Connective tissue growth factor is overexpressed in human hepatocellular carcinoma and promotes cell invasion and growth

Ming Xiu, Ya-Hui Liu, David R Brigstock, Fang-Hui He, Rui-Juan Zhang, Run-Ping Gao

AIM: To determine the expression characteristics of connective tissue growth factor (CTGF/CCN2) in human hepatocellular carcinoma (HCC) in histology and to elucidate the roles of CCN2 on hepatoma cell cycle progression and metastasis in vitro.

METHODS: Liver samples from 36 patients (who underwent hepatic resection for the first HCC between 2006 and 2011) and 6 normal individuals were examined for transforming growth factor β1 (TGF-β1) or CCN2 mRNA by in situ hybridization. Computer image analysis was performed to measure integrated optimal density of CCN2 mRNA-positive cells in carcinoma foci and the surrounding stroma. Fibroblast-specific protein-1 (FSP-1) and E-cadherin were examined to evaluate the process of epithelial to mesenchymal transition, α-smooth muscle actin and FSP-1 were detected to identify hepatic stellate cells, and CD34 was measured to evaluate the extent of vascularization in liver tissues by immunohistochemical staining. CCN2 was assessed for its stimulation of HepG2 cell migration and invasion using commercial kits while flow cytometry was used to determine CCN2 effects on HepG2 cell-cycle.

RESULTS: In situ hybridization analysis showed that TGF-β1 mRNA was mainly detected in connective tissues and vasculature around carcinoma foci. In comparison to normal controls, CCN2 mRNA was enhanced 1.9-fold in carcinoma foci (12.36 ± 6.08 vs 6.42 ± 2.35) or 9.4-fold in the surrounding stroma (60.27 ± 28.71 vs 6.42 ± 2.35), with concomitant expression of CCN2 and TGF-β1 mRNA in those areas. Epithelial-mesenchymal transition phenotype related with CCN2 was detected in 12/36 (33.3%) of HCC liver samples at the edges between carcinoma foci and vasculature. Incubation of HepG2 cells with CCN2 (100 ng/mL) resulted in more of the cells transitioning into S phase (23.85 ± 2.35 vs 10.94 ± 0.23), and induced a significant migratory (4.0-fold) and invasive (5.7-fold) effect. TGF-β1-induced cell invasion was abrogated by a neutralizing CCN2 antibody showing that CCN2 is a downstream mediator of TGF-β1-induced hepatoma cell invasion.

CONCLUSION: These data support a role for CCN2 in the growth and metastasis of HCC and highlight CCN2 as a potential novel therapeutic target.

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Key words: Connective tissue growth factor; Hepatocellular carcinoma; Hepatoma cell line; Migration; Invasion

Peer reviewer: Hikaru Nagahara MD, PhD, Professor, Aoyama Hospital, Tokyo Women’s Medical University, 2-7-13 Kita-Aoyama, Minatoku, Tokyo 107-0061, Japan

Xiu M, Liu YH, Brigstock DR, He FH, Zhang RJ, Gao RP. Connective tissue growth factor is overexpressed in human hepatocellular carcinoma and promotes cell invasion and growth. World J Gastroenterol 2012; 18(47): 7070-7078 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i47/7070.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i47.7070

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and the third most common cause of cancer death[1]. Although HCC resection plays an important role in improving prognosis of HCC, short survival has been observed in these patients due to high rate of recurrence and metastasis after surgery[6,9]. The mechanisms regulating tumor growth and metastasis of HCC are very complicated and still not fully understood.

HCC consists of a tumor parenchyma comprised of hepatoma cells and a surrounding microenvironment composed of various cell types such as hepatic stellate cells (HSCs), fibroblasts or inflammatory cells that produce a variety of tumor stroma components, including extracellular matrix (ECM) proteins, growth factors and cytokines[4,5]. In normal liver, HSCs are typically located in the perisinusoidal space of disse which lies between endothelial cells of sinusoids and hepatocytes. Following liver injury, HSCs become activated, undergo a phenotypic transformation into fibroblast-like cells or myofibroblasts, and migrate to sites of injury where they produce a provisional ECM to support parenchymal repopulation. In settings of chronic injury, this process proceeds unabated, leading to deposition of excessive amounts of ECM proteins and fibrillar collagens, resulting in fibrosis and scarring. This fibrotic environment supports the growth of hepatoma cells and HCC progression is dependent on an intricate cross-talk between the tumor and its surrounding environment[6-8]. Recent literature has highlighted a fundamental role of the tumor microenvironment in modulating the process of liver fibrosis, hepatocarcinogenesis, epithelial-mesenchymal transition (EMT), tumor invasion and metastasis[9,10]. A growing body of evidence from animal models and in vitro studies suggests that growth factors and matricellular proteins as well as resident HSCs play a key role in this stromal-tumor interaction)[5,10-12].

