รายงานวิจัยฉบับสมบูรณ์

โครงการ บทบาทของออโตแทกซินต่อพยาธิกำเนิดของพังผืดตับในโรคท่อน้ำดีตีบตัน

โดย ศาสตราจารย์ นายแพทย์สิทธิศักดิ์ หรรษาเวก

มิถุนายน 2561

สัญญาเลขที่ RSA5880019

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
จุฬาลงกรณ์มหาวิทยาลัย

Abstract (บทคัดย่อ)

Project Code : RSA5880019

(รหัสโครงการ)

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(ชื่อโครงการ)

Investigator : ศาสตราจารย์นายแพทย์สิทธิศักดิ์ หรรษาเวก

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(ระยะเวลาโครงการ)

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 130 ราย และกลุ่มควบคุมซึ่งมีสุขภาพดี 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 และภาวะเครียดออกซิเดชันอย่างมีนัยสำคัญทางสถิติ สรุปได้ว่า ระดับโปรตีนออโต้แทกซิน การแสดงออกระดับ mRNA ของยีนโอโต้แทกซิน ระดับ promoter methylation ของยีนออโต้แทกซิน ความยาวของเทโล

เมียร์ ระดับ global methylation และภาวะเครียดออกซิเดชัน มีความสัมพันธ์กับลักษณะอาการทางคลินิกของผู้ป่วยโรคท่อน้ำดีตีบตัน ซึ่งอาจจะนำมาใช้เป็นตัวบ่งชี้ทำนายความรุนแรงของโรคท่อน้ำดีตีบตันโดยเฉพาะในผู้ป่วยที่มีพังผืดตับ และช่วยทำให้ความเข้าใจกลไกและพยาธิกำเนิดของการเกิดพังผืดตับในโรคตับเรื้อรังรวมทั้งโรคท่อน้ำดีตีบตันได้ดียิ่งขึ้น

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. The etiology, pathogenesis, and factors modifying the disease progression remain largely mysterious. 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. 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 purpose of this study was to investigate autotaxin (ATX), relative telomere length (RLT), global DNA methylation and oxidative stress whether these biomarkers could be related to liver stiffness and outcome parameters of liver fibrosis in BA. One hundred and thirty postoperative BA patients and 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 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 chronic liver disease including biliary atresia.

Keywords : Biliary atresia, Autotaxin, Liver fibrosis, Global methylation, Telomere length
(คำหลัก)

Executive summary

This study showed that 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. Moreover, promoter hypomethylation and overexpression of ATX were inversely associated with jaundice status, hepatic dysfunction, and liver stiffness in BA patients. These findings suggest that the promoter hypomethylation and overexpression of ATX might play a contributory role in the pathogenesis of liver fibrosis in BA. BA patients had significantly shorter telomeres than healthy controls. The RTL in BA patients with jaundice was significantly lower than that of patients without jaundice. Alu and LINE-1 hypomethylation, and telomere shortening were found to be associated with elevated risk of BA. Furthermore, LINE-1 methylation was associated with liver stiffness in BA patients. Stratified analysis revealed negative correlations between Alu and LINE-1 methylation and 8-OHdG in BA patients. In contrast, positive relationships were identified between Alu and LINE-1 methylation and relative telomere length in BA patients. These findings suggest that retrotransposon hypomethylation is associated with plasma 8-OHdG and telomere length in BA.

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

Biliary atresia (BA) is a devastating cholestatic liver disorder. Autotaxin (ATX) has a profibrotic effect resulting from lysophosphatidic acid activity. The purpose of this study was to investugate ATX expression and ATX promoter methylation in peripheral blood leukocytes and liver tissues from BA patients and controls and examine their associations with outcome parameters in BA patients. A total of 130 subjects were registered. DNA was extracted from peripheral blood leukocytes and liver tissues of BA patients and from and age-matched healthy controls. ATX promoter methylation status was determined by bisulfite pyrosequencing. ATX expression was analyzed using quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay. Decreased methylation of specific CpGs were observed at the ATX promoter in BA patients (Figure 1).

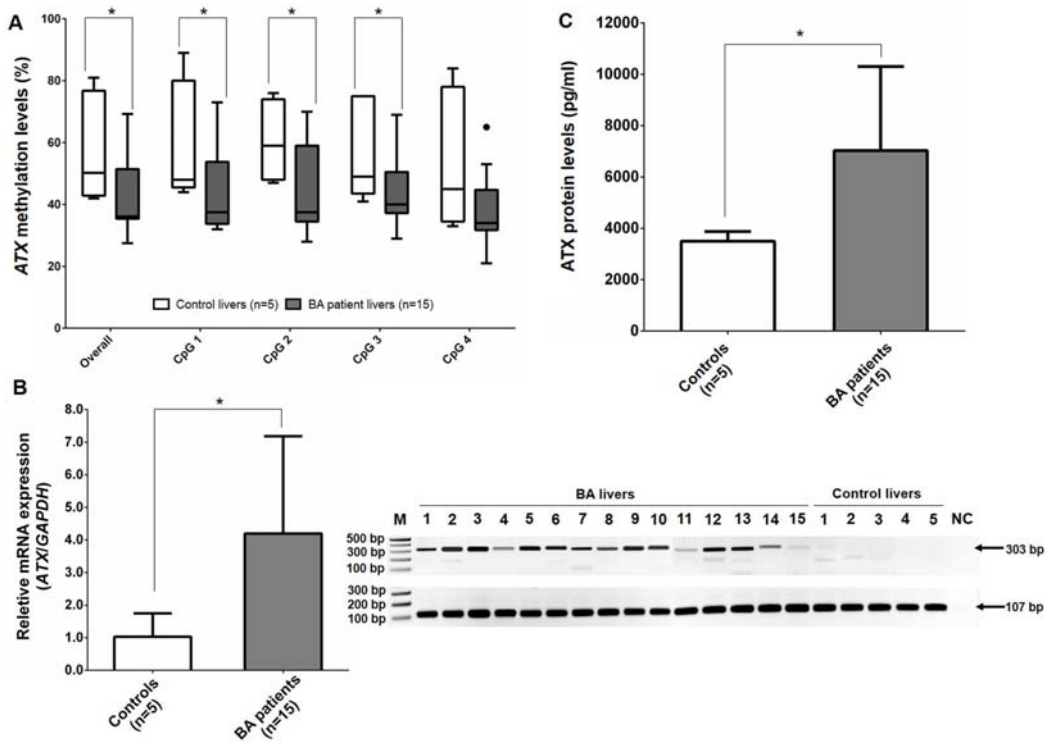

Figure 1. Distribution of the ATX promoter methylation, relative mRNA expression, and protein levels in liver tissue of BA patients and controls. (A) Decreased methylation levels of the *ATX* promoter in BA liver tissue samples. (B) Higher mRNA expression of *ATX* in BA cases and representative gel of *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.

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. Moreover, promoter hypomethylation and overexpression of ATX were inversely associated with jaundice status, hepatic dysfunction, and liver stiffness in BA patients (Table 1 and 2).

Table 1. 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)	0.07	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

Table 2. 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)	-0.04	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

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 (Figure 2). These findings suggest that the promoter hypomethylation and overexpression of ATX might play a contributory role in the pathogenesis of liver fibrosis in BA.

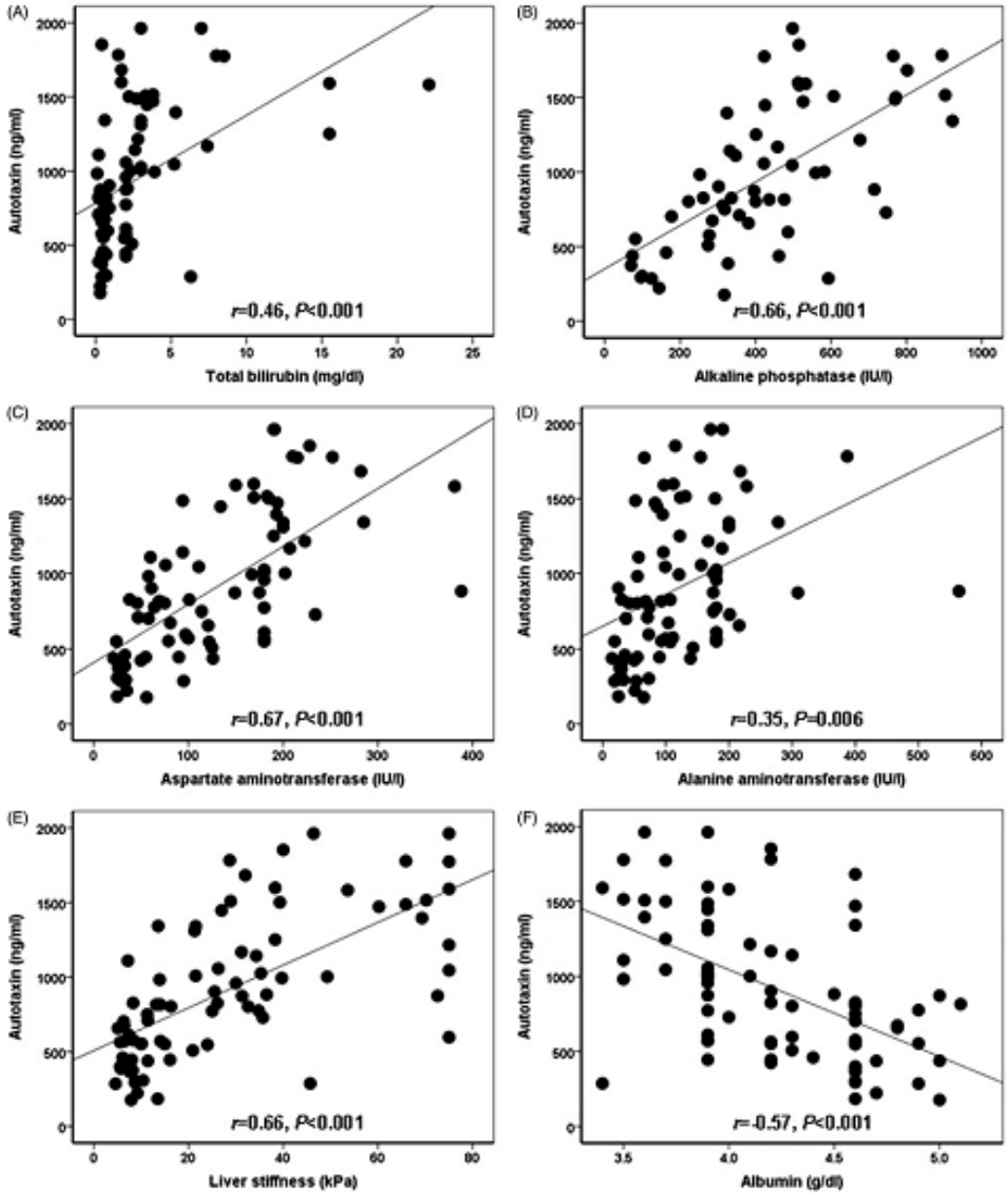

Figure 2. Scatter diagram and correlation analysis in biliary atresia patients. Serum autotoxin levels are correlated with total bilirubin (A), alkaline phosphatase (B), aspartate aminotransferase (C), alanine aminotransferase (D), liver stiffness (E), and albumin (F).

Alu and LINE-1 elements are retrotransposons with a ubiquitous presence in the human genome that result in genomic instability, especially 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). The objective of this study was to investigate correlations between global methylation, 8-OHdG, and relative telomere length (RTL), as well as reporting on Alu and LINE-1 hypomethylation in postoperative BA. BA patients had remarkably shorter telomeres than healthy controls ($P < 0.0001$) (Figure 3).

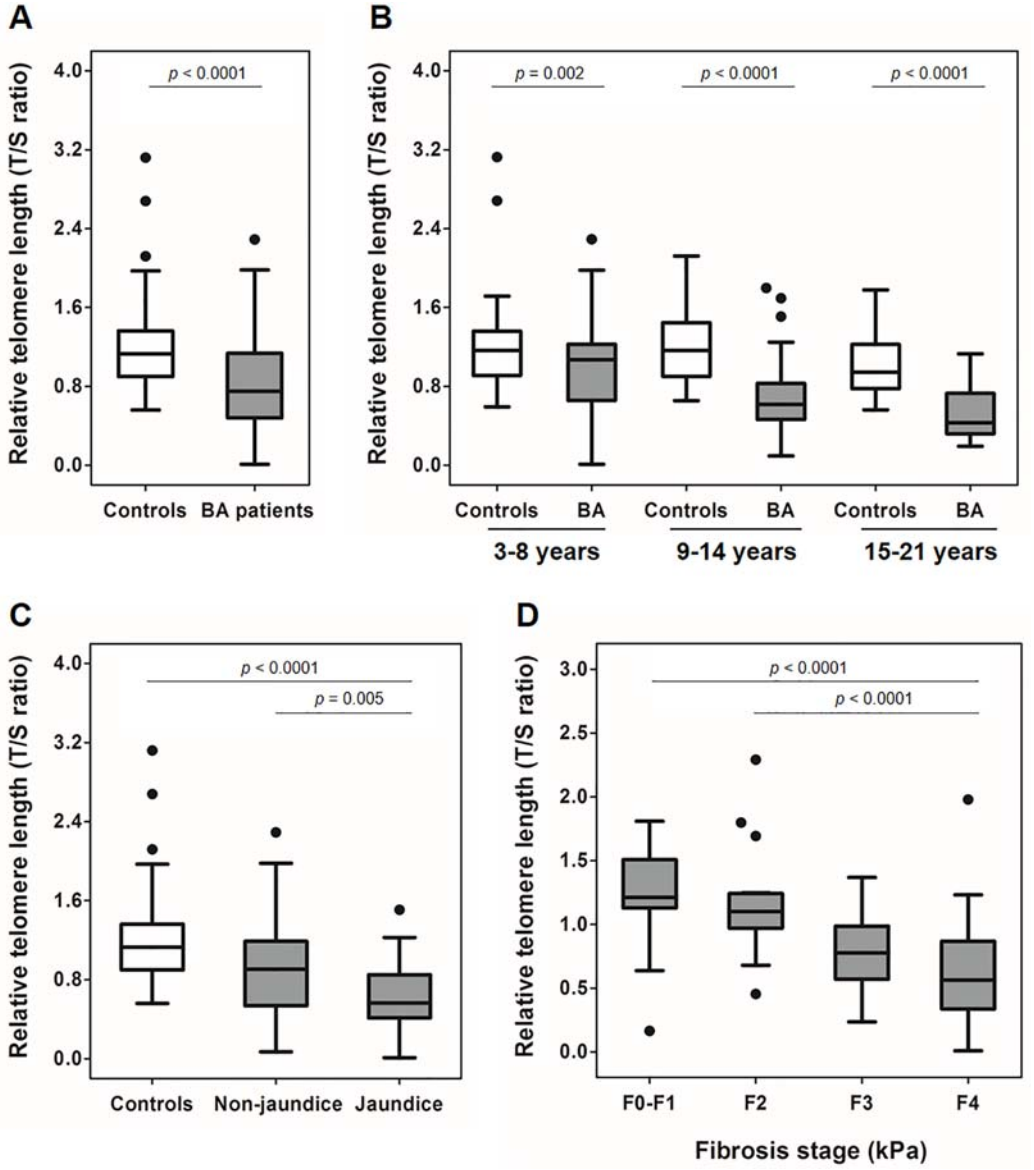

Figure 3 Box-plot illustrating telomere length distribution in subjects among different groups: The line through the middle of the boxes represents the median of T/S value and the top and bottom of each box represents the first and third quartiles. The lower and upper error bars are computed as the lower 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).

The RTL in BA patients with jaundice was significantly lower than that of patients without jaundice (P = 0.005). Alu and LINE-1 hypomethylation, and telomere shortening were found to be associated with elevated risk of BA (P < 0.0001) (Table 3 and 4).

Table 3. Association between global methylation and risk of BA. aUnconditional logistic regression analysis, adjusted for age and gender; *P*-value < 0.05 indicates statistical significance.

	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 (0.84–0.97)	< 0.001	0.88 (0.84–0.92)	< 0.0001
By median						
Low	78.07%	50.00%	4.07 (2.27–7.33)	< 0.0001	4.07 (2.27–7.32)	< 0.0001
High	21.93%	50.00%	1.00 (reference)		1.00 (reference)	
By tertile						
1st tertile	73.68%	33.33%	9.95 (4.54–21.80)	< 0.0001	9.98 (4.55–21.89)	< 0.0001
2nd tertile	14.46%	33.33%	2.53 (1.03–6.20)	0.04	2.51 (1.02–6.16)	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 (0.85–0.94)	< 0.0001	0.89 (0.85–0.94)	< 0.0001
By median						
Low	77.19%	50.00%	3.53 (1.88–6.61)	< 0.0001	3.51 (1.87–6.59)	< 0.0001
High	22.81%	50.00%	1.00 (reference)		1.00 (reference)	
By tertile						
1st tertile	62.28%	33.33%	6.46 (2.78–15.00)	< 0.0001	6.52 (2.79–15.27)	< 0.0001
2nd tertile	21.93%	33.33%	2.82 (1.17–6.82)	0.02	2.83 (1.17–6.88)	0.02
3rd tertile	15.79%	33.33%	1.00 (reference)		1.00 (reference)	
P-trend				< 0.0001		< 0.0001

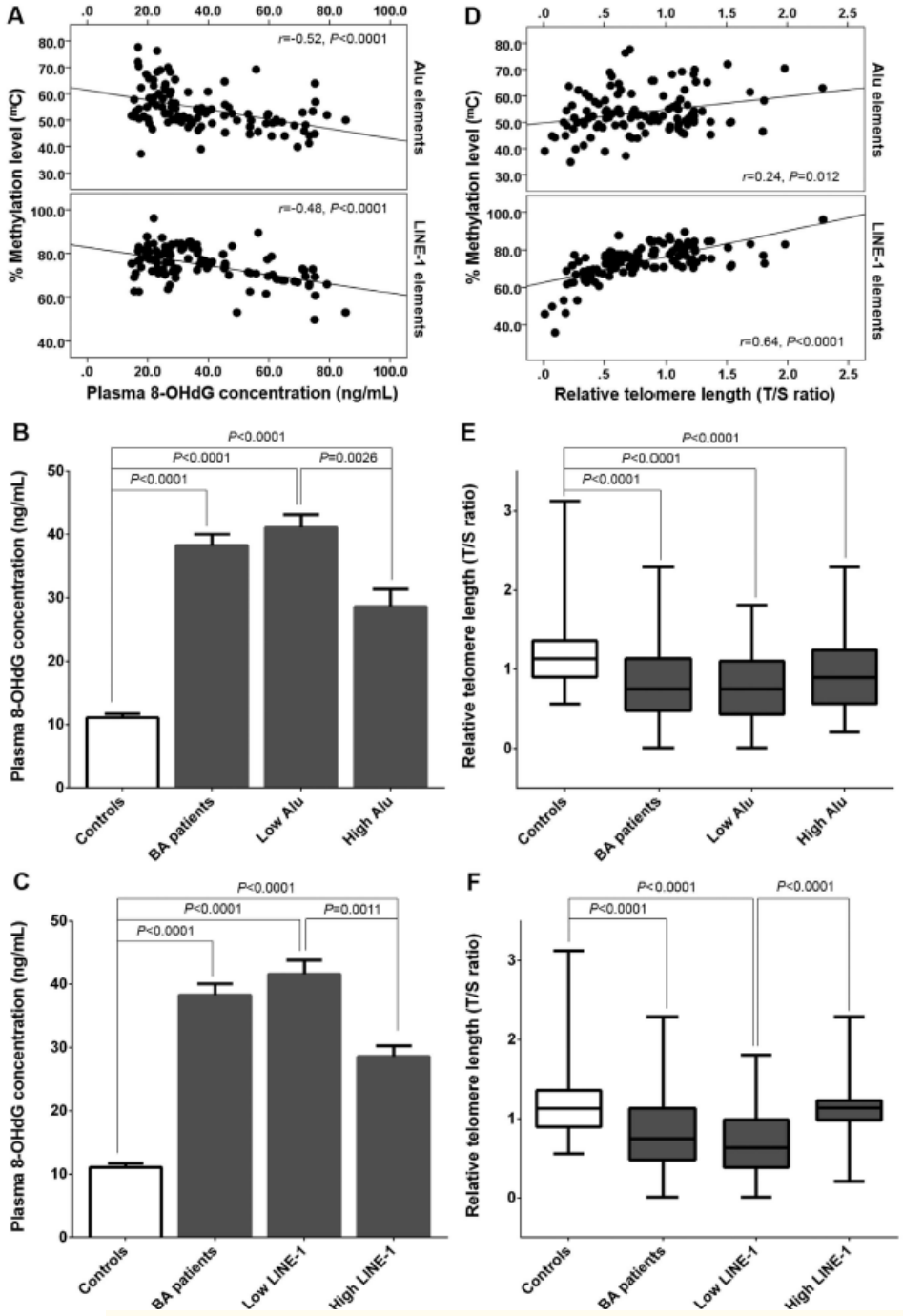

Figure 4. Relationships between global methylation, 8-hydroxy-2′-deoxyguanosine, and telomere length in BA. (A) negative correlations between Alu or LINE-1 methylation and 8-OHdG; (B) plasma 8-OHdG levels in BA patients with hypo- and hypermethylated status of Alu elements; (C) plasma 8-OHdG levels in 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.

Table 4. Multivariate linear regression analysis of global methylation estimates. 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.

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 (−1.10 to 5.78)	0.16
Liver stiffness (kPa)	0.03 (−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	0.02 (−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

Furthermore, LINE-1 methylation was associated with liver stiffness in BA patients (P < 0.0001). Stratified analysis revealed negative correlations between Alu and LINE-1 methylation and 8-OHdG in BA patients (P < 0.0001) (Figure 4). 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.

In conclusion, 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.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   1.1 Udomsinprasert W, Honsawek S, Anomasiri W, Chongsrisawat V, Vejchapipat P, Poovorawan Y. Serum autotaxin levels correlate with hepatic dysfunction and severity in postoperative biliary atresia. Biomarkers 2015;20:89-94.

   1.2 Udomsinprasert W, Poovorawan Y, Chongsrisawat V, Vejchapipat P, Zhan D, Honsawek S. Telomere Length in Peripheral Blood Leukocytes Is Associated with Severity of Biliary Atresia. PLoS One 2015;10:e0134689.

   1.3 Udomsinprasert W, Kitkumthorn N, Mutirangura A, Chongsrisawat V, Poovorawan Y, Honsawek S. Global methylation, oxidative stress, and relative telomere length in biliary atresia patients. Sci Rep. 2016 May 31;6:26969.

   1.4 Udomsinprasert W, Honsawek S, Jirathanathornnukul N, Chongsrisawat V, Poovorawan Y. Elevation of serum urokinase plasminogen activator receptor and liver stiffness in postoperative biliary atresia. World J Hepatol. 2016 Nov 28;8(33):1471-1477.

   1.5 Udomsinprasert W, Kitkumthorn N, Mutirangura A, Chongsrisawat V, Poovorawan Y, Honsawek S. Association between Promoter Hypomethylation and Overexpression of Autotaxin with Outcome Parameters in Biliary Atresia. PLoS One. 2017 Jan 4;12:e0169306.

   1.6 Honsawek S, Udomsinprasert W, Jirathanathornnukul N, Chongsrisawat V, Poovorawan Y. Elevated serum heat shock protein 70 and liver stiffness reflect hepatic dysfunction and severity in postoperative biliary atresia. Pediatr Surg Int. 2017 Aug;33:893-899.

   1.7 Homchan K, Chaiwatanarat T, Udomsinprasert W, Chongsrisawat V, Poovorawan Y, Honsawek S. Low bone mineral density and the severity of cholestasis in biliary atresia. World J Hepatol. 2017 Jun 8;9:746-751.

   1.8 Udomsinprasert W, Vejchapipat P, Klaikeaw N, Chongsrisawat V, Poovorawan Y, Honsawek S. Hepatic autotaxin overexpression in infants with biliary atresia. PeerJ. 2018. In press.

2. การนำผลงานวิจัยไปใช้ประโยชน์

   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

สามารถนำความรู้ที่ได้จากการศึกษาวิจัยไปใช้ในการพัฒนาการเรียนการสอนและสามารถสร้างนักวิจัยใหม่ ได้แก่ ดร.วันวิสาข์ อุดมสินประเสริฐ ซึ่งสำเร็จการศึกษาในระดับปริญญาเอก หลักสูตรวิทยาศาสตรดุษฎีบัณฑิต ชีวเคมีทางการแพทย์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และนางสาวกฤตภัค หอมจันทร์ ซึ่งสำเร็จการศึกษาในระดับปริญญาโท หลักสูตร วิทยาศาสตรมหาบัณฑิต วิทยาศาสตร์การแพทย์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
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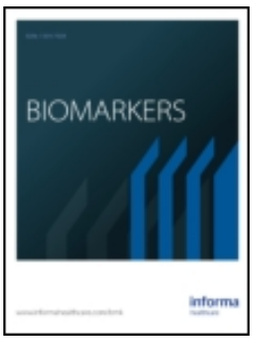

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RESEARCH ARTICLE

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 and disease severity in biliary atresia (BA).

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

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

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

Keywords

Autotaxin, biliary atresia, jaundice, liver stiffness, severity

History

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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 hypertension, hepatic failure, and ultimately die by the age of 2 years (Hartley et al., 2009). Surgical treatment which remains the standard of care for first line intervention for infants with BA is the Kasai portoenterostomy (Davenport, 2012). Failure of the Kasai procedure leaves liver transplantation as the only hope for survival (Hartley et al., 2009). 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 (A-Kader et al., 2003).

Autotaxin, also known as ENPP-2 (ectonucleotide pyrophosphatases/phosphodiesterase-2), is a 125 kDa secreted glycoprotein that belongs to the ENPP family (Yuelling & Fuss, 2008). It was originally characterized as an autocrine motility-stimulating factor from the conditioned medium of A2058 melanoma cells (Stracke et al., 1992). Since then, the increased expression of autotaxin has been shown in various malignant tumor growth and metastasis (Stracke et al., 1997). Autotaxin uniquely exhibits a lysophospholipase D (LPD) activity through which it hydrolyzes lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA) (Tokumura et al., 2002). Autotaxin is widely expressed in tissues such as brain, placenta or high endothelial venules (Fotopoulou et al., 2010; Iwasawa et al., 2009, Nakasaki et al., 2008). In heterozygous autotaxin-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 autotaxin is embryonic lethal due to blood vessel abnormalities, showing that autotaxin is responsible for the bulk of LPA production in blood (Tanaka et al., 2006; van Meeteren et al., 2006).

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 pro-fibrogenic factor in liver (Ikeda et al., 1998). 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

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in the production of abundant extracellular matrices (Wallace et al., 2008). Previous investigations have also suggested a connection between liver fibrosis and serum or plasma LPA and autotaxin was elevated in patients with chronic hepatitis C virus (HCV) infection (Nakagawa et al., 2011; Watanabe et al., 2007a). However, the origin and fate of serum autotaxin must be further studied and serum autotaxin 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 autotaxin 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 autotaxin, liver stiffness, and biochemical parameters in postoperative BA. We postulated that serum autotaxin would be elevated and associated with the disease severity and liver stiffness in BA patients, and to prove this hypothesis, we examined serum autotaxin and liver stiffness in BA patients compared with healthy controls. Therefore, the objective of this study was to analyze serum autotaxin levels collected from BA patients and to determine the possible correlations of serum autotaxin 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 ($TB \geq 2$ 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 autotaxin 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 autotaxin standards and serum samples were added into each well, which was pre-coated with a monoclonal antibody against autotaxin. After incubating for 2 h at room temperature, every well was washed thoroughly 4 times with wash buffer. Then, a horseradish peroxidase-conjugated polyclonal antibody specific for autotaxin was pipetted into each well and incubated for a further 2 h at room temperature. After 4 washes, substrate solution was pipetted into the wells and then the microplate was incubated for 30 min 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 autotaxin in the sample. Autotaxin concentration was determined by a standard optical density-concentration curve. Twofold serial dilutions of recombinant human autotaxin 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) $\times$ 100/platelet count (10^9/L) (Wai et al., 2003).

