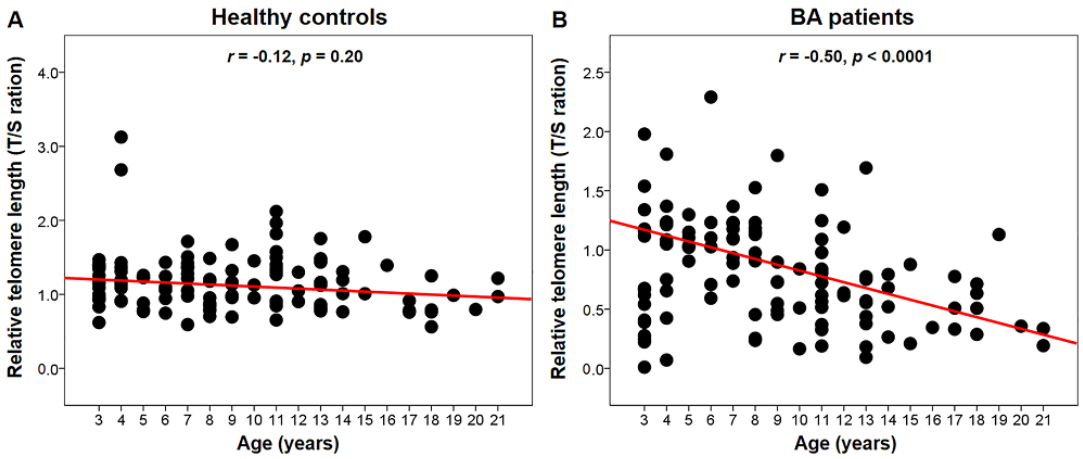

Figure 19. Scatter plot demonstrating correlation between relative telomere length of peripheral blood leukocytes and age in controls and BA patients. (A) Relative telomere length decrease with age in the controls; (B) Significant relative telomere length decrease with age in BA patients.

Table 15. Multiple linear regression analysis of telomere length estimates.

Variables	Relative telomere length	*P*-value
	Estimate *b* (95% CI)	
Age (years)	-0.023 (-0.039 to -0.007)	0.005
Gender	0.028 (-0.11 to 0.17)	NS
Total bilirubin (mg/dL)	0.001 (-0.024 to 0.025)	NS
AST (IU/L)	0.00038 (-0.002 to 0.001)	NS
ALT (IU/L)	0.001 (-0.001 to 0.002)	NS
ALP (IU/L)	0.000076 (-0.00025 to 0.0004)	NS
Liver stiffness (kPa)	-0.01 (-0.013 to -0.007)	<0.0001

Abbreviations: AST=aspartate aminotransferase; ALT=alanine aminotransferase; ALP=alkaline phosphatase; NS = not significant

Shorter telomere length as prognostic marker for liver cirrhosis

Since RTL is an independent prognostic indicator, we further investigated RTL as a predictor of the risk of liver cirrhosis in postoperative BA patients. We calculated the area under curve of the ROC curve, which was constructed using RTL values. Based on the ROC curve, the optimal cutoff value of RTL as a useful marker for discriminating BA patients with cirrhosis from non-cirrhosis BA patients was projected to be 0.58, which yielded a sensitivity of 76.6%, a specificity of 72%, and an AUC of 0.78 (95% CI: 0.70 to 0.86, *P*<0.0001) (Figure 20).

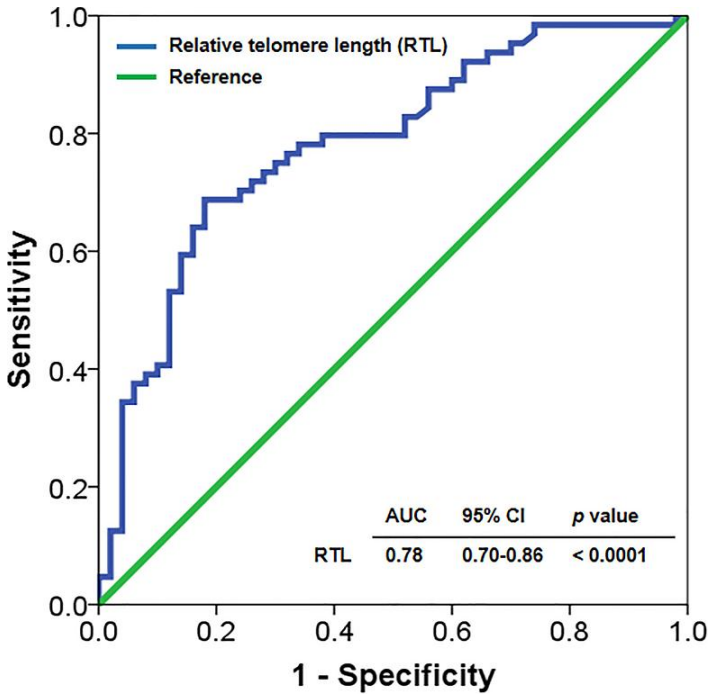

Figure 20. Receiver operating characteristic (ROC) curve representing diagnostic value of relative telomere length in biliary atresia patients with cirrhosis. The optimal cut-off value of relative telomere length at 0.58 as a marker discriminating between BA patients with and without cirrhosis.

Correlation between telomere length in peripheral blood leukocytes and liver tissue

Genomic DNA was prepared from matched peripheral blood leukocytes and liver tissue from 6 individuals with BA. Although the RTL was higher in leukocytes compared to liver tissue, the difference was not statistically significant (1.41±0.10 vs. 1.36±0.19, respectively), as shown in Figure 21A. Subsequent analysis demonstrated that there was a positive correlation between RTL in peripheral blood leukocytes and RTL in liver tissue (r=0.83, P<0.001; Figure 21B).

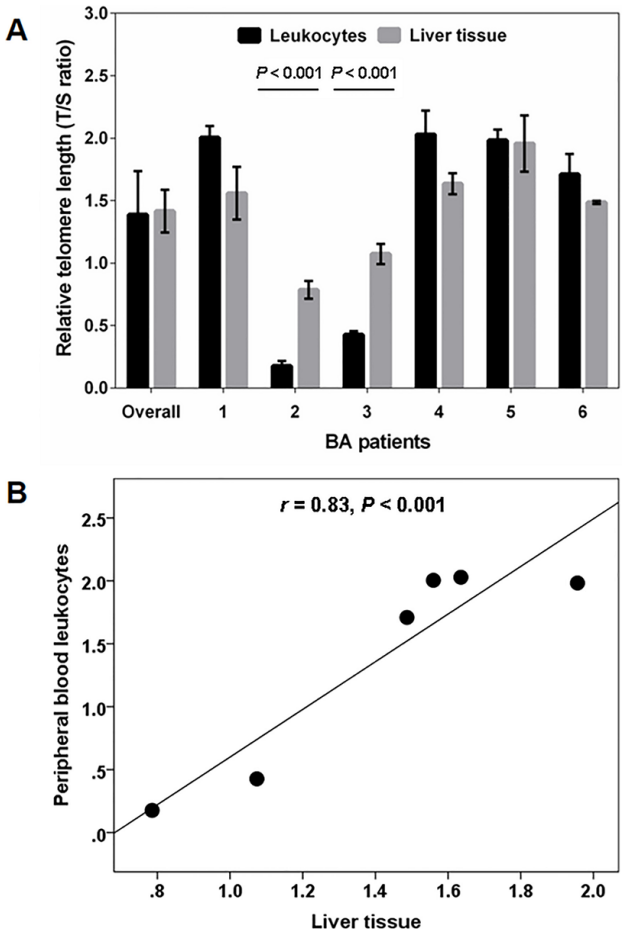

Figure 21. Telomere length distribution between peripheral blood leukocyte and liver tissue in BA patients. (A) Mean levels of relative telomere length for peripheral blood leukocytes and liver tissue in BA patients; (B) Correlation between relative telomere length in peripheral blood leukocytes and liver tissue in BA patients.

Discussion

In the current study, we examined the relative telomere length of peripheral blood leukocytes in BA patients and investigated the association of telomere length changes with the severity of BA. To the best of our knowledge, this study is the first to demonstrate a dramatically significant decrease in leukocyte RTL in BA patients, as compared to age matched healthy controls. In addition, advanced BA patients had substantially shorter telomeres than early-stage BA children. We observed that shortened telomere length was associated with a higher risk of liver cirrhosis in BA. Furthermore, RTL was found to be inversely correlated with age and liver stiffness. In contrast, we did not find any relationships between RTL and biochemical parameters such as AST, ALT, ALP, albumin, and total bilirubin in BA children. The ROC curve analysis showed that RTL could be a prognosis indicator for distinguishing BA patients with cirrhosis from non-cirrhosis patients. These findings confirm our hypothesis that a reduction in telomere length is associated with the severity of liver fibrosis in BA and that telomere length may serve as a non-invasive biomarker in determining cirrhosis progression in postoperative BA patients. The present study has also examined the relationship between leukocyte telomere length and liver telomere length in BA patients. We found that the relative telomere length was not significantly different between leukocytes and liver tissue. Further analysis revealed that RTL in peripheral blood leukocytes was positively correlated with RTL in liver tissue. Our observations are in agreement with a previous study that determined the correlation of leukocyte telomere length with telomere length in liver tissue. Dlouha and coworkers reported a significant direct correlation between leukocyte RTL and liver RTL in human autopsy material (135). The strong correlation of telomere length between peripheral blood leukocytes and liver tissue suggests that telomere length is relatively similar and that telomeres shorten at approximately similar rates. Our findings support the hypothesis that leucocyte telomere length might have potential as a possible non-invasive biomarker for monitoring the severity and progression of liver cirrhosis in post Kasai BA.

Aberration of the telomere complex might lead to chromosomal and genetic instability, contributing to cellular senescence or apoptosis and increasing the risk of malignancy (18). Telomere length evaluation is a possibly beneficial biomarker for investigating individual susceptibility for disorders in epidemiological studies because the balance of processes that abridge and elongate telomeres are largely genetically determined (136, 137). In addition, a growing body of epidemiological evidence in chronic liver diseases suggested that increased telomere attrition might be closely associated with a genetic risk of liver illness. Here, we report the attrition of telomere length in BA patients. RTL in BA patients was considerably shorter than that in unaffected volunteers, implying that telomere length reduction could be associated with a higher risk of liver cirrhosis in BA. To support this observation, we identified two pairs of female twins, of which only one of the twins was diagnosed with having BA. The twins diagnosed with BA had a shorter telomere length than the healthy twins. Moreover, the leukocyte RTL in the nineteen-year-old twin of set 2 was much lower than that in the nine-year-old twin of set 1. We also observed a significant inverse correlation between leukocyte telomere length and age in patients affected with BA. The explanation for this finding could be due to a progressive decline in leukocyte telomere length with ageing in BA patients.

