



Clinical significance of connective tissue growth factor in hepatitis B virus-induced hepatic fibrosis

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Abstract

AIM: To determine the utility of connective tissue growth factor (CCN2/CTGF) for assessing hepatic fibrosis in hepatitis B virus (HBV)-induced chronic liver diseases (CLD-B).

METHODS: Enzyme-linked immunosorbent assay was used to measure CCN2 in sera from 107 patients with chronic hepatitis B (CHB) and 39 patients with HBV-induced active liver cirrhosis and 30 healthy individuals. Liver samples from 31 patients with CHB, 8 patients with HBV-induced liver cirrhosis and 8 HBV carriers with normal liver histology were examined for transforming growth factor β -1 (TGF- β 1) or CCN2 mRNA levels by *in situ* hybridization, and computer image analysis was performed to measure integrated optical density (IOD) of CCN2 mRNA-positive cells in liver tissues. Histological inflammation grading and fibrosis staging were evaluated by H and E staining and Van Gieson's method.

RESULTS: Serum CCN2 concentrations were, respectively, 4.0- or 4.9-fold higher in patients with CHB or active liver cirrhosis as compared to healthy individuals ($P < 0.01$). There was good consistency between the levels of CCN2 in sera and CCN2 mRNA expression in liver tissues ($r = 0.87$, $P < 0.01$). The levels of CCN2 in sera were increased with the enhancement of histological fibrosis staging in patients with CLD-B ($r = 0.85$, $P < 0.01$). Serum CCN2 was a reliable marker for the assessment of liver fibrosis, with areas under the receiver operating characteristic (ROC) curves (AUC) of 0.94 or 0.85 for, respectively, distinguishing normal liver controls from patients with F1 stage liver fibrosis or discriminating between mild and significant fibrosis.

CONCLUSION: Detection of serum CCN2 in patients with CLD-B may have clinical significance for assessment of severity of hepatic fibrosis.

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Key words: Connective tissue growth factor; Liver fibrosis; Chronic hepatitis B; Chronic liver disease; Chronic hepatitis C

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INTRODUCTION

Hepatic fibrosis, characterized by an excessive accumu-

lation of extracellular matrix (ECM) components, is a common feature of many chronic liver diseases (CLD-B) and can ultimately lead to liver cirrhosis^[1,2]. Hepatitis B virus (HBV) is a predominant cause of chronic liver disease and presents a high risk of fibrosis progression^[3]. While the pathobiology of HBV-induced hepatic fibrosis has not been fully clarified, HBV presents a huge medical challenge because one third of the world's population has been infected and 350 million people are carriers of the virus. Hepatitis B is endemic in China (> 8% prevalence) and has caused epidemics in other parts of Asia and Africa.

Over last two decades, hepatic stellate cells (HSCs) have dominated studies exploring mechanisms of hepatic fibrosis^[4]. In response to chronic liver injury, quiescent HSCs become activated myofibroblast-like cells that express α -smooth muscle actin (α -SMA) and produce components of the ECM, including fibrillar collagens^[1,2]. This process is driven by a variety of growth factors, cytokines and matricellular proteins. Connective tissue growth factor (CCN2, also known as CTGF) is a secreted matricellular protein that is recognized increasingly as a central player in hepatic fibrosis^[5]. Previously, we have shown that CCN2 production and secretion is enhanced by transforming growth factor- β 1 (TGF- β 1) in rat HSC and exposure of HSCs to CCN2 induces cell adhesion, migration, and proliferation^[6,7]. We and others have shown that CCN2 induces expression of α -SMA or type I collagen in HSCs consistent with a role in activation and fibrogenesis^[8,9]. In addition, CCN2 also stimulates survival pathways in activated HSCs thereby prolonging their fibrogenic potential^[10]. In human or experimental liver fibrosis, CCN2 expression is higher than in normal liver, with strong correlation between hepatic CCN2 production and the degree of liver fibrosis^[11].

Given the central role of CCN2 in hepatic fibrosis, we investigated the levels of serum CCN2 of patients with HBV-induced CLD-B, and determined the potential clinical value of hepatic or serum CCN2 levels in diagnosing the severity of HBV-induced hepatic fibrosis.

MATERIALS AND METHODS

Ethics

This work was approved by First Hospital of Jilin University and was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Informed consent was obtained from all patients prior to sample collection.