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, $p < 0.001$). In addition, BA patients had significantly higher serum autotaxin levels than healthy controls (905.9 ± 53.6 versus 290.0 ± 37.1 ng/ml, $p < 0.001$), as shown in Figure 1.

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 autotaxin, 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, $p < 0.001$). As presented in Figure 2, serum autotaxin levels in BA patients with jaundice were substantially greater than those in BA patients without jaundice (1144.4 ± 72.2 versus 642.3 ± 54.2 ng/ml, $p < 0.001$).

Further analysis showed that serum autotaxin levels were markedly elevated in BA patients with PH than those without PH (1078.5 ± 62.8 versus 585.4 ± 65.6 ng/ml, $p < 0.001$) (Figure 3). Additionally, serum autotaxin 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 autotaxin were inversely correlated with serum albumin ($r = -0.57$, $p < 0.001$). Correlations between serum autotaxin, ALP,

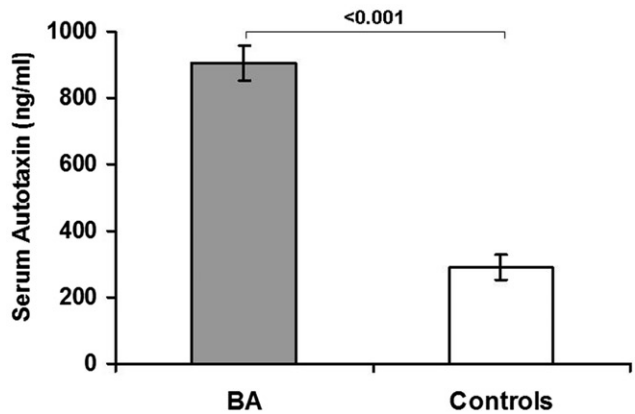

Figure 1. Comparison of serum autotaxin levels in postoperative biliary atresia patients and healthy controls. The data are expressed as mean ± SEM.

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
GGT (IU/l)	205.3 ± 20.5	–	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.

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
GGT (IU/l)	265.0 ± 28.9	128.3 ± 25.8	<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)	1144.4 ± 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.

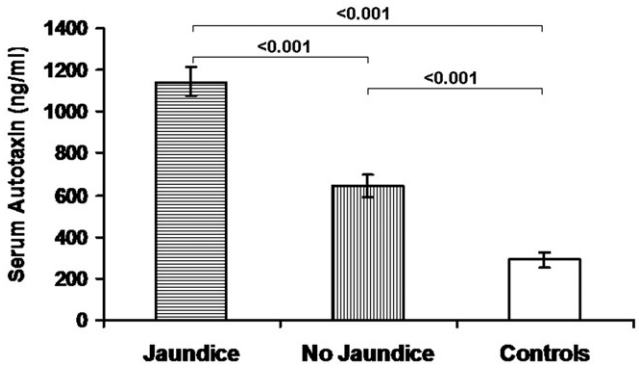

Figure 2. Comparison of serum autotaxin levels in biliary atresia patients with jaundice, biliary atresia patients without jaundice, and controls. The data are expressed as mean ± SEM.

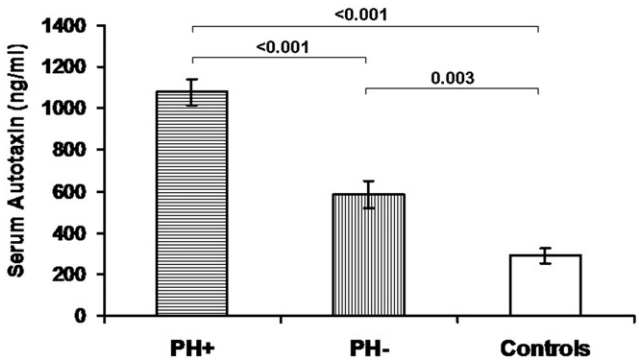

Figure 3. Comparison of serum autotaxin levels in biliary atresia patients with portal hypertension, biliary atresia patients without portal hypertension, and controls. The data are expressed as mean ± SEM.

AST, ALT, liver stiffness, and serum albumin are illustrated in Figure 4.

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 (Gressner & Weiskirchen, 2006). Progression of liver fibrosis is associated with an increased number of HSCs (Yamaoka et al., 1993). LPA, which appears to be the major biological effector of autotaxin, inhibits apoptosis, stimulates, and contracts rat HSCs (Ikeda et al., 1998). Therefore, one can speculate that elevated serum autotaxin 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 autotaxin, liver stiffness measurements, and biochemical parameters in BA patients after Kasai procedure.

In the present study, we demonstrated that serum autotaxin 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 autotaxin was positively correlated with serum total bilirubin, suggesting that serum autotaxin was associated with jaundice status in BA patients. We further found that elevated serum autotaxin 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 autotaxin and serum albumin. Thus, these findings indicate that autotaxin 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 autotaxin is elevated in BA patients compared with healthy controls. We also found that serum autotaxin was positively correlated with AST, ALP, TB, and liver stiffness, but negatively correlated with serum albumin. These results support that serum autotaxin is associated with jaundice status, hepatic dysfunction, and liver fibrosis in BA patients. Previous investigation has also indicated that autotaxin is a key enzyme for converting LPC to LPA and plasma LPA levels are correlated with the serum autotaxin activity in patients with chronic liver disease (Watanabe et al., 2007a). In agreement with our findings, Watanabe and colleagues demonstrated that serum autotaxin levels were elevated in patients with chronic hepatitis C (Watanabe et al., 2007a). A recent study using hepatectomized rats suggested that elevated autotaxin activity in rats with liver injury was caused by a decrease in autotaxin clearance (Watanabe et al., 2007b). In addition, Wu and coworkers further reported that the increased autotaxin expression was detected mainly in hepatocellular carcinoma (HCC) tissues compared to normal liver tissues and that autotaxin overexpression in HCC was specifically correlated with inflammation and liver cirrhosis (Wu et al., 2010).

It is notable that the more elevated serum autotaxin 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 autotaxin. A reduced first-pass effect in the liver may be responsible for the greater serum autotaxin in the patients with PH. In line with our findings, Pleli et al. have documented that serum autotaxin was associated with the stage of liver cirrhosis, the prevalence of esophageal varices, and portal hypertensive gastropathy, suggesting that serum autotaxin could be an indicator for the severity of liver disease and the prognosis of cirrhosis patients (Pleli et al., 2014).

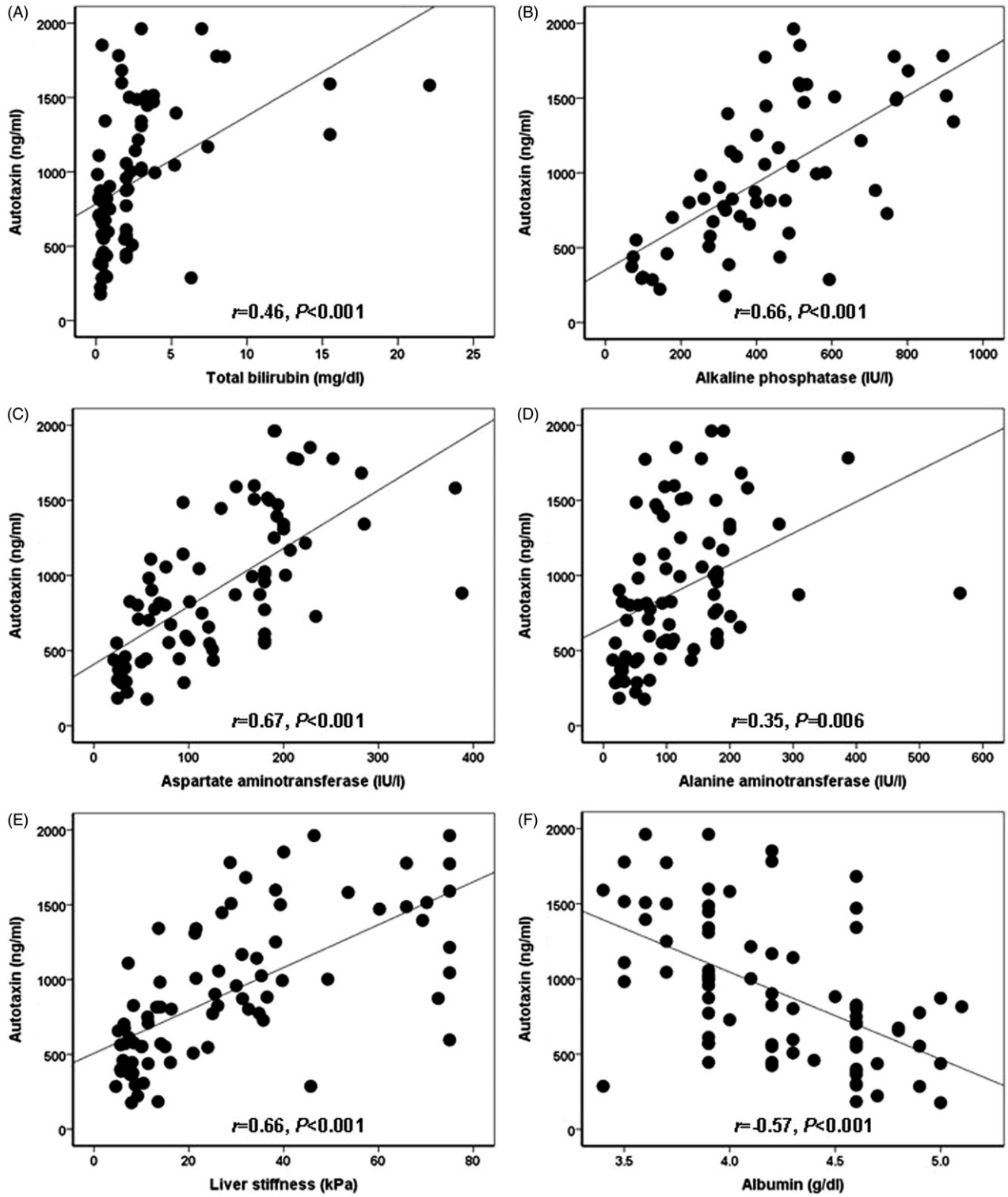

Figure 4. Scatter diagram and correlation analysis in biliary atresia patients. Serum autotoxin levels are correlated with total bilirubin (A), alkaline phosphatase (B), aspartate aminotransferase (C), alanine aminotransferase (D), liver stiffness (E), and albumin (F).

Several possible mechanisms may contribute to the significant elevation of serum autotaxin in BA patients, especially in those with a poor outcome. The elevated serum autotaxin is likely attributed to an increase in autotaxin production, a reduction in autotaxin 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 autotaxin by liver sinusoidal endothelial cells (LSEC) (Jansen et al., 2009). 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 (Muro et al., 1993). Thus, dysregulation of the LSEC phenotype is a critical step in liver fibrosis. This process may lead to a reduction in autotaxin clearance thereby increasing circulating autotaxin levels. Furthermore, other organs apart from the liver can produce and secrete autotaxin in systemic circulation. In recent years,

autotaxin expression has been evident in brain, lung, heart, liver, duodenum, adrenals, and skeletal muscle, indicating that autotaxin could be expressed in various tissues or organs (Stefan et al., 1999). The major sources of high serum autotoxin in this study may be extrahepatic organs. The higher autotaxin 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 autotaxin 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 autotaxin has not been determined. Additional immunohistochemical analysis of autotaxin hepatic expression could render more valuable information on the pathophysiologic role of autotaxin in BA.

In summary, the current evidence revealed that BA patients had significantly elevated serum autotaxin and liver stiffness values compared with healthy controls. Serum autotaxin 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 autotaxin than those without PH. Furthermore, serum autotaxin was associated with status of jaundice, hepatic dysfunction, and liver stiffness in post-operative BA. Based on these findings, serum autotaxin and liver stiffness measurements could serve as possible non-invasive 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 autotaxin 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 autotaxin in the pathogenesis of biliary atresia.

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Declaration of interest

This study has been facilitated by the Thailand Research Fund. The authors report no conflicts of interest. Wanvisa Udomsinprasert has been supported by the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship.

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ORIGINAL ARTICLE

Prospective Study

Low bone mineral density and the severity of cholestasis in biliary atresia

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Author contributions: Homchan K carried out laboratory work, collected the data, and analyzed the data; Udomsinprasert W collected blood samples and assisted in analysis of data; Chaiwatanarat T, Chongsrisawat V and Poovorawan Y examined all the patients and collected clinical data; Honsawek S designed the study, carried out laboratory work, analyzed the data, wrote the manuscript, and revised the manuscript for final submission.

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Informed consent statement: All study participants provided written informed consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at sittisak.h@chula.ac.th. Participants gave informed consent for data sharing.

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Abstract

AIM

To investigate the prevalence of osteopenia and osteoporosis in postoperative biliary atresia (BA) children and the association of bone mineral density (BMD) and biochemical parameters in postKasai BA subjects.

METHODS

A total of 70 patients with postKasai BA were enrolled in this prospective study. The patients were classified into two groups according to their jaundice status. BMD of the lumbar spine was analyzed using dual energy X-ray absorptiometry.

RESULTS

The prevalence of low bone mass (osteopenia and osteoporosis) in BA patients were 51.4% (36 out of 70). Ten patients (35.7%) in the jaundice group and 8 patients (19.0%) in the non-jaundice group had

osteopenia. Sixteen patients (57.1%) in the jaundice group and 2 patients (4.8%) in the no jaundice group had osteoporosis. In addition, lumbar spine BMD Z-score was substantially lower in the jaundice BA patients compared with non-jaundice patients. BA subjects with persistent jaundice had significantly lower serum 25-hydroxyvitamin D than those without jaundice. Further analysis revealed that lumbar spine BMD was correlated with age (r = 0.774, P < 0.001), serum albumin (r = 0.333, P = 0.005), total bilirubin (r = -0.476, P < 0.001), aspartate aminotransferase (r = -0.583, P < 0.001), alanine aminotransferase (r = -0.428, P < 0.001), and alkaline phosphatase(r = -0.456, P < 0.001).

CONCLUSION

Low BMD was associated with biochemical parameters reflecting the severity of cholestasis in postKasai BA patients.

Key words: Bone mineral density; Jaundice; Biliary atresia; Cholestasis; Severity

Core tip: Recent evidences have highlighted the importance of bone mineral density (BMD) in chronic liver disease including biliary atresia (BA). This study revealed that BA patients with persistent jaundice had significantly lower BMD and 25-hydroxyvitamin D than those without jaundice. Furthermore, lumbar spine BMD was correlated with hepatic dysfunction suggesting that low BMD was associated with outcome parameters reflecting the severity of cholestasis in postoperative BA patients.

Homchan K, Chaiwatanarat T, Udomsinprasert W, Chongsrisawat V, Poovorawan Y, Honsawek S. Low bone mineral density and the severity of cholestasis in biliary atresia. *World J Hepatol* 2017; 9(16): 000-000 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i16/000.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i16.000

INTRODUCTION

Biliary atresia (BA) is a progressive, idiopathic, necro-inflammatory process resulting in obliteration of the extrahepatic biliary tree resulting in intrahepatic cholestasis, hepatic fibrosis, biliary cirrhosis, and advanced chronic liver failure[1]. It is a rare disease, with the reported prevalence ranging from 1 in 5000 to 1 in 19000 live births[2]. It is the most common cause of neonatal jaundice for which surgery is indicated and also the most common indication for liver transplantation in children. The pathogenesis of BA has remained a mystery. Most of the causal theories include defects resulting from a viral infection or toxin exposure, defects in morphogenesis, genetic predisposition, defects in prenatal circulation and immune dysregulation[3-5].

Low bone mass is frequent in patients with chronic liver disorder including BA. Metabolic bone disease is a common disorder that can be found in patients with hepatic osteodystrophy, particularly those affected by chronic cholestasis[6,7]. Its etiology is complex and multifactorial and presents as osteopenia and osteoporosis which should be investigated and diagnosed early in patients with chronic liver disease in order to minimize the risk of fractures and improve their quality of life[8,9]. The purpose of this study was to determine bone mineral density (BMD) from postKasai BA children and to investigate the association of BMD and outcome parameters in postoperative BA patients.

MATERIALS AND METHODS

Patients

This investigation was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University and was conducted in compliance with the Declaration of Helsinki. All parents of BA children were informed of the study's objectives, and written informed consent was derived from the parents prior to the participants entering the study.

A total of 70 postKasai BA subjects (30 males and 40 females; mean age 7.6 ± 0.5 years) who attended the follow-up visit in Pediatric Liver Clinic at King Chulalongkorn Memorial Hospital were recruited in the present study. Among the 70 BA children in this study, none of them had any evidence of residual infection or ascending cholangitis or clotting abnormalities during venipuncture. None had experienced liver transplantation. To compare the clinical outcomes among BA subjects, they were allocated into two groups corresponding to their levels of serum total bilirubin (TB): Non-jaundiced group (TB < 2.0 mg/dL, n = 42) and persistently jaundiced group (TB ⩾ 2.0 mg/dL, n = 28).

Laboratory tests

Venous blood specimens were procured from each subject, centrifuged, and then kept at -80 ℃ until measurement. Liver function tests including TB, direct bilirubin, aspatate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were assessed using Hitachi 912 automated chemical analyzer at the central laboratory of our hospital. Serum 25-hydroxyvitamin D [25(OH)D] levels were analyzed using automated chemiluminescent immunoassay (Diasorin, Saluggia, Italy).

BMD assessments

Dual-energy X-ray absorptiometry scans (Hologic QDR 2000, Hologic Inc., Waltham, MA, United States) were performed on the lumbar spine (anteroposterior lumbar vertebrae L1-L4) of every subject for BMD assessments. BMD was reported as grams of mineral per square centimeter (g/cm^2) and Z-scores. Z-scores of BMD were expressed as numbers of standard deviations from the mean BMD of age matched norms. Children were

Table 1 Demographic data and laboratory parameters of biliary atresia patients based on status of jaundice

BA patients	Total	Jaundice	No jaundice	*P*-value
n	70	28	42	
Gender (male/female)	30:40	12:16	18:24	0.5
Age (yr)	7.6 ± 0.5	6.3 ± 0.8	8.6 ± 0.6	0.01
Albumin (g/dL)	3.9 ± 0.1	3.2 ± 0.3	4.3 ± 0.1	< 0.001
Total bilirubin (mg/dL)	3.8 ± 0.7	8.2 ± 1.5	0.9 ± 0.1	< 0.001
Direct bilirubin (mg/dL)	2.5 ± 0.6	5.8 ± 1.1	0.2 ± 0.1	< 0.001
AST (IU/L)	148.8 ± 13.7	235.9 ± 20.9	90.8 ± 11.3	< 0.001
ALT (IU/L)	133.3 ± 12.8	183.4 ± 18.4	99.8 ± 15.7	0.001
ALP (IU/L)	501.7 ± 36.3	681.6 ± 46.3	381.8 ± 43.3	< 0.001
25(OH)D (ng/mL)	25.3 ± 1.1	16.0 ± 1.8	30.1 ± 0.7	< 0.001
Lumbar BMD (g/cm^2)	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	< 0.001
Lumbar BMD Z-score	-1.2 ± 0.2	-2.3 ± 0.2	-0.4 ± 0.1	< 0.001

Data are expressed as mean and SEM. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BA: Biliary atresia; BMD: Bone mineral density; 25(OH)D: 25-hydroxyvitamin D.

categorized into normal, osteopenia, and osteoporosis based on World Health Organization (WHO) criteria. Osteoporosis was designated as a lumbar spine BMD equal to or exceeding 2.5 standard deviations (SD) below the average values (Z score $\leqslant$ -2.5). Osteopenia was designated as a lumbar spine BMD below 2.5 SD but above 1 SD under the average values (-2.5 < Z score < -1.0). Normal BMD was designated as a lumbar spine BMD equal to or below 1 SD under the average values (Z score $\geqslant$ -1.0).

Statistical analysis

Statistical analysis was performed using the statistical package for social sciences software, version 22.0 for Windows. All values are expressed as a mean ± standard error. Demographic and clinical data between groups were compared by χ^2 tests and unpaired Student's *t* tests, where appropriate. Comparisons of clinical data and biochemical markers among patients with normal, osteopenia, and osteoporosis were analyzed using one-way analysis of variance (ANOVA) with Tukey post hoc test if ANOVA showed significance. Correlations between numerical data were acquired using the Pearson correlation coefficient (*r*). A *P*-value < 0.05 indicated statistically significant.

RESULTS

Comparisons between BA subjects with and without persistent jaundice

Seventy postKasai BA patients were enrolled in this prospective study. The characteristics and laboratory parameters of BA children with persistent jaundice compared to BA children without jaundice are described in Table 1. Jaundice BA subjects had markedly lower serum albumin levels than non-jaundice BA children. On the other hand, serum bilirubin, AST, ALT, ALP were considerably higher in BA cases with jaundice than those without jaundice. Subsequent analysis demonstrated that lumbar spine BMD and serum 25-hydroxyvitamin D values of jaundice BA subjects were significantly lower than those of non-jaundice BA subjects ($P < 0.001$).

Correlation of lumbar spine BMD and outcome parameters in BA subjects

The prevalence of low bone mass (osteopenia and osteoporosis) in BA subjects were 51.4% (36 out of 70). Ten patients (35.7%) in the jaundice group and 8 patients (19.0%) in the non-jaundice group had osteopenia. Sixteen patients (57.1%) in the jaundice group and 2 patients (4.8%) in the no jaundice group had osteoporosis. Subsequently, BA patients were divided into tertiles based on the WHO criteria. The first tertile included 34 patients with BMD Z-scores from 0 to -1 (considered as normal), the second tertile included 18 patients with Z-scores from -1.0 to -2.5 (considered as osteopenia), and the third tertile included 18 patients with Z-score lower than -2.5 (considered as osteoporosis). There was no statistically significant difference in gender and age distribution among the three tertiles (Table 2). However, serum albumin, serum bilirubin, AST, ALT, serum 25(OH)D and lumbar spine BMD were significantly different between the three tertiles. Further analysis revealed that lumbar spine BMD was correlated with age ($r = 0.774$, $P < 0.001$), serum albumin ($r = 0.333$, $P = 0.005$), TB ($r = -0.476$, $P < 0.001$), AST ($r = -0.583$, $P < 0.001$), ALT ($r = -0.428$, $P < 0.001$), and ALP ($r = -0.456$, $P < 0.001$). The correlations between lumbar spine BMD, age, serum albumin, serum TB, AST, ALT, ALP are illustrated in Figure 1.

DISCUSSION

BA is a serious cholestatic liver disease in neonates. The obstruction of bile flow in BA results in worsening cholestasis, liver fibrosis and cirrhosis, which lead to portal hypertension and eventually end-stage liver failure in children. Early diagnosis and timely Kasai porto-enterostomy to restore bile flow can help avoid the need of liver transplantation during childhood in a number of patients[10]. Despite a number of extensive clinical research studies on BA, the etiology and pathogenesis of

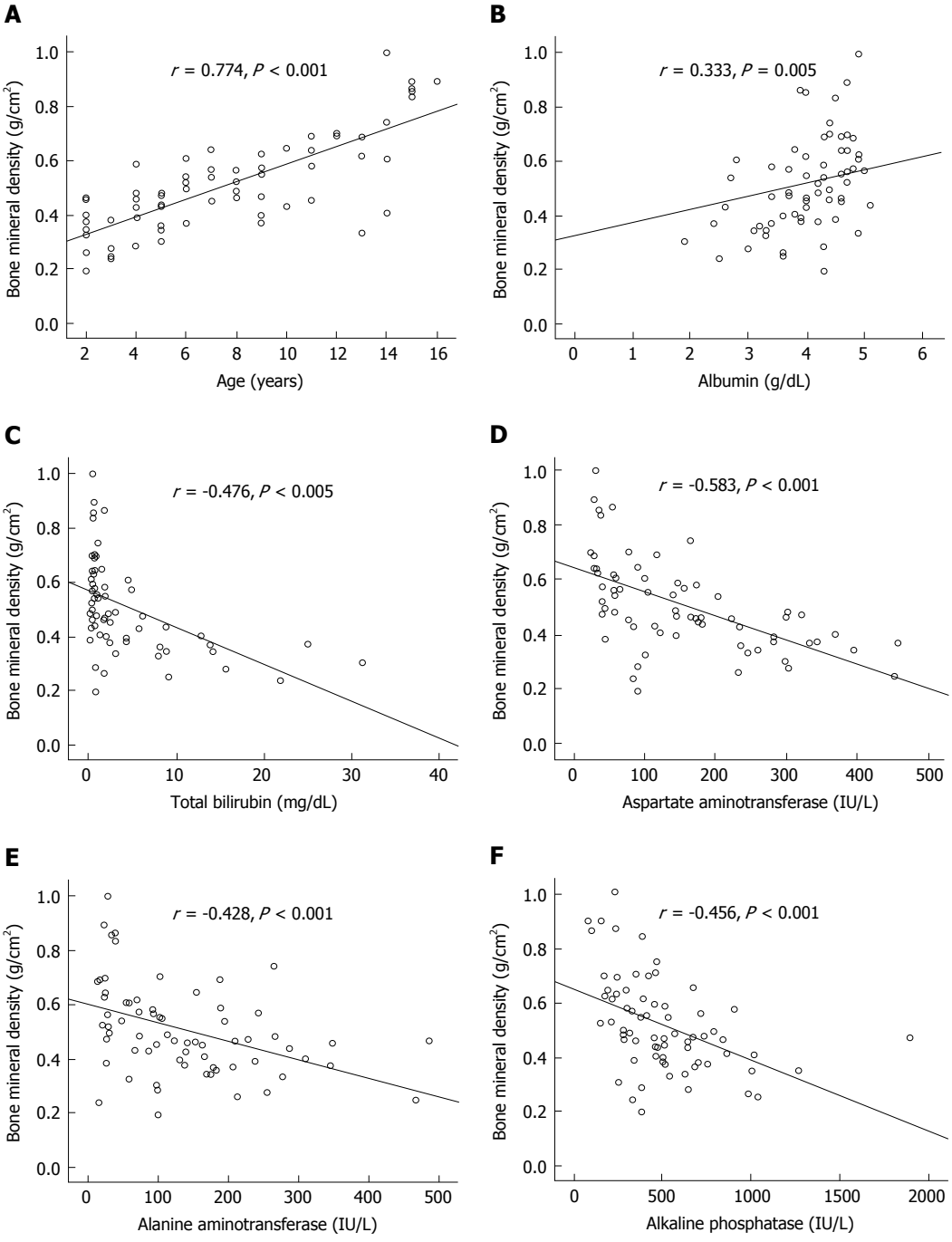

Figure 1 Scatter diagram and correlation analysis in biliary atresia patients. Lumbar spine bone mineral density are correlated with age (A), serum albumin (B), total bilirubin (C), aspartate aminotransferase (D), alanine aminotransferase (E), alkaline phosphatase (F).