In accordance with our finding, Kitada et al. investigated telomere length in chronic liver disorders and reported that telomere length was consistently shorter in liver tissue of patients with chronic liver diseases, when compared to control groups (84). Sanada and colleagues reported hepatocellular telomere length in 20 BA children using quantitative fluorescence in situ hybridization that normalized the telomere-centromere ratio in the liver biopsies of the BA group to be significantly smaller than that of the control group (86). The findings from our study, however, are partially in accordance with the previous research of Invernizzi and colleagues. They found that telomere length in peripheral blood mononuclear cells was not significantly different between patients with primary biliary cirrhosis (PBC) and unaffected volunteers; whereas, an excessive telomere shortening was observed in the advanced stage PBC patients, as compared to healthy controls (138). The reason for this discrepancy

remains unexplained. It may be attributed to a difference in methodology relating to measurement of telomere length between our study and the Invernizzi study.

In humans, decreasing telomere length is correlated with age. Telomere length has been extensively proven to be shorter in patients with age-related disorders than in unaffected volunteers. Its role in mediating age-related disease, however, has not yet been fully elucidated. Our findings also indicate that shortened telomere length in chronological age is significantly different between BA patients and age-matched controls. We further found that telomere shortening showed a trend of inverse association with age in BA patients, suggesting premature cellular ageing in BA children. This is consistent with previous investigations that reported telomere attrition to be negatively correlated with age-related diseases (139, 140).

Chronic liver damage induces regeneration and repair processes in hepatocytes, which leads to elevated cell turnover and ultimately results in excessive telomere shortening. When telomeres become critically shortened, they cause impairment of cell proliferation and senescence. Eventually, hepatocyte growth is arrested and/or senescence assumes a profibrogenic state, either or both trigger the activation of stellate cells by as yet uncertain mechanisms, leading to fibrogenesis in the liver (141). It is noteworthy that the RTL was considerably shorter in the advanced BA patients with jaundice, when compared with jaundice-free patients, indicating that RTL could be a non-invasive parameter reflecting the severity of biliary atresia.

The mechanism of shortened RTL in leukocytes is not easily addressed. Given the complexity of telomere biology, it merits thorough and complex investigation for clear understanding. Several plausible mechanisms either independently or in combination, may be speculated. In BA, the natural progressive decrease of telomere length with age could be accelerated by telomeric DNA damage due to oxidative stress, chronic inflammation, increased cellular turnover, and/or defects in telomere repair (18). Telomeric DNA sequences, rich in guanine residues, are likely more susceptible to oxidative stress, particularly by the formation of 8-hydroxy-2-deoxyguanosine (8-OHdG). Moreover, these could promote DNA double-strand breaks particularly at

telomeric regions resulting in the loss of the distal fragments of telomeric DNA and, thus, telomere shortening with each cell division (142). Inflammation triggers cellular proliferation and accelerates cell turnover, therefore facilitating telomere attrition due to the end-replication problem. A genetic predisposition must be taken into account. It is conceivable that a variety of these factors may act in concert to generate the phenomenon. The mechanism behind the connection of shortened RTL in leukocytes and liver cirrhosis in BA remains a mystery and requires further study.

This study revealed a significant inverse association between the RTL and liver stiffness in BA patients. Our findings further demonstrated that BA patients with severe fibrosis had increased telomere erosion, compared with BA patients with mild fibrosis, denoting that attrition of telomere length could drive the progression of liver cirrhosis in these patients. These findings are in agreement with results reported by Urabe and collaborators who found that increases in telomere shortening were correlated with the severity of fibrosis in patients diagnosed with human liver diseases (143). Our findings are also supported by a previous study by Wiemann et al. (85) which demonstrated that telomere shortening in hepatocytes and senescence were associated with fibrotic scarring in human cirrhosis. Thus, the erosion of telomere length is believed to be an indicator of cirrhosis progression and telomere shortening to be an important cause in the pathogenesis of chronic liver injury in BA.

The current research acknowledges certain limitations that should be noted. First, we evaluated telomere length in BA patients, but did not measure the activity of telomerase enzymes. As such, we were not able to determine the effects of telomerase activation and the dynamics of telomere length relating to BA in these results. Second, this study was cross-sectional in its design. Therefore, cause-and-effect associations could not be determined. Prospective longitudinal studies are necessary to investigate the association between telomere shortening and the severity of BA. Third, since this study was carried out with only Thai participants, the results may not be generalizable among other ethnic groups. Fourth, since BA is a sporadic disorder, this study reduced the number of BA subgroups, which diminished the power of the

statistics. For this reason, the sample size of BA needs to be increased in order to reach an unequivocal conclusion. Accordingly, the mechanisms behind the connection of shortened RTL in leukocytes and liver cirrhosis in BA remain unknown and need further investigation.

Conclusion

This study supports the association between short telomere length in leukocytes and higher risk of liver cirrhosis in BA. In addition, RTL in peripheral blood leukocytes was associated with disease severity, showing that BA patients with advanced-stage exhibit excessive telomere shortening. These observations indicate that telomere length measurement might serve as an important predictor of BA patients at high risk of cirrhosis. Prognostic telomere length value as a biomarker for future risk of hepatic impairment in BA needs to be confirmed in a longitudinal study. Further understanding of the pathogenesis of BA will provide new therapeutic approaches to the treatment of this disorder.

CHAPTER VII

GLOBAL METHYLATION, OXIDATIVE STRESS, AND RELATIVE TELOMERE LENGTH IN BILIARY ATRESIA PATIENTS

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Abstract

Alu and LINE-1 elements are retrotransposons with a ubiquitous presence in the human genome that can cause genomic instability, specifially relating to telomere length. Genotoxic agents may induce methylation of retrotransposons, in addition to oxidative DNA damage in the form of 8-hydroxy-2′-deoxyguanosine (8-OHdG). Methylation of retrotransposons induced by these agents may contribute to biliary atresia (BA) etiology. Here, we investigated correlations between global methylation, 8-OHdG, and relative telomere length, as well as reporting on Alu and LINE-1 hypomethylation in BA patients. Alu and LINE-1 hypomethylation were found to be associated with elevated risk of BA (OR=4.07; 95% CI: 2.27–7.32; *P*<0.0001 and OR=3.51; 95% CI: 1.87–6.59; *P*<0.0001, respectively). Furthermore, LINE-1 methylation was associated with liver stiffness in BA patients (β coefficient=−0.17; 95% CI: −0.24 to −0.10; *P*<0.0001). Stratified analysis revealed negative correlations between Alu and LINE-1 methylation and 8-OHdG in BA patients (*P*<0.0001). In contrast, positive relationships were identified between Alu and LINE-1 methylation and relative telomere length in BA patients (*P*<0.0001). These findings suggest that retrotransposon hypomethylation is associated with plasma 8-OHdG and telomere length in BA patients.

Introduction

Biliary atresia (BA) is one of the most common causes of neonatal cholestatic liver disease. BA is characterized by a progressive idiopathic fibrosclerotic cholangiopathy that results in obliteration of the extrahepatic biliary tree. Although effective bile flow can be established by Kasai portoenterostomy, the majority of BA patients will ultimately develop severe cholestasis, liver cirrhosis, and end-stage liver disease (1). The etiologies of BA have not been well established. However, several theories have been proposed to explain the pathogenesis of BA, including viral infections, toxins, and immunologic insults; notably, the interplay between environmental and genetic factors (87). Growing evidence suggests that epigenetic variation can be elicited by viruses, toxins, and genetic defects (88), which may have relevance in the development of BA.

DNA methylation, one type of epigenetic change, is a reversible modification of cytosine residues in the genome through the addition of a methyl group to cytosine nucleotides. This variation is an important mechanism in regulating expression of human genes, maintenance of genomic stability, and telomere length (89). A substantial portion of methylation sites throughout the human genome are found in repetitive sequences and transposable elements, such as Alu or short interspersed nuclear element (SINE) and long interspersed nuclear element-1 (LINE-1). Alu and LINE-1 are major components of non-long terminal repeat retrotransposons, comprising approximately 11% and 17% of the human genome, respectively (20). Because repetitive DNA sequences account for over 40% of methylation in the genome, DNA methylation measured in retrotransposon elements has served as a useful proxy for global DNA methylation (90). Alu and LINE-1 elements are usually heavily methylated in normal cells, thus maintaining transcriptional inactivation and inhibiting retrotransposition. Hypomethylation of these elements is hypothesized to facilitate genomic instability by resulting in retrotransposition of transposable elements, dysregulation of DNA repair genes (21, 22), and altered expression of important genes (23). Previous studies have highlighted relationships between global hypomethylation

and several human diseases (24, 26, 27). Methylation of these elements also makes them susceptible to oxidative stress (91), which may be a possible factor associated with biliary atresia.

Oxidative stress constitutes the majority of DNA damage in human cells, which is due mainly to excess production of reactive oxygen species (ROS) (92). Generation of ROS can lead to a wide range of DNA lesions, including base deletions, mutations, DNA strand breakage, chromosomal rearrangements, and cross-linking with proteins (93). Oxidative DNA damage can modify epigenetic alterations by multiple mechanisms. One form of DNA damage induced by oxidative stress is the change in genomic base to species like 8-hydroxy-2′-deoxyguanosine (8-OHdG). 8-OHdG is able to interfere with the ability of DNA to function as a substrate for the DNA methyltransferases (DNMTs), leading to global DNA hypomethylation and subsequent genomic instability (94). Alu and LINE-1 may be critical elements in chromosome and genomic stability and may be induced by an increase in oxidative stress, leading to genomic instability and DNA damage. As such, these elements may contribute to the pathophysiology of BA. To date, there has been no evidence regarding the possible association between global methylation and oxidative DNA damage in BA patients. This information could improve our understanding of the relationship between epigenetic alteration-mediated DNA damage and BA etiology. Interestingly, epigenetic mechanism appears to be an important component of telomere regulation. Several studies have reported that hypomethylation of subtelomeric regions was related to telomere length and that these regions might be important to epigenetic regulation in telomere maintenance (28, 29), thereby establishing a possible etiologic link between global DNA methylation and telomere length in BA patients.

