Hepatology Research

Hepatology Research 2012 doi: 10.1111/hepr.12015

Short Communication

Correlation of connective tissue growth factor with liver stiffness measured by transient elastography in biliary atresia

Sittisak Honsawek,[1]* Wanvisa Udomsinprasert,[1] Chintana Chirathaworn,[2] Wilai Anomasiri,[1] Paisarn Vejchapipat[3] and Yong Poovorawan[4]

Departments of [1]Biochemistry, [2]Microbiology, [3]Surgery, and [4]Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok, Thailand

Aim: Biliary atresia (BA) is a neonatal liver disease defined as chronic progressive fibrotic obliteration of extrahepatic bile ducts. The objective of this study was to determine the association of serum connective tissue growth factor (CTGF) with clinical outcome and liver stiffness measurement.

Methods: Eighty-two BA patients post-Kasai operation and 28 healthy controls were recruited. BA patients were categorized into two groups based on their portal hypertension (PH) status. Serum CTGF levels were determined by enzyme-linked immunosorbent assay. Liver stiffness scores were measured by transient elastography.

Results: BA patients had greater CTGF levels (905.9 ± 57.7 vs 238.3 ± 23.5 pg/mL, $P < 0.001$) and higher liver stiffness values than controls (28.2 ± 2.6 vs 5.0 ± 0.5 kPa, $P < 0.001$). Serum CTGF levels were remarkably elevated in BA patients with PH compared to those without PH (1092.4 ± 73.9 vs 582.6 ± 45.7 pg/mL, $P < 0.001$). Furthermore, BA patients with PH had significantly higher liver stiffness values compared to those without PH (37.3 ± 3.0 vs 10.6 ± 1.1 kPa, $P < 0.001$). Additionally, serum CTGF was positively correlated with liver stiffness ($r = 0.875$, $P < 0.001$) and total bilirubin ($r = 0.462$, $P < 0.001$). There was an inverse correlation between serum CTGF and serum albumin ($r = –0.579$, $P < 0.001$).

Conclusion: High serum CTGF was associated with a poor outcome in BA patients. Accordingly, serum CTGF and transient elastography may serve as non-invasive biomarkers reflecting the disease severity in postoperative BA patients.

Key words: biliary atresia, connective tissue growth factor, jaundice, liver stiffness

INTRODUCTION

BILIARY ATRESIA (BA), a chronic inflammatory disorder of bile ducts in neonate, is characterized by progressive fibrosclerotic cholangiopathy resulting in partial or complete obstruction of bile flow. Despite successful Kasai portoenterostomy, a significant number of BA patients develop severe liver fibrosis, biliary cirrhosis, and die within a few years.[1] The progression of BA results in portal hypertension (PH) with severe complications including gastroesophageal varices, splenomegaly and progressive ascites.[2] Nonetheless, the exact pathophysiology of liver fibrosis or cirrhosis in BA patients remains to be solved.

Connective tissue growth factor (CTGF or CCN2) is a member of a family of growth factors termed the CCN (cystein-rich 61 [*Cyr61*], CTGF, nephroblastoma overexpressed [*Nov*]) gene family.[3] This family consists of six distinct members: *CYR61* (*CCN1*), *CTGF* (*CCN2*), *NOV* (*CCN3*), *WISP-1* (*wnt-1*-inducible gene, *CCN4*), *WISP-2* (*CCN5*) and *WISP-3* (*CCN6*).[4] Each member comprises four distinct structural modules as follows: insulin-like growth factor-binding protein module, von Willebrand factor type C repeat module, thrombospondin type 1 repeat module, and carboxy-terminal cystein-knot module.[5] Indeed, CTGF exerts diverse cellular functions including proliferation, differentiation, extracellular

Correspondence: Dr Sittisak Honsawek, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Bangkok 10330, Thailand. Email: sittisak.h@chula.ac.th
Received 19 April 2012; revision 23 October 2012; accepted 26 October 2012.

matrix synthesis and the migration of various cell types, and its overproduction is proposed to play a crucial role in pathways that result in fibrosis.[6]

In recent years, the expression of CTGF in BA liver significantly elevated in comparison with normal liver, and significant correlations were shown between CTGF mRNA and protein levels and the severity of liver fibrosis by *in situ* hybridization and immunohistochemical staining analyses.[7,8] Serum CTGF levels were significantly upregulated in BA patients and also were related to the progression of liver dysfunction.[9] The correlations of several cytokines and liver stiffness have been previously demonstrated in BA patients.[10,11] However, until recently, the study of association between serum CTGF and liver stiffness measured by transient elastography (TE) in BA has received little attention. Hence, the objective of this study was to analyze serum CTGF levels in postoperative BA patients and to investigate the possible association of serum CTGF, liver stiffness scores and clinical outcome in BA patients after Kasia procedure.