BA are largely unknown.

In the recent years, serum 25-hydroxyvitamin D level was decreased in BA patients with low BMD[11]. Additionally, circulating leptin and osteoprotegerin levels has been shown to be correlated with BMD and the presence of jaundice in BA, suggesting that leptin and osteoprotegerin could play a pontential role in maintaining bone mass of BA patients[12,13].

The current study showed that postoperative BA patients with jaundice had significantly lower lumbar spine BMD than those without jaundice. Moreover, we have illustrated that the prevalence rates of osteopenia and osteoporosis in jaundiced BA subjects were higher in comparison with those in non-jaundiced children. Further analysis revealed an inverse association between lumbar spine BMD and serum TB and liver synthetic function. The explanation for these findings may be attributable to decreased osteoblastic function or increased osteoclastic resorption in BA patients. It has been documented that osteoblast proliferation was inhibited by unconjugated bilirubin *in vitro* and by the serum of jaundiced patients, indicating that bilirubin might have a direct effect on bone metabolism[14,15]. A number of BA cases eventually become advanced stage of liver disease and pediatric

Table 2 Comparison of clinical characteristics and laboratory parameters among biliary atresia patients with normal, osteopenic, and osteoporotic bone mineral density Z-scores at the lumbar spine

Characteristics	Normal	Osteopenia	Osteoporosis	*P*-value
n	34	18	18	
Gender (male/female)	15/19	7/11	8/10	0.3
Age (yr)	8.2 ± 0.7	7.7 ± 1.1	6.5 ± 1.0	0.4
Albumin (g/dL)	4.1 ± 0.2	4.0 ± 0.1	3.3 ± 0.2	< 0.05
Total bilirubin (mg/dL)	1.0 ± 0.2	2.8 ± 0.7	10.0 ± 2.1	< 0.001
Direct bilirubin (mg/dL)	0.4 ± 0.1	1.6 ± 0.5	7.3 ± 1.7	< 0.001
AST (IU/L)	95.6 ± 13.7	177.1 ± 24.8	221.2 ± 31.2	< 0.001
ALT (IU/L)	104.2 ± 18.2	164.6 ± 23.7	156.8 ± 25.1	< 0.001
ALP (IU/L)	429.1 ± 55.7	538.4 ± 55.2	602.3 ± 71.3	0.08
25(OH)D (ng/mL)	33.2 ± 0.7	26.3 ± 0.5	14.3 ± 1.5	< 0.01
25(OH)D (ng/mL)	33.2 ± 0.7	26.3 ± 0.5	14.3 ± 1.5	< 0.01
Lumbar BMD (g/cm^2)	0.6 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	< 0.001

Data are expressed as mean and SEM. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BA: Biliary atresia; BMD: Bone mineral density; 25(OH)D: 25-hydroxyvitamin D.

liver transplantation is the treatment strategy of choice for improving quality of life in BA children. Recent study has reported that successful liver transplantation could improve biochemical markers of bone formation and resorption suggesting acceleration of growth process in BA children[16]. However, the connection between cholestasis and low bone mass in BA patients merits further investigations.

Some caveats need to be acknowledged regarding the current study. First, the number of patients and controls enrolled in the present study was relative small. This could reduce the statistical power of these results. Accordingly, prospective longitudinal study with a larger population is warranted to elucidate the exact relationship between BMD, outcome parameters, and the severity in BA subjects. Secondly, inadequate measurement of plausible confounding factors including comorbidities needed to be taken under advisement. Moreover, another limitation of our study is the lack of Child–Pugh and Model for End-Stage Liver Disease (MELD) scores. Future study is also required to evaluate the Child-Pugh and MELD values for predicting of chronic liver disease severity. Ultimately, the paucity of quantitative bone histomorphometry analysis which may render evidence as to whether bone was correlated with BMD data. Therefore, more research will be needed in order to better comprehend the precise role of bone mass in the severity of postKasai BA.

To summarize, the current study demonstrated that BA subjects with persistent jaundice had significantly lower BMD than those without jaundice. Additionally, lumbar spine BMD was correlated with hepatic dysfunction suggesting that low BMD was associated with outcome parameters reflecting the severity of cholestasis in postKasai BA patients.

COMMENTS

Background

Biliary atresia (BA) is a severe congenital cholestatic liver disease with an unknown etiology. Metabolic bone disorder (osteopenia and osteoporosis) can be complicated by existing chronic liver diseases including BA. There is evidence that serum markers of bone metabolism correlated with the degree of jaundice in BA.

Research frontiers

In recent years, much research has revealed that vitamin D deficiency is associated with the severity of hepatic fibrosis or reduced bone mineral density (BMD) in patients with chronic liver disease. This study showed that lumbar spine BMD and 25-hydroxyvitamin D level in BA patients with jaundice were lower than those without jaundice. Moreover, low BMD was associated with serum bilirubin and liver function.

Innovations and breakthroughs

Jaundiced BA patients showed significantly lower lumbar spine BMD and 25-hydroxyvitamin D than in non-jaundiced BA patients. Additionally, lumbar spine BMD correlated with hepatic function markers, which reflect the severity of cholestasis in postKasaiBA patients.

Applications

BMD could be used to assist clinicians in assessing the progression of cholestasis. This study highlights the need of vitamin D supplementation and its potential in maintaining bone mass in persistently jaundiced BA children.

Terminology

BMD is the amount of bone mineral per unit volume of the bone tissue and is used as an indirect parameter of bone health. BMD measurements of the patients are generally compared to those from age-matched population and are expressed as Z-score. Osteopenia is defined as Z-score between -1 and -2.5, and osteoporosis as Z-score < -2.5.

Peer-review

A very interesting study to explore the prevalence of osteopenia and osteoporosis in post-Kasai BA children and the association of bone mineral density and biochemical parameters in postoperative BA patients.

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PLOS ONE

RESEARCH ARTICLE

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 (AUC = 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 [2]. 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 [3, 4]. 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 [5]. 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 [6]. 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 [7, 8], including a variety of cancers and chronic liver disorders, such as liver hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [9–11]. 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 [12]. 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 can 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 into a non-jaundice group (TB $<$ 2 mg/dl) and a persistent jaundice group (TB $\geq$ 2 mg/dl), according to serum total bilirubin (TB). 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: $\geq$ 17.3 kPa), as previously described by Corpechot et al. [13].

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. [14]. 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 (l 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′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTG GGTTTGGGTT-3′; telomere reverse 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCC

TTACCCT-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 (l 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 min, followed by 40 cycles of 15 sec at 95°C and 1 min 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) [15].

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 1. 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 ($p < 0.0001$).

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 Fig 1A. Given that telomere length is age-related, we classified

Table 1. 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

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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 Fig 1B.

In stratified analysis according to jaundice status, BA patients were divided into persistent jaundice and non-jaundice groups (Table 2). 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 ($p < 0.0001$) (Fig 1C).

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: $\geq$ 17.3 kPa, n = 69). Table 3 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 (Fig 1D).

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 Fig 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

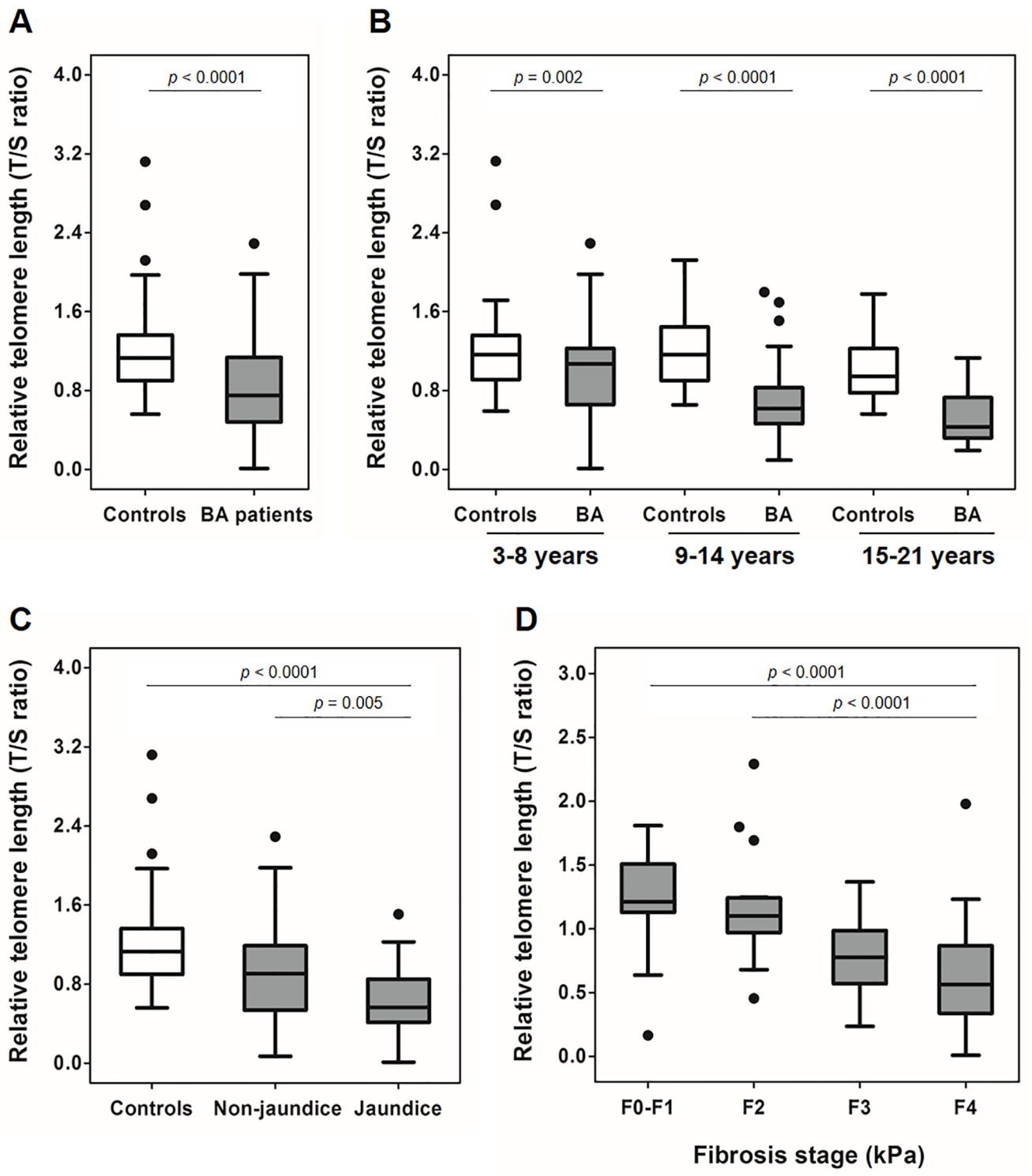

Fig 1. Box-plot illustrating telomere length distribution in subjects among different groups: The line through the middle of the boxes represents the median of T/S value and the top and bottom of each box represents the first and third quartiles. The lower and upper error bars are computed as the lower 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).

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Table 2. 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

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(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 4, 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.

Association between telomere length and clinical characteristics

The association between RTL and age in BA patients and healthy controls is shown in Fig 3. 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

Table 3. 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 ± 0.078	NS
Total bilirubin (mg/dl)	1.39 ± 0.86	1.34 ± 0.65	1.46 ± 0.50	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	0.0020
ALT (IU/l)	59.26 ± 14.38	74.52 ± 15.03	91.06 ± 13.67	128.46 ± 12.50	0.0060
ALP (IU/l)	300.26 ± 42.75	292.47 ± 53.97	386.23 ± 77.22	501.49 ± 43.92	0.010
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; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; NS = not significant

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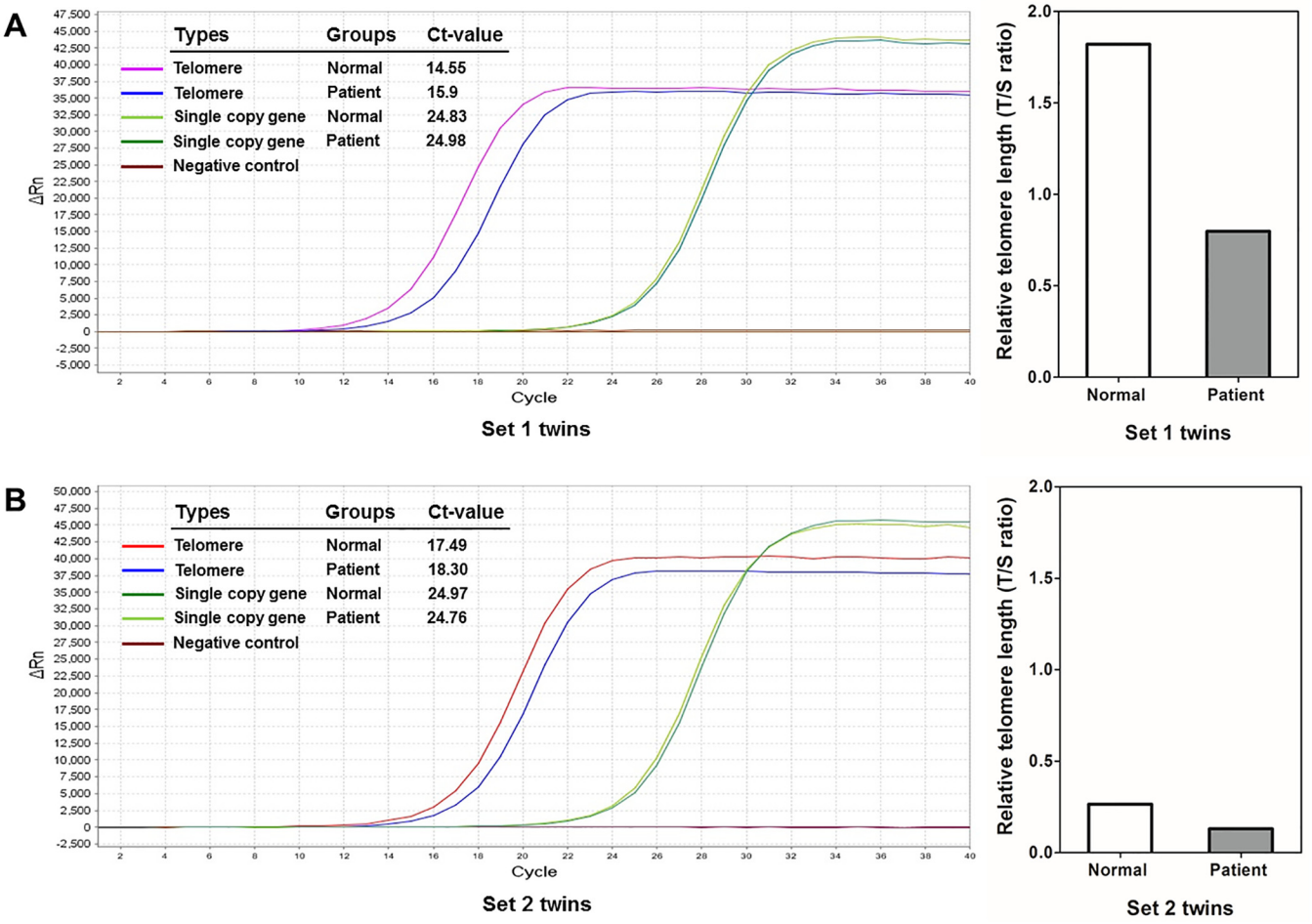

Fig 2. Telomere length assessment finding in two sets of twins by BA discordance: (A) Amplification plot and relative telomere length analysis in nine-year-old twin girls who were discordant for BA (set 1); (B) Amplification plot and relative telomere length analysis in nineteen-year-old twin women affected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

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Table 4. Logistic regression analysis of association between relative telomere length and risk of biliary atresia.

			Unadjusted		Adjusted[a]	
RTL	**BA**	**Controls**	**OR (95% CI)**	***p*-value**	**OR (95% CI)**	***p*-value**
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

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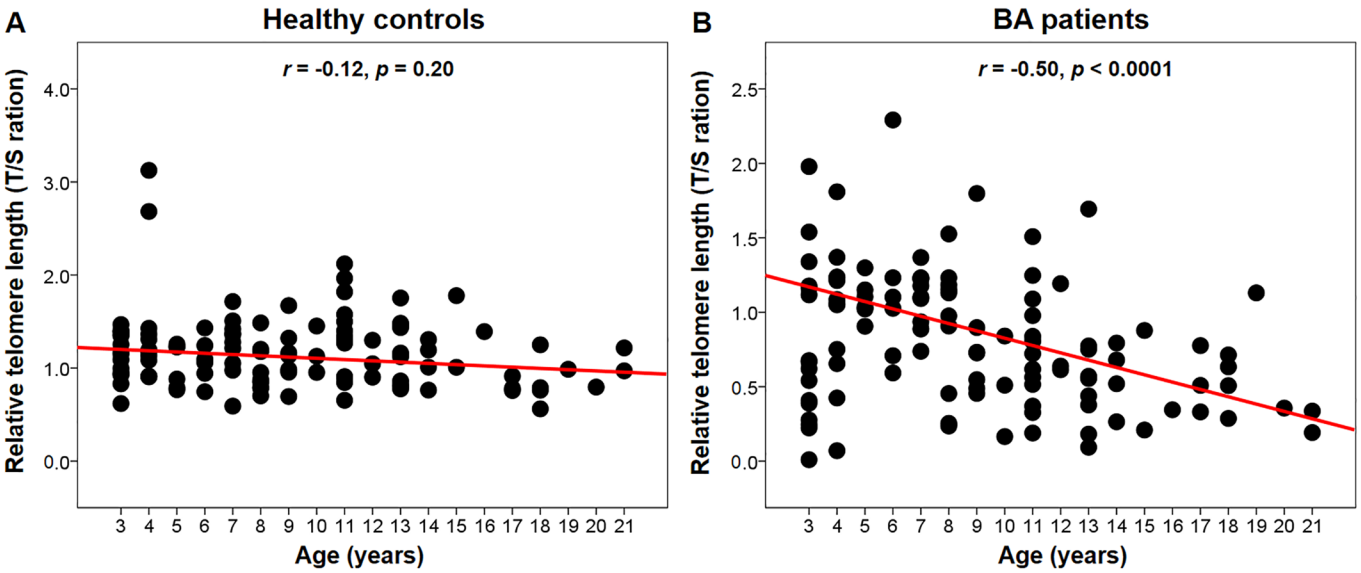

Fig 3. Scatter plot demonstrating correlation between relative telomere length of peripheral blood leukocytes and age in controls and BA patients: (A) Relative telomere length decrease with age in the controls; (B) Significant relative telomere length decrease with age in BA patients.

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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 5). Interestingly, liver stiffness was found to be associated with a reduction in relative telomere length after adjusting for age and gender (b = -0.01, $p < 0.0001$).

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 (AUC) 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, $p < 0.0001$) (Fig 4).

Table 5. 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

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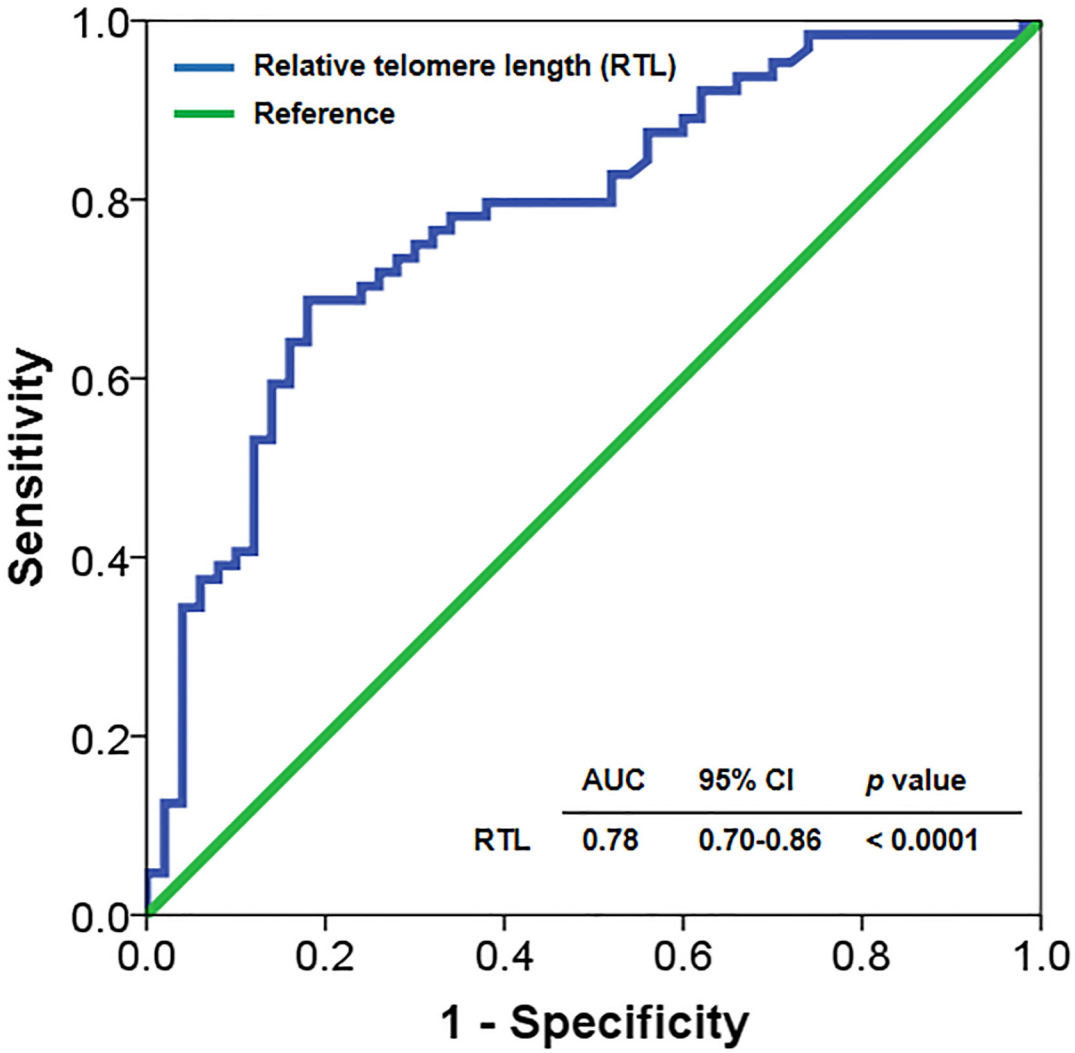

Fig 4. Receiver operating characteristic (ROC) curve representing diagnostic value of relative telomere length in biliary atresia patients with cirrhosis: The optimal cut-off value of relative telomere length at 0.58 as a marker discriminating between BA patients with and without cirrhosis.

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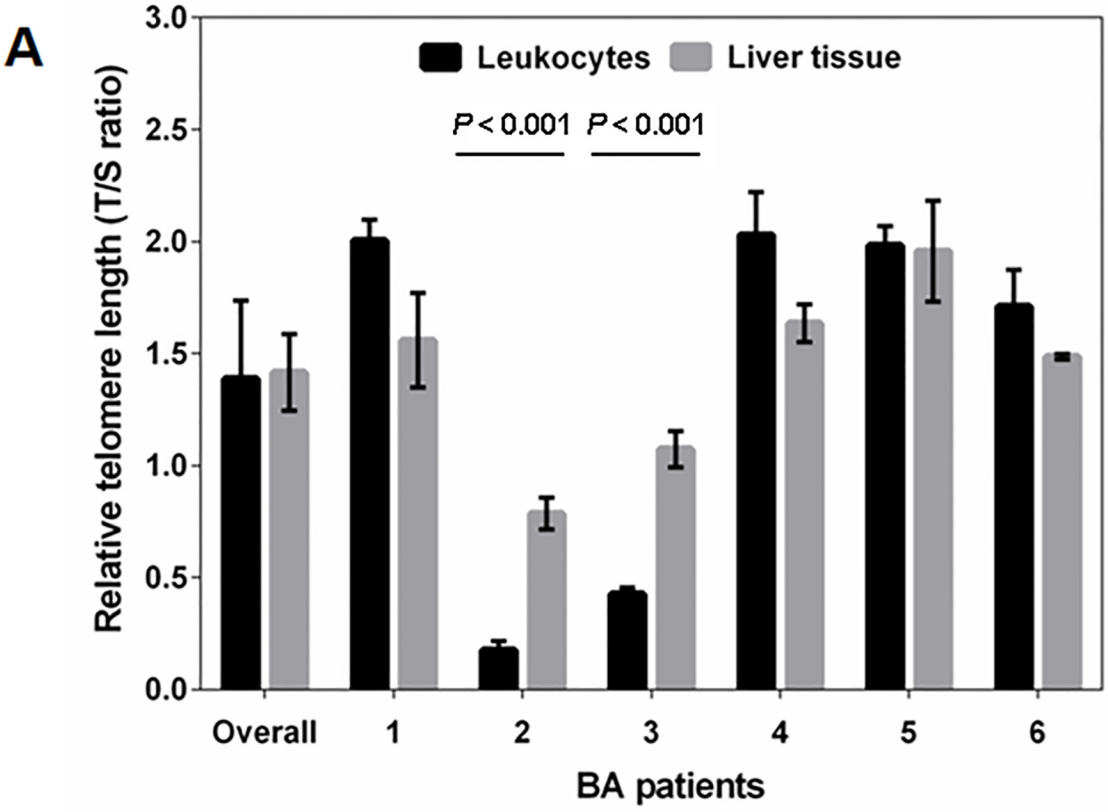
Correlation between telomere length in peripheral blood leukocytes and liver tissue in BA

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 Fig 5A. Subsequent analysis demonstrated that there was a positive correlation between RTL in peripheral blood leukocytes and RTL in liver tissue ($r = 0.83$, $p < 0.001$; Fig 5B).

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.