Connective tissue growth factor (CTGF/CCN2) is a cysteine-rich matricellular protein that has been implicated in regulating diverse processes in vivo, including angiogenesis, embryogenesis, chondrogenesis, fibrogenesis, and tumorigenesis[13]. CCN2 is transcriptionally activated by transforming growth factor β1 (TGF-β1) through a mechanism that involves activation of Smad binding elements and a unique TGF-β response element in the CCN2 promoter. Enhanced expression of CCN2 by TGF-β1 has been demonstrated in many cell types including HSCs and hepatoma cells[14,15]. Numerous studies have demonstrated that CCN2 is a downstream mediator of TGF-β1-induced ECM production in HSCs, pancreatic stellate cells and osteoblasts[16,17]. Recently, down-regulation of CCN2 by either the TGF-β1 inhibitor LY2109761 or CCN2 siRNA appeared to inhibit the growth of HCC in culture or xenograft models[18].

In this study, we have characterized CCN2 expression in human HCC, its association with EMT, and its effects on hepatoma cell migration, invasion or cell cycle progression.

MATERIALS AND METHODS

Clinical data

Thirty-six HCC specimens were obtained by surgical resection from May 2006 to June 2011. Samples obtained from the peripheral areas of the tumors were selected for this study. The patients with HCC consisted of 26 men and 10 women, mean age 56.4 years (range: 27-71 years). According to Pittsburgh modified TNM criteria, the patients were classified as small hepatocellular carcinoma (n = 20), solitary large hepatocellular carcinoma (n = 10) and nodular hepatocellular carcinoma (n = 6). Six normal liver specimens were obtained from patients undergoing hepatic resection due to a single cavernous hemangioma and the normal liver tissues were taken at the greatest distance from the location of the angio cavernoma. The tissue samples used as controls were histologically analyzed and clearly shown to have no inflammation or fibrosis. All specimens were examined under a light microscope after haematoxylin and eosin (HE) staining or Masson’s trichome staining.

In situ hybridization

ISH was performed using digoxigenin-labeled sense or anti-sense probes for CCN2 or TGF-β1 (Boster Biotechnology Co. Ltd., Wuhan, China). In brief, liver samples from HCC patients were formaldehyde-fixed and paraffin-embedded. The tissue sections (5 μm) were deparaffinized, rehydrated with phosphate buffered solution (PBS), digested with pepsin (30 μg/mL) for 10 min at 37 °C, fixed in 4% paraformaldehyde in PBS and washed in 3 × SSC. The samples were pre-hybridized at 42 °C for 2 h, and hybridization was performed overnight at 42 °C with sense or anti-sense probes. After hybridization, unbound probe was removed by washing in 2 × SSC, 0.5 × SSC and then 0.2 × SSC at 37 °C for 2 h. The tissue sections were incubated at 37 °C for 1 h with biotinylated mouse anti-digoxigenin, followed by addition of streptavidin-biotin-peroxidase complex for
20 min. The slides were then developed with 3-amino-9-ethylcarbazole (Boster Biotechnology). Ten random images (original magnification × 400) of each slide underwent computer image analysis using Image-Pro Plus 6.0 software to assess the integrated optimal density (IOD) of TGF-β1 or CCN2-positive cells in liver tissues.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded sections (5 μm) were de-waxed and re-hydrated. Sections were incubated overnight at 4 ℃ with rabbit anti-human E-cadherin polyclonal antibody (Boster Biotechnology Co. Ltd., Wuhan, China), mouse anti-human cluster of differentiation 34 monoclonal antibody (Maixin Bio, Fuzhou, China), rabbit anti-human CCN2 polyclonal antibody (Santa Cruz, Heidelberg, Germany) or rabbit anti-human fibroblast-specific protein-1 (FSP-1) polyclonal antibody (Millipore Corporation, Billerica, MA, United States). Sections were washed in PBS and incubated at room temperature for 10 min with biotinylated goat anti-mouse and anti-rabbit IgG (Maixin Bio, Fuzhou, China). After washing with PBS, sections were incubated with streptavidin-peroxidase (Maixin Bio, Fuzhou) for 10 min and then developed with diaminobenzidine or 3-amino-9-ethylcarbazole.