Clinical data

Serum was collected from 146 patients with CLD-B who were either outpatients or inpatients at First Hospital (Jilin University, Changchun, China) from June 2008 to September 2011. Of the 146 patients, there were 107 cases with chronic hepatitis B (CHB) (69 men and 38 women; mean age: 43.9 years, range: 18-76 years), 39 cases with active liver cirrhosis (27 men and 12 women; mean age:

41.9 years, range: 26-68 years). As determined using the Child-Pugh score to assess the severity of liver cirrhosis, 25 cases were class A and 14 cases were class B.

Serum was collected from 30 healthy individuals (18 men and 12 women; mean age: 33.6 years, range: 19-56 years) for controls. Serum and liver samples were collected from 8 HBV carriers who had histological normal livers (6 men and 2 women; mean age: 28.6 years, range: 19-38 years), and were studied as normal controls for the assessment of liver fibrosis of CLD-B patients. Patients and HBV carriers meet the diagnostic criteria for chronic HBV infection^[12].

Liver tissue samples from 39 CLD-B patients and 8 HBV carriers were obtained using a percutaneous needle. The length of each sample was more than 1.5 cm. There were 31 cases with CHB (23 men and 8 women; mean age: 29.8 years, range: 18-50 years) and 8 cases with HBV-induced active liver cirrhosis (6 men and 2 women; mean age: 36.6 years, range: 26-54 years). Importantly, initial studies showed that levels of serum CTGF, Collagen I, Collagen III or aminotransferases in CLD-B patients receiving a liver biopsy were not significantly different as compared to CLD-B patients without a biopsy.

CCN2 enzyme linked immunosorbent assay

Sera were stored at -70 °C for 1 to 6 mo before analysis. The level of CCN2 in sera from 146 patients with CLD-B, 8 HBV carriers and 30 healthy individuals were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to manufacturer's protocols (USCN Life Science and Technology Co, TX, United States). Briefly, microtiter wells were pre-coated for 2 h at 37 °C, with 100 μ L of each standard or 1:20 dilutions of sera. The plates were then developed by sequential addition of biotinylated anti-CCN2 antibody, avidin-conjugated horseradish peroxidase and tetramethylbenzidine substrate solution, and the color reaction was measured at 450 nm.

Grading and staging of liver biopsies

Liver tissue samples from 31 CHB patients, 8 patients with HBV-induced liver cirrhosis and 8 HBV carriers were individually fixed, paraffin-embedded and subjected to H and E staining or Van Gieson's method to determine histological inflammation and fibrosis which were scored using the Metavir system. Fibrosis was staged on a 4-point scale (F0: No fibrosis; F1: Minimal fibrosis; F2: Fibrosis with a few septa; F3: Numerous bridging fibrosis without cirrhosis; F4: Cirrhosis or advanced severe fibrosis). F1-F2 was defined as mild fibrosis and F3-F4 as significant fibrosis. Inflammation was graded on a four-point scale from A0, which indicated no inflammatory activity, up to A3, which indicated severe activity.

In situ hybridization

In situ hybridization (ISH) was performed using digoxigenin-labeled sense or anti-sense probes for CCN2 or TGF- β 1 (Boster Biotechnology Co. Ltd. Wuhan, China). In