While methylation of retrotranposon elements has been investigated in relation to a variety of disorders, little is known about the association of global DNA methylation and the exact patho-etiology of BA. We hypothesize that epigenetic alterations in the form of global DNA methylation, may be associated with outcome parameters and telomere length in BA patients. Accordingly, the primary aim of the

present study was to assess methylation levels and patterns of Alu and LINE-1 elements in peripheral blood leukocytes from BA patients and age-matched healthy controls using quantitative combine bisulfite restriction analysis (qCOBRA). We further investigated whether Alu and LINE-1 methylation levels were associated with hepatic dysfunction, oxidative stress, and relative telomere length in BA patients. Additionally, we examined the association between Alu and LINE-1 methylation and risk of BA. Further understanding of global DNA methylation, oxidative damage, and telomere length would shed light on the role of epigenetic aberrations play in the etiology of BA and may ultimately support the development of effective strategies.

Materials and Methods

Study participants

The study protocol conformed to the ethical standards outlined in the Declaration of Helsinki and was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University. All participants, parents, or legal guardians were fully informed regarding the study protocol and procedures prior to participating in the study. Written informed consent was obtained from all patients and from parents or legal guardians of patients younger than 18 years of age.

This case-control study consisted of 114 BA patients and 114 age-matched unaffected volunteers with no underlying liver disease. All BA patients were diagnosed by intraoperative cholangiography and were surgically treated with Kasai portoenterostomy. Healthy controls who participated in an evaluation of hepatitis B vaccine and attended the Well Baby Clinic at King Chulalongkorn Memorial Hospital for vaccination had normal physical findings and no underlying disease. In addition, two pairs of monozygotic girl twins with BA discordance were enrolled in this study. We classified BA patients according to serum total bilirubin (TB) into either the non-jaundice group (TB<2 mg/dL; n=77) or the persistent jaundice group (TB≥2 mg/dL; n=37). BA patients were stratified according to severity of liver fibrosis into either the mild fibrosis group (F0-F2: 0-9.7 kPa; n=32) or the severe fibrosis group (F3-F4: >9.7 kPa;

n=82). Based on severity of hepatic dysfunction [aspartate aminotransferase (AST) value], BA patients were also categorized as either early-stage (AST<100 IU/L; n=56) or late-stage (AST≥100 IU/L; n=58).

Blood samples from participants were collected in ethylenediaminetetraacetic acid (EDTA) tubes to facilitate isolation of plasma and leukocytes and were then stored at −80 ℃ until analysis.

Clinical assessments of outcomes

All liver function analyses, including TB, AST, alanine aminotransferase (ALT), alkaline phosphatase (ALP), and albumin were performed on a Roche Hitachi 912 chemistry analyzer (Roche Diagnostics, Basel, Switzerland). Measurement of liver stiffness by transient elastography was performed using a Fibroscan (EchoSens, Paris, France). Briefly, assessments were performed by placing a Fibroscan transducer probe on the intercostal space at the area of the right lobe of the liver. Measurements were then performed until 10 validated results were obtained with a success rate of at least 80%. The median value of 10 validated scores represented the elastic modulus measurement of the liver, which was expressed in kilopascals (kPa).

Alu and LINE-1 methylation analysis

Genomic DNA was extracted from peripheral blood leukocytes using GE Healthcare DNA Purification Kit (Buckinghamshire, UK). Extracted DNA (50 ng; concentration: 2.5 ng/μL) was treated by EZ DNA Methylation Gold Kit (Zymo Research, Orange, CA, USA), according to manufacturer's protocol.

DNA methylation was quantitated by qCOBRA using previously described primers and conditions (144). Primers used for COBRA Alu and COBRA LINE-1 amplifications were, as follows: Alu forward primer 5′-GGRGRGGTGGTTTARGTTTGTAA-3′; Alu reverse primer 5′-CTAACTTTTTATATTTTTAATAAAAACRAAATTTCACCA-3′; LINE-1 forward primer 5′-GTTAAAGAAAGGGGTGAYGGT-3; and, LINE-1 reverse primer 5′-AATACRCCRTTTCTTAAACCRATCTA-3′. Both PCRs were functioned in a final volume of

10 μL, containing 2.5 ng of bisulfite-treated DNA, 10X PCR buffer, 25 mM MgCl2, 200 mM dNTPs, 20 μM primers, and 0.5 U Taq DNA polymerase (HotStar, Qiagen, Valencia, CA, USA). PCR cycling conditions started with a 95 °C incubation for 15 min, followed by 40 cycles of 95 °C for 45 sec, then 57 °C (for Alu) or 55 °C (for LINE-1) for 45 sec and 72 °C for 45 sec, and finally 72 °C for 7 min. After PCR amplification, Alu amplicons (133 bp) were subsequently digested with 2 U *TaqI* in *TaqI* buffer (MBI Fermentas, Burlington, Canada), while LINE-1 amplicons (92 bp) were digested with 2 U *TaqI* and 8 U *TasI* in NEBuffer 3 (New England Biolabs, Ontario, Canada). Both digestion reactions were incubated at 65 °C overnight, followed by separation on an 8% non-denaturing polyacrylamide gel. Gels were then stained with ethidium bromide and band intensities were analyzed by Molecular Imager Gel Doc using Image Lab Software (Bio-Rad, Begoniastraat, Belgium).

Both qCOBRA Alu and qCOBRA LINE-1 were stratified into four patterns depending on methylation status of two CpG dinucleotides, as follows: hypermethylation ($^{m}C^{m}C$), partial methylation ($^{m}C^{u}C$ and $^{u}C^{m}C$), and hypomethylation ($^{u}C^{u}C$). Methylation levels and patterns of both Alu and LINE-1 were measured to determine the precise percentage of methylated CpG dinucleotides. For Alu methylation analysis, we measured the percentage of Alu methylation levels and patterns in each group based on the intensity of the COBRA-digested Alu products. DNA fragments derived from enzymatic digestion of COBRA-Alu products were divided into six fragments of 133, 90, 75, 58, 43, and 32 bp, which represented different methylation states. Percentage of each methylation pattern was estimated, as follows: A=intensity of the 133 bp fragment divided by 133; B=intensity of the 58 bp fragment divided by 58; C=intensity of the 75 bp fragment divided by 75; D=intensity of the 90 bp fragment divided by 90; E=intensity of the 43 bp fragment divided by 43; and, F=intensity of the 32 bp fragment divided by 32. The percentage of each Alu element methylation pattern was then calculated, as follows: percentage of Alu methylation level ($\%^{m}C$) = 100 × (E + B)/(2A + E + B + C + D); percentage of hypermethylated loci ($\%^{m}C^{m}C$) = 100 × F/(A + C + D + F); percentage of both partially methylated loci

(%$^{u}C^{m}C$) = 100 × C/(A + C + D + F); (%$^{m}C^{u}C$) = 100 × D/(A + C + D + F); and, percentage of hypomethylated loci (%$^{u}C^{u}C$) = 100 × A/(A + C + D + F).

For LINE-1 methylation analysis, DNA fragments from enzymatic digestion for qCOBRA LINE-1 were separated into fie fragments: 92 bp, 60 bp, 50 bp, 42 bp, and 32 bp. Th number of CpG dinucleotides was determined by dividing each band intensity by the length (bp) of the double-stranded DNA fragment, as follows: A=92 bp fragment intensity/92; B=60 bp fragment intensity/56; C=50 bp fragment intensity/48; D=42 bp fragment intensity/40; E=32 bp fragment intensity/28; and, F = [(D + E) – (B – C)]/2. LINE-1 methylation levels were calculated using the number of CpG dinucleotides according to the following formulas: LINE-1 methylation level percentage (%^{m}C) = 100 × (A + 2C + F)/(2A + 2B + 2C + 2F); hypermethylated loci percentage (%$^{m}C^{m}C$) = 100 × (C/2)/[(C/2) + A + B + F]; both of partially methylated loci percentage (%$^{u}C^{m}C$) = 100 × F/[(C/2) + A + B + F); (%$^{m}C^{u}C$) = 100 × A/[(C/2) + A + B + F); and, hypomethylated loci percentage (%$^{u}C^{u}C$) = 100 × B/[(C/2) + A + B + F]. DNA samples from HeLa, Jurkat, and Daudi cell lines were used as positive controls to normalize inter-assay variations in all experiments.

Quantitation of 8-hydroxy-2-deoxyguanosine

Plasma 8-OHdG levels were quantitatively determined from venous blood samples using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit (Trevigen, Gaithersburg, MD, USA), according to manufacturer's instructions. Antibodies specific to 8-OHdG generated by the entire immunogen were utilized. Twofold serial dilutions of 8-OHdG standard with a concentration of 0.89–56.7 ng/mL were used as standards. Intra-assay and inter-assay precision were less than 10% and 15%, respectively. The sensitivity of this assay was 0.57 ng/mL.

Telomere length measurement

Telomere length in genomic DNA was estimated by applying a quantitative real-time polymerase chain reaction (PCR) method originally described by Cawthon (2002). Briefly, PCRs were performed using StepOnePlus™ Real-Time PCR System (Applied

Biosystems, Foster City, CA, USA) with SYBR Green florescence (RBC Bioscience, Taipei, Taiwan). Relative telomere length was measured according to the ratio of the telomere repeat copy number (T) to the single-copy gene copy number (S) in each given sample. In each sample, the quantity of telomere repeats and the quantity of single-copy genes were normalized to a reference DNA sample (from a single individual).

Statistical analysis

All statistical analyses were performed using SPSS Statistics version 22.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance between clinical parameters of healthy controls and BA groups was determined by Student's *t*-test. Kolmogorov-Smirnov test and quantile-quantile (q-q) plot were used to evaluate Alu and LINE-1 methylation levels for normal distribution. Given that Alu and LINE-1 methylation levels were found not to be normally distributed, significance of changes in these methylations was calculated by Mann-Whitney *U* test or Kruskal-Wallis H test for continuous variables. Unconditional logistic regression models were used to estimate associations between methylation of Alu or LINE-1 and BA risk using odds ratio (OR) and 95% confidence interval (CI), with adjustments for confounding factors including age and gender. We used linear regression models to evaluate potential predictors of Alu or LINE-1 methylation levels as continuous variables. Spearman's rank correlation coefficient test was used to estimate relationships between global methylation, telomere length, and circulating 8-OHdG levels. Data were expressed as mean ± standard error of the mean (SEM). All statistical tests were based on two-tailed probability, with *P*-values less than 0.05 considered statistically significant.

Results

Clinical characteristic of study subjects

Baseline demographic characteristics of participants in this analysis are listed in Supplementary Table 1. Of 228 participants enrolled in this study, 114 patients were diagnosed with BA (57.89% female and 42.11% male) and 114 were healthy controls

(56.14% female and 43.86% male). There were no significant differences in age or gender between BA patients and healthy controls. However, BA patients had significantly higher liver stiffness, AST, and ALT values than controls (P<0.0001).