**Flow cytometry**

Two milliliter of 1 × 10⁷/mL HepG2 cell suspension were placed to each well of 6-well tissue culture plates in 12% FBS-DMEM and incubated for 12 h. The cells were then cultured in 0.5% FBS-DMEM for 24 h. The cell culture medium was exchanged with 0.5% FBS-DMEM and the cells were incubated for another 24 h in the absence or presence of 100 ng/mL CCN2 (Bioventor, Modrice, Czech Republic) or 20 ng/mL TGF-β1 (Peprotech, Rehovot, Israel). The cells were then trypsinized with 0.25% trypsin and washed in PBS. HepG2 cell-cycle progression was determined by resuspending the cells at 1 × 10⁶ cells/mL in PBS, fixing the cells with 75% ethanol overnight, and then staining the cells with 0.1 mg/mL propidium iodide in a 0.1% sodium citrate/0.1% Triton X-100 solution in the presence of 0.2 mg/mL Rnase for 30 min at room temperature in the dark. Analysis of cellular DNA content after cell staining with propidium iodide was performed by flow cytometry at an excitation wavelength of 488 nm. The distribution of cells in three major phases of the cycle (G0/G1, S, G2/M) was analyzed using CellQuest software (BD Biosciences, San Jose, CA, United States).

**Migration assay**

HepG2 migration studies were performed using a 12-well companion plate into which inserts were placed that had an uncoated membrane with 5-μm pores (Millipore, Billerica, MA). HepG2 cells, detached from stock dishes with 1 mmol/L EDTA, were suspended in DMEM and placed at 3 × 10⁶ cells per insert for 6 h in the absence or presence of CCN2 (100 ng/mL) or TGF-β1 (20 ng/mL) in the lower chamber of the well. Cells that adhered to the membrane were fixed with 100% methanol and stained with May-Grunwald's Giemsa. The numbers of HepG2 cells on the upper or lower surfaces were individually counted in 10 randomly selected microscopic fields at a magnification of 400 ×. The rate of migration of HepG2 cells was expressed as a migration index (% defined as follows: (number of cells on the undersurface of membrane/total number of cells on both surface of the membrane) × 100.

**Invasion assay**

A HepG2 cell invasion assay was performed in a 24-well plate with ECMatrixTM cell culture inserts (Chemicon, United States). The inserts contained an ECMatix-coated polycarbonate membrane with 8 μm pores which was rehydrated with 300 μL DMEM for 2-h at room temperature. 300 μL HepG2 cells in DMEM (5 × 10⁵ cells/mL) were added to each insert while 500 μL DMEM containing CCN2 (100 ng/mL) or TGF-β1 (20 ng/mL) were added to the lower chamber. After 24 h, adherent cells were fixed with 100% methanol and stained with staining solution. The numbers of non-invaded cells on the upper surface and those of invaded cells on the under surface were counted in 10 randomly selected microscopic fields at a magnification of 400 ×. The cell invasion index (%) was defined as follows: (number of cells on the undersurface of membrane/total number of cells on both surface of the membrane) × 100.

**Ethical approval**

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.

**Statistical analysis**

Statistical analysis of the data was performed using SPSS 13.0 for Windows (SPSS Inc, Chicago, IL, United States). The values reported represent the mean ± SD of the measurements. Differences were analyzed statistically with paired sample Student’s t-test and P < 0.05 was considered significant.

**RESULTS**

**Localization of CCN2 mRNA in HCC**

At the light microscopic level, all HCC samples tested in this study had multiple foci of carcinoma by HE or Masson’s trichrome staining. The carcinoma foci were separated or wrapped by their surrounding stroma. Collagen bundles were present in the stroma (Figure 1A) while CD34, a specific marker of vascular endothelial cells, was detected in the vasculature surrounding the stroma and in a few small blood vessels within tumor foci (Fig-
Computer image analysis of CCN2 mRNA expression in comparison to normal controls revealed a 9.4-fold increase in stromal expression (60.27 ± 28.71 vs 6.42 ± 2.35, \(P < 0.01\)) and a 1.9-fold increase in expression in carcinoma foci (12.36 ± 6.08 vs 6.42 ± 2.35, \(P < 0.01\), Table 1).