METHODS

THE PRESENT STUDY was approved by the Ethics Committee on Human Research of Faculty of Medicine, Chulalongkorn University, and was conducted in compliance with the 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 obtained from the parents prior to the children entering the study.

Study population

Eighty-two BA patients (44 girls and 38 boys; mean age, 9.0 ± 0.6 years) and 28 healthy children (14 girls and 14 boys; mean age, 9.6 ± 0.8 years) were recruited in the present study. All BA children had undergone hepatic portojejunostomy with Roux-en-Y (original Kasai operation). None of them had any symptoms and signs of suspected infection or ascending cholangitis at the time of blood sampling. None 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. Serum samples were taken during their routine follow up between October 2009 and July 2011. The duration of follow up after the Kasai operation was 8.5 ± 1.0 years. BA children were categorized into two groups based on their PH and splenomegaly status. PH was indicated by the presence of esophageal varices as diagnosed by endoscopy. A palpable spleen below the costal margin on physical examination (supine palpation) was considered splenomegaly. There were 52 BA patients with esophageal varices and 48 with splenomegaly.

Laboratory methods

Samples of peripheral venous blood were collected from every participant, and were stored at −70°C for subsequent analysis. Double-blind quantitative measurement of serum CTGF was performed with a Human CTGF ELISA Development Kit (Peprotech, Rocky Hill, NJ, USA), a quantitative sandwich enzyme immunoassay using a purified rabbit antibody against human CTGF precoated onto an enzyme-linked immunosorbent assay (ELISA) plate. Following four washes in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (Sigma Chemical, St Louis, MO, USA), the plate was blocked with 300 μL/well of 1% bovine serum albumin in PBS, for 1 h at room temperature. Serum samples were applied to the plate following blocking, alongside a standard curve, from 4000 pg/mL down in doubling dilutions, constructed from a stock recombinant human CTGF. Samples and standards were incubated (100 μL/well) at room temperature for 2 h following which the plate was washed a further three times with wash buffer. Detection of bound CTGF was performed using 100 μL/well of biotinylated detection antibody at a concentration of 0.5 μg/mL for 2 h at room temperature. After a further four washes, the plate was incubated with a 1:2000 dilution of avidin–horseradish peroxidase conjugate for 30 min at room temperature. Finally, the plate was washed four times and 100 μL of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) liquid substrate was added to the wells and color developed in proportion to the amounts of bound CTGF. Absorbance was detected by spectrophotometry, at 405 nm, with wavelength correction set at 650 nm. The CTGF concentration was examined by a standard optical density concentration curve (range, 31.5–4000 pg/mL). Serum hyaluronic acid (HA) level was analyzed using a competitive inhibition-based ELISA as described previously.[12] The liver function tests including serum albumin, total bilirubin (TB), direct bilirubin (DB), aspartate 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. The AST-to-platelets ratio index (APRI) was calculated as follows: (AST / upper limit of normal) × 100 / platelet count (10^9/L).[13]

Liver stiffness measurement

Transient elastography measured the liver stiffness 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 with patients lying in a dorsal decubitus position with maximum abduction of the right arm. A recent study reported that the upper limit of normal in TE increases significantly with age.[14] The S-probe (5 MHz, 5-mm diameter) was used for examination. The measurement depth is 15–40 mm with the S1-probe and 20–50 mm with the S2-probe. 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 SPSS software ver. 16.0 for Windows (SPSS, Chicago, IL, USA). Comparisons of demographic and clinical parameters between groups were performed using the χ^2-test and Student's unpaired *t*-test. Correlation between numerical data was obtained using Pearson correlation coefficient (*r*). Sensitivity, specificity, receiver–operator curve (ROC) and area under the receiver–operator curve (AUROC) were also determined. Data were expressed as mean ± standard error of the mean. $P < 0.05$ was considered statistically significant.