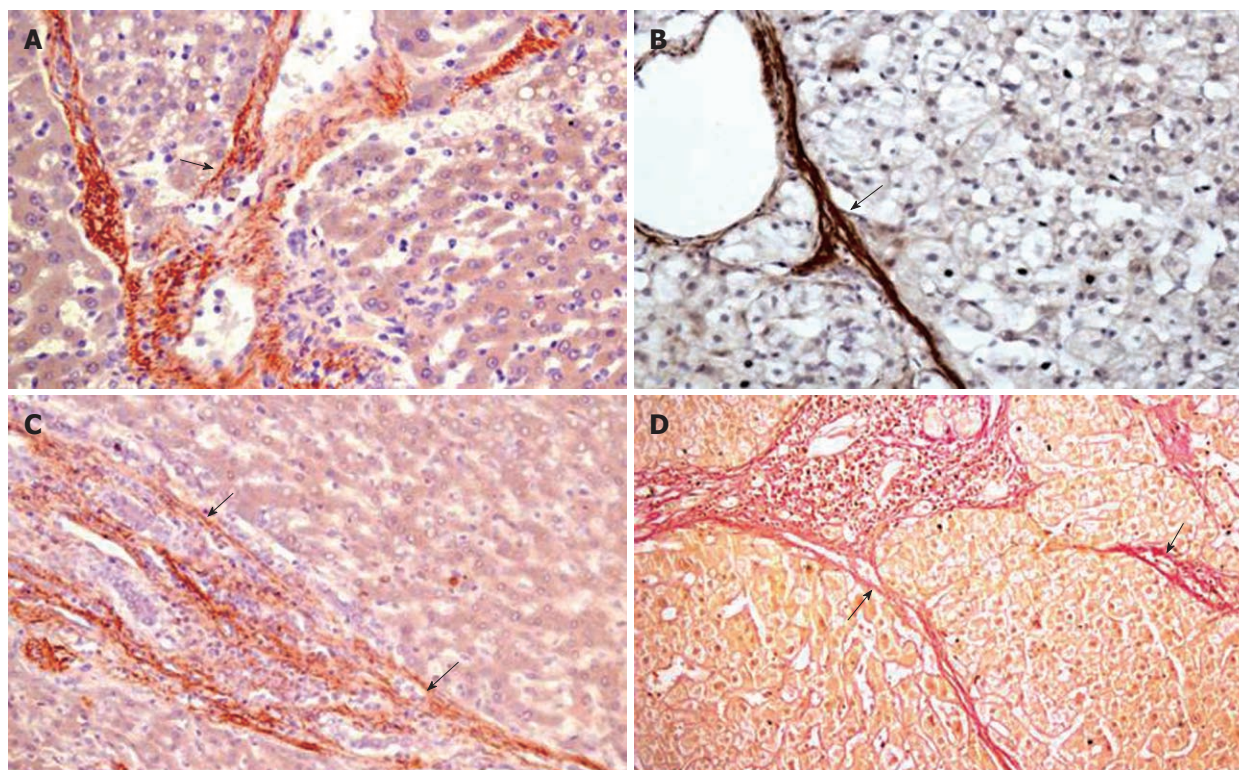


Figure 1 Production of connective tissue growth factor or transforming growth factor β -1 in fibrous septa of hepatitis B virus-infected livers. Connective tissue growth factor mRNA (A) or protein (B) were detected by *in situ* hybridization (ISH) or immunohistochemistry respectively, transforming growth factor β -1 mRNA (C) was detected by ISH while collagen bundles (D) were stained red using Van Gieson's method. Original magnification, $\times 200$ in A, B, C and D. Examples of positively stained cells or structures in each panel are arrowed.

brief, liver tissue samples from CLD-B patients were formaldehyde-fixed and paraffin-embedded. The tissue sections ($5\ \mu\text{m}$) were deparaffinized, rehydrated with PBS, digested with pepsin ($30\ \mu\text{g}/\text{mL}$) for 10 min at $37\ ^\circ\text{C}$, fixed in 4% paraformaldehyde in PBS and washed in $3\times\text{SSC}$. The samples were pre-hybridized at $40\ ^\circ\text{C}$ for 2 h, and hybridization was performed overnight at $40\ ^\circ\text{C}$ with sense or anti-sense probes. After hybridization, excess probes were removed by sequential washing in twice concentrated ($2\times$) saline-solution citrate buffer (SSC), $0.5\times\text{SSC}$ and then $0.2\times\text{SSC}$ at $37\ ^\circ\text{C}$ for 2 h. The tissue sections were incubated at $37\ ^\circ\text{C}$ for 1 h with biotinylated mouse anti-digoxigenin, followed by addition of the streptavidin-biotin-peroxidase complex for 20 min. The slides were then developed with 3-amino-9-ethylcarbazole (Boster Biotechnology). Ten random images (original magnification $\times 400$) of each slide underwent computer image analysis using Image-Pro Plus 6.0 software to assess the integrated optical density (IOD) of CCN2-positive cells in liver tissues.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections ($5\ \mu\text{m}$) were de-waxed and re-hydrated. Sections were incubated overnight at $4\ ^\circ\text{C}$ with mouse anti-human α -SMA monoclonal antibody (Zhongshan Goldbridge Biotechnology, Beijing, China) or rabbit anti-human CCN2 polyclonal antibody (Santa Cruz, Heidelberg, Germany) or rabbit anti-human

F4/80 polyclonal antibody (Spring Bioscience, United States). Sections were washed in PBS and incubated at room temperature for 10 min with biotinylated *goat anti-mouse* and *rabbit IgG* (Maixin Bio, Fuzhou, China). After washing with PBS, sections were incubated with streptavidin-peroxidase (Maixin Bio, Fuzhou, China) for 10 min and then developed with diaminobenzidine or 3-amino-9-ethylcarbazole.