**Relationship between CCN2 expression and EMT**

Having shown the expression and localization of CCN2 mRNA, we next investigated the relationship between CCN2 and the occurrence of EMT, the latter of which was assessed by staining for either (1) E-cadherin which is expressed in normal epithelial cells but is absent or only weakly expressed in high-metastatic cancer cells[8] or (2) FSP-1 which is a marker of fibroblasts and epithelial

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**Figure 1** Expression and distribution characteristics of transforming growth factor \(\beta\)1 or connective tissue growth factor mRNA in human hepatocellular carcinoma. A: Masson’s trichrome stain for collagen (blue) in hepatocellular carcinoma (HCC); B: Immunohistochemical detection of CD34 in vascular endothelial cells (brown) in HCC; C: Normal liver showing connective tissue growth factor (CCN2) mRNA expressed in connective tissue around the veins; D: Absence of staining of HCC when the in situ hybridization probes were omitted; E: HCC stained for transforming growth factor \(\beta\)1 mRNA, showing reactivity in connective tissue and around the carcinoma foci; F: Overexpression of CCN2 mRNA in connective tissue and vascular endothelial cells (black arrow) in HCC. Original magnification, × 100 in A and E, × 200 in B, C, D and F. CF: Carcinoma foci; CT: Connective tissue.
cancer cells with EMT features. CCN2 mRNA (Figure 2A) or protein (Figure 2B) were detected in hepatoma cells at the edge of carcinoma foci by ISH or immunohistochemistry respectively. E-cadherin was present in areas that were adjacent to the carcinoma foci but it was only very weakly detectable in hepatoma cells within the carcinoma foci (Figure 2C). At the edges between carcinoma foci and the vasculature, hepatoma cells were FSP-1-positive in 33.3% (12/36) of HCC sections (Figure 2D). As CCN2 has been proposed as a key regulatory cytokine in tumor-stroma crosstalk in an animal model of HCC[7], this aspect was investigated in our clinical specimens. As expected, CCN2 mRNA expression occurred in (1) fibroblast-like or myofibroblast-like HSC marked by expression of FSP-1 or α-smooth muscle actin (α-SMA) (Figure 2D-F); (2) hepatoma cells (Figure 2G) located along the border between tumor foci and vasculature; Since CCN2 mRNA was detected in hepatoma cells that were FSP-1-positive (Figure 2D and H), this finding supports a role for CCN2 in autocrine or paracrine regulation of EMT in human hepatoma cells.

**Effect of CCN2 on hepatoma cell cycle distribution**

Cell cycle analysis was assessed by flow cytometry of propidium iodide-stained HepG2 cells after treatment of the cells with 100 ng/mL CCN2 for 24 h. As shown in Table 2, approximately 53% of HepG cells were in G0/G1, 11% were in the S, and 34% were in G2/M after serum starvation of the cells in medium containing 0.5% FCS. After CCN2 stimulation, a relatively lower proportion of cells (41%) were in the G0/G1 phase (41.01 ± 4.45 vs 53.56 ± 2.51, P < 0.05), and a higher proportion (23.8%) were in S phase (23.85 ± 2.35 vs 10.94 ± 0.23, P < 0.05), as compared to control cells. However, TGF-β1 stimulation did not significantly alter the distribution of phases of the cell cycle as compared to control cells.

**CCN2 induces hepatoma cell migration and invasion**

To determine effects of CCN2 on HepG2 cell migration, we used a chemotaxis assay in which the migration of HepG2 cells from the upper chamber of a culture insert was assessed following addition of CCN2 to the lower chamber. As shown in Figure 3A, CCN2 induced HepG2 cell migration across the polyethylene membrane in the culture insert. Cell migration was also promoted by TGF-β1.

Since CCN2 expression was associated with EMT in hepatoma cells (see above), we further examined whether CCN2 played a role in HepG2 cell invasion through ECM using an ECMMatrix culture insert. As shown in Figure 3B, HepG2 invasion across the ECM layer was stimulated by either CCN2 or TGF-β1, the latter of which was blocked by anti-CCN2 antibody but not normal IgG. These data indicate that HepG2 cell invasion is stimulated by CCN2 directly or by TGF-β1-mediated CCN2 production.

**DISCUSSION**

In this study, we determined the expression and distribution of CCN2 in HCC, its relationship to HCC-associated EMT, and the role of CCN2 in stimulating hepatoma migration, invasion, or cell cycle progression in *vivo*. Our findings can be summarized as follows: (1) In comparison to normal controls, CCN2 mRNA expression was enhanced, respectively, 9.4- or 1.9-fold in tumor stroma or carcinoma foci; (2) A concomitant expression of CCN2 and TGF-β1 mRNA was found in these areas; (3) CCN2 expression in the stroma, hepatoma cells or HSCs was correlated with expression of markers of EMT; (4) CCN2 promoted HepG2 cell migration, invasion or cell cycle progression; and (5) CCN2 neutralizing antibody blocked TGF-β1-induced HepG2 cell invasion.