Hypomethylation of Alu and LINE-1 elements in biliary atresia

In order to explore potential epigenetic alterations resulting from global methylation in BA, we measured Alu and LINE-1 methylation in peripheral blood leukocytes of BA patients and age-matched healthy controls. Figure 22A reveals the distribution of Alu methylation levels in BA patients and controls in box plot format. Median Alu methylation level in BA patients was significantly lower than in healthy controls (P<0.0001). LINE-1 methylation levels were also found to be lower in BA patients than in healthy controls (P<0.0001) (Figure 22B).

We further investigated methylation patterns of Alu and LINE-1 elements in BA patients and healthy controls. Median percentages of each Alu methylation pattern are shown in Figure 22C. Interestingly, we observed significant elevation of hypomethylation pattern ($^{u}C^{u}C$) at Alu elements in BA patients, as compared to healthy controls (P<0.0001). Similarly, BA patients demonstrated higher methylation of partial methylation patterns ($^{u}C^{m}C$ and $^{m}C^{u}C$) than the control group (P=0.0037 and P=0.0035, respectively). In contrast, the percentage of hypermethylation pattern ($^{m}C^{m}C$) was significantly decreased in BA patients (P<0.0001). BA patients had significantly reduced LINE-1 methylation of both hypermethylation pattern ($^{m}C^{m}C$) and partial methylation pattern ($^{u}C^{m}C$), as compared to controls (P<0.0001 and P<0.0001, respectively) (Figure 22D). However, the percentage of partial methylation pattern ($^{m}C^{u}C$) at LINE-1 elements was significantly higher in BA patients than in unaffected controls (P<0.0001). This was not observed in LINE-1 methylation of hypomethylation pattern in a comparison between cases and controls.

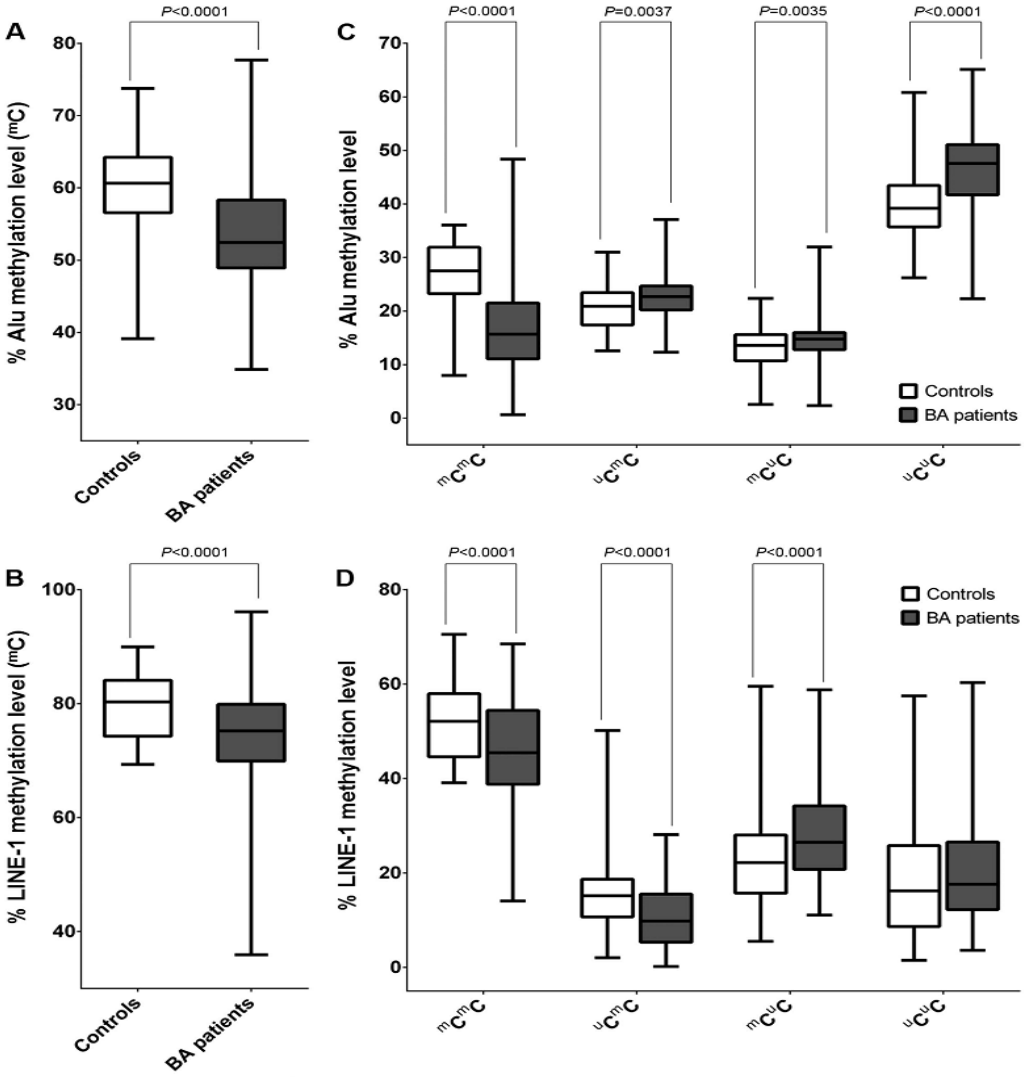

Figure 22. Methylation levels and patterns of Alu and LINE-1 elements in controls and BA patients. (A) Alu methylation levels; (B) LINE-1 methylation levels; (C) Alu methylation patterns; (D) LINE-1 methylation patterns.

When disease severity was considered, BA patients were classified according to liver fibrosis status and hepatic dysfunction marker (AST value). Alu methylation levels in the different subgroups were remarkably lower than in controls (P<0.0001); however, there were no significant differences in Alu methylation between early-stage (mild fibrosis and low AST value) and late-stage (severe fibrosis and high ATS value) BA patients (Figure 23A, B). Notably, LINE-1 hypomethylation was observed in advanced

BA patients with severe fibrosis and high AST value, when compared with patients with early-stage disease (*P*=0.001 and *P*=0.019, respectively) (Figure 23C, D).

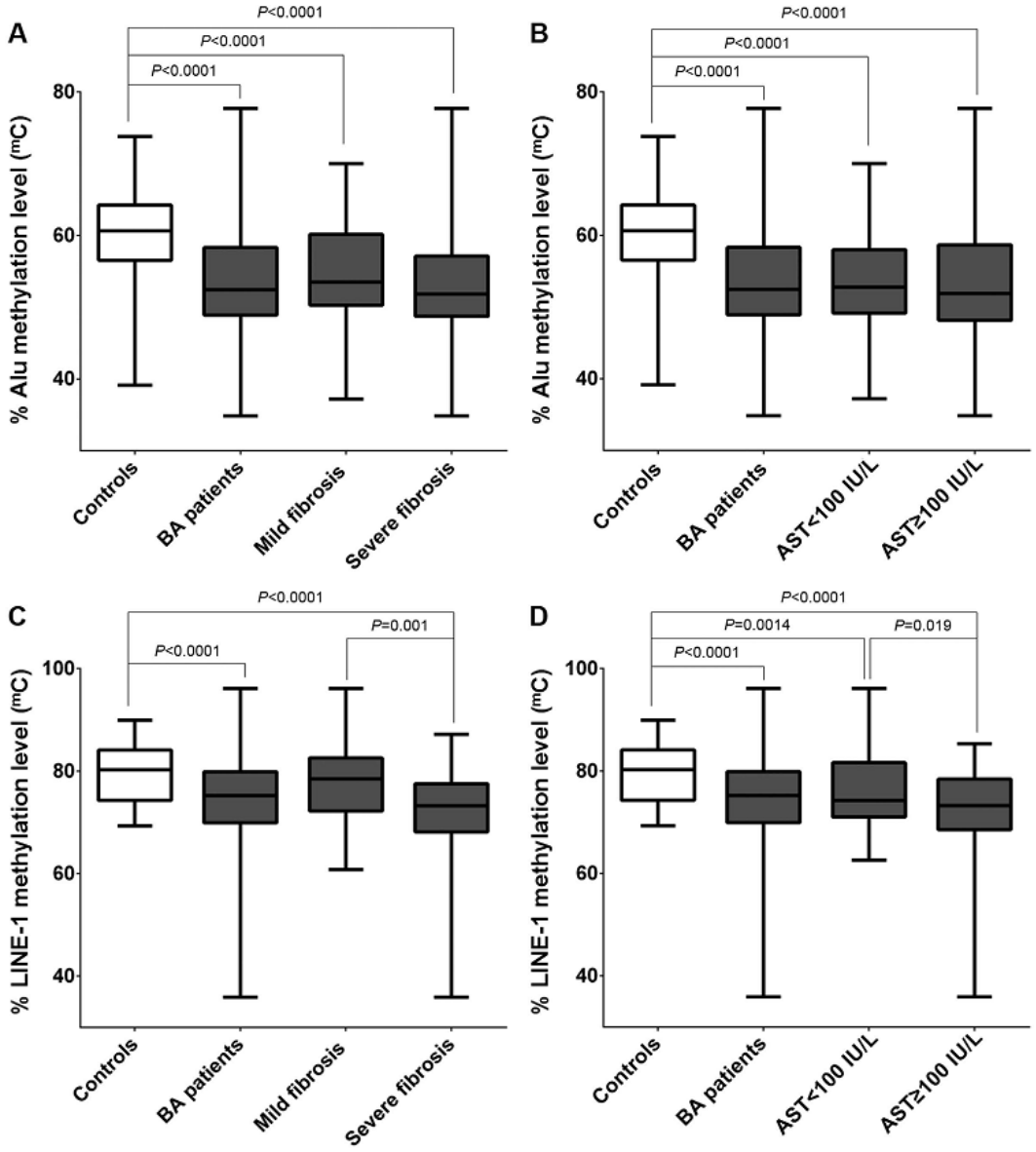

Figure 23. Alu and LINE-1 methylation levels among groups. (A) Alu methylation level in controls and BA according to fibrosis status; (B) severity of hepatic injury; (C) LINE-1 methylation level in control and BA according to fibrosis status; (D) severity of hepatic injury.

Alu and LINE-1 hypomethylation in monozygotic twins discordant for biliary atresia

Subsequently, we investigated Alu and LINE-1 methylation levels in two sets of monozygotic twins discordant for BA. Set 1: the patient was a nine-year-old girl who was diagnosed with BA, while her sister was born healthy and remains so to date. Expectedly, this case demonstrated slightly lower Alu methylation level than control (58.37% vs. 59.62%, respectively). Set 2: the patient was a nineteen-year-old woman diagnosed as BA, with a twin sister who is healthy and has normal liver function tests. We also observed a slight reduction in Alu methylation level in this case when compared to control (57.28% vs. 57.84%, respectively). This effect was restricted to LINE-1 methylation level comparisons.