Statistical analysis

The values reported represent the median [95% confidence interval (CI)] of the measurements. Statistical analysis of the data was performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, United States). The nonparametric Wilcoxon signed ranks test was used for pair-wise comparison of groups and Spearman's rank correlation analysis was used to determine the relationship between two variables. Areas under the receiver operating characteristic (ROC) curves (AUC) were calculated for comparing the accuracy of the CCN2 in sera in different subgroups.

RESULTS

Localization of CCN2 mRNA or protein in HBV-induced chronic liver disease

In normal livers, only mild CCN2 mRNA staining was detected in portal tracts and there was no staining in the

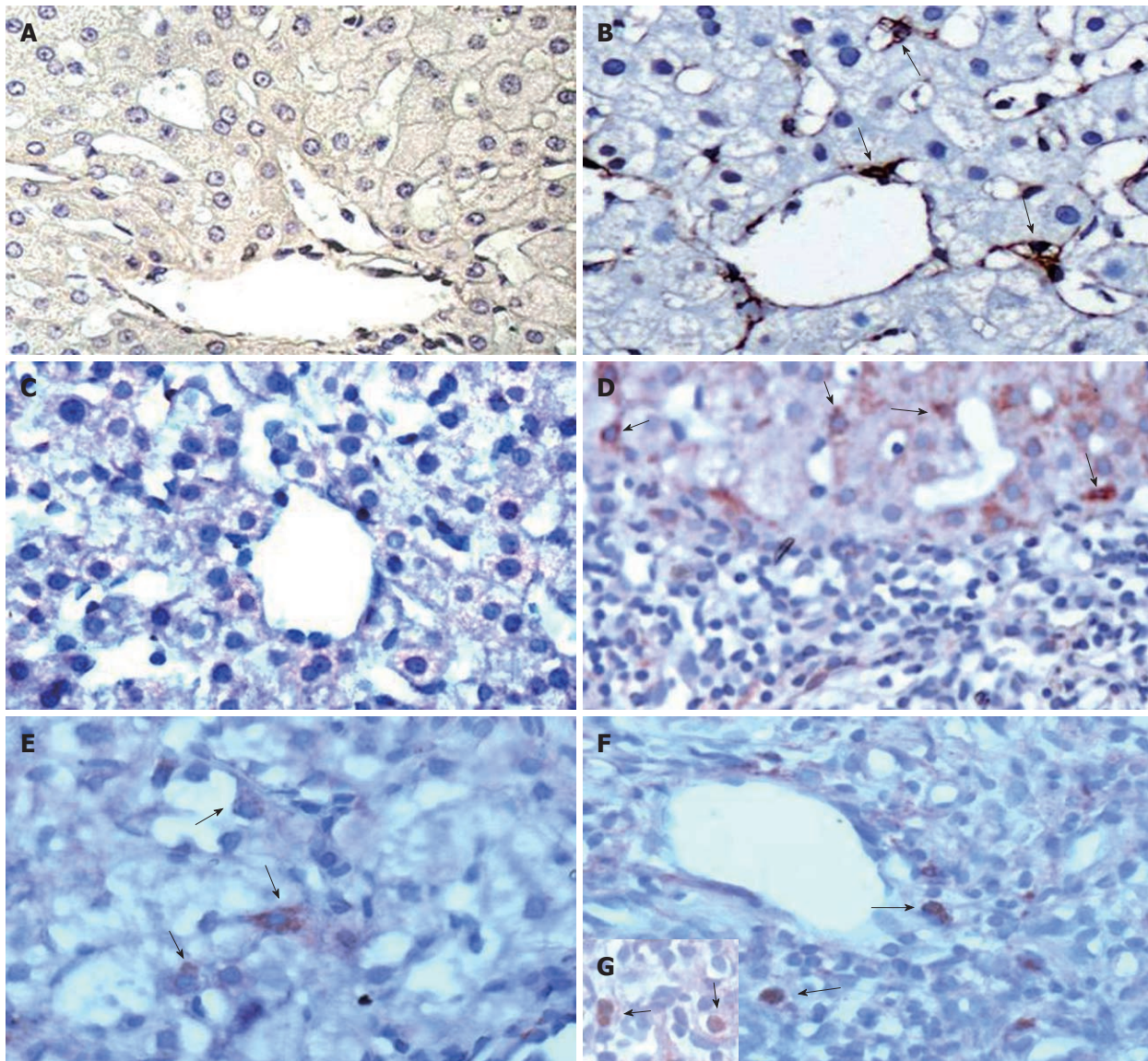


Figure 2 Cellular localization of key fibrotic markers in hepatitis B virus-induced liver fibrosis. α -SMA-positive hepatic stellate cells (HSCs) were not detectable in hepatitis B virus carriers who had normal liver histology (A) but were present in chronic hepatitis B (CHB) liver (B). In CHB liver samples, there was no staining when the *in situ* hybridization probes were omitted (C) but their inclusion demonstrated the presence of either connective tissue growth factor mRNA in activated HSCs (D) or transforming growth factor β -1 mRNA in activated HSCs (E) or Kupffer cells (F). F4/80 antigen-positive Kupffer cells (G). Original magnification, $\times 400$ in A-G. Examples of positively stained cells in each panel are arrowed.

central vein or lobule (data not shown). However, CCN2 mRNA and protein were localized to fibrotic septa in CLD-B patients with hepatic fibrosis (Figure 1A and B) or cirrhosis (data not shown), and the pattern of CCN2 staining was well correlated with the distribution of collagen fibers (Figure 1D). By comparison to normal livers, CHB patients demonstrated strong α -SMA-positive staining in presumptive activated HSC (Figure 2A and B) and these cells also stained strongly for CCN2 mRNA (Figure 2C and D).

Since CCN2 acts downstream of TGF- β 1 to drive fibrosis^[13], we examined the expression and distribution of TGF- β 1 mRNA in the liver of CLD-B patients. TGF- β 1 mRNA was detected in the fibrotic septa of patients with hepatic fibrosis (Figure 1C) or cirrhosis (data not