The initiation, growth and progression of HCC are dependent on an intricate crosstalk between tumor and stroma[7]. Components of the microenvironment that surround hepatoma cells, including ECM protein and growth factors as well as its constituent non-tumor cells, are critical for HCC growth and metastasis[8,9]. It has previously been demonstrated that TGF-β1 plays an important role in HCC by stimulating fibrogenic remodeling of the liver and contributing to tumor progression[20]. Produced downstream of TGF-β1, CCN2 participates in a variety of pathophysiological processes, including formation of fibrous scar, angiogenesis, tumor growth[21-23]. Expression of CCN2 is related to recurrence, metastasis and poor prognosis in human HCC and pancreatic cancer[24-26]. Increasing evidence supports a role for CCN2 in mediating the matrigenic actions of TGF-β1 in numerous cell types, especially those that have specialized role in ECM production such as HSC, pancreatic stellate cells and osteoblasts[14-17]. In experimental HCC models,
LY2109761, an inhibitor of TGF-β1, was shown to inhibit neo-angiogenesis, tumor growth and progression by down-regulation of CCN2 production\(^6,7\). Our results show that human HCC is characterized by enhanced CCN2 expression in tumor stroma and tumor cells, as well as its co-expression with TGF-β1. Collectively these data suggest that CCN2 is an important component of the tumor-stroma axis and likely plays an important role in the development and progression of HCC.

EMT is a cellular program characterized by loss of cell adhesion, repression of E-cadherin expression, rearrangement of the cellular cytoskeleton, upregulation of matrix remodeling factors and increased cell mobility\(^27\). EMT is essential for numerous developmental processes including embryo implantation, embryogenesis, organ development and fibrosis\(^28-30\). Over past decade, the EMT process has been increasingly recognized to occur during the progression of various carcinomas such as HCC or pancreatic adenocarcinoma\(^31,32\). Recent studies have shown that TGF-β1 or hepatocyte growth factor induces EMT in HCC cell lines or in animal models\(^32,33-34\). CCN2 has been demonstrated to promote human renal tubular epithelial cells to mesenchymal transition characterized by down-regulation of E-cadherin and up-regulation of α-SMA\(^35\). In this study we showed that hepatoma cells with EMT characteristics (downregulation of E-cadherin and upregulation of FSP-1) were located at the tumor border and were positive for CCN2,
which was also detected in tumor- or stroma- associated HSCs. Taken together, these results support a role for HSC- or HCC-derived CCN2 in the process of EMT.

Tumor cell invasion and metastasis are complex and multistage processes that occur after cancer cells have undergone genotypic and phenotypic alterations resulting in the acquisition of a matrix-degrading and migratory phenotype[36]. Tumor cell migration is necessary at the initiation of the metastatic cascade, at which time the tumor cells leave the primary site and migrate in response to, and toward, specific external chemotactic factors such as growth factors and ECM proteins, growth factors and cytokines. A growing body of literature has highlighted a fundamental role of the tumor microenvironment in modulating the process of hepatocarcinogenesis, epithelial-mesenchymal transition, tumor invasion and metastasis. Connective tissue growth factor (CTGF/CCN2) is a downstream mediator of transforming growth factor β1 (TGF-β1)-induced ECM production in HSCs and hepatoma cells. CCN2 has been previously implicated in cancer metastasis and invasion in various tumors. However, the role of CCN2 in human HCC remains largely unknown.

**Research frontiers**

To date, there have been a limited number of HCC studies using in vitro cell culture, animal models or human clinical specimens to address the roles of CCN2 in regulation of tumor growth and metastasis. In this study, the authors determined the expression characteristics of CCN2 in human HCC by histology and elucidated the roles of CCN2 on hepatoma cell migration, invasion and cell cycle progression. Furthermore, the authors described the relationship between the overexpression of CCN2 in human HCC and its association with epithelial-mesenchymal transition (EMT).

**Applications**

These studies suggest that CCN2 plays important roles in tumor growth and metastasis in human HCC and highlight CCN2 as a potential novel therapeutic target.

**Peer review**

This paper provided some aspects on understanding EMT of HCC and mecha-
nisms on HCC migration and invasion. The result is interesting and suggest that a role for CCN2 in the growth and metastasis of HCC and highlight CCN2 as a potential novel therapeutic target.

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