Association between global methylation and risk of BA

Using unconditional logistic regression models, we evaluated Alu or LINE-1 methylation levels as an independent risk factor of BA. As shown in Table 16, this study demonstrated that overall Alu and LINE-1 methylation were inversely associated with risk of BA (OR: 0.88, 95% CI: 0.84–0.92; $P<0.0001$ and OR: 0.89, 95% CI: 0.85–0.94; $P<0.0001$, respectively). After adjusting for age and gender, a 4.07-fold (95% CI: 2.27–7.32) higher risk of BA was observed among individuals with lower Alu methylation below the median distribution in the controls, compared with individuals with higher Alu methylation ($P<0.0001$), consistent with LINE-1 methylation analysis (OR: 3.51, 95% CI: 1.87–6.59; $P<0.0001$). We further evaluated a significant dose-response association between Alu or LINE-1 hypomethylation and increased BA risk. Compared with individuals in the highest Alu methylation tertile (third tertile), individuals in the lowest tertile (fist tertile) were associated with a 9.98-fold increased risk of BA (P-trend<0.0001). In addition, there was a significant dose-response association between the lowest LINE-1 methylation tertile and increased risk of BA (P-trend<0.0001). Specifically, when using the third tertile (the highest tertile) as the reference group, adjusted ORs for the first and second tertile were 6.52 (95% CI: 2.79–15.27) and 2.83 (95% CI: 1.17–6.88), respectively.

Table 16. Association between global methylation and risk of BA.

	BA	Controls	Unadjusted OR	*P*-value	Adjusted[a] OR	*P*-value
			OR (95% CI)		OR (95% CI)	
Alu elements						
Overall	100.00%	100.00%	0.88 (0.84 to 0.97)	<0.001	0.88 (0.84 to 0.92)	<0.0001
By median						
Low	78.07%	50.00%	4.07 (2.27 to 7.33)	<0.0001	4.07 (2.27 to 7.32)	<0.0001
High	21.93%	50.00%	1.00 (reference)		1.00 (reference)	
By tertile						
1st tertile	73.68%	33.33%	9.95 (4.54 to 21.80)	<0.0001	9.98 (4.55 to 21.89)	<0.0001
2nd tertile	14.46%	33.33%	2.53 (1.03 to 6.20)	0.04	2.51 (1.02 to 6.16)	0.04
3rd tertile	12.28%	33.33%	1.00 (reference)		1.00 (reference)	
***P*-trend**				<0.0001		<0.0001
LINE-1 elements						
Overall	100.00%	100.00%	0.90 (0.85 to 0.94)	<0.0001	0.89 (0.85 to 0.94)	<0.0001
By median						
Low	77.19%	50.00%	3.53 (1.88 to 6.61)	<0.0001	3.51 (1.87 to 6.59)	<0.0001
High	22.81%	50.00%	1.00 (reference)		1.00 (reference)	
By tertile						
1st tertile	62.28%	33.33%	6.46 (2.78 to 15.00)	<0.0001	6.52 (2.79 to 15.27)	<0.0001
2nd tertile	21.93%	33.33%	2.82 (1.17 to 6.82)	0.02	2.83 (1.17 to 6.88)	0.02
3rd tertile	15.79%	33.33%	1.00 (reference)		1.00 (reference)	
***P*-trend**				<0.0001		<0.0001

[a]Unconditional logistic regression analysis, adjusted for age and gender; *P*-value<0.05 indicates statistical significance

Correlation between global methylation and clinical parameters

To further determine possible correlations between Alu and LINE-1 methylation, as well as biochemical variables in BA patients, we performed multiple linear regression analysis with adjustments for confounding variables. Relationships between global DNA methylation and clinical outcomes are presented in Table 17. There were no statistically significant associations between Alu methylation and clinical outcomes in BA patients. In contrast, a reduction in LINE-1 methylation was found to

be associated with increased liver stiffness (β coefficient = −0.17, 95% CI: −0.24 to −0.10; *P*<0.0001).

Table 17. Multivariate linear regression analysis of global methylation estimates.

Variables	Alu methylation[a]		LINE-1 methylation[a]	
	β coefficients (95% CI)	*P*-value	β coefficients (95% CI)	*P*-value
Age (years)	-0.12 (-0.49 to 0.25)	0.52	-0.14 (-0.51 to 0.24)	0.50
Gender	-1.47 (-4.72 to 1.79)	0.37	2.40 (-1.098 to 5.78)	0.16
Liver stiffness (kPa)	0.032 (-0.04 to 0.10)	0.38	-0.17 (-0.24 to -0.10)	<0.0001
TB (mg/dL)	-0.14 (-0.69 to 0.42)	0.63	0.27 (-0.29 to 0.84)	0.34
AST (IU/L)	0.00 (-0.03 to 0.04)	0.96	0.021 (-0.02 to 0.07)	0.30
ALT (IU/L)	0.00 (-0.03 to 0.04)	0.85	-0.01 (-0.05 to 0.02)	0.46
ALP (IU/L)	0.00 (-0.01 to 0.01)	0.99	0.00 (-0.01 to 0.01)	0.76
Albumin (g/dL)	1.03 (-0.80 to 2.86)	0.27	-1.13 (-3.02 to 0.77)	0.24

[a]Unconditional logistic regression analysis, adjusted for age, gender, liver stiffness, total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and albumin; *P*-value<0.05 indicates statistical significance

Increased 8-hydroxy-2-deoxyguanosine levels

To assess levels of oxidative DNA damage in BA, we measured circulating 8-OHdG concentrations in 114 BA patients and 53 healthy controls. Mean plasma 8-OHdG value in BA patients was considerably higher than unaffected controls (*P*<0.0001) (Figure 24A). In analyses stratified by disease severity, BA patients were categorized based on jaundice status, fibrosis status, and hepatic dysfunction marker (AST value). Elevated plasma 8-OHdG concentrations was found in advanced BA patients with persistent jaundice, severe fibrosis, and advanced stage of hepatic injury, as compared to healthy controls (*P*<0.0001, *P*<0.0001, and *P*<0.0001, respectively). Contrariwise, no significant differences in 8-OHdG concentrations were noted in comparison with concentrations in patients with early-stage and late-stage, as demonstrated in Figure 24B–D.

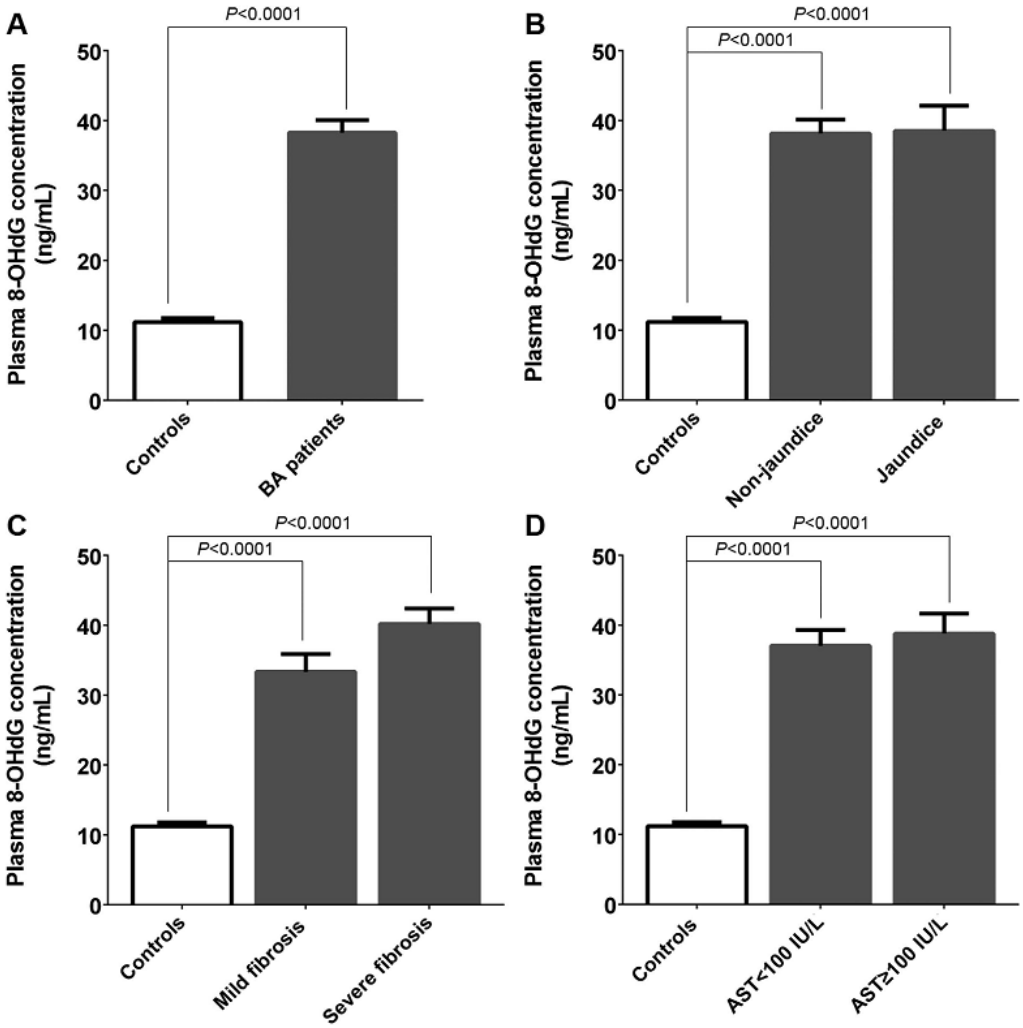

Figure 24. Plasma 8-hydroxy-2-deoxyguanosine levels of subjects among groups. (A) plasma 8-OHdG levels in BA patients and healthy controls; (B) BA patients with and without jaundice; (C) BA subgroups, including mild fibrosis (F0-F2) and severe fibrosis (F3–F4); (D) early-stage or late-stage of hepatic dysfunction in BA patients based on AST value.

Relationships between global methylation, oxidative DNA damage, and telomere length

Given that epigenetic modifications in global methylation may be involved in telomere elongation and may be induced by oxidative DNA damage, we evaluated

associations between methylation of Alu or LINE-1, plasma 8-OHdG, and telomere maintenance. A significantly negative association between Alu methylation and plasma 8-OHdG was observed in BA patients (r=−0.52, P<0.0001). Moreover, LINE-1 methylation was inversely correlated with plasma 8-OHdG in BA patients (r=−0.48, P<0.0001), as represented in Figure 25A. To better understand the relationship between global methylation and oxidative DNA damage, we also separately evaluated BA patients with global hypomethylation or hypermethylation. Mean plasma 8-OHdG concentrations in BA patients with Alu hypomethylation were remarkably higher than both patients with Alu hypermethylation and healthy controls (P=0.0026 and P<0.0001, respectively) (Figure 25B). Patients with LINE-1 hypomethylation had consistently significantly higher plasma 8-OHdG concentrations than patients with hypermethylation of LINE-1 elements and unaffected controls (P=0.0011 and P<0.0001, respectively) (Figure 25C).