shown) and was localized to activated HSCs in the lobule (Figure 2E) and Kupffer cells (Figure 2F and G) within inflammatory area in the livers of CHB patients.

Serum levels of CCN2 in HBV-induced chronic liver diseases

Since CCN2 is synthesized with a signal peptide and can exist extracellularly in soluble or matrix-associated forms, we examined serum from CLD-B patients for the presence of CCN2 protein by ELISA. As shown in Table 1, serum CCN2 concentrations were, respectively, 4.0- or 4.9-fold higher in patients with CHB or active liver cirrhosis as compared to healthy individuals ($P < 0.01$). There was no difference in the serum CCN2 levels between the HBV carriers and healthy individuals.

Table 1 Serum connective tissue growth factor concentrations in patients with chronic liver diseases ($\mu\text{g/L}$)

Group	<i>n</i>	Median (95% CI)
Healthy control	30	2.2 (1.6-2.8)
HBV carrier	8	2.2 (1.5-2.9)
Chronic hepatitis B	107	8.8 (6.0-12.3)
Active liver cirrhosis	39	10.9 (7.0-14.6)

HBV: Hepatitis B virus.

Table 2 Relationship between fibrosis stage and hepatic or serum connective tissue growth factor content

Fibrosis stage	<i>n</i>	Serum CCN2 ($\mu\text{g/L}$) median (95% CI)	Hepatic CCN2 mRNA (IOD) median (95% CI)
Normal control	8	2.2 (1.5-2.9)	6.0 (3.9-8.8)
F1	11	6.8 (5.0-8.9)	19.4 (12.3-26.4)
F2	9	8.9 (7.1-10.7)	25.6 (13.9-34.8)
F3	11	9.4 (7.3-12.0)	31.9 (19.7-44.6)
F4	8	10.1 (8.2-12.1)	39.6 (25.5-52.8)

Normal Control samples were from hepatitis B virus carriers and had normal liver histology; F1-F2: Mild fibrosis; F3-F4: Significant fibrosis. CCN2: Connective tissue growth factor; IOD: integrated optimal density.

CCN2 production as a function of severity of fibrosis or inflammation

Having shown that hepatic and serum CCN2 concentrations were higher in CLD-B patients than in healthy individuals, we next investigated if there was a correlation between CCN2 and fibrosis stage. As shown in Table 2, serum concentrations and hepatic content of CCN2 increased in proportion to the severity of fibrosis; Spearman's rank correlation analysis showed that correlation coefficients were 0.85 and 0.89 (both $P < 0.01$), respectively. However, the levels of CCN2 in sera were not correlated with the degree of inflammation in CHB patients.