RESULTS

THERE WERE NO significant differences with respect to age or sex between the BA patients and normal children. However, serum CTGF levels were considerably higher in BA patients compared with healthy children (905.9 ± 57.7 vs 238.3 ± 23.5 pg/mL, $P < 0.001$). BA patients had significantly more pronounced serum hyaluronic acid levels than controls (50.1 ± 5.5 vs 24.0 ± 1.5 ng/mL, $P = 0.001$). In addition, liver stiffness scores in BA patients were substantially higher than those in controls (28.2 ± 2.6 vs 5.0 ± 0.5 kPa, $P < 0.001$).

We subsequently classified BA patients into two groups according to their PH status. As illustrated in Table 1, BA patients with PH had significantly higher TB, AST, ALT, ALP, APRI, hyaluronic acid and liver stiffness values compared to those without PH. Moreover, serum CTGF levels of patients with PH were remarkably higher than those of patients without PH (1092.4 ± 73.9 vs 582.6 ± 45.7 pg/mL, $P < 0.001$). ROC analysis showed a good discriminating power of serum CTGF for PH/esophageal varices in BA patients, with a cut-off value of 695.0 pg/mL and an AUROC of 0.853 (95% confidence interval, 0.768–0.937). Similarly, BA patients with splenomegaly displayed significantly

Table 1 Comparison between biliary atresia patients with and without portal hypertension

Variables	BA Patients with PH ($n = 52$)	BA Patients without PH ($n = 30$)	*P*-value
Age (years)	9.1 ± 0.8	8.9 ± 0.9	0.3
Sex (female : male)	28:24	16:14	0.4
Albumin (g/dL)	4.1 ± 0.1	4.4 ± 0.1	<0.001
Total bilirubin (mg/dL)	3.3 ± 0.6	0.4 ± 0.1	<0.001
Direct bilirubin (mg/dL)	2.8 ± 0.6	0.2 ± 0.0	0.001
AST (IU/L)	149.9 ± 11.0	44.0 ± 4.2	<0.001
ALT (IU/L)	142.9 ± 12.8	43.8 ± 6.2	<0.001
ALP (IU/L)	524.6 ± 25.1	214.7 ± 18.6	<0.001
Platelet count (10^3/mm^3)	143.5 ± 11.5	192.5 ± 15.6	0.01
APRI	3.6 ± 0.4	0.9 ± 0.1	<0.001
CTGF (pg/mL)	1092.4 ± 73.9	582.6 ± 45.7	<0.001
Hyaluronic acid (ng/mL)	49.0 ± 5.3	38.1 ± 5.4	<0.05
Liver stiffness (kPa)	37.3 ± 3.0	10.6 ± 1.1	<0.001

The data are expressed as mean ± standard error of the mean.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; APRI, AST-to-platelets ratio index; AST, aspartate aminotransferase; BA, biliary atresia; CTGF, connective tissue growth factor; PH, portal hypertension.