A
Leukocytes
Liver tissue
P < 0.001
P < 0.001
Relative telomere length (T/S ratio)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Overall
1
2
3
4
5
6
BA patients

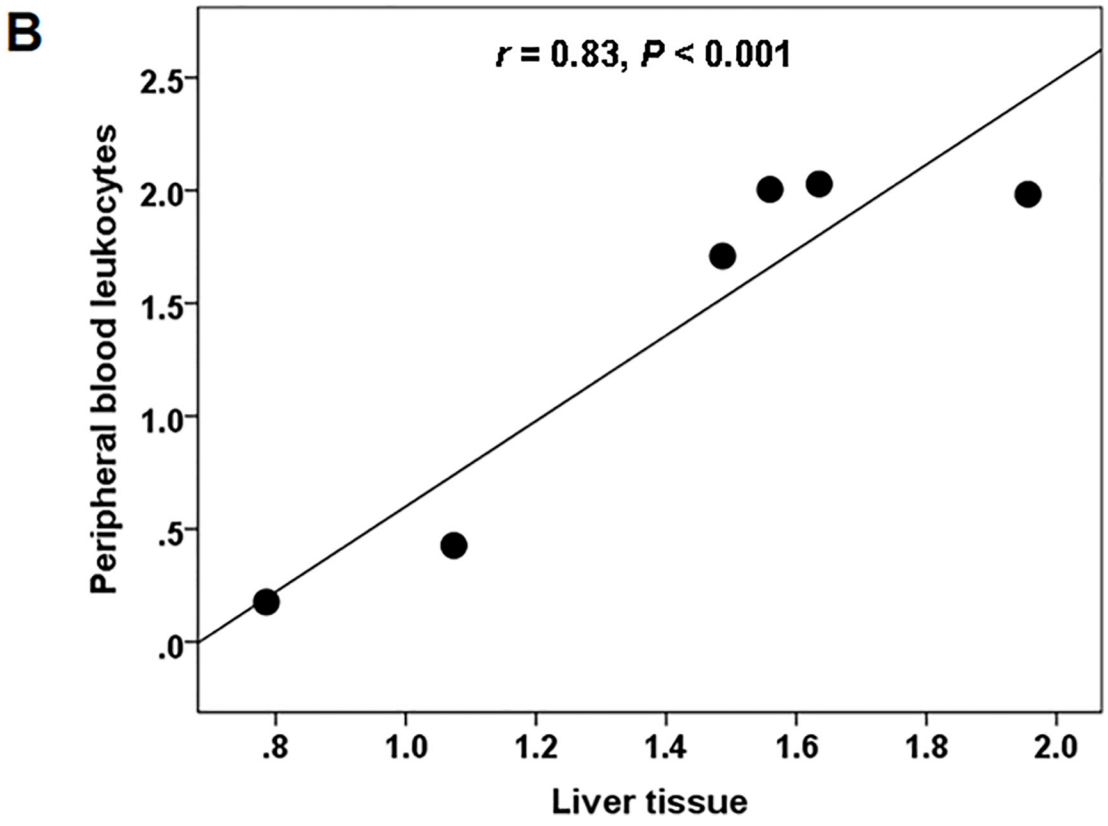

B
r = 0.83, P < 0.001
Peripheral blood leukocytes
2.5
2.0
1.5
1.0
.5
.0
.8
1.0
1.2
1.4
1.6
1.8
2.0
Liver tissue

Fig 5. 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.

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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 [16]. 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 [6]. 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 [17, 18]. 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 [10]. 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 [12]. 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 [19]. 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 [20, 21].

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 [22]. 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 [23]. Telomeric DNA sequences, rich in guanine residues, are likely more susceptible to oxidative stress, particularly by the formation of 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 [24]. 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 [25]. Our findings are also supported by a previous study by Wiemann et al. which demonstrated that telomere shortening in hepatocytes and senescence were associated with fibrotic scarring in human cirrhosis [26]. 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.

Supporting Information

S1 Table. Relative telomere length (T/S ratio) distribution in the study participants.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: WU YP SH. Performed the experiments: WU YP DZ SH. Analyzed the data: WU YP VC PV DZ SH. Contributed reagents/materials/analysis tools: WU YP VC PV DZ SH. Wrote the paper: WU YP SH.

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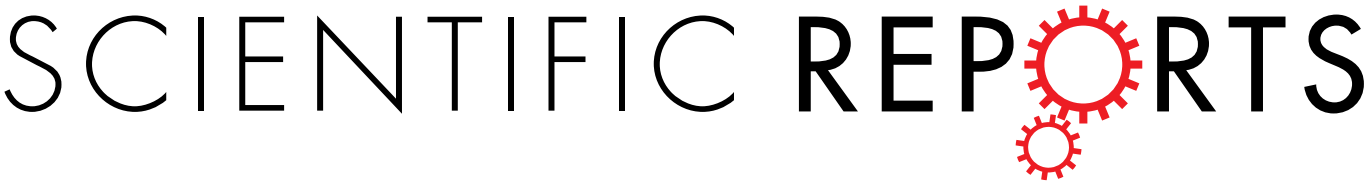

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Global methylation, oxidative stress, and relative telomere length in biliary atresia patients

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Alu and LINE-1 elements are retrotransposons with a ubiquitous presence in the human genome that can cause genomic instability, specifically 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.

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[2]. Growing evidence suggests that epigenetic variation can be elicited by viruses, toxins, and genetic defects[3], 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[4]. 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[5]. 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[6]. 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[7,8], and altered expression of important genes[9].

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Previous studies have highlighted relationships between global hypomethylation and several human diseases[10–13]. Methylation of these elements also makes them susceptible to oxidative stress[14], 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)[15]. 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[16]. 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[17]. 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[18,19], 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.

Results

Characteristics 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 1A 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 ($P < 0.0001$) (Fig. 1B).

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 Fig. 1C. 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) (Fig. 1D). 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.

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 (Fig. 2A,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) (Fig. 2C,D).

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.

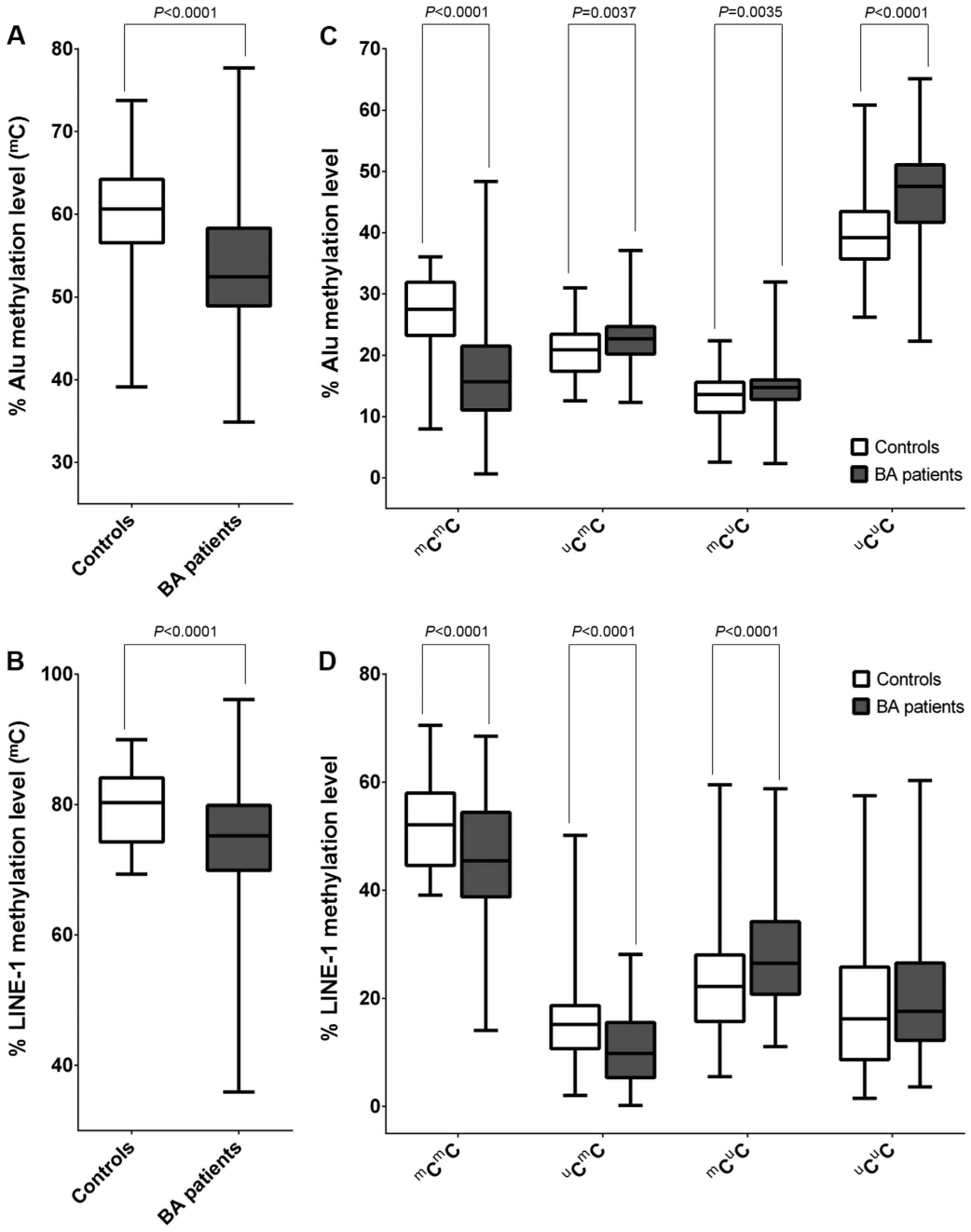

Figure 1. Methylation levels and patterns of Alu and LINE-1 elements in controls and BA patients. (**A**) Alu methylation levels; (**B**) LINE-1 methylation levels; (**C**) Alu methylation patterns; (**D**) LINE-1 methylation patterns.

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 1, 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 (first 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.

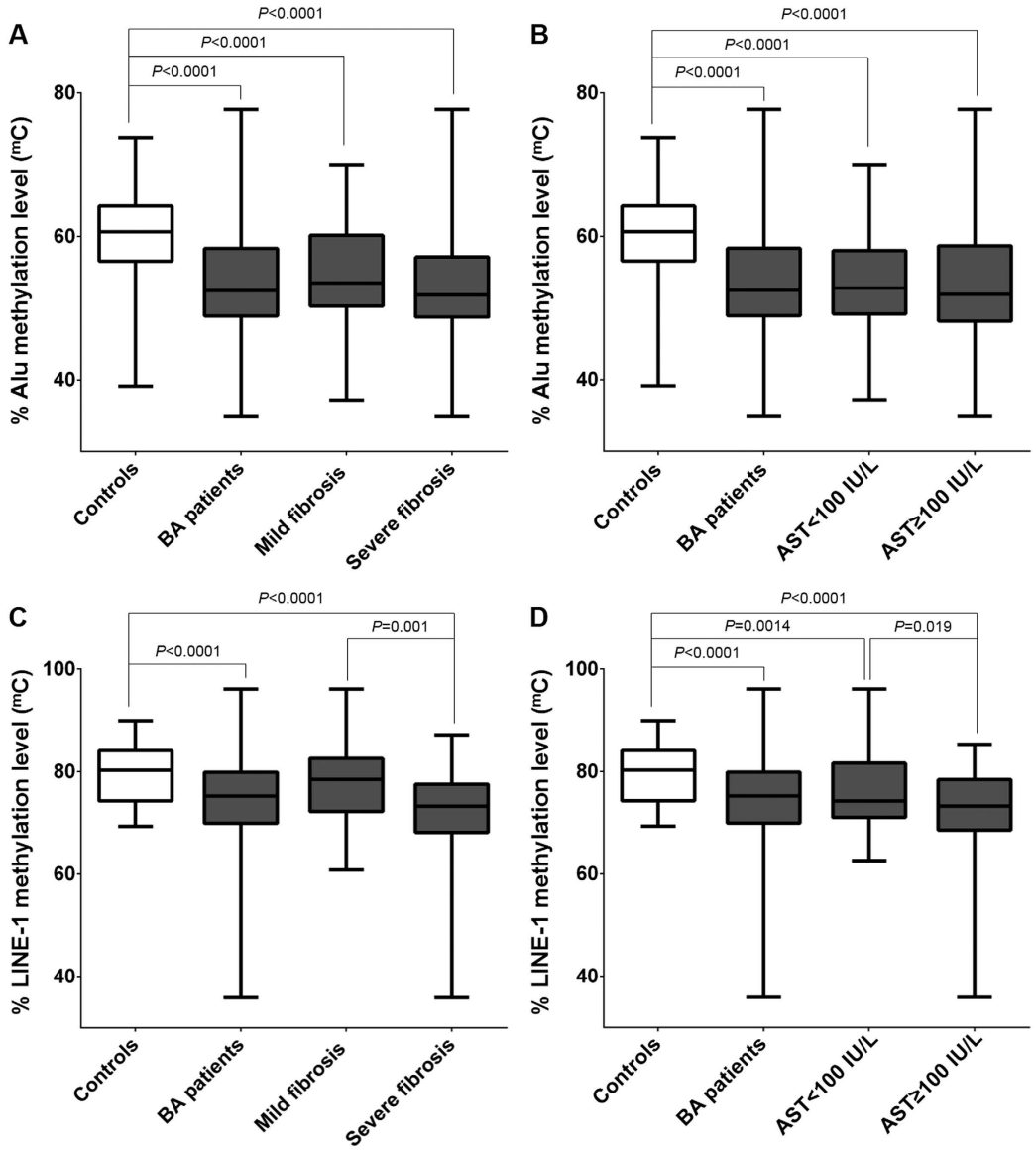

Figure 2. Alu and LINE-1 methylation levels among groups. (**A**) Alu methylation level in controls and BA according to fibrosis status; (**B**) severity of hepatic injury; (**C**) LINE-1 methylation level in control and BA according to fibrosis status; (**D**) severity of hepatic injury.

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 2. 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; $P < 0.0001$).

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 ($P < 0.0001$) (Fig. 3A). 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 Fig. 3B–D.

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

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

Table 1. Association between global methylation and risk of BA. [a]Unconditional logistic regression analysis, adjusted for age and gender; *P*-value < 0.05 indicates statistical significance.

	Alu methylation[a]		LINE-1 methylation[a]	
Variables	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 (−1.10 to 5.78)	0.16
Liver stiffness (kPa)	0.03 (−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	0.02 (−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

Table 2. Multivariate linear regression analysis of global methylation estimates. [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.

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 Fig. 4A. 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) (Fig. 4B). 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) (Fig. 4C).

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 ($r = 0.64$, $P < 0.0001$) (Fig. 4D). 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 (Fig. 4E,F).

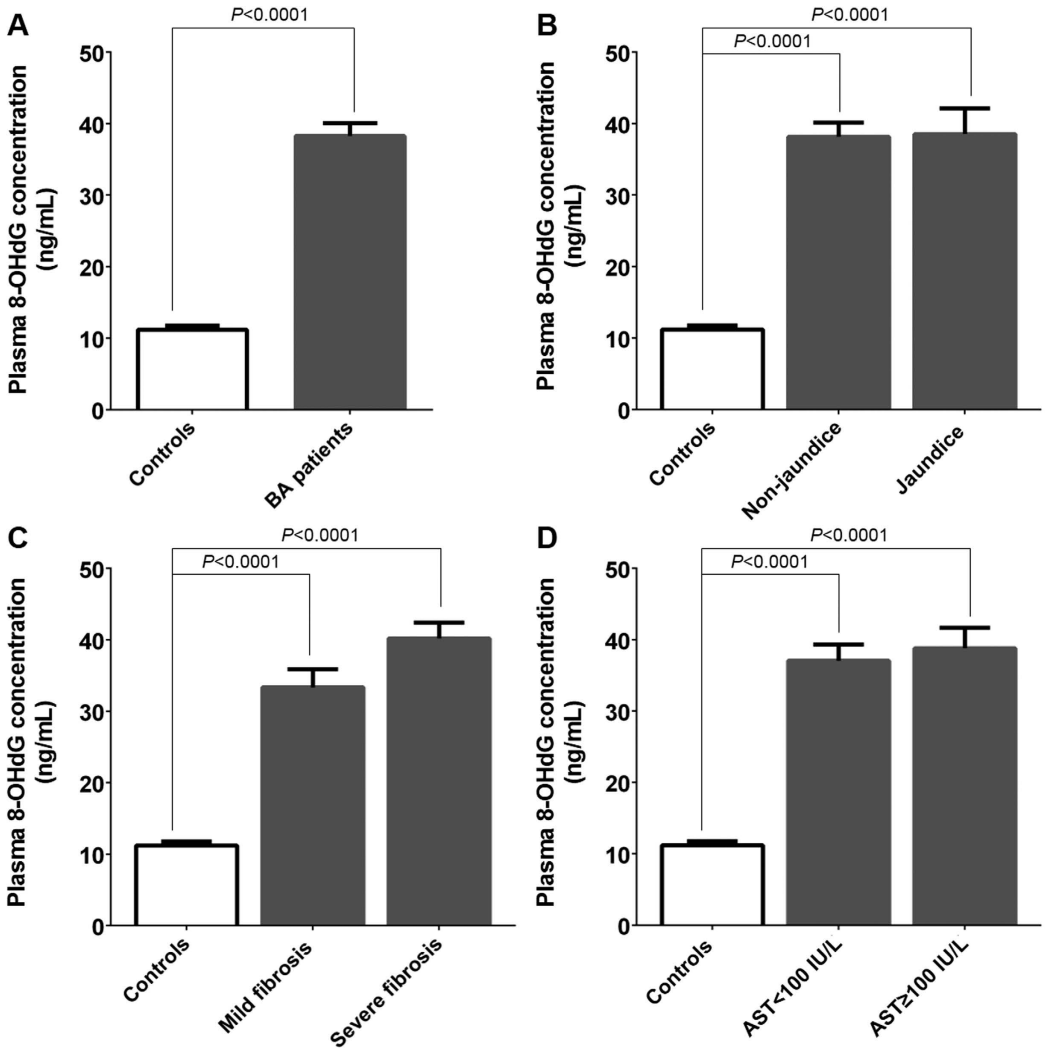

Figure 3. Plasma 8-hydroxy-2′-deoxyguanosine levels of subjects among groups. (**A**) plasma 8-OHdG levels in BA patients and healthy controls; (**B**) BA patients with and without jaundice; (**C**) BA subgroups, including mild fibrosis (F0-F2) and severe fibrosis (F3–F4); (**D**) early-stage or late-stage of hepatic dysfunction in BA patients based on AST value.

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[20]. 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[10] and patients with glioma cancer[11]. Furthermore, LINE-1 methylation has been reported in hepatocellular carcinoma patients[12,13]. 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[21,22]. Transcription of LINE-1 elements has been shown to contribute to transcriptional regulation of human development genes and cell differentiation[23,24]. 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[25].

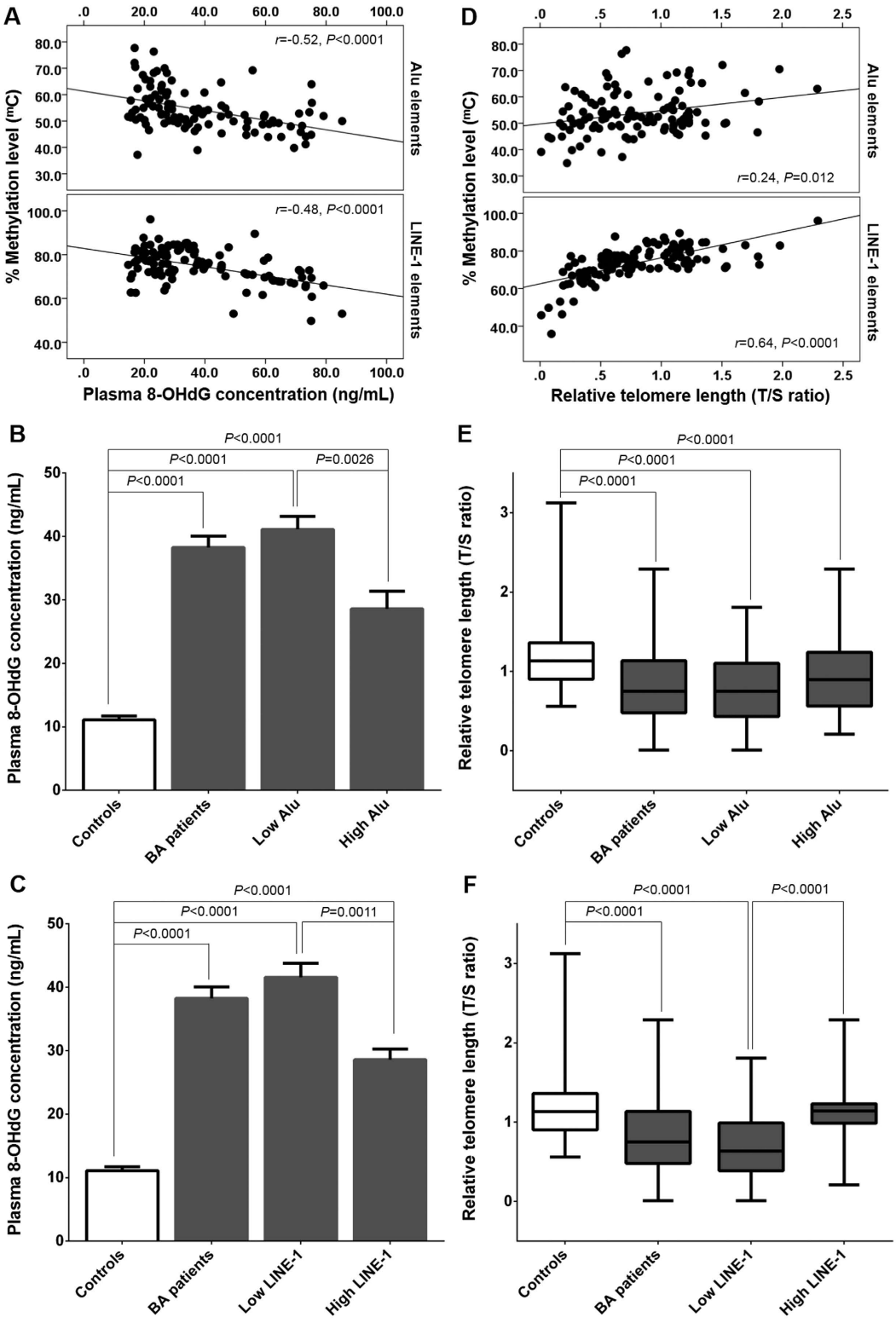

Figure 4. Relationships between global methylation, 8-hydroxy-2′-deoxyguanosine, and telomere length in BA. (**A**) negative correlations between Alu or LINE-1 methylation and 8-OHdG; (**B**) plasma 8-OHdG levels in BA patients with hypo- and hypermethylated status of Alu elements; (**C**) plasma 8-OHdG 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.

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[26]. Tiao *et al.* also reported that hepatic 8-OHdG expression in early-stage BA patients was substantially greater than in patients with choledochal cyst[27]. 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[28]. Telomeres are repeated DNA sequences of TTAGGG and an associated protein complex at chromosome ends that are essential for maintaining chromosome integrity[29]. 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[30]. Our recent study has provided evidence for telomere shortening in age-associated biliary atresia[31]; 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[32], 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[33]. Wong *et al.* recently reported positive relationships between both Alu and LINE-1 methylation levels and telomere length[34]. 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[35].

The limitation of this study should be considered. First, measurement of global methylation was performed with DNA from peripheral blood leukocytes, which may not reflect methylation levels in tissue-specific liver cells; however, global methylation in leukocyte DNA has been shown to be associated with BA development[36]. 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. Thus, 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.

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 ($TB \geq 2$ 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 ($AST \geq 100$ 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 °C 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[37]. Primers used for COBRA Alu and COBRA LINE-1 amplifications were, as follows: Alu forward primer 5′-GGRGRGGTGGTTTARGTTTGTAA-3′; Alu reverse primer 5′-CTAACTTTTTATATTTTTAATAAAAA CRAAATTTCACCA-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 $MgCl_2$, 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)/(2 A + 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 five fragments: 92 bp, 60 bp, 50 bp, 42 bp, and 32 bp. The 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[38]. Briefly, PCRs were performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green fluorescence (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.

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Author Contributions

W.U., N.K., A.M., Y.P. and S.H. conceived and designed the experiments. W.U., N.K., A.M. and Y.P. performed the experiments. W.U., N.K., A.M., V.C., Y.P. and S.H. analyzed the data. W.U., N.K., A.M., Y.P. and S.H. contributed reagents/materials/analysis tools. W.U. and S.H. wrote the paper. All authors reviewed the manuscript.

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RESEARCH ARTICLE

Association between Promoter Hypomethylation and Overexpression of Autotaxin with Outcome Parameters in Biliary Atresia

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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 polymerase chain reaction 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.

decision to publish, or preparation of the manuscript.

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATX, autotaxin; AUC, area under the ROC curve; BA, biliary atresia; CI, confidence interval; CpG, cytosine–guanine dinucleotide; DNMTs, DNA methyltransferases; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ENPP, ectonucleotide pyrophosphatase/ phosphodiesterase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cell; IFN-γ, interferon gamma; kPa, kilopascals; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPD, lysophospholipase D; OR, odds ratio; PCR, polymerase chain reaction; QPCR, quantitative real-time polymerase chain reaction; ROC, Receiver operating characteristic; TB, total bilirubin.

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 [1]. BA patients initially develop neonatal jaundice due to hepatic cholestasis and progress to liver fibrosis, which leads to cirrhosis [2]. 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 [3]. 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 [4].

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) [5]. LPA acts via activation of at least six different G-protein-coupled receptors to influence a number of biological processes [6]. 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 [7, 8]. LPA can induce hepatic stellate cell (HSC) proliferation, stimulate their contraction, and inhibit their apoptosis [9]. 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 [10]. Recent studies have also suggested a connection between liver fibrosis and circulating LPA, and serum ATX was elevated in chronic hepatitis C patients [11, 12]. Importantly, the ATX-LPA axis has been shown to be upregulated in human HCC, suggesting that ATX possibly plays an important role in inflammation-related liver fibrogenesis [13, 14]. 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 [15]. Methylation of cytosine–guanine dinucleotide (CpG) residues catalysed by DNA methyltransferases (DNMTs) within the promoter and enhancer regions leads to suppression of gene expression and DNA methylation alterations can be elicited by viruses and genetic defects [16–18]. DNA hypomethylation may result in the development of several autoimmune disorders, such as systemic lupus erythematosus and rheumatoid arthritis [19, 20]. 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 [21]. However, the hypomethylation of the interferon gamma (IFN-γ) gene promoter may be responsible for increased IFN-γ expression in BA infants [22]. 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 [23]. 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 ($<$9.7 kPa, n = 22) and fibrotic BA ($>$9.7 kPa, n = 43). We also classified BA patients according to serum total bilirubin (TB) into either the non-jaundice group (TB$<$2 mg/dl, n = 41) or the persistent jaundice group (TB$\geq$2 mg/dl, n = 24). Based on the severity of hepatic injury (aspartate aminotransferase, AST value), BA patients were divided into either the early-stage group (AST$<$100 IU/l, n = 36) or late-stage group (AST$\geq$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 underwent liver biopsies 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 polymerase chain reaction (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′-TAGGT ATTGTAGGGGGTGGGAA-3′; reverse primer biotinylated-5′-ACCTTTAACAAAACACA CACATAACC-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′-CGTGGCTGGGAGT GTACTAA-3′; *ATX* reverse primer 5′AGAGTGTGTGCCACAAGACC-3′, as previously described [8]; *DNMT1* forward primer 5′- CAGGCCCAATGAGACTGACA-3′, and *DNMT1* reverse primer 5′- GTGGGTGTTCTCAGGCCTGTAG-3′; *GAPDH* forward primer 5′-GTGAAGGTCGGAGTCAACGG-3′, and *GAPDH* reverse primer 5′-TCAATGAAGGGGTC ATTGATGG-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* and *DNMT1* 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 possible parameter 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 all analyses.