We further examined correlations between changes in Alu or LINE-1 methylation and telomere length in BA patients and observed a weak positive correlation between Alu methylation and telomere length (r=0.24, P=0.012). Correspondingly, LINE-1 methylation levels were positively associated with telomere length (r=0.64, P<0.0001) (Figure 25D). We also compared relative telomere length in BA patients with Alu or LINE-1 hypomethylation and hypermethylation. Subsequent analysis showed that BA patients with LINE-1 hypomethylation had significantly shorter telomere length than those with LINE-1 hypermethylation and healthy controls (P<0.0001). This effect did not vary in comparison between patients with Alu hypomethylation and hypermethylation (Figure 25E, F).

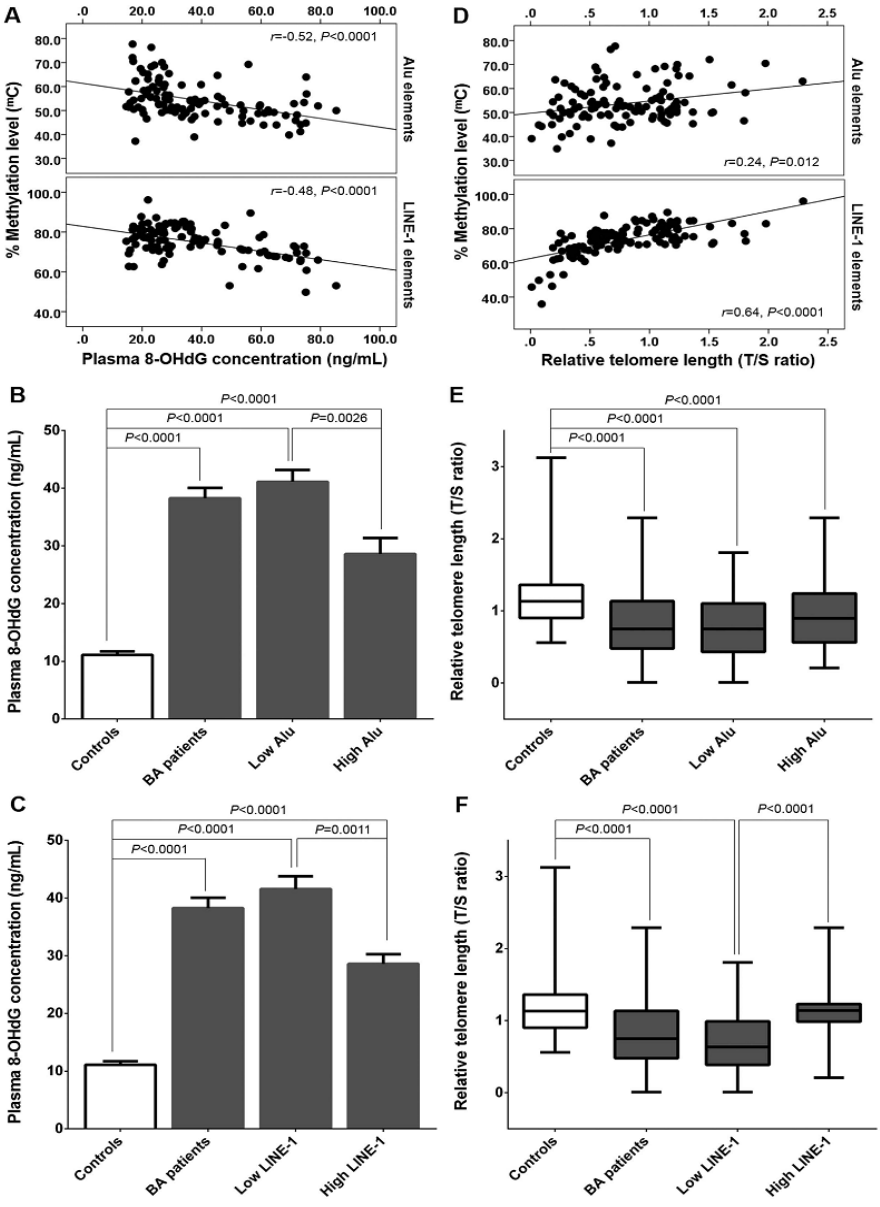

Figure 25. Relationships between global methylation, 8-hydroxy-2-deoxyguanosine, and telomere length in BA. (A) negative correlations between Alu or LINE-1 methylation and 8-OHdG; (B) plasma 8-OHdG levels in BA patients with hypo- and hypermethylated status of Alu elements; (C) plasma 8-OHdG levels in BA patients with hypo- and hypermethylated status of LINE-1 elements; (D) positive associations between Alu or LINE-1 methylation and telomere length; (E) relative telomere length in BA patients with hypo- and hypermethylated status of Alu elements; (F) relative telomere length in BA patients with hypo- and hypermethylated status of LINE-1 elements.

Discussion

This study investigated the effectiveness of repetitive elements methylation in peripheral blood leukocytes as a proxy for global methylation in postoperative BA patients. We found that Alu and LINE-1 elements were robustly hypomethylated in BA patients, as compared to healthy controls. Reduction of both Alu and LINE-1 methylation levels was also associated with increased risk of BA. Importantly, LINE-1 methylation was associated with poor outcomes in BA patients. Moreover, Alu and LINE-1 methylation levels were significantly related with oxidative DNA damage and relative telomere length. These findings support the notion that there exists epigenetic mechanism associated with genomic instability in the pathogenesis of BA.

Methylation of retrotransposable elements has been shown to be associated with global genomic methylation. Hypomethylation in these elements may increase their activity as retrotransposon sequences, resulting in genomic alterations and more mutations by several different mechanisms (145). To our knowledge, this is the first study to explore relationships between Alu or LINE-1 methylation, oxidative DNA damage, telomere length, and hepatic dysfunction in BA patients. Here, we report hypomethylation of both Alu and LINE-1 elements in BA patients, which was supported by decreased Alu methylation levels in two BA patients compared to those in their respective monozygotic twin sisters. In accord with our findings, Alu hypomethylation has been observed in post-menopausal women with osteoporosis (24) and patients with glioma cancer (25). Furthermore, LINE-1 methylation has been reported in hepatocellular carcinoma patients (26, 27). It is well known that LINE-1 elements encode enzymes that allow them to replicate and insert themselves into different genomic regions, altering transcription and translation into functional proteins (138, 146). Transcription of LINE-1 elements has been shown to contribute to transcriptional regulation of human development genes and cell differentiation (147, 148) and Han et al., 2005). Our observation regarding association of LINE-1 methylation with poor outcome in BA patients supports prior evidence that epigenetic modifications play important roles in BA etiology (73).

The current study showed that plasma 8-OHdG concentrations were significantly higher in BA patients than in controls. Consistent with this finding, previous study demonstrated that 8-OHdG, oxidative stress marker, was highly expressed in liver tissues of BA patients (149). Tiao et al. also reported that hepatic 8-OHdG expression in early-stage BA patients was substantially greater than in patients with choledochal cyst (150). Subsequent analysis revealed elevation of plasma 8-OHdG in BA patients with both Alu and LINE-1 hypomethylation. Furthermore, Alu and LINE-1 methylation levels were inversely correlated with plasma 8-OHdG levels in BA patients.

Previous investigation has documented the role of global DNA methylation in the variability of telomere length (19). Telomeres are repeated DNA sequences of TTAGGG and an associated protein complex at chromosome ends that are essential for maintaining chromosome integrity (17). With each cell division, telomeres shorten due to the inability of DNA polymerases to replicate the ends of linear molecules and also due to nucleolytic degradation, oxidative DNA damage, and inflammation (151). Our recent study has provided evidence for telomere shortening in age-associated biliary atresia31; however, this causal relation remains largely unknown. Epigenetic mechanism also appears to be an important component of telomere length regulation. Importantly, DNA hypomethylation, especially in subtelomeric DNA repeats, was associated with telomere shortening that may result from mutation in the DNA methyltransferase 3b gene (29), suggesting a regulatory role of DNA methylation on telomere length. In this study, we showed positive correlations between Alu and LINE-1 methylation with telomere length in BA patients. In agreement with these findings, LINE-1 methylation was positively associated with telomere length in dyskeratosis congenital (152). Wong et al. recently reported positive relationships between both Alu and LINE-1 methylation levels and telomere length (153). Notably, we found that BA patients with LINE-1 hypomethylation had significantly shorter telomere length than those with LINE-1 hypermethylation. Given their sequence contexts, LINE-1 elements comprise a greater number of bases in subtelomeric regions across the genome than do Alu elements (154).

The limitation of this study should be considered. First, measurement of global methylation was performed with DNA from peripheral blood leukocytes, which may not reflct methylation levels in tissue-specific liver cells; however, global methylation in leukocyte DNA has been shown to be associated with BA development (80). Second, white blood cell differentials were not measured in the present study. Peripheral blood leukocytes contain a heterogeneous mixture of cell types, each cell population contributing its own unique methylation and telomere length to the final analysis. Therefore, further studies on differential analyses of white blood cells will be necessary in order to validate that apparent differences in global methylation and/or telomere length are not in fact differences in leukocyte cell type composition. Additionally, because the subjects in this study are from hospital-based participants rather than the general population, there might be some risk of selection bias if they had any differences in terms of the studied exposures. Moreover, the timing of blood draws varied with respect to time since diagnosis and treatment, which introduces uncertainty regarding correlations between clinical outcomes and Alu hypomethylation. This, the associations identified in leukocyte DNA may represent either causal, consequential or coincidental relationships. Longitudinal or prospective cohort studies will be needed to verify the risk-effect of global hypomethylation on BA susceptibility. Furthermore, DNA methylation level estimations may be confounded by other factors such as environmental exposures, parental smoking, socioeconomic status, ethnicity, body mass index, and lifestyle habits. Unfortunately, such information would be unavailable due to limitations of records accessibility. Therefore, residual confounding might still exist. To address these challenges, future studies should collect prospective measurements of these data to preclude bias and reverse causation. Lastly, sample size of BA subgroups was relatively small. This factor diminished the power of statistics, resulting in a failure to observe significant differences of Alu methylation among BA subgroups. Larger studies with various ethnic groups/races are warranted to evaluate the differences between subgroups.