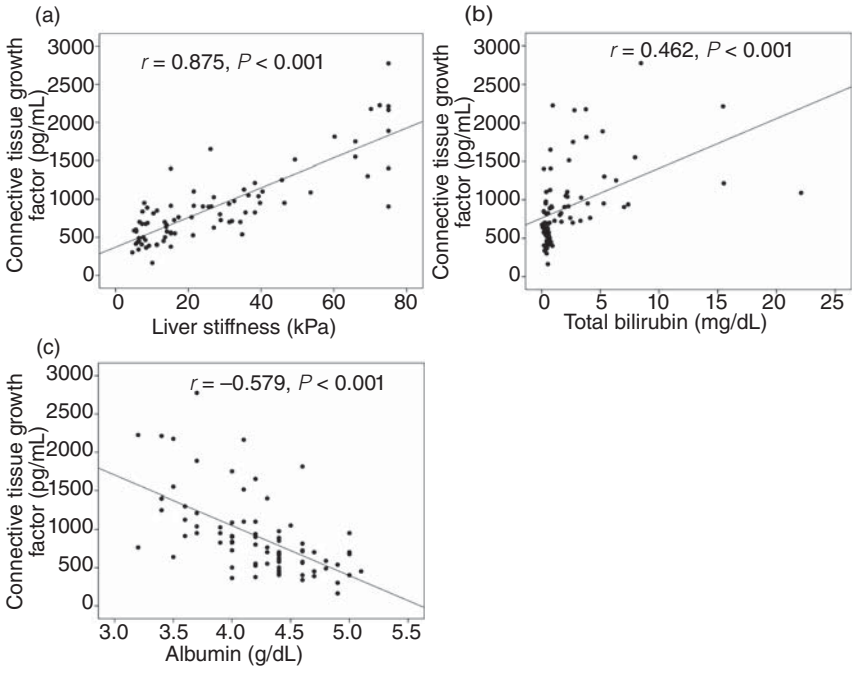

Figure 1 Correlation of connective tissue growth factor (CTGF) with liver stiffness, total bilirubin and albumin. (a) CTGF was positively correlated with liver stiffness ($r = 0.875$, $P < 0.001$) and (b) total bilirubin ($r = 0.462$, $P < 0.001$), (c) and negatively correlated with albumin ($r = -0.579$, $P < 0.001$).

higher serum CTGF (1130.2 ± 77.2 vs 589.2 ± 42.0 pg/mL, $P < 0.001$) and liver stiffness values (39.4 ± 3.1 vs 10.8 ± 1.0 kPa, $P < 0.001$) than those without splenomegaly.

Further analysis demonstrated that serum CTGF levels were positively correlated with liver stiffness measurements ($r = 0.875$, $P < 0.001$) and TB ($r = 0.462$, $P < 0.001$) (Fig. 1a,b). Conversely, there was an inverse correlation between serum CTGF and serum albumin levels ($r = -0.579$, $P < 0.001$) (Fig. 1c).

DISCUSSION

BILIARY ATRESIA REMAINS one of the most serious liver disorders in children. The etiopathogenic mechanism of BA has remained a mystery and therapeutic options are unsatisfactory. Despite early diagnosis and successful hepatoportoenterostomy, the great majority of BA patients eventually develop liver fibrosis and PH with severe complications such as gastroesophageal varices, splenomegaly and progressive ascites.[1,2] Therefore, the assessment of fibrogenic progression in BA is undoubtedly important.

Connective tissue growth factor has an overarching biologic significance as a regulator of multiple cellular functions such as cell adhesion, mitogenesis, chemotaxis, proliferation, differentiation, neovascularization, apoptosis and cell survival, not only in liver but also other organs. Published reports on serum CTGF from various clinical stages of BA have not received much attention. This study has been aimed to investigate the association of serum CTGF concentrations, liver stiffness scores and clinical parameters in BA patients after Kasai operation.

The present study revealed that serum CTGF levels in BA patients were substantially higher than those in healthy controls. Several possible mechanisms could be responsible for the significant elevation of CTGF in BA children. The increased CTGF may result from the decreased CTGF clearance, the enhanced CTGF synthesis or both. Moreover, extrahepatic organs can produce and secrete CTGF in systemic circulation. The precise mechanism from our observation needs additional investigation. In this study, we use TE or FibroScan as a diagnostic assessment of liver stiffness because it is a reproducible, rapid and non-invasive method for measuring the degree of liver fibrosis, which can be completed in the outpatient setting.[15] Further analysis showed that serum CTGF was positively correlated with liver stiffness measurement using TE and serum total bilirubin but negatively correlated with serum albumin.

It is notable that the more elevated serum CTGF was evident in BA patients with PH. PH is also a consequence of advanced hepatic fibrosis that obstructs sinu-

soidal blood flow. In this regard, we revealed that a serum CTGF level of more than 695.0 pg/mL had a sensitivity of 83% and a specificity of 80% to predict PH/esophageal varices in BA patients. These findings indicate that serum CTGF could be a possible indicator for ongoing deterioration of liver function and development of varices and, therefore, become a biochemical parameter for monitoring the severity of postoperative BA.

Previously, CTGF has been documented to play a critical part in a variety of liver diseases, including chronic hepatitis, liver fibrosis and hepatocellular carcinoma.[16–18] In line with our findings, El-Bassiouni *et al*. stated that CTGF was upregulated in liver specimens from patients with chronic hepatitis, liver cirrhosis and hepatocellular carcinoma.[18] Moreover, hepatic overexpression of CTGF has been illustrated in BA liver tissues.[7,8] In addition, Tamatani *et al*. reported that serum CTGF levels were significantly higher in BA patients compared with healthy controls.[9] Future clinical studies will yield more essential information on the pathophysiological mechanisms of CTGF in BA.

We recognize that CTGF has an overarching biologic function. Therefore, age-specific normal serum levels of CTGF and hyaluronic acid seem to be different and are a limitation of this study. The further development of reference values for serum CTGF and hyaluronic acid on a large number of healthy children of various age groups are needed to minimize this limitation.