Results

Characteristics of study participants

The baseline clinical characteristics of study participants are summarized in Table 1. 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 1. 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 are 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

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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. Fig 1 illustrates a

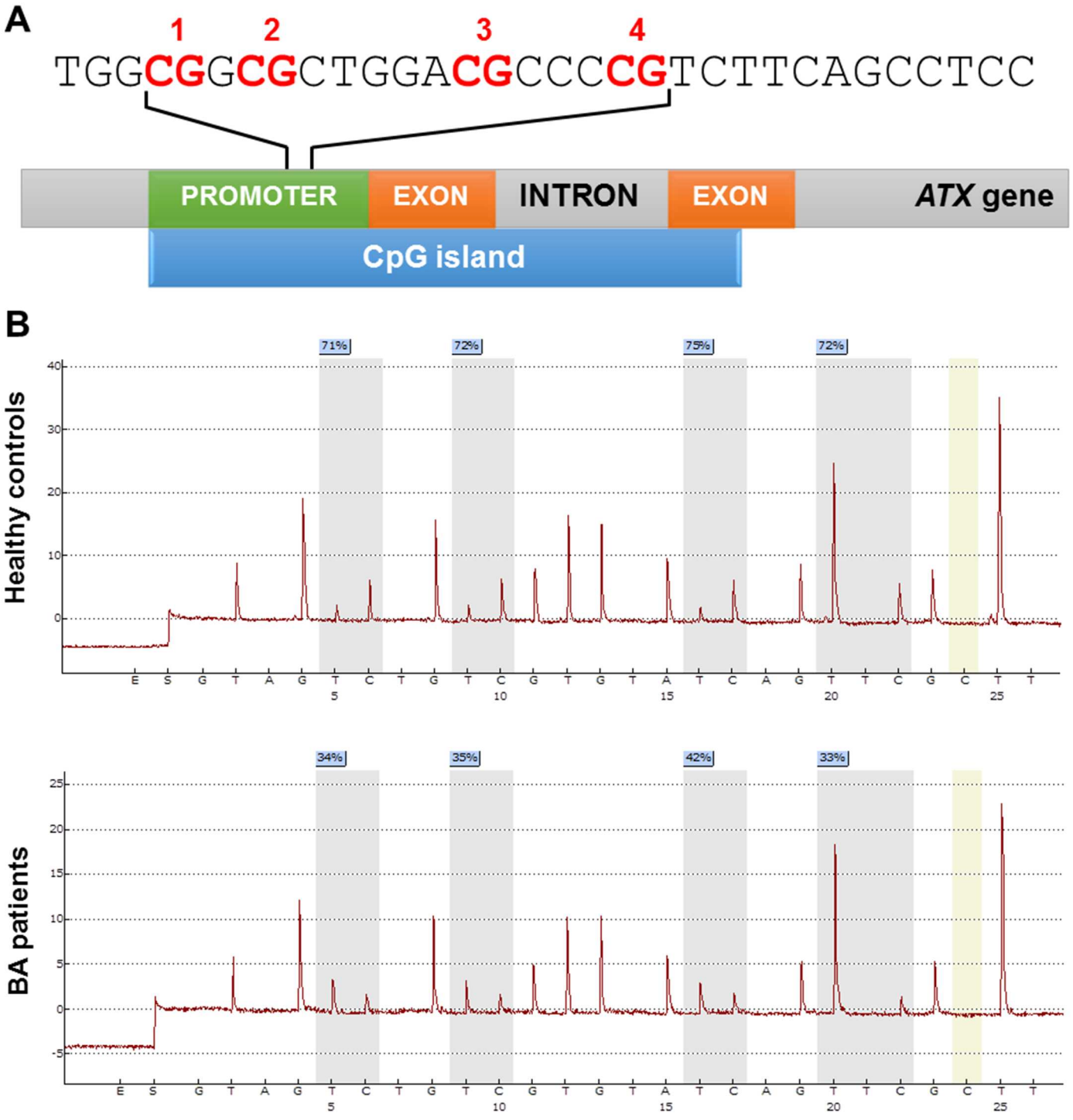

Fig 1. DNA methylation at four CpG islands within *ATX* gene promoter. (A) Schematic diagram representing CpG islands at the *ATX* promoter showing the four CpG sites. (B) Pyrosequencing results in healthy controls and BA patients.

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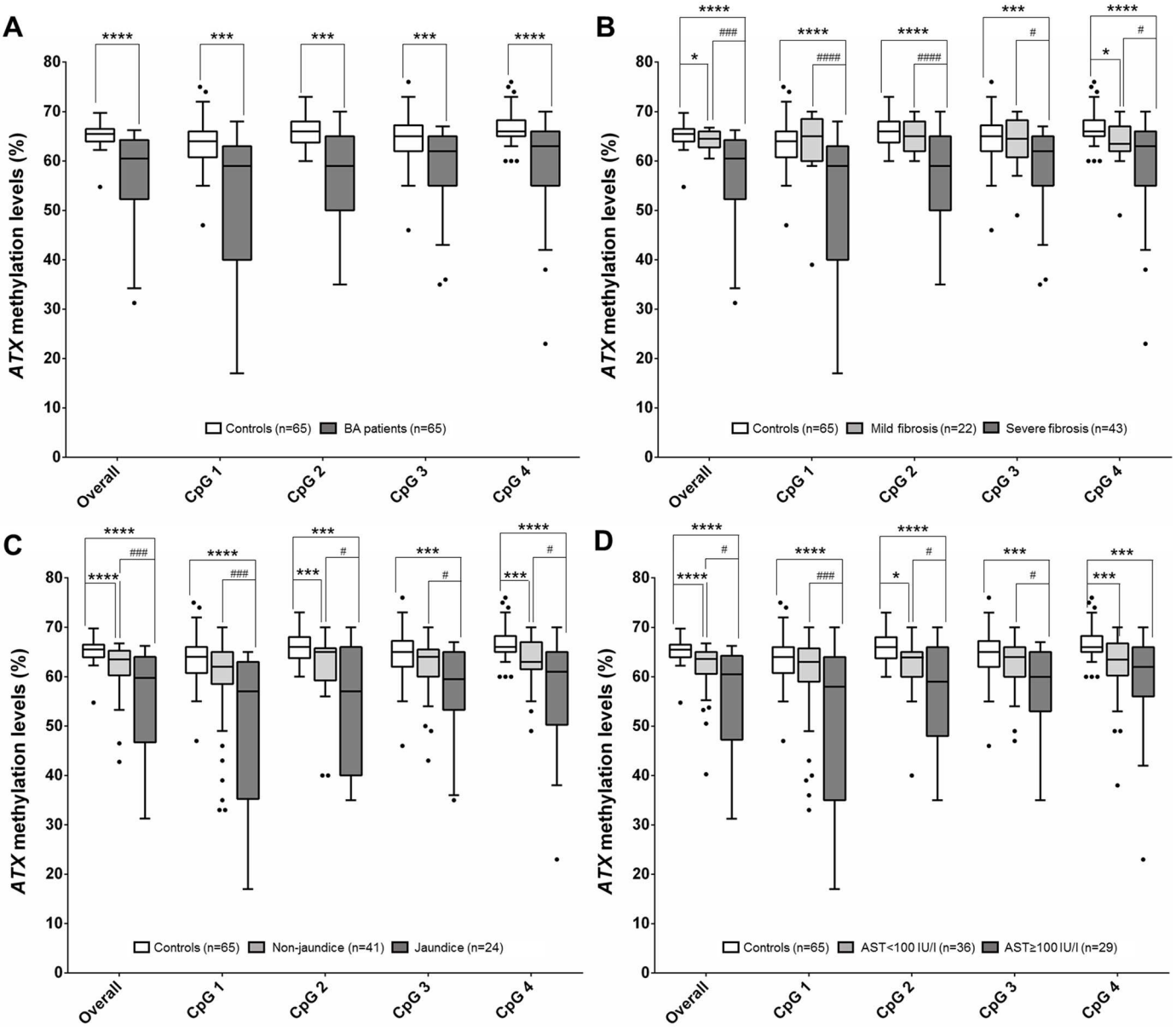

Fig 2. Box-plot illustrating methylation levels of the *ATX* promoter in peripheral blood leukocytes of subjects among different groups. (A) Methylation levels of the *ATX* promoter at four CpG sites in BA patients and healthy controls. (B) Methylation levels of *ATX* promoter at four CpG sites in BA 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.

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schematic representation of four CpG sites within the *ATX* gene promoter region. Overall, methylation levels at the *ATX* promoter were significantly lower in the BA group than healthy controls ($P<0.0001$). 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: $P<0.0001$ (Fig 2A).

In analyses stratified by disease severity, we classified BA patients according to fibrosis, jaundice status, and hepatic dysfunction marker (AST value) (Table 2). Overall, decreased

Table 2. Characteristics of biliary atresia patients among different groups according to fibrosis stage, jaundice status, and AST value.

	BA patients (n = 65)								
	Mild fibrosis	Severe fibrosis	*P*-value	Non-jaundice	Jaundice	*P*-value	Low AST	High AST	*P*-value
	(F0-F2, n = 22)	(F3-F4, n = 43)		(TB<2 mg/dl, n = 41)	(TB≥2 mg/dl, n = 24)		(<100 IU/l, n = 36)	(≥100 IU/l, n = 29)	
Age (years)	8.30±0.81	8.63±0.65	0.77	8.19±0.60	9.25±4.30	0.34	8.35±0.73	8.72±0.71	0.72
Gender (female: male)	14:8	21:22	0.11	23:18	12:12	0.79	20:16	15:14	0.81
Liver stiffness (kPa)	7.03±0.33	35.18±3.28	<0.0001*	14.70±1.50	51.89±4.54	<0.0001*	14.12±2.16	40.64±4.19	<0.0001*
TB (mg/dl)	0.40±0.04	2.92±0.56	<0.0001*	0.62±0.070	5.47±0.95	<0.0001*	0.64±0.11	3.89±0.77	<0.0001*
AST (IU/l)	43.05±5.95	137.40±9.99	<0.0001*	79.09±9.70	170.05±8.93	<0.0001*	51.12±4.01	174.79±8.39	<0.0001*
ALT (IU/l)	44.40±7.24	119.31 ±11.25	<0.0001*	86.35±12.43	115.05±8.38	0.060	53.68±6.04	145.50±13.78	<0.0001*
ALP (IU/l)	194.35 ±25.88	460.49 ±31.10	<0.0001*	306.83±32.04	520.00±35.48	<0.0001*	262.85±27.21	503.32±38.76	<0.0001*
Albumin (g/dl)	4.41±0.27	4.16±0.09	0.27	4.40±0.14	3.91±0.09	0.018*	4.48±0.16	3.98±0.10	0.011*

Data are 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

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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) (Fig 2B–2D).

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 3). 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 3).

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 ($P = 0.0096$) (Fig 3A). 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) (Fig 3B–3D).

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

Table 3. 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*)	0.08	0.11	0.13	-0.08	-0.02
	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	0.02
	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)

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

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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 4).

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 [23]. 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 *ATX* 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 3). Subsequent analysis revealed that serum ATX levels were correlated with biochemical parameters in BA patients (Table 5).

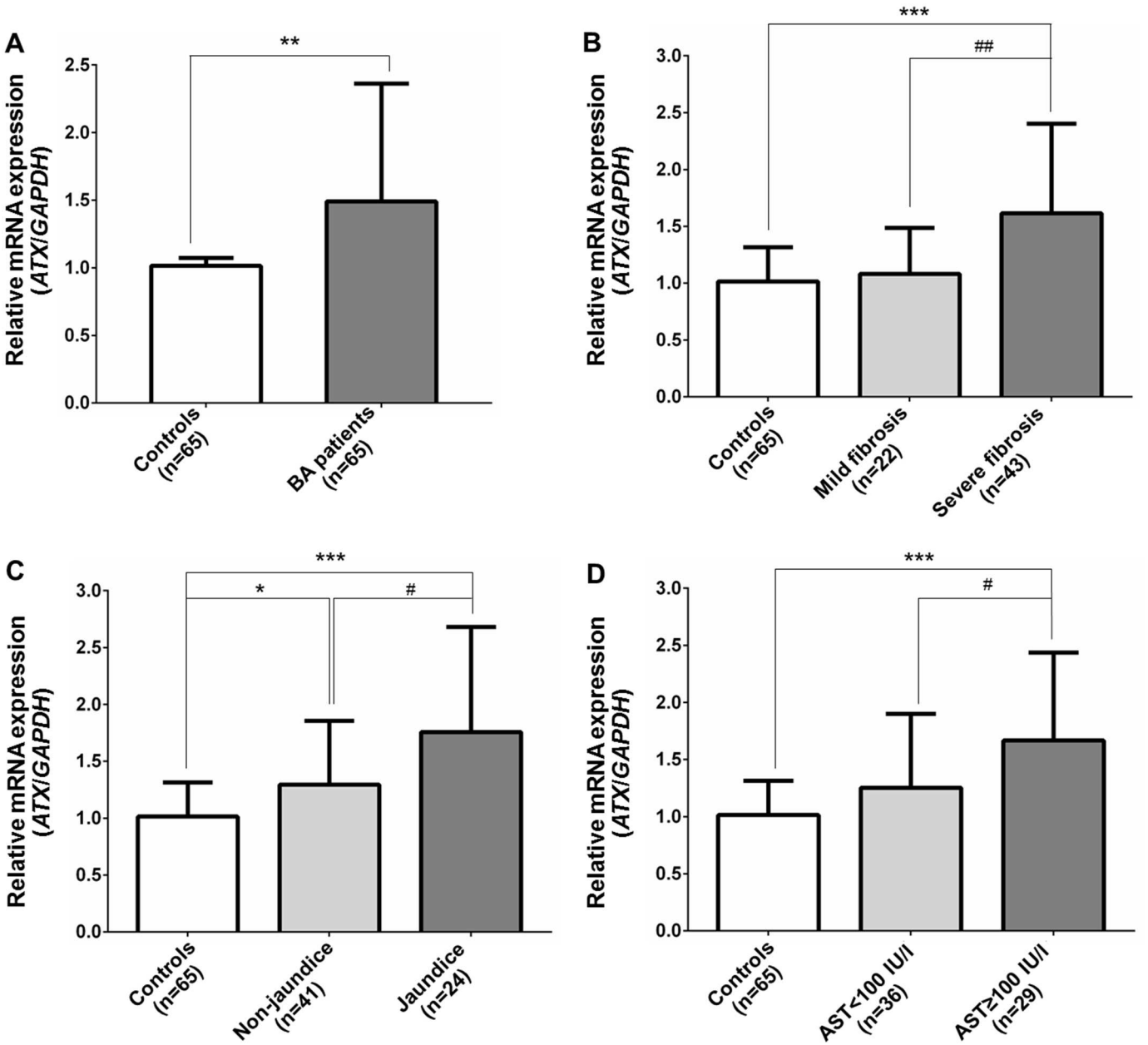

Fig 3. *ATX* mRNA expression in peripheral blood leukocytes of subjects among different groups. (A) Relative *ATX* mRNA expression in BA patients and healthy controls. (B) Relative *ATX* mRNA expression in BA patients with mild and severe fibrosis. (C) Relative *ATX* mRNA expression in BA patients with 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.

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ATX promoter hypomethylation increased its expression in liver tissue samples

To determine whether *ATX* promoter methylation in genomic DNA reflects epigenetic alterations in liver tissues, we examined methylation levels of the *ATX* promoter in the liver samples 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

Table 4. 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)	0.07	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

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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 (Fig 4A).

Table 5. 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)	-0.04	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

doi:10.1371/journal.pone.0169306.t005

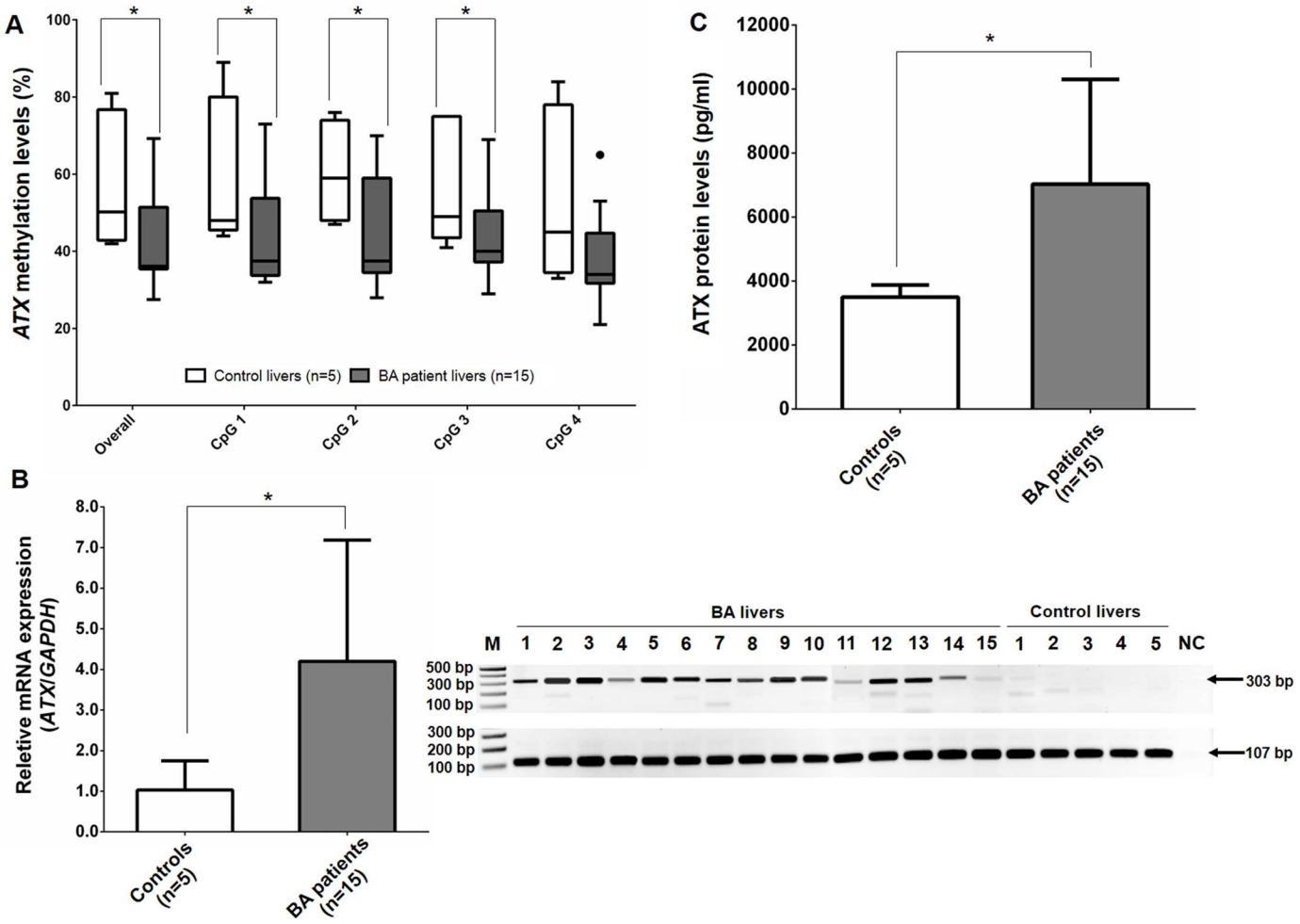

Fig 4. Distribution of the *ATX* promoter methylation, relative mRNA expression, and protein levels in liver tissue of BA patients and controls. (A) Decreased methylation levels of the *ATX* promoter in BA liver tissue samples. (B) Higher mRNA expression of *ATX* in BA cases and representative gel of *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.

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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 ($P<0.05$) (Fig 4B). Moreover, ATX protein levels were significantly higher in the BA livers, when compared to the non-BA control livers ($P<0.05$) (Fig 4C).

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 ($P<0.05$) (Fig 5A). 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,

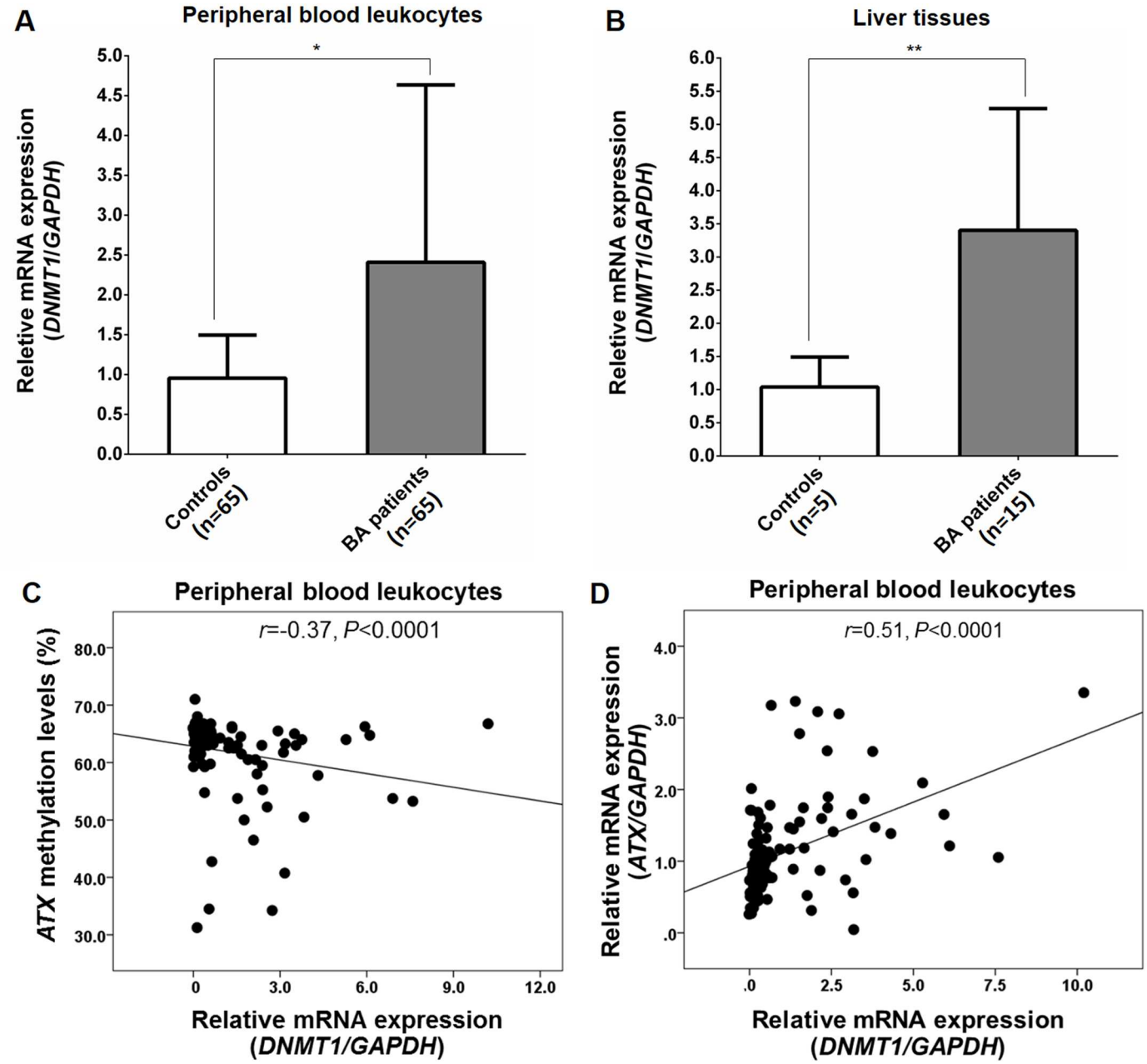

Fig 5. *DNMT1* mRNA expression in peripheral blood leukocytes and livers of BA patients and controls. (A) Relative *DNMT1* mRNA expression in peripheral blood leukocytes from BA patients and healthy controls. (B) Relative *DNMT1* mRNA expression in liver tissue samples from BA patients and healthy controls. (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.

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relative *DNMT1* mRNA expression in BA livers was significantly increased when compared with that in control livers as shown in Fig 5B ($P<0.01$). 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 ($r = -0.37$, $P<0.0001$) (Fig 5C) but *DNMT1* mRNA expression was positively correlated with

ATX mRNA expression in peripheral blood leukocytes of BA patients ($r = 0.51$, $P<0.0001$) (Fig 5D).

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 biomarker 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 (Fig 6A–6E).

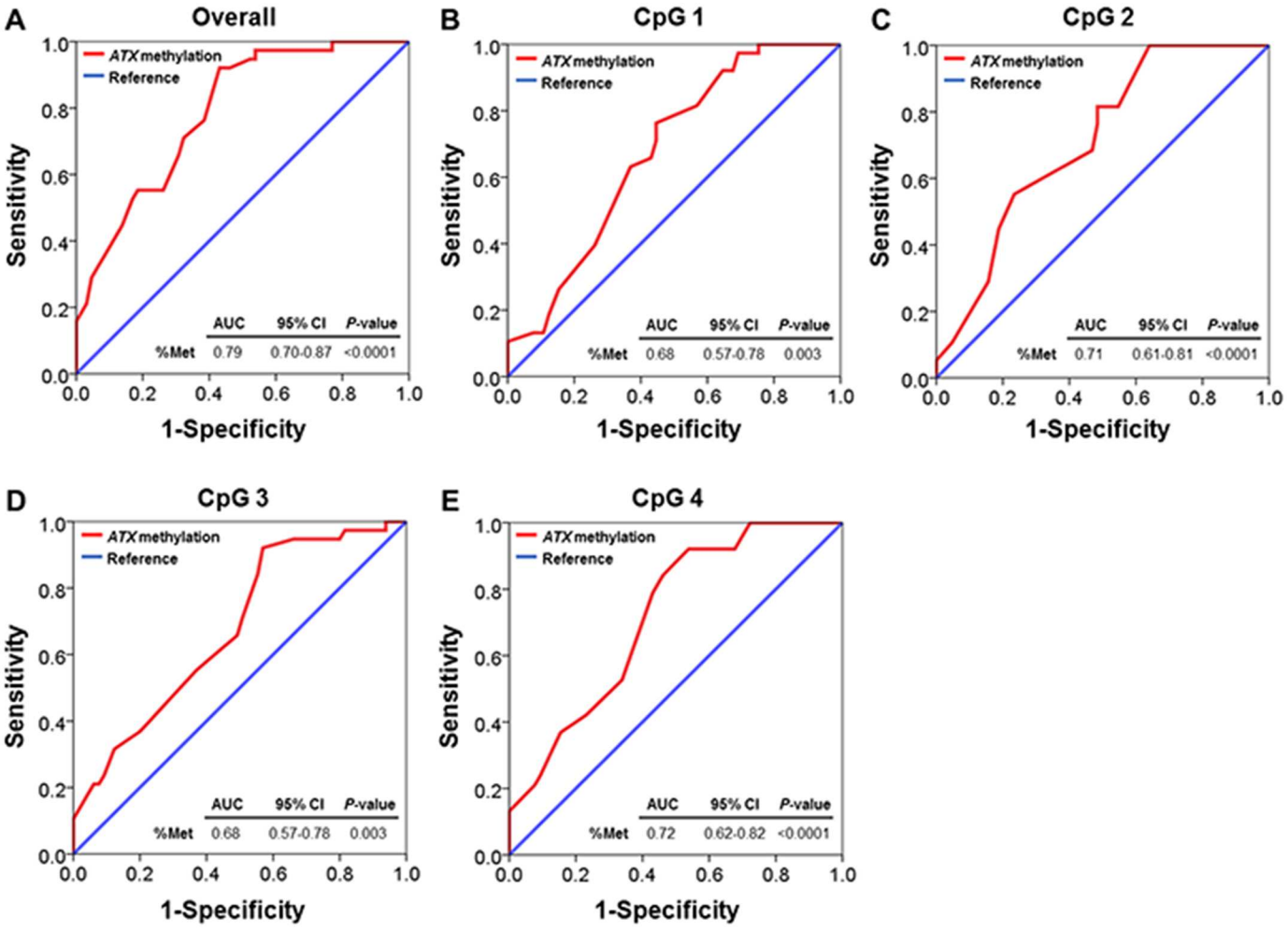

Fig 6. Receiver operating characteristic (ROC) curve representing the cutoff value of *ATX* promoter methylation in BA patients. (A) Overall *ATX* promoter methylation. (B) *ATX* promoter methylation at CpG 1 site. (C) *ATX* promoter methylation at CpG 2 site. (D) *ATX* promoter methylation at CpG 3 site. (E) *ATX* promoter methylation at CpG 4 site.