Diagnostic performance of serum CCN2

We further analyzed the diagnostic performance of serum CCN2 for assessing liver fibrosis using the ROC curves. Calculation of the areas under ROC curves (AUC) showed that serum CCN2 could be used to distinguish either normal liver controls from patients with F1 stage liver fibrosis (AUC = 0.94) or mild fibrosis (F1/F2) from significant fibrosis (F3/F4) (AUC = 0.85) (Figure 3).

DISCUSSION

Chronic HBV infection can cause hepatic fibrosis and eventually cirrhosis. Over the last few years, HBV infection has been studied extensively *in vitro* with the finding that expression of the HBV X protein (HBx) in hepatocytes results in paracrine activation and proliferation of human or rat HSC resulting in their increased expression of collagen I, CCN2, α -SMA, matrix metalloproteinase-2, or TGF- β ^[14,15]. Although hepatocytes serve as a suitable host for HBV and permit viral replication and

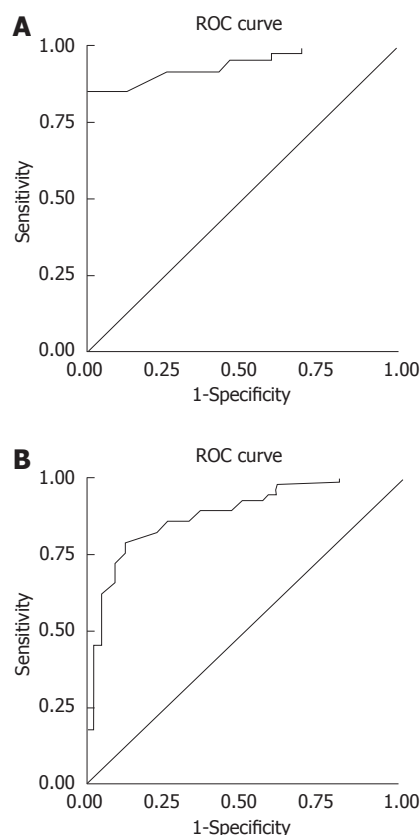


Figure 3 Receiver operating characteristic curve of connective tissue growth factor. Receiver operating characteristic (ROC) curves of connective tissue growth factor distinguishing normal liver controls from patients with F1 stage liver fibrosis (A) or discriminating between mild and significant fibrosis (B) with areas under the ROC curves of 0.94 or 0.85, respectively.

antigen production, HBV can also transiently infect and replicate in human HSCs which directly increases the production of collagen type in the cells^[3,16]. Collectively, these findings have shown that activation of fibrogenic pathways in HSC following HBV infection of either hepatocytes or HSC is a key event in HBV-mediated hepatic fibrosis. In this regard, CCN2 has emerged as a potential key fibrogenic mediator in response to HBV in as much as CCN2 supports HSC activation, promotes HSC proliferation and survival, and acts downstream of TGF- β to drive HSC collagen production^[6,7,10,13,17]. In this study, we showed that CCN2 mRNA and protein were expressed at high levels by myofibroblasts (including presumptive activated HSC) in fibrotic septa in CLD-B patients with hepatic fibrosis or cirrhosis, and that increased levels of hepatic or circulating CCN2 were associated with severe fibrosis. This result is consistent with previous observations by others^[11].

Activation of Kupffer cells, the resident macrophage population of the liver, serves as a central determinant of the liver's response to injury and repair, and the resulting inflammatory reaction is an important prerequisite for HSC activation and progression to hepatic fibrosis^[18,19]. Macrophage-derived TGF- β 1 has been identified as a potential paracrine stimulator of HSC activation^[20] and addition of TGF- β antibodies to Kupffer cell conditioned medium inhibits its ability to induce expression of

CCN2, collagen I and TIMP-1 when added to cultured HSC^[21]. In the present study, both TGF- β 1 and CCN2 mRNA were detected in presumptive activated HSCs, while TGF- β 1 mRNA alone was detected in Kupffer cells within inflamed areas of livers from CHB patients. These data support the notion that TGF- β 1 upregulates CCN2 production in HSCs *via* paracrine and autocrine pathways, and further enhance the effects of CCN2 during fibrogenesis. This is supported by *in vitro* studies showing that CCN2 is a downstream mediator of TGF- β 1-induced collagen I production in human HSCs^[16].