To sum up, this study reported that, independent of risk factors, hypomethylation of retrotransposable DNA elements in peripheral blood leukocytes

was associated with shorter telomeres, elevated oxidative DNA damage, and a higher risk of BA. Accordingly, hypomethylation of retrotransposable DNA elements in peripheral blood leukocytes may serve as a potential biomarker for BA susceptibility. Examinations to elucidate whether genome-wide methylation in peripheral blood reflects epigenetic changes in liver tissue will be essential to elicit and identify the role of epigenetics in BA. Future research in both gene-specific methylation and potential underlying mechanisms related to retrotransposon methylation will help to elucidate the effect of epigenetic alterations in BA etiology, potentially yielding new diagnostic and therapeutic approaches in BA.

CHAPTER VIII

DISCUSSION AND CONCLUSION

Biliary atresia (BA) is the most common cause of pediatric end-stage liver disease that destroys the extrahepatic bile ducts and disrupts bile flow. With a poorly defined disease pathogenesis, primary treatment consists of the surgical removal of duct remnants followed by Kasai portoenterostomy (KPE). However, even with restoration of bile flow at the time of surgery, the majority of patients with BA develop end stage liver disease with biliary-type cirrhosis and eventually require liver transplantation. A definitive diagnosis of BA is often delayed, and currently requires invasive procedures including liver biopsy and intra-operative cholangiography. New noninvasive diagnostic indicators may speed diagnosis, thereby improving patient outcome following KPE. Consequently, the mechanisms of pathogenesis need to be clarified to identify new therapeutic targets for slowing the progression of disease and delaying the need for liver transplant. In the present study, we investigated the effect of cytokines on the pathogenesis of biliary injury and fibrosis that may serve as novel biomarkers. Our novel findings of contributors for hepatic dysfunction in BA include: (1) elevated serum autotaxin (ATX) was correlated with hepatic dysfunction in BA; (2) transcription and translation of *ATX* gene were increased in BA patients, and (3) methylation status of *ATX* promoter was significantly decreased related to liver fibrosis. Moreover, the molecular mechanisms of cellular senescence have been reported in the pathogenesis of biliary diseases. Due to telomere shortening, DNA damage, inflammation, and other forms of cell stress all function as stimuli for cellular senescence, (4) we therefore explored several indicators for cellular senescence including telomere length, global DNA methylation, and oxidative DNA damage in BA patients as evidenced by telomere shortening related to an elevated risk of liver cirrhosis in BA. Furthermore, (5) global DNA hypomethylation as an indicator for

genomic instability was correlated with high oxidative DNA damage and telomere shortening in BA patients.

It is well known that BA is a rapidly progressive fibrosis of the biliary tree and the liver. Despite extensive research efforts, it still remains uncertain when BA starts and how the ongoing deterioration of the liver can be presented. For decades, several cytokines have been proposed to play an important role in the regulation of hepatic fibrogenesis. As previously described in chapter III, progression of liver fibrosis is associated with hepatic stellate cells (HSCs) proliferation. Autotaxin has an effect on hepatic fibrogenesis via the enzymatic production of lysophosphatidic acid. LPA has been reported to inhibit apoptosis, stimulate, and contract rat HSCs (12). Hence, one can speculate that elevated serum ATX levels could be a biochemical indicator for activation of HSCs during the development of liver fibrosis. In the present study, we demonstrated that serum ATX concentration was significantly higher in BA patients than in healthy controls. Subsequent analysis in this study showed associations of serum ATX concentration with jaundice status, hepatic dysfunction, and liver fibrosis in BA patients. The results presented here are similar to the previously reported by Watanabe et al. when they investigated serum ATX in patients with chronic hepatitis C (57). They reported an increased ATX levels in the circulation from patients with chronic hepatitis C and observed a positive correlation between serum ATX levels and liver fibrosis. The authors concluded that serum ATX may be associated with fibrosis in liver disease. In 2014, Pleli et al. studied relationships between circulating ATX concentration and severity of cirrhosis as well as prognosis of cirrhotic patients (104). They illustrated that elevated serum ATX levels were associated with cirrhotic stage and LPA levels in cirrhotic patients. Collectively, these studies suggested that serum ATX could be used as an indicator for the severity of liver disease and the prognosis of cirrhosis patients.

Several possible mechanisms may contribute to the significant elevation of serum ATX in BA patients, especially in those with a poor outcome. The elevated serum ATX is likely attributed to an increase in ATX production, a reduction in ATX

clearance from the circulation or a combination of both. Furthermore, the decreased clearance could be caused by reduced uptake of ATX by liver sinusoidal endothelial cells (LSEC) (105). Lack of LSEC fenestration and formation of an organized basement membrane resulting in the capillarization of liver sinusoids, not only precedes fibrosis, but is also permissive for HSCs activation and fibrosis (106). Thus, dysregulation of the LSEC phenotype is a critical step in liver fibrosis. This process may lead to a reduction in ATX clearance thereby increasing circulating ATX levels. Additionally, other organs apart from the liver can produce and secrete ATX in systemic circulation. Over decade ago, ATX expression has been evident in brain, lung, heart, liver, duodenum, adrenals, and skeletal muscle, indicating that ATX could be expressed in various tissues or organs (44). The major sources of high serum ATX in this study may be extrahepatic organs. The elevated ATX levels could be regarded as indicating hepatic injury and cholestasis in BA children.

Whilst this study revealed a direct association between an increase in circulating ATX and the pathogenesis of BA, tissue expression of ATX is needed to determine for more valuable information on the pathophysiologic role of ATX in BA. Hence, we confirmed mRNA and protein expression of ATX in liver biopsies from BA children, choledochal cysts (CC), and healthy controls, as previously described in chapter IV. Our results demonstrated that *ATX* mRNA was upregulated in liver samples of infants with BA when compared with CC controls. This is in line with Wu et al. findings demonstrating overexpression of *ATX* in liver tissues of patients with hepatocellular carcinoma (103). Besides up-regulation of *ATX* mRNA in the livers of infants with BA, it has shown an increase in hepatic protein expression of ATX. Although our immunohistochemistry data showed positive cytoplasmic ATX expression in the inflammatory cells and the biliary epithelial cells, its expression did not correlate with biochemical abnormalities in BA patients; thereby, its diagnostic or prognostic applications remain limited at the moment. Nonetheless, supporting the results presented here, Vejchapipat et al. reported no association between hepatic expression of inducible nitric oxide synthase and clinical outcomes in BA children (118). Whereas, Honsawek et al. observed associations of cyclooxygenase-2 expression with clinical

outcomes including serum total bilirubin, AST, and ALT in BA infants (119). A partial explanation for no association of hepatic ATX expression with BA outcomes might be associated with the nature of BA disease itself. Thus, it is reasonable to postulate that an increase in *ATX* expression from BA livers might be a defensive response of the body to fight against hepatic impairment, or simply a compensatory response to ATX sensitivity resistance which leads to its compensatory up-regulation. The results from ATX mRNA and protein expression suggest that ATX might be related to the progression of liver injury in BA. Based on these findings, serum ATX and liver stiffness measurements could serve as possible noninvasive biomarkers reflecting the disease severity and the development of liver fibrosis in the post Kasai BA patients. Given that elevated ATX levels contribute to the pathogenesis of liver fibrosis in BA, we hypothesized that the hypomethylation of *ATX* promoter region could upregulate the *ATX* expression in BA patients.

Modifications in DNA methylation can be caused by drugs, viruses or genetic factors, leading to suppression of gene expression. DNA hypomethylation has been shown to result in development of autoimmune diseases (52) and inhibiting lymphocyte differentiation (155). Importantly, in 2004 Zhang et al. identified a set of genes that are known to be regulated by DNA methylation (156). They observed an upregulation of those genes in the liver from BA patients. The present study provides further evidence of the primary role that epigenetic modifications assume in influencing level of gene expression in BA (chapter V). This revealed specific CpGs within the *ATX* promoter were hypomethylated in both peripheral blood leukocytes and liver tissues from BA patients, which was supported by significantly elevated *ATX* expression and a corresponding increase in ATX protein levels. The *ATX* promoter hypomethylation also correlated negatively with the severity of clinical parameters and hepatic fibrosis (AST, ALP, and liver stiffness). This suggests that epigenetic mechanisms could play a possible role in the regulation of *ATX* expression regarding hepatic dysfunction and/or hepatic fibrosis. It seems plausible that the selected promoter regions of *ATX* contain the coding sequence, thus affecting the transcription of *ATX* gene. These findings support prior evidence that DNA methylation is an

important determinant of BA (73), as well as a stimulator of stellate cells and progressive hepatic fibrosis in animal model (127). A number of potential explanations have been proposed regarding these findings observed in this part. Methylation of CpG sites at the promoter region may limit access to the binding of different transcription factors to DNA, thus hindering transcriptions that mediate specific molecular pathways in the pathogenesis of BA and that may be influenced by those transcription factors. In addition, determinants of hypomethylation known to be involved in various diseases have been associated with altered DNA methyltransferase activity, histone modifications, and exogenous insults including diet, environment, non-coding RNAs, and defection of DNA repair system (88). Similarly, potential causes of BA, including viral infections, toxins, and genetic defects, may influence alterations in DNA methylation. Other environmental factors with diverse epigenetic backgrounds could also be implicated. It is possible that epigenetic variations might be a contributory factor associated with etiology of BA.

Although several plausible theories of viral, toxic, genetic, and immunological etiologies have been generally considered, the exact pathophysiology of biliary atresia remains mysterious. Interestingly, emerging evidence suggests the effect of cellular senescence in the regulation of biliary growth and injury (157-159). A number of factors including telomeric dysfunction, DNA damage, and oxidative stress, persuade senescence in division competent cells. As mentioned in chapter VI, this study showed a reduction of relative telomere length (RTL) in both of peripheral blood leukocytes and liver biopsies from BA patients when compared to age-matched healthy controls. In addition, leukocyte RTL was associated with age and degree of fibrosis in BA patients. Albeit, previously there have been little information regarding telomere dysfunction as a mechanism leading to BA disease, a study by Sanada et al. investigated telomere length in the livers from BA infants (86). They reported normalized telomere/centromere ratios to be significantly reduced in BA livers when compared to healthy controls. Moreover, Wiemann et al. revealed that telomere shortening in hepatocytes and senescence were associated with fibrotic scarring in human cirrhosis (85). Although the role of telomere shortening in BA has not been well established,

these studies suggest that telomere length might be an indicator of cirrhosis progression and telomere shortening may play an essential part in the pathogenesis of chronic liver injury in BA.