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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 [23]. 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* comprise 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 [24]. 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 [25]. 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 autotaxin 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 [15], as well as a stimulator of stellate cells and progressive hepatic fibrosis in animal models [26]. 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 [27]. Since serum levels of ATX and LPA have been correlated with the development of liver fibrosis [12], 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 [28]. Wu *et al.* reported extensive evidence of *ATX* overexpression associated with progression of inflammation and liver cirrhosis in HCC patients [14], 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 [7, 11, 12]. Moreover, serum ATX was increased in hepatocellular carcinoma with liver fibrosis, nonalcoholic fatty liver disease, and cholestatic disorders [8, 29, 30]. ATX expression was also shown to be augmented in chronic cholestatic diseases such as primary biliary cholangitis and primary sclerosing cholangitis [31]. 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 elusive 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 [10]. 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 [32]. 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 [8, 10]. 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 result from 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 [10]. 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 [33]. 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 liver fibrosis in 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 potential 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.

Supporting Information

S1 Table. *ATX* promoter methylation distribution in the study participants.
(DOC)

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Supervision: NK AM YP SH.

Validation: WU NK YP SH.

Visualization: WU NK YP SH.

Writing – original draft: WU SH.

Writing – review & editing: WU SH.

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ORIGINAL ARTICLE

Prospective Study

Elevation of serum urokinase plasminogen activator receptor and liver stiffness in postoperative biliary atresia

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Author contributions: Honsawek S designed the study; Udomsinprasert W, Honsawek S and Jirathanathornnukul N performed the research; Udomsinprasert W, Honsawek S, Jirathanathornnukul N, Chongsrisawat V and Poovorawan Y analyzed the data; Chongsrisawat V and Poovorawan Y examined all the patients and collected the clinical data; Udomsinprasert W and Honsawek S wrote the paper; Honsawek S revised the manuscript for final submission.

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Abstract

AIM

To investigate serum urokinase-type plasminogen activator receptor (uPAR) and liver stiffness in biliary atresia (BA) and examine the correlation of circulating uPAR, liver stiffness, and clinical outcomes in postoperative BA children.

METHODS

Eighty-five postKasai BA children and 24 control subjects were registered. Circulating uPAR was measured using enzyme-linked immunosorbent essay. Liver stiffness was analyzed using transient elastography.

RESULTS

BA children had significantly greater circulating uPAR and

liver stiffness scores than control subjects ($P < 0.001$). Circulating uPAR and liver stiffness were substantially higher in jaundiced BA children than non-jaundiced BA children ($P < 0.001$). In addition, circulating uPAR was positively associated with serum aspartate aminotransferase ($r = 0.507$, $P < 0.001$), alanine aminotransferase ($r = 0.364$, $P < 0.001$), total bilirubin ($r = 0.559$, $P < 0.001$), alkaline phosphatase ($r = 0.325$, $P < 0.001$), and liver stiffness scores ($r = 0.508$, $P < 0.001$).

CONCLUSION

Circulating uPAR and liver stiffness values were greater in BA children than healthy controls. The increased circulating uPAR was associated with liver dysfunction in BA. As a consequence, serum uPAR and liver stiffness may be used as noninvasive biomarkers indicating the progression of liver fibrosis in postKasai BA.

Key words: Biliary atresia; Jaundice; Liver stiffness; Severity; Urokinase plasminogen activator receptor

Core tip: Urokinase plasminogen activator receptor (uPAR) is known to be a substantial factor in the etiopathogenesis of hepatic inflammation and liver fibrogenesis. This study is the first to show that circulating uPAR is more elevated in biliary atresia (BA) children than in control subjects, and that circulating uPAR is correlated with the degree of jaundice and liver fibrosis in biliary atresia. Elevated serum uPAR is positively correlated with the severity of liver stiffness in postKasai BA children. Hence, serum uPAR could be used as a biological parameter indicating the progression and prognosis of liver fibrosis in BA children.

Udomsinprasert W, Honsawek S, Jirathanathornnukul N, Chongsrisawat V, Poovorawan Y. Elevation of serum urokinase plasminogen activator receptor and liver stiffness in postoperative biliary atresia. *World J Hepatol* 2016; 8(33): 1471-1477 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i33/1471.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i33.1471

INTRODUCTION

Biliary atresia (BA) is a severe chronic cholestatic liver disease of unknown etiology in young infants. The estimated incidence of BA varies from 1 in 8000 to 1 in 20000 live births, with a high frequency in Asians[1]. Affected newborns exhibit evidence of biliary obstruction within the first few months of life. BA is manifested by impaired liver function and fibroinflammatory obliterative cholangiopathy of both intrahepatic and extrahepatic bile ducts[2,3]. Extrahepatic BA is the most common form of ductal cholestasis. BA patients initially develop neonatal jaundice due to hepatic cholestasis and progress to hepatic fibrosis, which result in biliary cirrhosis[1-3]. Even though no medical therapies exist, sequential treatment strategy involving surgical Kasai portoenterostomy and liver transplantation is the only option for the most affected children. Nonetheless the precise pathogenesis of BA has yet to be determined, a number of theories regarding the etiology of BA include toxin exposure, virus-mediated inflammation, abnormal inflammatory response, defective morphogenesis, genetic mutation, and immunological dysregulation[4].

Urokinase-type plasminogen activator receptor (uPAR, CD87) is a cellular membrane receptor that attachs to urokinase-type plasminogen activator (uPA) with high affinity, through promoting the pericellular activation of plasminogen[5]. The involvement of uPA, its receptor (uPAR), and plasminogen activator inhibitor-1 (PAI-1) in regulation of cell adhesion, migration, proliferation, differentiation, and cell survival has recently demonstrated[6]. uPAR is expressed by a wide range of immune cells and endothelial cells, which contribute to the etiopathogenesis of hepatic inflammation and liver fibrogenesis[7,8]. Once inflammation is activated, uPAR is released from the cell membrane by proteolytic enzymes to produce soluble uPAR[9]. In recent years, previous studies have investigated that elevated circulating uPAR levels have been observed in acute liver failure, chronic liver diseases, and nonalcoholic fatty liver diseases[10-12].

It has been previously shown that certain cytokines and growth factors play possible parts in the etiopathology of biliary atresia[13-16]. The measurements on circulating uPAR and liver stiffness of BA have never been documented. We hypothesized that circulating uPAR and liver stiffness could be more elevated in BA patients than in control subjects and circulating uPAR would be associated with the disease severity and clinical outcomes in postKasia biliary atresia. Hence, the purpose of the current research is to determine circulating uPAR and liver stiffness measurements and to investigate the plausible correlation of circulating uPAR, liver stiffness, and clinical outcomes in postoperative biliary atresia children.

MATERIALS AND METHODS

The present study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University, and was conducted in compliance with the ethical guidelines of the Declaration of Helsinki. All parents of children were informed of the study's purpose and of any interventions involved in the current study. Written informed consent was derived from the parents prior to the subjects entering the study.

Study population

Eighty-five BA children (39 girls and 46 boys with mean age of 9.0 ± 0.6 years) and 24 normal control subjects (11 girls and 13 boys with mean age of 8.5 ± 0.5 years) were enrolled in the study. None of them had undergone

liver transplantation. Healthy controls attending the Well Baby Clinic at our institution for vaccination had normal physical findings and no underlying disease. BA children were classified into two groups according to their serum total bilirubin (TB): Non-jaundiced BA children (TB < 2 mg/dL, n = 46) and persistent jaundiced BA children (TB ⩾ 2 mg/dL, n = 39).

Laboratory methods

Samples of peripheral venous blood were collected from every participant, and were kept at -80 ℃ for subsequent measurement. The quantitative assessment of serum uPAR was performed by using commercially available enzyme-linked immunosorbent essay (Quantikine, R and D Systems, Minneapolis, MN, United States). According to the manufacturer's protocol, recombinant human uPAR standards and serum samples were added into each well, which has been pre-coated with specific antibody to uPAR. After incubating for 2 h at room temperature, every well was washed thoroughly with wash buffer. Then, uPAR conjugate was pipetted into each well and incubated for 2 h at room temperature. After 4 washes, substrate solution was added into the wells and the microplate was incubated for 30 min at room temperature with protection from light. Lastly, the reaction was stopped by the stop solution and the optical density was determined using an automated microplate reader at 450 nm. A standard optical density-concentration curve was drawn for the determination of uPAR concentration. The liver function tests including serum albumin,TB, direct bilirubin, aspatate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were measured using a Hitachi 912 (Roche Diagnostics, Basel, Switzerland) automated machine at the central laboratory of our hospital.

Liver stiffness measurement

Transient elastography (Fibroscan, Echosens, Paris, France) measured the liver stiffness between 25 to 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 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 executed by using the SPSS version 22.0 statistical software package (SPSS Inc., Chicago, IL, United States). Comparisons of demographic and clinical outcomes between groups were performed using χ^2 and Student's unpaired t-test when appropriate. Correlation between numerical data was obtained using Pearson's correlation coefficient (r). Data were presented as mean ± SEM of the mean. A two-tailed P-value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Comparison between BA children and control subjects

Eighty-five postoperative biliary atresia children and 24 ethnically matched unaffected volunteers were prospectively recruited in the current work. The baseline features of BA children and control subjects are presented in Table 1. There was no significant difference of age and gender between case and control groups. However, circulating uPAR values were substantially greater in BA children than in control subjects (6085.9 ± 400.7 pg/mL *vs* 4754.5 ± 294.9 pg/mL, P = 0.01) (Figure 1). Moreover, BA group had notably greater liver stiffness values than control group (28.7 ± 2.7 kPa *vs* 4.1 ± 0.2 kPa, P < 0.001).

Differences between jaundiced group and non-jaundiced group of BA children

BA children were subdivided into jaundiced group (n = 39) and non-jaundiced group (n = 46). The clinical characteristics and biochemical features of patients according to jaundice status are illustrated in Table 2. Jaudiced BA children exhibited remarkably greater serum uPAR levels than non-jaundiced BA children (7373.5 ± 684.6 pg/mL *vs* 4994.2 ± 400.9 pg/mL, P = 0.003) (Figure 2). Furthermore, mean liver stiffness measurement of jaundiced BA group was greatly increased compared with that of non-jaundiced BA group (46.2 ± 3.7 kPa *vs* 13.9 ± 2.0 kPa, P < 0.001).

Subsequent investigation revealed that circulating uPAR was directly associated with serum AST (r = 0.507, P < 0.001), ALT (r = 0.364, P < 0.001), TB (r = 0.559, P < 0.001), ALP (r = 0.325, P < 0.001), and liver stiffness values (r = 0.508, P < 0.001) in BA children (Figure 3). However, circulating uPAR concentration was negatively associated with serum albumin level (r = -0.666, P < 0.001) (Figure 3).

DISCUSSION

Biliary atresia is a chronic progressive fibroinflammatory liver disorder with mysterious etiology. The etiopathology of BA currently remains elusive and it seems that multiple factors may contribute to the development of BA. Yet today, Kasai operation has been proved as the most effective option of surgical treatment. Without surgery, children with biliary atresia will finally die due to biliary cirrhosis and liver failure[1]. Recently, circulating uPAR levels have been shown to be involved in chronic liver disorders, including chronic hepatitis B and C, liver cirrhosis, and hepatocellular carcinoma[17-20]. Based on

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

Variables	BA (n = 85)	Controls (n = 24)	P value
Age (yr)	9.0 ± 0.6	8.5 ± 0.5	0.2
Gender (female:male)	39:46	11:13	0.4
Albumin (g/dL)	4.2 ± 0.1	-	NA
Total bilirubin (mg/dL)	2.7 ± 0.4	-	NA
Direct bilirubin (mg/dL)	2.3 ± 0.4	-	NA
AST (IU/L)	143.7 ± 11.9	-	NA
ALT (IU/L)	137.1 ± 12.5	-	NA
ALP (IU/L)	449.2 ± 34.0	-	NA
Liver stiffness (kPa)	28.7 ± 2.7	4.1 ± 0.2	< 0.001
uPAR (pg/mL)	6085.9 ± 400.7	4754.5 ± 294.9	0.01

The data was expressed as mean ± SEM. BA: Biliary atresia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; uPAR: Urokinase-type plasminogen activator receptor; NA: Not applicable.

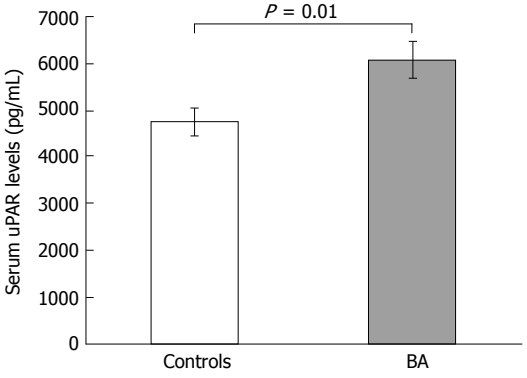

Figure 1 Comparison of serum urokinase-type plasminogen activator receptor levels in biliary atresia patients and healthy controls. uPAR: Urokinase-type plasminogen activator receptor; BA: Biliary atresia.

Table 2 Comparison of biliary atresia patients without and with jaundice

Variables	BA patients with jaundice (n = 39)	BA patients without jaundice (n = 46)	P-value
Age (yr)	9.5 ± 0.9	8.6 ± 0.9	0.4
Gender (female:male)	18:21	21:25	0.5
Albumin (g/dL)	3.8 ± 0.1	4.5 ± 0.1	< 0.001
Total bilirubin (mg/dL)	5.1 ± 0.7	0.5 ± 0.1	< 0.001
Direct bilirubin (mg/dL)	4.5 ± 0.6	0.2 ± 0.1	< 0.001
AST (IU/L)	210.4 ± 17.2	84.7 ± 10.2	< 0.001
ALT (IU/L)	195.9 ± 19.9	85.1 ± 10.7	< 0.001
ALP (IU/L)	599.7 ± 52.8	313.0 ± 32.0	< 0.001
Liver stiffness (kPa)	46.2 ± 3.7	13.9 ± 2.0	< 0.001
uPAR (pg/mL)	7373.5 ± 684.6	4994.2 ± 400.9	0.003

The data are expressed as mean ± SEM. BA: Biliary atresia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; uPAR: Urokinase-type plasminogen activator receptor; NA: Not applicable.

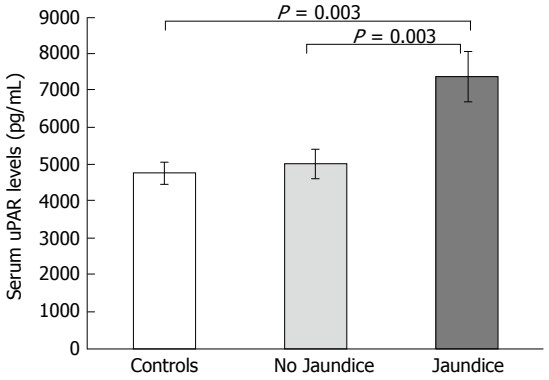

Figure 2 Comparison of serum urokinase-type plasminogen activator receptor levels in biliary atresia patients without jaundice and with jaundice. uPAR: Urokinase-type plasminogen activator receptor.

our experience, there is no report about circulating uPAR and hepatic fibrosis in various degrees of postoperative biliary atresia.

The present study is the first to show that circulating uPAR and liver fibrosis values were significantly higher in children suffering from BA than in control subjects. Additionally, circulating uPAR in jaundiced BA children was markedly increased with respect to that in non-jaundiced BA children. Elevated circulating uPAR levels were directly associated with total bilirubin, AST, ALT, ALP in post Kasai BA children, suggesting that circulating uPAR is related to degree of jaundice BA children. Furthermore, the degree of jaundice is possibly linked to the severity of intrahepatic biliary obliteration. Both AST and ALT are extensively used as biochemical parameters of hepatic abnormality indicating liver cell injury. Hence, the findings imply that uPAR could have a plausible role in the mechanism of liver cell injury in postoperative biliary atresia, and it would be associated with the severity of bile duct obliteration.

The present investigation demonstrated that circulating uPAR was more pronounced in biliary atresia children than control subjects. In accordance with this observation, Sjöwall *et al*[10] reported that circulating uPAR was increased in subjects with non-alcoholic fatty liver disease and associated with the severity of fibrosis. Moreover, uPAR expressions in liver tissue samples have been documented in subjects with hepatocellular carcinoma as shown by Morita *et al*[21]. In addition, Zimmermann *et al*[12] reported that circulating uPAR was substantially elevated in subjects with chronic liver diseases compared with controls and were closely correlated with liver function and fibrosis.

In light of our findings, certain hypotheses could explain high circulating uPAR in jaundiced biliary atresia children. Firstly, the release of uPAR in the injured liver could be accountable for the increased circulating uPAR. Secondly, the elevation of circulating uPAR may be ascribed to the unbalance between uPAR synthesis and uPAR clearance. The reduction of uPAR destruction in BA children with liver fibrosis may lead to the elevated circulating uPAR. Decreased pre-systemic hepatic metabolism might explain the increased uPAR levels in serum BA children with hepatic dysfunction. Besides, other tissues outside the liver could synthesize and

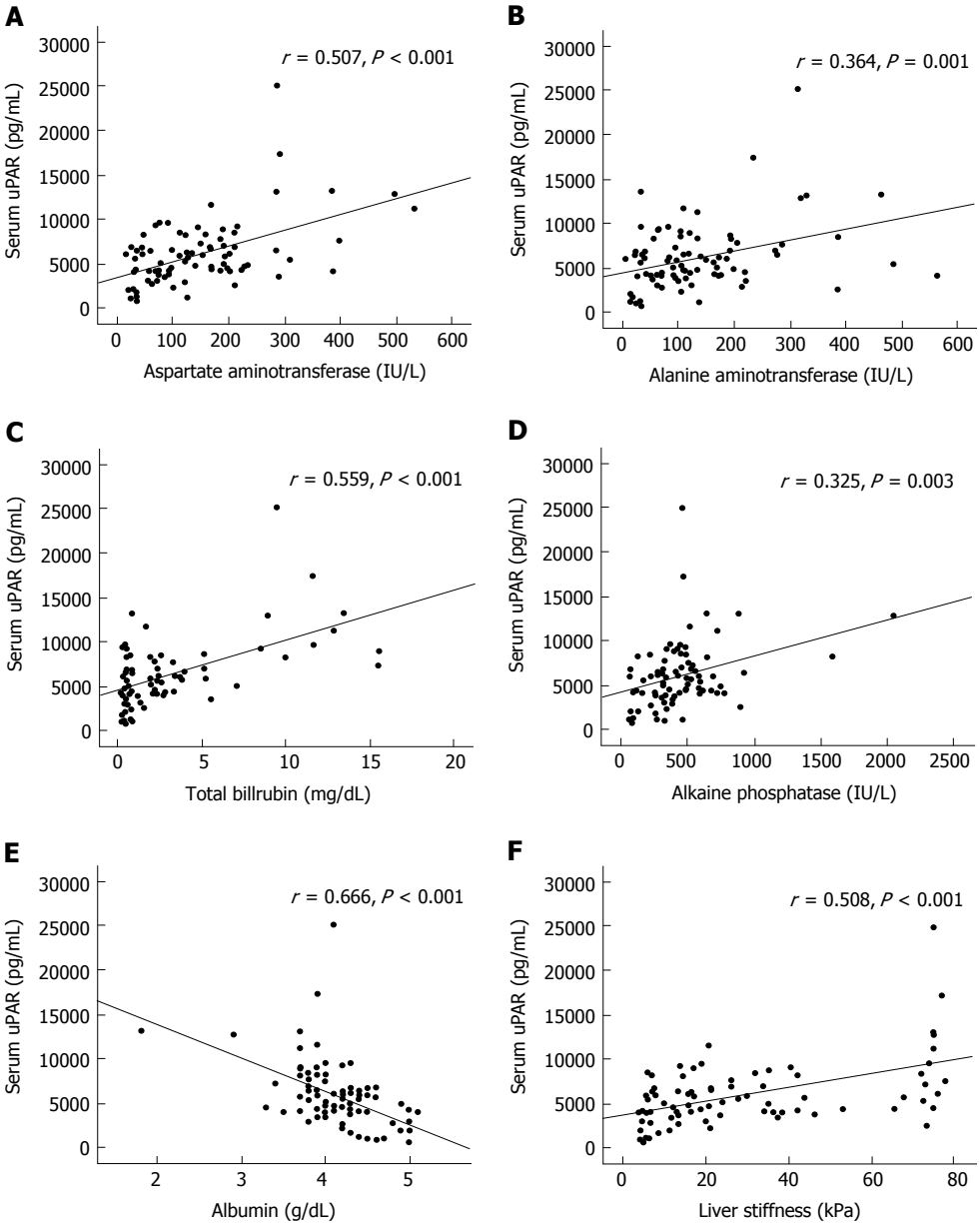

Figure 3 Scatter diagram and regression analysis in biliary atresia patients. uPAR levels are correlated with (A) serum aspartate aminotransferase (B) serum alanine aminotransferase (C) serum total bilirubin (D) serum alkaline phosphatase (E) serum albumin and (F) liver stiffness. uPAR: Urokinase-type plasminogen activator receptor.

release uPAR into the blood. The rising serum level of uPAR is likely attributable to the results of hepatocellular injury and further liver fibrosis. Whether increment of serum uPAR in BA children indicates low destruction, high production, or both remain obscure. Additional research will be needed to clarify the molecular basis leading to increased circulating uPAR.

Several caveats need to be acknowledged in this study. First, relatively small sample size of enrolled subjects limits the statistical power of our findings. Second, the cross-sectional study precludes definite information regarding causal relationships. In addition, inadequate assessment of various confounders such as comorbidity must be considered. To address these challenges, future studies should collect prospective measurements of these data to preclude bias and reverse causation. Moreover, the present investigation was restricted to the subjects under follow-up at our institution. Accordingly, our results may not be generalized across different populations. Finally, hepatic expression of uPAR has not been investigated. Further studies on immunohistochemistry of uPAR from liver tissues might provide better knowledge on molecular mechanisms of uPAR in biliary atresia.

To sum up, our study illustrated that circulating uPAR and liver stiffness measurement were markedly higher in biliary atresia children than in control subjects. Circulating uPAR was more elevated in jaundiced BA children compared to non-jaundiced BA children. Furthermore, elevated serum uPAR was correlated with hepatic dysfunction and outcome parameters. Circulating uPAR

and liver stiffness values might be used as noninvasive biological markers indicating the progression and prognosis of hepatic fibrosis in postoperative biliary atresia children. Although underlying mechanisms of the cause and effect relationships remain elusive, there is abundant room for the definite role of uPAR in the etiopathogenesis of hepatic fibrosis in BA.

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COMMENTS

Background

Biliary atresia (BA) is a severe chronic cholestatic liver disease of unknown etiology in young infants. The exact pathogenesis of BA remains a matter of debate. Circulating urokinase plasminogen activator receptor (uPAR) has arisen as a promising biochemical marker of certain disorders, such as liver injury and fibrosis. Although recent reports suggest a potential applicability for the measurement of circulating uPAR in liver fibrosis, the assessments on circulating uPAR and liver stiffness of BA have never been documented.

Research frontiers

Recent evidences demonstrate the significance of urokinase plasminogen activator receptor in hepatitis, liver fibrosis, and liver failure. The current study shows that circulating uPAR levels are more elevated in BA children than in control subjects. Moreover, uPAR level is correlated with liver stiffness, and clinical outcomes in postoperative BA.

Innovations and breakthroughs

BA children exhibited significantly higher circulating uPAR and liver stiffness values than control subjects. Circulating uPAR and liver stiffness values were more pronounced in jaundiced BA children than in non-jaundiced BA children. Additionally, elevated circulating uPAR levels were associated with hepatic dysfunction and clinical outcomes.

Applications

Increased circulating uPAR and liver stiffness values were was associated with hepatocellular dysfunction in postKasai children affected with BA. As a consequence, circulating uPAR and liver stiffness measurements could be used as noninvasive biological markers indicating the progression and prognosis of liver fibrogenesis in BA children.

Terminology

uPAR also known as CD87, is a multidomain membrane protein that has a role in the regulation of cell migration, proliferation, and survival and is expressed by diverse immune cells and endothelial cells, which contribute to the etiopathogenesis of hepatic inflammation and liver fibrogenesis.

Peer-review

Great paper that needs to be published. uPAR is known to be a substantial factor in the etiopathogenesis of hepatic inflammation and liver fibrogenesis.

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ORIGINAL ARTICLE

Elevated serum heat shock protein 70 and liver stiffness reflect hepatic dysfunction and severity in postoperative biliary atresia

Sittisak Honsawek[1] · Wanvisa Udomsinprasert[1] · Napaphat Jirathanathornnukul[1] · Voranush Chongsrisawat[2] · Yong Poovorawan[2]

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Abstract
Background Biliary atresia (BA) is a severe chronic liver disease characterized by progressive obstructive cholangiopathy of biliary tract. Heat shock protein 70 (HSP70) is involved in protecting cells against a wide variety of stress and plays a protective role in tissue damage. The purpose of this study was to investigate serum HSP70 and liver stiffness in BA and determine the association of serum HSP70, liver stiffness, and outcome parameters in post-Kasai BA patients.
Methods One hundred post-Kasai BA patients and 40 controls were enrolled. Serum HSP70 levels were analyzed using enzyme-linked immunosorbent assay. Liver stiffness values were assessed by transient elastography.
Results BA patients had significantly higher serum HSP70 and liver stiffness values than controls. Serum HSP70 and liver stiffness values were markedly elevated in BA patients with jaundice compared to those without jaundice ($P < 0.001$). Furthermore, serum HSP70 was more elevated in BA children with portal hypertension than those without portal hypertension (35.1 ± 2.1 vs. 27.9 ± 2.5 ng/mL, $P < 0.001$). Moreover, serum HSP70 was positively correlated with serum aspartate aminotransferase ($r = 0.491$, $P < 0.001$), alanine aminotransferase ($r = 0.448$, $P < 0.001$), total bilirubin ($r = 0.303$, $P = 0.002$), alkaline phosphatase ($r = 0.414$, $P < 0.001$), and liver stiffness values ($r = 0.455$, $P < 0.001$). There was a negative correlation between serum HSP70 and serum albumin ($r = -0.434$, $P = 0.001$).
Conclusion Serum HSP70 and liver stiffness values were higher in BA patients than controls. The increased serum HSP70 was correlated with hepatic dysfunction in BA. Consequently, serum HSP70 and liver stiffness could serve as non-invasive parameters reflecting the severity in post-Kasai BA.

Keywords Biliary atresia · Heat shock protein 70 · Jaundice · Liver stiffness · Severity

Introduction

Biliary atresia (BA) is a chronic progressive fibroinflammatory liver disorder with mysterious etiology [1]. BA is characterized by impaired liver function and obstructive cholangiopathy of both intrahepatic and extrahepatic bile ducts [2, 3]. Extrahepatic BA is the most common form of ductal cholestasis. Affected newborns exhibit evidence of biliary obstruction within the first few months of life. The etiopathogenesis of BA currently remains obscure and it seems that multiple factors may contribute to the development of BA. Thus far, Kasai operation has been proved as the most effective option of surgical treatment. If left untreated, BA children will ultimately die due to biliary cirrhosis and liver failure. Several theories regarding the etiology of BA include toxin exposure, virus-mediated inflammation, abnormal inflammatory response, defective morphogenesis, genetic mutation, and immunological dysregulation [4].