CCN2 is a secreted protein that has been detected in several human body fluids including serum, cerebrospinal fluid, follicular fluid, uterine fluid, or urine^[22]. This has led to examination of the potential utility of evaluating CCN2 concentrations in highly accessible fluids such as serum or plasma for non-invasive diagnostic evaluation of the extent or severity of various fibrotic pathologies. Thus, serum levels of CCN2 have been shown to be correlated with the extent of systemic skin sclerosis and severity of pulmonary fibrosis in human subjects^[23] and to serve as a biomarker of progressive kidney fibrosis in chronic allograft nephropathy in a clinical and experimental study^[24]. Studies on circulating CCN2 levels in assessment of hepatic fibrosis have just begun to gain momentum and are founded on the well documented over-expression of CCN2 in fibrotic livers due to its production by multiple cell types (including HSC, hepatocytes, biliary epithelial cells). An early study reported as association between elevated CCN2 serum levels and progression of hepatic fibrosis in biliary atresia^[25] while a more recent investigation demonstrated significantly elevated serum levels of CCN2 in patients with chronic hepatitis and cirrhosis that were well correlated with the progression of hepatic fibrosis^[26-28]. In the present research, we found that increased CCN2 concentrations were present in the serum of patients with CHB and HBV-induced cirrhosis. Serum CCN2 levels were consistent with those in liver tissue and were strongly correlated with the stage of hepatic fibrosis. Taken together, our data indicate that CCN2 is a potential valuable biomarker of HBV-induced hepatic fibrosis, and further support the classification of CCN2 as class I fibrosis biomarker, defined as one that is derived from changes of the fibrogenic cell types and which reflects the activity of the fibrogenic and/or fibrolytic process^[29].

Finding the best method to evaluate and diagnose the stage of liver fibrosis continues to be a challenge^[30]. Although liver biopsy is a gold-standard procedure for determining the grade of liver inflammation and stage of fibrosis^[30-33], there are well recognized difficulties including complications, high hospital expenses^[30,34], false sample recording^[35], contra-indications during the procedure, and dependence on the pathologists' skills in examining samples. Serum fibrosis tests with AUCs ranging from 0.85 to 0.90 have been proposed as good biochemical markers with high diagnostic value^[36,37]. In our research, serum CCN2 was valuable not only in distinguishing normal liver controls from patients with F1 stage liver fibro-

sis but also in distinguishing between mild and significant liver fibrosis. We therefore propose that further studies are warranted to further evaluate the potential utility of serum CCN2 as a biomarker of liver fibrosis in HBV-induced CLD-B.

COMMENTS

Background

Millions of individuals around the world are infected with hepatitis B virus (HBV), resulting in chronic liver disease. In many cases, affected individuals suffer from hepatic fibrosis, a highly debilitating pathology in which the normal cellular architecture and function in the liver are severely compromised through the deposition of collagen and other insoluble extracellular matrix molecules. This process is driven by connective tissue growth factor (CCN2) which is known to be produced at high levels in fibrotic livers and which acts to drive fibrogenic pathways in hepatic stellate cells (HSCs), a principal fibrotic cell type in the liver.

Research frontiers

Currently, parameters used to assess liver fibrosis are inaccurate. There is optimism that measurement of CCN2 levels in either the livers or serum of affected patients will have useful diagnostic or prognostic value.

Innovations and breakthroughs

To date, there have been a limited number of studies regarding the value of serum CCN2 for assessment of hepatic fibrosis. In this study, the authors employed more systemic detection techniques to evaluate the relationship among serum CCN2 levels, hepatic CCN2 content and liver fibrosis severity in patients with chronic liver diseases. Furthermore, the authors described the expression characteristics of CCN2 in liver tissues and its role and mechanism in HBV-induced hepatic fibrosis.

Applications

These studies suggest that serum CCN2 concentrations are a reliable diagnostic indicator of HBV-induced liver fibrosis and that CCN2 can be used a part of the platform for evaluation of the severity of liver fibrosis.

Terminology

CCN2: a pro-fibrogenic molecule that is over-expressed in many fibrotic diseases and which stimulates collagen synthesis in HSC.

Peer review

This is an interesting and important issue in the utility of CCN2 for assessing hepatic fibrosis. Correlations of the serum levels of CCN2 in HBV infected patients with hepatic fibrosis have been well documented in literature.

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