In conclusion, the current study showed that serum CTGF was strongly correlated with liver stiffness and hepatic dysfunction in BA. Serum CTGF and transient elastography could serve as non-invasive biomarkers for determining the disease severity in the follow up of BA patients post-Kasai procedure. Prospective studies with a longitudinal design are required to determine the disease progression and define the exact role of CTGF in BA.

ACKNOWLEDGMENTS

THIS INVESTIGATION HAS been supported by the Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University, the Thailand Research Fund, the Commission on Higher Education, the National Research Council of Thailand, and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1155A). The authors gratefully acknowledge Dr Maneerat Chayanupatkul for kind and valuable assistance in data and sample collection. We are also indebted to Ms Thamolwan Mowong for additional data collection in this study.

REFERENCES

1 Hartley JL, Davenport M, Kelly DA. Biliary atresia. *Lancet* 2009; **374:** 1704–13.
2 Erlichman J, Hohlweg K, Haber BA. Biliary atresia: How medical complications and therapies impact outcome. *Expert Rev Gastroenterol Hepatol* 2009; **3:** 425–34.
3 Takigawa M, Nakanishi T, Kubota S, Nishida T. Role of CTGF/HCS24/ecogenin in skeletal growth control. *J Cell Physiol* 2003; **194:** 256–66.
4 Perbal B. CCN proteins: Multifunctional signalling regulators. *Lancet* 2004; **363:** 62–4.
5 Bork P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett* 1993; **327:** 125–30.
6 Moussad EE, Brigstock DR. Connective tissue growth factor: What's in a name? *Mol Genet Metab* 2000; **71:** 276–92.
7 Narkewicz MR, Kasaragod A, Lucia MS, Pflummer S, Sokol RJ, Stenmark KR. Connective tissue growth factor expression is increased in biliary epithelial cells in biliary atresia. *J Pediatr Surg* 2005; **40:** 1721–5.
8 Kobayashi H, Hayashi N, Hayashi K, Yamataka A, Lane GJ, Miyano T. Connective tissue growth factor and progressive fibrosis in biliary atresia. *Pediatr Surg Int* 2005; **21:** 12–6.
9 Tamatani T, Kobayashi H, Tezuka K *et al*. Establishment of the enzyme-linked immunosorbent assay for connective tissue growth factor (CTGF) and its detection in the sera of biliary atresia. *Biochem Biophys Res Commun* 1998; **251:** 748–52.
10 Honsawek S, Chayanupatkul M, Chongsrisawat V, Vejchapipat P, Poovorawan Y. Increased osteopontin and liver stiffness measurement by transient elastography in biliary atresia. *World J Gastroenterol* 2010; **16:** 5467–73.
11 Honsawek S, Chayanupatkul M, Chongsrisawat V *et al*. Serum adiponectin and transient elastography as non-invasive markers for postoperative biliary atresia. *BMC Gastroenterol* 2011; **11:** 1–7.
12 Kongtawelert P, Ghosh P. A method for the quantitation of hyaluronan (hyaluronic acid) in biological fluids using a labeled avidin-biotin technique. *Anal Biochem* 1990; **185:** 313–8.
13 Wai CT, Greenson JK, Fontana RJ *et al*. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology* 2003; **38:** 518–26.
14 Engelmann G, Gebhardt C, Wenning D *et al*. Feasibility study and control values of transient elastography in healthy children. *Eur J Pediatr* 2012; **171:** 353–60.
15 Yoshioka K, Kawabe N, Hashimoto S. Transient elastography: Applications and limitations. *Hepatol Res* 2008; **38:** 1063–8.

16 Zhang D, Wang NY, Yang CB *et al*. The clinical value of serum connective tissue growth factor in the assessment of liver fibrosis. *Dig Dis Sci* 2010; **55:** 767–74.
17 Guo-Qiu W, Nai-Feng L, Xiao-Bo V *et al*. The level of connective tissue growth factor in sera of patients with hepatitis B virus strongly correlates with stage of hepatic fibrosis. *Viral Immunol* 2010; **23:** 71–8.
18 El-Bassiouni NE, Nosseir MM, Madkour ME *et al*. Role of fibrogenic markers in chronic hepatitis C and associated hepatocellular carcinoma. *Mol Biol Rep* 2012; **39:** 6843–50.