Heat shock proteins (HSPs), firstly characterized as heat-inducible gene products, are a highly conserved

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family of stress response proteins that function as molecular chaperones, mediating the folding of cellular proteins, preventing protein aggregation, and targeting improperly folded proteins to specific degradative pathways [5]. Expression of HSPs is increased under environmental, physiological, and pathophysiological stress; indeed, the exposure of cells to physical or chemical stress leads to elevated production of HSPs, especially the heat shock protein 70 (HSP70) family [6]. HSP70 is involved in protecting cells against a wide variety of stress. Additionally, HSP70 plays a protective role in tissue damage: it is synthesized in the liver and spleen as an acute-phase reactant and is secreted into the circulation to promote the removal of dead cells [7]. Recently, it has been reported that elevated serum HSP70 levels have been evident in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [8].

It has been recently demonstrated that several cytokines and growth factors play plausible roles in the etiopathogenesis of biliary atresia [9–12]. The measurements on serum HSP70 and liver stiffness of BA have never been reported. We postulated that serum HSP70 and liver stiffness could be increased and correlated with the severity in BA patients. Accordingly, the present research aimed to examine serum HSP70 and liver stiffness measurements and to investigate the correlation of serum HSP70, liver stiffness, and outcome parameters in post-Kasia BA.

Materials and methods

The current study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University, and was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. All parents of children were informed of the study's purpose and of any interventions involved in this study. Written informed consent was acquired from the parents prior to the children entering the study.

Study population

One hundred BA patients (50 boys and 50 girls with mean age of 8.1 ± 0.5 years) and 40 healthy children (18 boys and 22 girls with mean age of 7.9 ± 0.2 years) were registered in this study. None of them had undergone liver transplantation. Healthy controls attending the Well Baby Clinic at King Chulalongkorn Memorial hospital for vaccination had normal physical findings and no underlying disease. BA patients were classified into two groups according to their serum total bilirubin (TB). According to their jaundice status, BA children were divided into a non-jaundiced group (TB <2 mg/dL, $n = 58$) and a persistent jaundiced group (TB ≥ 2 mg/dL, $n = 42$). Furthermore, portal hypertension (PH) was assessed by the presence of ascites and/or esophageal varices observed on endoscopy. Forty-eight children had no portal hypertension but the remaining 52 did.

Laboratory methods

Samples of peripheral venous blood were collected from every participant, and were stored at −80 °C for further analysis. Double-blind quantitative assessment of serum HSP70 was performed using commercially available enzyme-linked immunosorbent essay (ELISA) (Quantikine, R&D Systems, Minneapolis, MN, USA). According to the manufacturer's protocol, recombinant human HSP70 standards and serum samples were added into each well, which has been pre-coated with specific antibody to HSP70. After incubating for 2 h at room temperature, every well was washed thoroughly with wash buffer. Then, HSP70 conjugate was pipetted into each well and incubated for 2 h at room temperature. After 4 washes, substrate solution was added into the wells and the microplate was incubated for 30 min at room temperature with protection from light. Lastly, the reaction was stopped by the stop solution and the optical density was determined using an automated microplate reader at 450 nm. A standard optical density–concentration curve was drawn for the determination of HSP70 concentration. The liver function tests including serum albumin, total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), prothrombin time (PT), and international normalized ratio (INR) were measured using a Hitachi 912 (Roche Diagnostics, Basel, Switzerland) automated machine at the central laboratory of our hospital.

Liver stiffness measurement

Transient elastography (Fibroscan, Echosens, Paris, France) measured the liver stiffness between 25 to 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 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 ten validated results had been obtained with a success rate of at least 80%. The median value of ten 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 22.0 statistical software package (SPSS Inc., Chicago, IL, USA). 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 (SEM). All the P values <0.05 based on a two-tailed test were considered statistically significant.

Results

Comparison between BA children and control subjects

One hundred postoperative BA children and 40 ethnically matched unaffected volunteers were prospectively recruited in the present study. The baseline features of BA children and control subjects are displayed in Table 1. There was no significant difference of age and gender between case and control groups. However, serum HSP70 levels were significantly higher in BA children than in control subjects (31.3 ± 1.9 vs. 23.2 ± 3.9 ng/mL, $P = 0.02$) (Fig. 1). Furthermore, BA group had remarkably greater liver stiffness values than control group (27.4 ± 2.4 vs. 4.2 ± 0.2 kPa, $P < 0.001$).

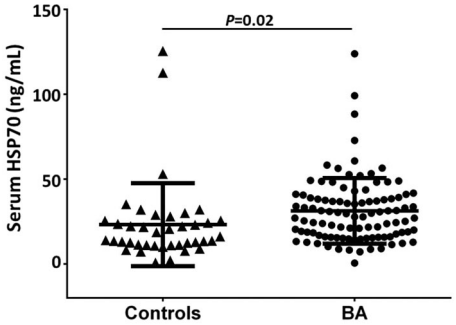

Fig. 1 Comparison of serum HSP70 levels in post-Kasai BA children and healthy controls. The data were expressed as mean ± SD

Comparison between jaundiced group and non-jaundiced group of BA children

Biliary atresia children were subdivided into jaundiced group ($n = 42$) and non-jaundiced group ($n = 58$). The clinical characteristics and biochemical features of patients according to jaundice status are demonstrated in Table 2. Jaundiced BA children exhibited notably higher serum HSP70 levels than non-jaundiced BA children (39.8 ± 2.6 vs. 25.1 ± 2.4 ng/mL, $P < 0.001$) (Fig. 2). Additionally, mean liver stiffness measurement of jaundiced BA group was substantially elevated compared with that of non-jaundiced BA group (46.8 ± 3.6 vs. 13.3 ± 1.7 kPa, $P < 0.001$).

Subsequent analysis revealed that circulating HSP70 was more elevated in BA children with PH than those without PH (35.1 ± 2.1 vs. 27.9 ± 2.5 ng/mL, $P < 0.001$)

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

Variables	BA ($n = 100$)	Controls ($n = 40$)	P value
Age (years)	8.1 ± 0.5	7.9 ± 0.2	0.2
Gender (female: male)	50:50	22:18	0.4
Albumin (g/dL)	4.3 ± 0.1	–	NA
Total bilirubin (mg/dL)	2.5 ± 0.4	–	NA
Direct bilirubin (mg/dL)	2.1 ± 0.3	–	NA
AST (IU/L)	138.1 ± 11.0	–	NA
ALT (IU/L)	134.6 ± 11.4	–	NA
ALP (IU/L)	429.0 ± 30.3	–	NA
Platelet count (10^3/mm^3)	150.1 ± 13.3	–	NA
Prothrombin time (s)	12.4 ± 0.2	–	NA
INR	1.1 ± 0.0	–	NA
APRI	2.9 ± 0.4	–	NA
Liver stiffness (kPa)	27.4 ± 2.4	4.2 ± 0.2	<0.001
HSP70 (ng/mL)	31.3 ± 1.9	23.2 ± 3.9	0.02

The data were expressed as mean ± SEM

BA biliary atresia, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *APRI* aspartate aminotransferase to platelets ratio index, *HSP70* heat shock protein 70, *NA* not applicable, *INR* international normalized ratio

Table 2 Comparison between biliary atresia patients with and without jaundice

Variables	BA patients with jaundice ($n = 42$)	BA patients without jaundice ($n = 58$)	P value
Age (years)	8.2 ± 0.8	8.0 ± 0.7	0.5
Gender (female: male)	21:21	29:29	0.5
Albumin (g/dL)	3.8 ± 0.1	4.5 ± 0.1	<0.001
Total bilirubin (mg/dL)	5.1 ± 1.0	0.6 ± 0.1	<0.001
Direct bilirubin (mg/dL)	4.5 ± 0.6	0.3 ± 0.1	<0.001
AST (IU/L)	215.7 ± 17.0	79.9 ± 8.3	<0.001
ALT (IU/L)	201.3 ± 19.5	84.5 ± 9.1	0.005
ALP (IU/L)	599.5 ± 50.0	299.5 ± 26.5	<0.001
Platelet count (10^3/mm^3)	111.0 ± 19.3	174.5 ± 16.9	0.007
Prothrombin time (s)	13.3 ± 0.3	11.8 ± 0.2	<0.001
INR	1.2 ± 0.0	1.0 ± 0.0	<0.001
APRI	4.9 ± 0.5	1.6 ± 0.3	<0.001
Liver stiffness (kPa)	46.8 ± 3.6	13.3 ± 1.7	<0.001
HSP70 (ng/mL)	39.8 ± 2.6	25.1 ± 2.4	<0.001

The data were expressed as mean ± SEM

BA biliary atresia, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *APRI* aspartate aminotransferase to platelets ratio index, *HSP70* heat shock protein 70, *INR* international normalized ratio

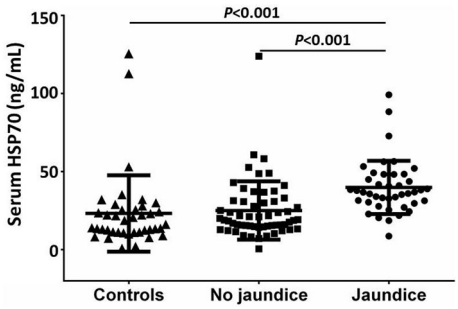

Fig. 2 Comparison of serum HSP70 levels in jaundiced BA children, non-jaundiced BA children, and controls. The data were expressed as mean ± SD

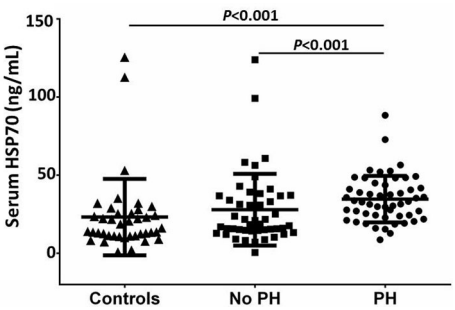

Fig. 3 Comparison of serum HSP70 levels in BA children with PH, BA children without PH, and controls. The data were expressed as mean ± SD

(Fig. 3). Moreover, serum HSP70 was positively associated with serum AST ($r = 0.491$, $P < 0.001$), ALT ($r = 0.448$, $P < 0.001$), TB ($r = 0.303$, $P = 0.002$), ALP ($r = 0.414$, $P < 0.001$), and liver stiffness values ($r = 0.455$, $P < 0.001$) in BA children (Fig. 4). However, serum HSP70 was negatively associated with serum albumin level ($r = -0.434$, $P = 0.001$) (Fig. 4).

Discussion

Biliary atresia (BA) is a severe chronic cholestatic liver disease of unknown etiology in young infants [1]. BA patients initially develop neonatal jaundice due to hepatic cholestasis and progress to hepatic fibrosis leading to biliary cirrhosis [1–3]. Although no medical therapies exist, sequential treatment strategy involving surgical Kasai portoenterostomy and liver transplantation is the only option for the most affected children. The precise pathogenesis of biliary atresia remains a matter of debate. In recent years, serum HSP70 has been shown to be involved in chronic liver disorders, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [8]. Based on our experience, there have never been any studies regarding serum HSP70 and hepatic fibrosis in various degrees of post-Kasai BA.

The current study is the first to reveal that serum HSP70 and liver fibrosis values were significantly greater in BA children than in control subjects. Furthermore, serum HSP70 in jaundiced BA children was substantially increased with respect to that in non-jaundiced BA children. Increased serum HSP70 levels were directly correlated with total bilirubin, AST, ALT, ALP in postoperative

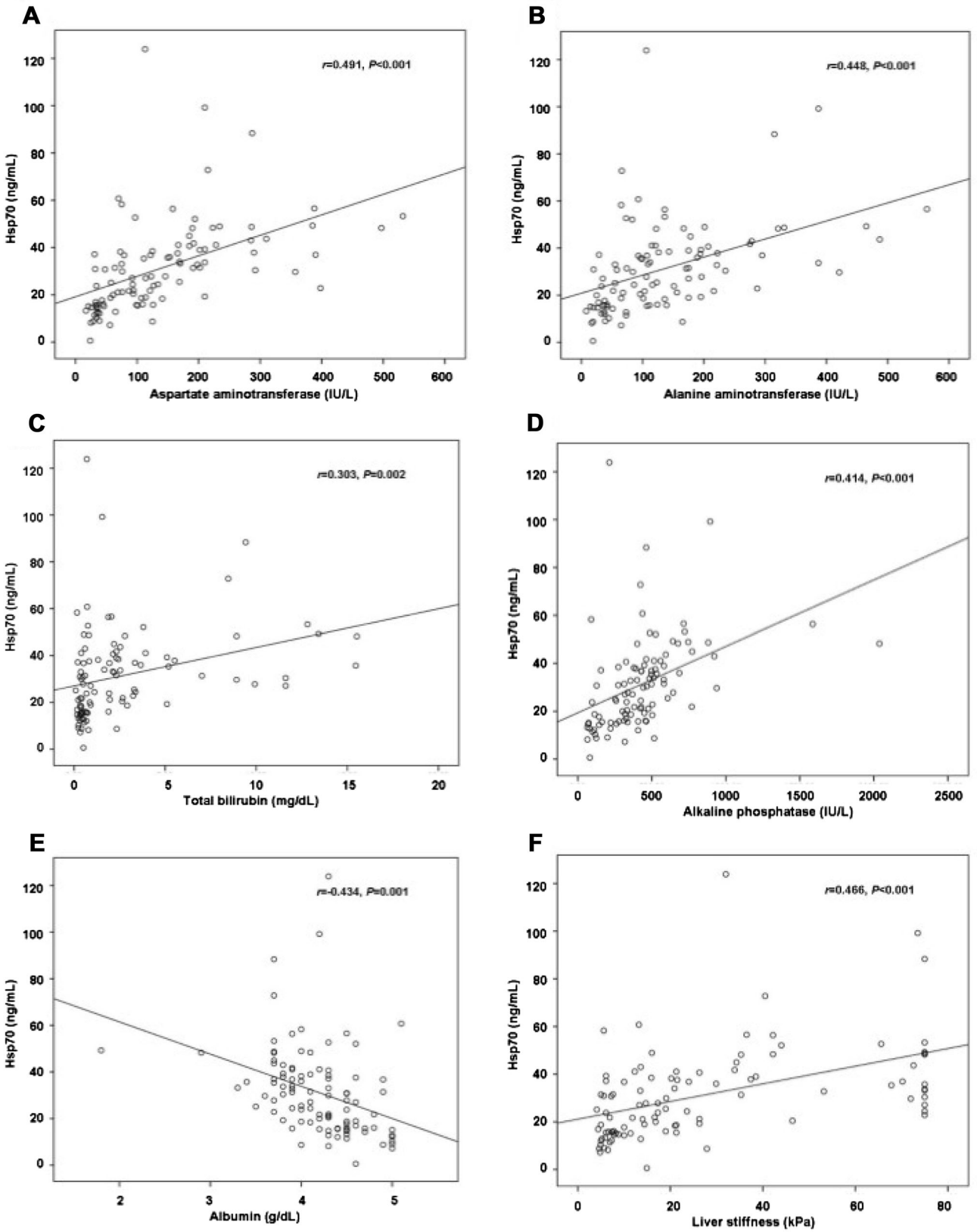

Fig. 4 Scatter diagram and correlation analysis in biliary atresia patients. Serum HSP70 levels are correlated with **a** serum aspartate aminotransferase, **b** serum alanine aminotransferase, **c** serum total bilirubin, **d** serum alkaline phosphatase, **e** serum albumin, and **f** liver stiffness

BA children, implying that serum HSP70 is related to degree of jaundice in BA children. In addition, the degree of jaundice is possibly linked to the severity of intrahepatic biliary obliteration. Both AST and ALT are extensively used as biochemical parameters of hepatic abnormality indicating liver cell injury. Therefore, the findings suggest that HSP70 may play a vital role in the mechanism of liver cell injury in postoperative biliary atresia, and it would be associated with the severity of bile duct obliteration. Serum HSP70 might be applied as a simple, affordable, non-invasive biomarker for determining the hepatic dysfunction in BA.

The present investigation showed that serum HSP70 was more pronounced in BA children than control subjects. In line with this observation, it has been documented that HSP70 expression was more elevated in advanced hepatocellular carcinoma than in early hepatocellular carcinoma [13]. In addition, Molvarec and colleagues reported that increased serum HSP70 levels seemed to reflect systemic inflammation, oxidative stress, and hepatocellular injury in preeclampsia [14]. Furthermore, immunohistochemical study revealed that HSP70 was observed in both advanced and early hepatocellular carcinoma and was less likely to be positive in the regenerative nodule in cirrhotic liver [15].

In light of our findings, certain hypotheses could explain high serum HSP70 in jaundiced BA children. Firstly, the release of HSP70 in the injured liver could be accountable for the increased circulating HSP70. Secondly, the elevation of serum HSP70 may be attributable to the unbalance between HSP70 synthesis and HSP70 clearance. The reduction of HSP70 destruction in BA children with liver fibrosis could result in the elevated circulating HSP70. Decreased pre-systemic hepatic metabolism could explain the increased HSP70 levels in serum BA children with hepatic dysfunction. Besides, other tissues outside the liver could synthesize and release HSP70 into the blood. The rising serum level of HSP70 is likely ascribed to the results of hepatocellular injury and further liver fibrosis. Whether increment of serum HSP70 in BA children indicates low destruction, high production, or both remain uncertain. Higher circulating HSP70 in BA children with PH may be ascribed to a decreased first-pass effect or portal-systemic shunting. Further research will be necessary to clarify the molecular basis leading to increased circulating HSP70.

In the current study, there was no difference in serum HSP70 between non-jaundiced BA cases and control subjects. No difference in circulating HSP70 was evidenced between BA children without PH and control subjects. These data imply that the elevated circulating HSP70 may be an epiphenomenon of bile duct obliteration or hepatocellular damage rather than biliary atresia by itself, as it is known that even non-jaundiced BA subjects have advancing disease process as observed by their ongoing abnormal hepatic enzymes. It can be pointed out that circulating HSP70 is increased in jaundiced BA subjects and advanced hepatic dysfunction from unsuccessful Kasai portoenterostomy and that circulating HSP70 levels were comparable in non-jaundiced BA children who achieved adequate biliary drainage after surgery and control subjects. The exact reason for this observation is still enigmatic, but this would be due to a variety of different subjects within the group in our study. The potential mechanisms of high circulating HSP70 in BA are worth further investigation.

Certain limitations should be acknowledged in the present study. Firstly, the numbers of patients and controls were relatively small. This may reduce the statistical power of these results. Secondly, the cross-sectional design of this study excludes definite information relating the causal relationship. Thirdly, inadequate measurement of some confounding factors including co-morbidity should be beared in mind. Further experimental investigation should collect prospective measurements of these data to prevent reverse causality bias. Moreover, one source of weakness in this study was the paucity of pediatric end stage liver disease (PELD) scores. More research is also required to evaluate the PELD value for predicting of chronic liver disease severity. Besides, high circulating HSP70 levels have been shown to be associated with inflammation or stress condition and these may affect the analysis [14, 16]. Furthermore, this study was limited to the participants under follow-up at our hospital. Consequently, our findings might not be applied to participants in other populations. Lastly, liver expression of HSP70 has not been determined. Additional researches on immunohistochemical study of hepatic HSP70 could offer better understanding on the molecular basis of HSP70 in post-Kasai BA.

To summarize, this investigation showed that serum HSP70 and liver stiffness values were significantly higher in BA cases than in control participants. Serum HSP70 levels were more pronounced in jaundiced BA cases than in non-jaundiced BA cases. Additionally, increased serum HSP70 levels were associated with liver stiffness and clinical outcomes in post-Kasai BA. Serum HSP70 and liver stiffness could be considered as non-invasive parameters identifying the disease severity of liver fibrogenesis in post-Kasai BA subjects. Considerably more work will need to be done to determine the importance of HSP70 in the pathogenesis of liver fibrogenesis in biliary atresia.

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Compliance with ethical standards

Conflict of interest The authors declare that the authors have no conflict of interest.

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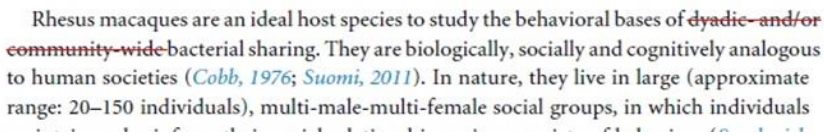
Rhesus macaques are an ideal host species to study the behavioral bases of ~~dyadic- and/or community-wide~~ bacterial sharing. They are biologically, socially and cognitively analogous to human societies (*Cobb, 1976*; *Suomi, 2011*). In nature, they live in large (approximate range: 20–150 individuals), multi-male-multi-female social groups, in which individuals

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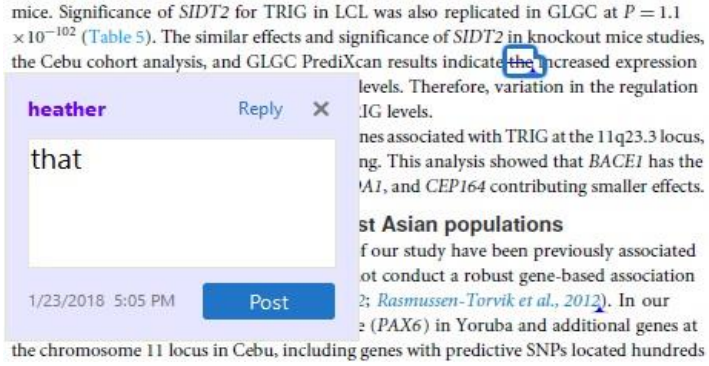
mice. Significance of *SIDT2* for TRIG in LCL was also replicated in GLGC at $P = 1.1 \times 10^{-102}$ (Table 5). The similar effects and significance of *SIDT2* in knockout mice studies, the Cebu cohort analysis, and GLGC PrediXcan results indicate the increased expression levels. Therefore, variation in the regulation IG levels.
nes associated with TRIG at the 11q23.3 locus, ng. This analysis showed that *BACE1* has the *A1*, and *CEP164* contributing smaller effects.
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Hepatic autotaxin overexpression in infants with biliary atresia

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ABSTRACT

Background. Autotaxin (ATX) is a secreted glycoprotein that is involved in the development of hepatic fibrogenesis via the enzymatic production of lysophosphatidic acid. The aim of this study was to investigate hepatic expression of ATX in biliary atresia (BA) compared with non-BA liver controls and to examine the association between ATX expression and clinical outcome in BA.

Methods. Liver specimens from BA infants ($n = 20$) were compared with samples from infants who underwent liver biopsy for reasons other than BA ($n = 14$) served as controls. Relative mRNA and protein expression of ATX were quantified using real-time polymerase chain reaction (PCR) and immunohistochemistry. Masson's Trichrome staining was performed to determine the degree of liver fibrosis.

Results. Quantitative real-time PCR demonstrated overexpression of *ATX* mRNA in BA livers. In immunohistochemical evaluation, ATX was positively stained on the hepatic parenchyma and the biliary epithelium in BA patients, as compared to non-BA controls. The immunostaining score of ATX in BA livers was also significantly higher than that observed in non-BA livers ($P < 0.001$). Subgroup analysis revealed that ATX expression in the patients with poor outcome was significantly greater than in those with good outcome ($P = 0.03$). Additionally, there was a positive correlation between hepatic ATX expression and Metavir fibrosis stage in BA livers ($r = 0.79$, $P < 0.001$).

Discussion. This study found that mRNA and protein expression of ATX was increased in BA livers. High hepatic ATX expressionat the time of Kasai operation was associated with liver fibrosis and outcome in BA, suggesting that ATX may serve a role as a promising biomarker of the prognosis in biliary atresia.

Subjects Gastroenterology and Hepatology, Pediatrics

Keywords Biliary atresia, Autotaxin, Immunohistochemistry, Quantitative real-time polymerase chain reaction, Liver

INTRODUCTION

Biliary atresia (BA) is a neonatal cholestasis disease that is characterized by fibrosclerosing and inflammatory obliteration of the biliary tracts, which leads to progressive liver damage (*Hartley, Davenport & Kelly, 2009*). Kasai portoenterostomy (KPE), the primary treatment for BA, establishes good bile flow and facilitates long-term survival. However and even after timely KPE, a number of infants are at risk of developing new biliary obstruction that could lead to chronic cholestasis, increased fibrosis, cirrhosis, and eventually to end-stage liver disease. As such, BA is the leading cause of liver transplantation in children. Although the precise pathogenesis of BA remains elusive, possible etiologies include viral infection, toxins, chronic inflammatory or immune-mediated bile duct injury, and abnormalities in bile duct development (*Bezerra, 2005*). Increased understanding of what causes inflammatory cholangiopathy in BA could lead to therapies aimed at protecting the intrahepatic biliary system from inflammation-mediated fibrosis. However, the molecular mechanisms involved in the pathogenesis of liver fibrosis in BA have not yet been fully and clearly established. Hepatic fibrosis is a reversible physiologic and pathologic event, and the possible role of cytokine-mediated pathogenesis in this disorder is of great interest to many researchers.

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 from lysophosphatidylcholine (*Tokumura et al., 2002*). ATX-LPA signaling has been implicated in multiple biological and pathophysiological processes, including vasculogenesis, cholestatic pruritus, tumor progression, and fibrosis via 6 distinct G-protein-coupled LPA receptors (LPAR1-6) (*Umezu-Goto et al., 2002*). *Ikeda et al. (2003)* Ikeda et al., identified a potential link between the ATX-LPA axis and liver fibrosis when they found that intradermal LPA induces hepatic stellate cell (HSC) proliferation, stimulates their contraction, and inhibits their apoptosis. HSCs are known as prototypic profibrogenic cells in the hepatic parenchyma. After transformation into myofibroblasts in response to a liver injury, HSCs start to produce abundant extracellular matrices and profibrogenic cytokines, such as ATX-derived LPA. In addition, both LPA and ATX concentrations were increased in chronic hepatitis C patients with liver fibrosis (*Watanabe et al., 2007*). The *ATX-LPA* axis has also been reported to be up-regulated in human hepatocellular carcinoma (*Park et al., 2011*; *Wu et al., 2010*), thereby establishing the possible influence of ATX in inflammation-related hepatic fibrosis disorders like biliary atresia.

Although circulating ATX levels have been shown to be associated with liver fibrosis, there is limited information on ATX expression in liver tissue and regarding the association between ATX expression and BA outcomes. Accordingly, the aim of this study were to investigate mRNA and protein expression of ATX in liver tissues from BA patients compared with non-BA controls and to evaluate whether hepatic ATX expression is associated with outcome parameters in BA infants.

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 (IRB No. 549/57). All parents of children were fully informed regarding the study protocol and procedures prior to the children entering the study. Written informed consent was obtained from the participants' parents.

Perioperative liver biopsies were obtained from 20 BA infants at the time of KPE and 14 non-BA patients at the Department of Surgery, King Chulalongkorn Memorial Hospital during the July 2005 to July 2007 study period. All BA infants were invited to participate in this study based on the following criteria: (1) diagnosis of type 3 (uncorrectable) isolated BA and they underwent Kasai procedure; (2) availability of clinical details and long term follow-up after surgery; and (3) availability of archived glass slides or paraffin blocks of wedge liver specimens taken at KPE. Infants diagnosed with BA or non-BA were included based on clinical, cholangiographic, and histologic findings. Non-BA patients with no history of immune-mediated diseases served as controls. Non-BA control samples were collected from 6 patients with choledochal cyst, 4 patients with thalassemia, 3 patients with neuroblastoma, and 1 patient with hepatoblastoma. Liver biopsies taken from non-BA controls were obtained during procedures that were required for medical reasons. Liver specimens from age-matched healthy controls could not be obtained due to ethical concerns about harvesting liver tissue from healthy infants.