Chronic liver injury induces regeneration and repair processes in hepatocytes, which leads to elevated cell turnover and ultimately results in excessive telomere shortening. When telomeres become critically shortened, they cause impairment of cell proliferation and senescence. Eventually, hepatocyte growth is arrested and/or senescence assumes a profibrogenic state, either or both trigger the activation of stellate cells by as yet uncertain mechanisms, leading to fibrogenesis in the liver (141). It is noteworthy that the RTL was considerably shorter in the advanced BA patients with jaundice, when compared with jaundice-free patients, indicating that RTL could be a non-invasive parameter reflecting the severity of biliary atresia.

The mechanism of shortened RTL in leukocytes is not easily addressed. Given the complexity of telomere biology, it merits thorough and complex investigation for clear understanding. Several possible mechanisms either independently or in combination, may be speculated. In BA, the natural progressive decrease of telomere length with age could be accelerated by telomeric DNA damage due to oxidative stress, chronic inflammation, increased cellular turnover, and/or defects in telomere repair (18). Telomeric DNA sequences, rich in guanine residues, are likely more susceptible to oxidative stress, particularly by the formation of 8-hydroxy-2′-deoxyguanosine (8-oxodG). Moreover, these could promote DNA double-strand breaks particularly at telomeric regions resulting in the loss of the distal fragments of telomeric DNA and, thus, telomere shortening with each cell division (142). Inflammation triggers cellular proliferation and accelerates cell turnover, therefore facilitating telomere attrition due to the end-replication problem. A genetic predisposition must be taken into account. It is conceivable that a variety of these factors may act in concert to generate the phenomenon. The mechanism behind the connection of shortened RTL in leukocytes and liver cirrhosis in BA remains a mystery and requires further study. Interestingly,

previous study has documented the role of global DNA methylation in the variability of telomere length (19).

Methylation of retrotransposable elements associates with global DNA methylation. Hypomethylation in these elements may increase their activity as retrotransposon sequences, resulting in genomic alterations and more mutations by several different mechanisms (145). Epigenetic mechanism, especially DNA methylation also appears to be an important component of telomere length regulation as suggested by Yehezkel et al. They reported the association between telomere shortening and DNA hypomethylation, especially in subtelomeric DNA repeats (29). This might result from mutation in the DNA methyltransferase 3b gene. Based on this knowledge, we hypothesized that global DNA hypomethylation could be involved in the pathogenesis of BA and correlate with telomere length as well as oxidative DNA damage. As the above mentioned in chapter VII, the results in this study revealed hypomethylation of Alu and LINE-1 elements in peripheral blood leukocytes from BA patients. Reduction of both Alu and LINE-1 methylation levels was also associated with increased risk of BA, suggesting that global methylation and potentially other forms of epigenetic modification might be useful in predicting the risk of BA. These findings are consistent with prior studies that reported Alu hypomethylation in post-menopausal women with osteoporosis (24) and patients with glioma cancer (25). Moreover, LINE-1 hypomethylation was found in patients with hepatocellular carcinoma (HCC) as investigated by Tangkijvanich et al. when they compared LINE-1 methylation levels in the circulations from patients with HCC and non-neoplastic group (27). In 2009, Lee et al. also reported similar finding of LINE-1 hypomethylation when they studied LINE-1 methylation in liver biopsies from patients with HCC and non-neoplastic patients (155).

LINE-1 elements encode enzymes that allow them to replicate and insert themselves into different genomic regions, altering transcription and translation into functional proteins (146, 160). Transcription of LINE-1 elements has been shown to contribute to transcriptional regulation of human development genes and cell differentiation (147, 148). In this study LINE-1 methylation in BA patients was inversely

correlated with severity of liver stiffness, but there was no association between Alu methylation and outcome parameters in BA patients. This could represent an alteration in gene expression that might have a mechanistic role in BA development. The exact reason for this discrepancy between LINE-1 and Alu remains unexplained. It might be attributable to a difference in the sequence context of the two elements, with LINE-1 preferring AT-rich regions; whereas, Alu elements tend to incorporate in regions of the genome with high GC content. As such, methylation of these elements could be distinctly regulated. The result regarding association of LINE-1 methylation with poor outcomes in BA patients supports prior evidence that epigenetic modifications are an important factor in BA etiology (73). Furthermore, there is substantial evidence suggesting an important role of IFN-γ in bile duct injury in BA disease (161). Importantly, the release of inflammatory cytokine-like IFN-γ can be dependent on transcription factors binding within retrotransposable elements (162). Therefore, it is possible that other specific inflammatory exposures previously known to be associated with the pathogenesis of BA might lead to epigenetic changes, such as hypomethylation of retrotransposable elements.

Chronic inflammation can cause biliary duct injuries in BA, eventually resulting in accelerated production of active oxygen (149). As a general rule, oxidative stress is a consequence of reactive oxygen species (ROS) accumulation influencing DNA lesions. Given its role as the most common cause of genomic lesions, 8-OHdG was generally applied as a biomarker of oxidative DNA damage. In support of this hypothesis, the current study demonstrated that plasma 8-OHdG levels were significantly higher in BA patients than in controls. In the above mentioned inflammatory response study by Asakawa et al., the investigators also determined that oxidative stress, notably 8-OHdG, was highly expressed in liver tissues of BA patients (149). Similar to previous study by Tiao et al., they also investigated 8-OHdG expression in BA livers and reported that 8-OHdG protein expression was substantially increased in early stage BA patients, as compared with patients who received liver transplantation or who had choledochal cyst (150). Based on strong evidence gained from studies involving the role of ROS-induced oxidative stress in altering the epigenetic involvement, 8-OHdG could induce

DNA hypomethylation by inhibiting the capacity of DNA methyltransferases (DNMTs) to bind and methylating DNA at nearby cytosine bases (94). This may contribute to changes in global DNA methylation, such as on Alu or LINE-1 sequences. To prove this finding, we compared 8-OHdG concentrations in hypomethylation and hypermethylation status of Alu and LINE-1 elements in BA patients and found elevation of 8-OHdG concentrations in BA patients with both Alu and LINE-1 hypomethylation. Moreover, Alu and LINE-1 methylation levels were inversely correlated with plasma 8-OHdG levels in BA patients. This suggests that Alu and LINE-1 hypomethylation might have an effect on genomic instability and that this elements-caused damage is associated with BA, which is possibly mediated by increased oxidative DNA damage.

The mechanisms underlying changes of retrotransposon methylation in peripheral blood leukocytes from BA patients are still poorly understood, with a number of possible explanations for these associations observed here having been suggested. Alu and LINE-1 hypomethylation may result from the effects of an inflammatory response or increased oxidative stress-mediated genomic instability, which might be a critical factor in the pathophysiology of BA. An alternative explanation is that hypomethylation of two transposable elements could be due to both genetic and environmental factors. Global methylation changes have been indicated to be directly related to genetic control regarding genes controlling one-carbon metabolism or DNMTs activity (163). As a result, it is also possible to postulate that Alu and LINE-1 elements may provide a link between such environmental or genetic factors and BA.

The present study acknowledges certain limitations that should be noted. First, these cross-sectional associations do not allow conclusions to be drawn with respect to direction of causation, and the potential for confounding cannot be dismissed. To further address this question of cause or consequence, prospective cohort or experimental studies will help to elucidate any relationships. Secondly, because the subjects in this study are from hospital-based participants rather than the general population, there might be some risk of selection bias if they had any differences in

terms of the studied exposures. Moreover, the timing of blood draws varied with respect to time since diagnosis and treatment, which introduces uncertainty regarding these relationships presented here. Other limitations would be that white blood cell differentials were not measured in the present study. Peripheral blood leukocytes contain a heterogeneous mixture of cell types, each cell population contributing its own unique methylation and telomere length to the final analysis. Therefore, further studies on differential analyses of white blood cells will be necessary in order to validate that apparent differences in global DNA methylation and/or telomere length are not in fact differences in leukocyte cell type composition. Based on telomere length study, we evaluated telomere length in BA patients, but did not measure the activity of telomerase enzymes. As such, we were not able to determine the effects of telomerase activation and the dynamics of telomere length relating to BA in these results. Furthermore, DNA methylation level estimations may be confounded by other factors such as environmental exposures, parental smoking, socioeconomic status, ethnicity, body mass index, and lifestyle habits. Unfortunately, such information would be unavailable due to limitations of records accessibility. Accordingly, residual confounding might still exist. To address these challenges, future studies should collect prospective measurements of these data to preclude bias and reverse causation. Lastly, sample size of BA subgroups was relatively small. This factor diminished the power of statistics, resulting in a failure to observe significant differences of Alu methylation among BA subgroups. Larger studies with various ethnic groups/races are warranted to evaluate the differences between subgroups.

In summary, this study is the first to document that circulating ATX levels, hepatic ATX expression, relative telomere length, global DNA methylation, and oxidative DNA damage have been identified in postoperative patients with BA. Here, this study reported that high serum ATX was associated with status of jaundice, hepatic dysfunction, and liver stiffness in postoperative BA. In addition, the current study provides evidence for up-regulation of *ATX* mRNA in liver specimens of BA patients compared to those in controls. Study confirming the up-regulation of *ATX* in BA liver samples was performed with immunocytochemical detection of ATX antigens within

the liver bile duct epithelia and the hepatocytes. Moreover, this study supports the association between short telomere length in leukocytes and higher risk of liver cirrhosis in BA. Relative telomere length in peripheral blood leukocytes was also associated with disease severity, showing that BA patients with advanced-stage exhibited excessive telomere shortening. Additionally, this study reported that, independent of risk factors, hypomethylation of retrotransposable DNA elements was associated with shorter telomeres, elevated oxidative DNA damage, and a higher risk of BA. Based on these findings, combinations of circulating ATX levels, hepatic ATX expression, relative telomere length, global DNA methylation, and oxidative DNA damage could serve as possible noninvasive biomarkers reflecting the disease severity and the development of liver fibrosis in the post Kasai BA patients. Although underlining mechanisms of the cause-and-effect relationships are not entirely elucidated, there is abundant room for further research regarding the potential role of these contributory factors-mediated hepatic dysfunction in BA. Further understanding of the pathogenesis of BA will provide new therapeutic approaches to the treatment of chronic liver disorder including biliary atresia.

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APPENDIX

VITA

Miss Wanvisa Udomsinprasert was born on the 28th of July 1988 in Bangkok, Thailand. She is the daughter of Mrs. Suthalinee Saekham and Mr. Keerati Udomsinprasert. She graduated with a Bachelor's degree in Medical technology from Chulalongkorn University in 2010 and graduated with a Master's degree in Medical science with outstanding academic performance award from Faculty of Medicine, Chulalongkorn University in 2011. She also pursued her Doctoral degree at Chulalongkorn University in 2012.

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