Demographic and clinical data collected at the time of KPE included age, albumin, total bilirubin (TB), and alanine aminotransferase (ALT). Laboratory investigations were performed on a Roche Hitachi 912 chemistry analyzer (Roche Diagnostics, Basel, Switzerland). In order to associate ATX hepatic expression with outcome at 6 months post-Kasai in BA, the infants were divided into good outcome and poor outcome based on their levels of serum TB, ALT, and clinical findings. Nine patients with good outcome had good bile flow after KPE. The stool color turned from pale to yellowish for 6 months following successful surgery. The serum TB returned to normal with satisfactory liver function (TB < 2 mg/dL, ALT < 100 IU/L). Another 11 patients with poor outcome had cholestasis after 6 months KPE and severe liver dysfunction (TB ≥ 2 mg/dL, ALT ≥ 100 IU/L).

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

Of 20 BA infants and 14 non-BA controls, 15 BA livers and 5 non-BA liver specimens (choledochal cyst) were snap-frozen in liquid nitrogen-cooled isopentane and stored at $-80\,^{\circ}C$ and only available for *ATX* mRNA expression. Total RNA was isolated from liver biopsies using RNeasy Mini Kit (Qiagen, Hilden, Germany) with cDNA that was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Inc., Foster City, CA, USA). Real-time PCR was performed using QPCR Green Master Mix 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 (*Kondo et al., 2014*); *GADPH* forward primer 5′-GTGAAGGTCGGAGTCAACGG-3′; and, *GADPH* reverse primer 5′-TCAATGAAGGGGTCATTGATGG-3′. Real-time PCR 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.

Masson's Trichrome staining

Masson's Trichrome staining was conducted according to the manufacturer's protocol (Genmed Scientifics, Wilmington, DE). The collagen fiber was stained blue, the nuclei were stained black, and the background was stained red. Liver fibrosis was evaluated according to the Metavir grading system (*Bedossa & Poynard, 1996*) as follows: F0, no fibrosis; F1, mild fibrosis in the portal area; F2, mild bridging fibrosis in the adjacent portal area; F3, severe bridging fibrosis in the adjacent portal area; and F4, cirrhosis and annular fibrosis with nodule formation.

Immunohistochemical analysis for protein expression of ATX

All liver specimens of 20 BA patients and 14 non-BA controls were 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 tissue sections were analyzed by a pathologist who was blinded to patient clinical status and diagnosis. Immunoreactivity of ATX in the biliary epithelium and the parenchyma was semi-quantitatively analyzed for percentage of positive cells and intensity of staining. A percentage of positive cells <1% was scored as 0; 1%-25% as 1; >25%–50% as 2; >50%–75% as 3; and, >75% as 4. Intensity of ATX immunostaining was determined using the following staining scores: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. Final results were scored by the total score [total scores = ((score of positive cell+score of intensity) × 100)/maximum score of both parameters]. Using the aforementioned staining scores, the positive areas of ATX positive cells were determined by measuring five randomly selected microscopic fields (400×) on each slide.

Statistical analysis

All statistical analyses were performed using SPSS Statistics version 22.0 (SPSS, Inc., Chicago, IL, USA). Demographic and clinical characteristics between groups were evaluated using Chi-square tests and unpaired Student's *t*-tests where appropriate. The comparisons of ATX expression between groups were performed by Mann–Whitney *U*-tests. Correlations were analyzed by Spearman's rank correlation. The curves for survival were drawn according to the Kaplan–Meier analysis with end points of death. The differences of survival curves were determined using log-rank test. Data are presented

Table 1 Demographic and clinical characteristics of BA patients and non-BA controls.

Characteristics	BA patients ($n = 20$)	Non-BA controls ($n = 14$)	*P*-value
Age (days)	91.1 ± 7.0	897.5 ± 315.2	<0.01
Gender (female:male)	12:8	8:6	0.6
Albumin (g/dL)	4.1 ± 0.1	4.2 ± 0.3	0.3
Total bilirubin (mg/dL)	12.2 ± 0.8	NA	–
ALT (IU/L)	191.8 ± 25.8	NA	–
Hepatic ATX expression (%)	50.0 ± 5.9	13.4 ± 5.0	<0.001

Notes.

Data presented as mean ± SEM.

P-value < 0.05 indicates a statistically significant difference in clinical data between BA patients and non-BA controls at the time of Kasai portoenterostomy (KPE).

Abbreviations: BA, biliary atresia; ALT, alanine aminotransferase; ATX, autotaxin; NA, Data not available; SEM, standard error of the mean.

as mean ± standard error of the mean (SEM). A P-value < 0.05 was considered to be statistically significant for differences and correlations.

RESULTS

Clinical characteristics of study participants

Baseline characteristics of BA infants and non-BA controls are summarized in Table 1. There was no statistically significant difference in gender ratio between BA patients and non-BA controls. All non-BA participants had no clinical jaundice. The diagnosis of non-BA subjects included 6 choledochal cysts, 4 thalassemias, 3 neuroblastomas, and 1 hepatoblastoma.

Relative mRNA expression of ATX

To identify mRNA expression of *ATX* in infants with BA, relative *ATX* mRNA expression was quantified by real-time polymerase chain reaction (PCR) in liver biopsies from BA patients ($n = 15$) and non-BA controls ($n = 5$). Relative *ATX* mRNA expression was found to be significantly higher in BA livers than non-BA liver controls ($P < 0.05$) (Figs. 1A; 1B).

Immunohistochemistry analysis of ATX protein expression

Immunohistochemical evaluation for ATX protein expression was performed in both BA and non-BA liver tissues. Representative immunohistochemical findings of ATX are illustrated in Fig. 2. In congenital BA liver specimens, overexpression of ATX was detectable in the hepatic parenchyma, biliary epithelial cells, and cells of the surrounding connective tissue. In contrast, ATX expression was only scarcely evident in non-BA control livers, being demonstrated as faint cytoplasmic staining (Fig. 2A). The distribution of ATX in positive cells was classified as cytoplasm-localized pattern.

In order to compare expression levels of ATX protein between BA patients and non-BA controls, staining intensity and percentage of ATX positive cells were assessed by visual scoring method. In BA livers at the time of KPE, hepatic ATX protein expression was significantly higher than that in non-BA controls when measured by the total score of

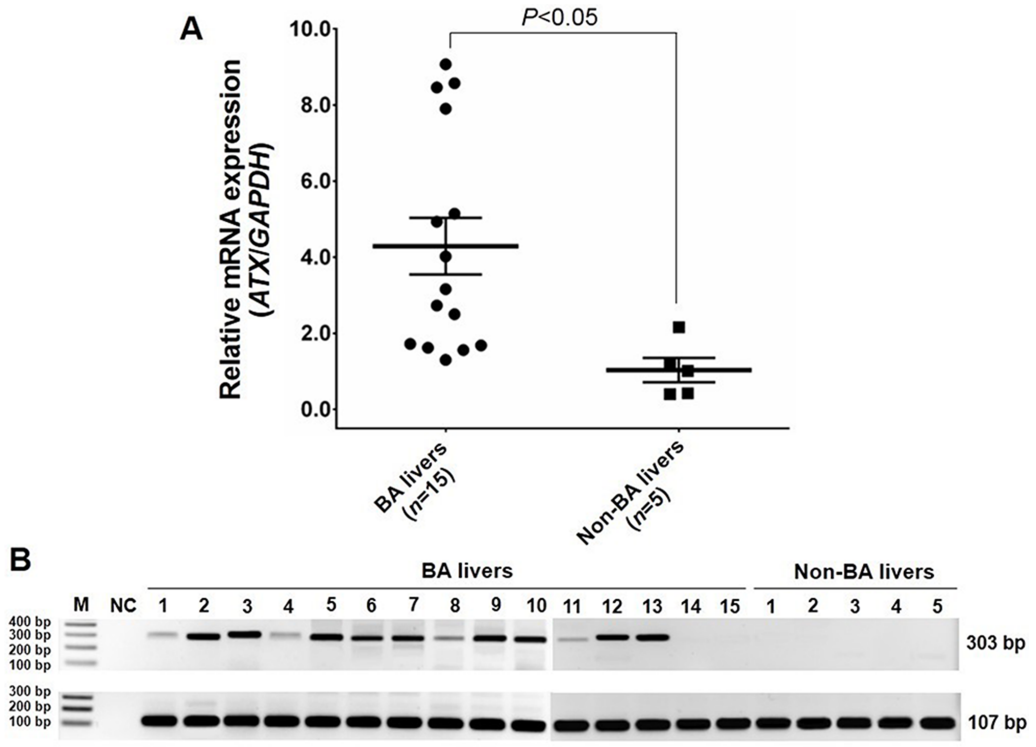

Figure 1 Relative *ATX* mRNA expression between BA livers and non-BA liver controls. (A) Up-regulated mRNA expression of *ATX* normalized by *GAPDH* in livers from BA infants. (B) Representative gel of *ATX* and *GAPDH* products from real-time PCR analysis. Abbreviations: M, molecular weight marker; NC, non-template control.

Full-size DOI: 10.7717/peerj.5224/fig-1

staining on histologic liver sections ($P < 0.001$) (Fig. 3A). There was no association between ATX mRNA and protein expression in BA livers.

Hepatic ATX protein expression in BA subgroups

To determine whether hepatic ATX protein expression would be associated with poor outcomes in BA patients, we classified BA children according serum TB, liver enzymes, and clinical findings at 6 months post-Kasai into patients with poor outcome ($n = 11$) and patients with good outcome ($n = 9$). Table 2 demonstrates the clinical characteristics of the BA subgroups based on clinical outcome at 6 months post-operation. Subsequent analysis demonstrated the mean immunoreactive score of ATX protein expression in BA patients with poor outcome was significantly greater than in patients with good outcome ($P = 0.03$) (Fig. 3B). We further analyzed the correlation between hepatic ATX protein expression and markers of liver function in BA patients. The results showed no association between hepatic ATX protein expression and liver function parameters in BA infants at the time of KPE.

Increased hepatic ATX protein expression and liver fibrosis

The portal areas illustrated various degrees of fibrosis, but fibrous septa were generally broad with lobular extension (Fig. 4A). The liver fibrosis grade was F0 in 5 cases,

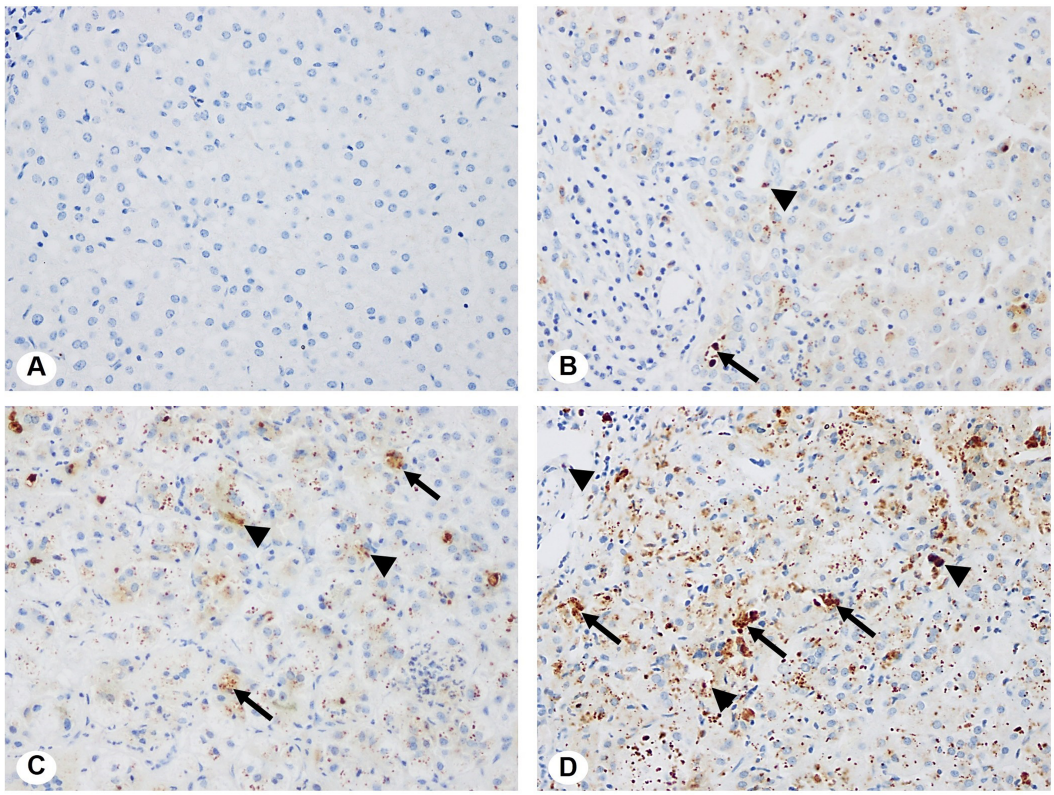

Figure 2 Immunohistochemical staining for ATX protein expression. Specific staining of ATX protein is represented by brown coloration. Expression of ATX in BA livers was observed mostly in the hepatic parenchyma (arrows) and biliary epithelium (arrowheads). ATX staining scores were defined, as follows. (A) 0 = no expression of ATX in a liver used as a control. (B) 1 = mild expression of ATX in BA liver. (C) 2 = moderate expression of ATX in BA liver. (D) 3 = strong expression of ATX in BA liver. (Original magnifications 400×).

Full-size DOI: 10.7717/peerj.5224/fig-2

Table 2 Demographic and clinical characteristics of BA patients based on clinical outcome at 6 months post-Kasai.

Characteristics	BA patients with poor outcome ($n = 11$)	BA patients with good outcome ($n = 9$)	*P*-value
Age at operation (days)	98.2 ± 11.3	77.0 ± 4.9	0.1
Gender (female:male)	6:5	6:3	0.4
Albumin (g/dL)	3.5 ± 0.2	4.1 ± 0.1	<0.05
Total bilirubin (mg/dL)	5.7 ± 1.7	0.4 ± 0.1	<0.001
ALT (IU/L)	140.2 ± 27.2	82.8 ± 16.2	0.01
Hepatic ATX expression (%)	61.4 ± 5.9	36.1 ± 9.4	0.03

Notes.

Data presented as mean ± SEM.

P-value < 0.05 indicates a statistically significant difference in clinical data between BA patients with poor outcome and good outcome at 6 months post-Kasai.

Abbreviations: BA, biliary atresia; ALT, alanine aminotransferase; ATX, autotaxin; SEM, standard error of the mean.

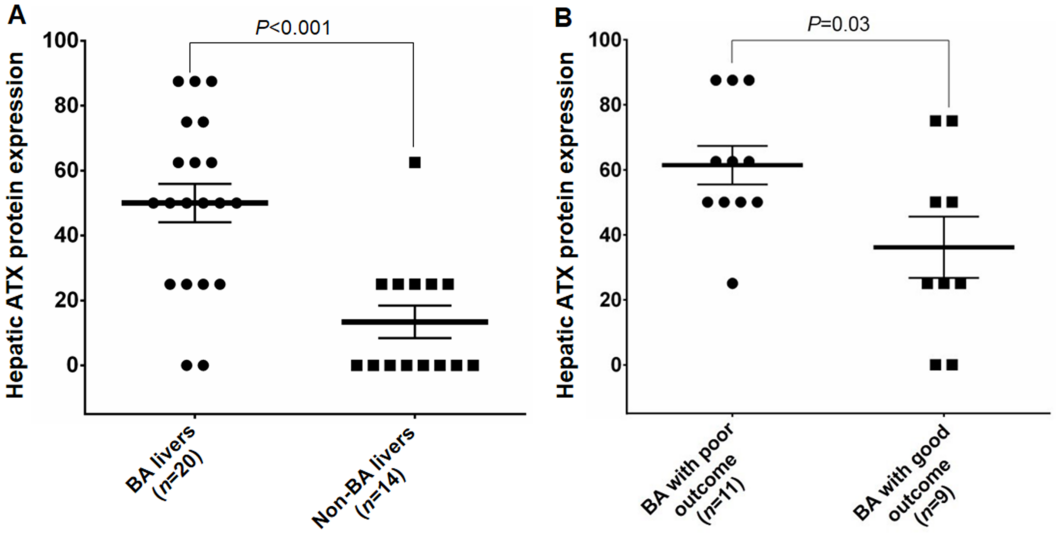

Figure 3 **Hepatic ATX protein expression in study subjects between different groups.** (A) Hepatic ATX expression in BA patients and non-BA patients. (B) Hepatic ATX expression in BA patients with poor outcome and good outcome.

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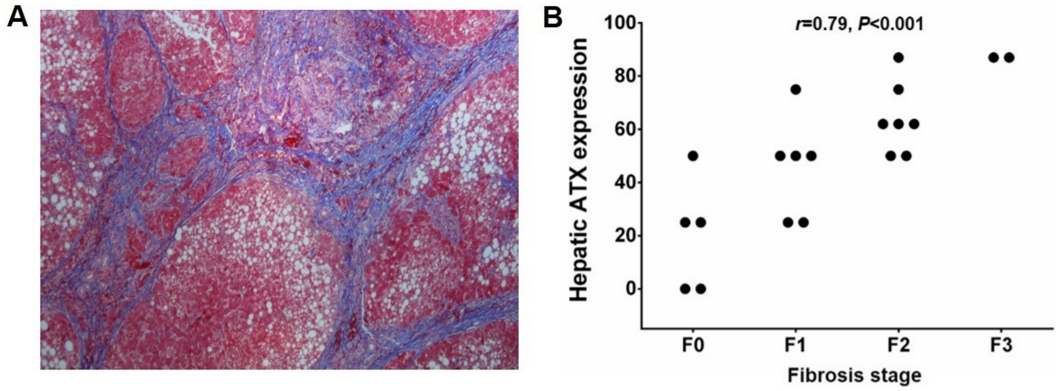

Figure 4 **Hepatic ATX expression and liver fibrosis.** (A) Histopathological analysis of liver samples in BA (Masson Trichrome staining, magnifications 100×). (B) Hepatic ATX protein expression correlated positively with Metavir fibrosis stage in BA ($r = 0.79$, $P < 0.001$).

Full-size DOI: 10.7717/peerj.5224/fig-4

F1 in 6 cases, F2 in 7 cases, and F3 in 2 cases. As shown in Fig. 4B hepatic ATX protein expression was positively correlated with Metavir fibrosis stage in BA; ($r = 0.79$, $P < 0.001$).

Survival curve analysis

We performed Kaplan–Meier analysis to investigate the overall survival curve of all 20 BA children. The 10-year survival rate with native livers of all BA children were estimated 80%, as shown in Fig. 5A. When stratified into low and high ATX protein expression using the cut-off value of 50%, the overall survival rates at 10 years were 91.7% for those

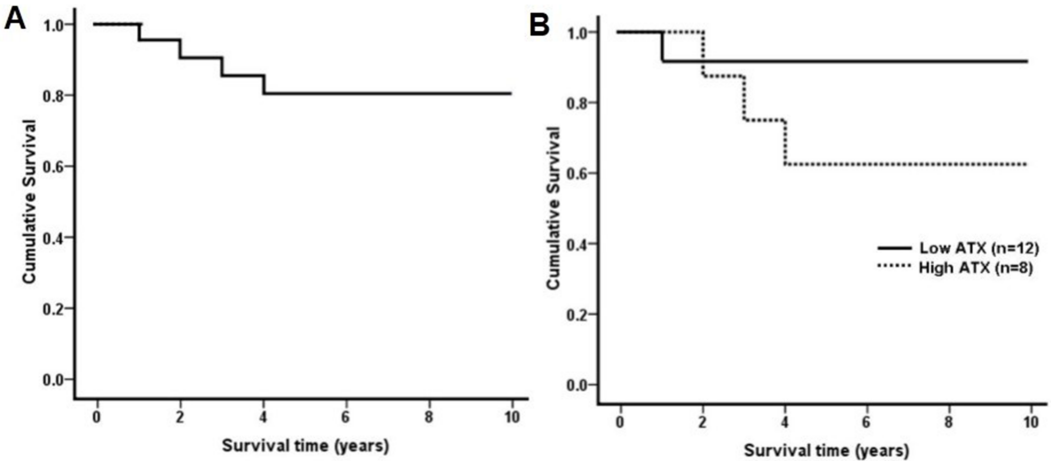

Figure 5 Kaplan–Meier survival curve of BA children after KPE over 10 years. (A) The overall survival curve of 20 BA reveals that 10-year survival rates with native livers are 80%. (B) Survival curve comparisons display that BA children with low ATX expression ($n = 12$) have 10-year survival greater than those with high ATX expression ($n = 8$) (log-rank, $X^2 = 2.17$, $P = 0.14$).

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with low expression and 62.5% for those with high expression. Survival rate was greater in BA patients with low ATX expression than those with high ATX expression (log-rank, $X^2 = 2.17$, $P = 0.14$) (Fig. 5B).

DISCUSSION

Despite extensive research efforts, the understanding of mechanisms that regulate biliary atresia (BA) progression following Kasai portoenterostomy (KPE) remains unclear. 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 unclear, several cytokines have been implicated in the regulation of hepatic fibrogenesis (*Kanzler et al., 1999*; *Williams et al., 2000*; *Farrington et al., 2010*; *Xiao et al., 2015*; *Iordanskaia et al., 2015*; *Klemann et al., 2016*). In a previous study, we reported association between elevation of circulating autotaxin (ATX) and poor outcomes in BA patients—especially severity of fibrosis (*Udomsinprasert et al., 2015*). This is important evidence that supports the hypothesis that ATX may serve a role as a potential biomarker of the prognosis in BA. In the present study, we investigated mRNA and protein expression of ATX in liver biopsies from BA infants compared with non-BA controls, and we found up-regulated *ATX* mRNA in BA patients. We then performed immunohistochemical analysis to determine protein expression of ATX and found an intense increase in ATX staining in BA infants, predominantly in the hepatic parenchyma and biliary epithelium at the time of KPE.

The biological outcome of ATX has been shown to induce a variety of inflammatory phenomena via LPA activity, and its role in disease pathophysiology has been verified in several diseases (*Umezu-Goto et al., 2002*), making ATX-derived LPA signaling an attractive therapy. Indeed, emerging evidence suggests that the liver is the main source of

ATX metabolism in both human and animal models (*Ikeda & Yatomi, 2012*). Additionally, ATX expression has been detected on all types of liver cells, including the biliary epithelium (*Kremer et al., 2010*). This suggests the possibility of ATX having a regulatory role in the liver. Our result demonstrated that *ATX* mRNA expression was up-regulated in the livers of BA infants when compared with non-BA liver controls. This finding is consistent with a previous investigation that reported overexpression of *ATX* in liver tissues of patients with hepatocellular carcinoma (HCC) (*Cooper et al., 2007*), suggesting that up-regulated *ATX* expression is associated with hepatic damage and liver fibrosis. In addition to up-regulation of *ATX* mRNA expression in the livers of BA infants, an increase in hepatic protein expression of ATX was also demonstrated. A recently published report also confirmed that overexpression of ATX protein was specifically associated with inflammation and cirrhosis in HCC patients (*Wu et al., 2010*). This was in agreement with our finding, implying that ATX may play a role in inflammation that is related to progressive BA. Furthermore, our immunohistochemistry data demonstrated positive cytoplasmic ATX expression in inflammatory cells and biliary epithelial cells. Indeed, hepatic ATX expression varied between different stages of cholestasis. This study also revealed that there was a positive correlation between hepatic ATX expression and the degree of fibrosis, suggesting the possibility of ATX as a predictive tool for discriminating between good and poor prognosis of clinical outcome in postoperative BA. Consistent with our finding, *Wunsch et al. (2016)* have demonstrated elevated ATX expression in chronic cholestatic diseases. In addition, a recent study revealed that high ATX expression was detected in hepatocellular carcinoma and was correlated with histological grade and survival rate (*Memet et al., 2017*). 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 reflect a defensive response by the body to fight against hepatic impairment, or may simply be a compensatory response to ATX, which leads to its compensatory up-regulation.

The potential significance of elevated ATX expression in BA remains unclear. The aberrant production of ATX may result in the altered activation of LPA signaling pathways via G-protein-coupled LPA-receptors, and may not be limited to activation of 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 and they may contribute to the prognosis of BA. For this reason, multiple factors with potentially fibrogenic activities in the liver have been evaluated due to their effects on HSC 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 via Rho/Rho kinase activation (*Ikeda et al., 2003*; *Yanase et al., 2003*). Although ATX may not play a primary role in the pathogenesis of liver fibrosis, it may accelerate fibrogenesis by stimulating the proliferation of HSCs in patients with liver fibrosis via its ability to produce LPA. This hypothesis has been supported by the recent observation that specific ATX transgenic overexpression and/or gene disruption from hepatocytes in mice models of chronic liver injury established a liver profibrotic role for ATX/LPA (*Kaffe et al., 2017*). A more recent study by *Bain et al. (2017)* found that a selective ATX inhibitor (PAT-505) markedly reduced liver fibrosis in mouse models. From those findings, we observe that hepatic ATX

expression was associated with an adverse clinical outcome in BA, which lends support to the hypothesis that inhibiting ATX as part of an antifibrotic model could serve as a novel therapeutic approach for treatment of hepatic fibrosis in BA patients. Taken together, the aforementioned findings suggest that the aberrant expression of ATX may be used as a promising biomarker for predicting the progression and prognosis of biliary atresia after Kasai portoenterostomy. Further experiments that isolate biliary epithelial cells and HSCs from BA livers will be required to determine the precise biological and pathological significance of the findings and observations presented in this report.

This study has some mentionable limitations. The most notable limitation is the fact that we were unable to obtain age-matched liver tissue from healthy infants due to ethical considerations. The limited availability of frozen liver biopsies from non-BA controls could have posed significant challenges to the study. Second, the sample size of our study population is relatively small. This is due, in large part, to the fact that BA is a relatively rare disorder. The limited number of subjects makes it challenging to show significant correlations of all parameters in BA patients. Future larger scale, multicenter studies should be conducted to verify our conclusions. Another caveat is the lack of data regarding the circulating ATX levels, total serum bile salt levels, and cholestatic pruritis. We recognize that these could be addressed by prospective longitudinal multicenter cohorts. Further research of costaining on BA liver specimens will identify the cellular fractions expressing ATX. Finally, 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 whether it is simply a compensatory response to the disease.

CONCLUSIONS

The current study presents evidence of the up-regulation of *ATX* mRNA expression in liver specimens of BA patients, as compared to specimens from livers of non-BA controls. ATX was expressed not only in the hepatic parenchyma, but also in biliary epithelial cells of BA infants at the time of KPE. These findings suggest that ATX expression could be related to liver fibrosis and outcome 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.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Wanvisa Udomsinprasert conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Paisarn Vejchapipat and Sittisak Honsawek conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Naruemon Klaikeaw conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Voranush Chongsrisawat conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Yong Poovorawan conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft, provide samples and clinical data.

Human Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

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 (IRB No. 549/57).

All supplemental information will be made available for download exactly as they were supplied. This link to the SI will only work when the article is published.

Data Availability

The following information was supplied regarding data availability:

The raw data/code is included in the manuscript in the supplements.

The raw data are provided in a Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.5224#supplemental-information.

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