Int J Clin Exp Pathol 2017;10(2):1860-1865
www.ijcep.com /ISSN:1936-2625/IJCEP0044619

Original Article

Correlation between the stromal activated hepatic stellate cells and angiogenesis in human hepatocellular carcinoma

Yi Lu*, Jianliang Xu*, Boxuan Zhou, Nan Lin, Ruiyun Xu, Yunbiao Ling

*Department of Hepatobiliary Surgery, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong Province, China. *Equal contributors.*

Received November 19, 2016; Accepted November 28, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Angiogenesis is important during the progression of hepatocellular carcinoma (HCC). In murine models, activated hepatic stellate cells (HSCs) are reported to be capable of promoting tumor angiogenesis. The effect of activated HSCs on angiogenesis in human HCC is not clear. Here, we elucidated the correlation between activated HSCs and angiogenesis in human HCC. We analyzed the activated HSCs in HCC samples and the correlation with patients' clinicopathologic features and tumor angiogenesis using immunohistochemical methods. We found that activated HSCs are mainly located in the blood sinus of HCC. The density of activated HSCs and microvessels was higher when the HCC was poorly differentiated or developed from liver cirrhosis. There was a positive correlation between the density of activated HSCs and microvessel density in HCC. In conclusion, Activated HSCs existed in human HCC stroma, the density of which was positively correlated with tumor microvessel density, indicating that activated HSCs participate in promoting angiogenesis in HCC.

Keywords: Activated hepatic stellate cells, hepatocellular carcinoma, angiogenesis

Introduction

Hepatocellular carcinoma (HCC) is a common and fatal malignant cancer with high mortality. There is high rate of recurrence and metastasis in patients with HCC even after radical surgery [1, 2]. HCC is rich in blood supply and angiogenesis is critical for the tumor growth and invasion [3, 4]. The current treatments for blocking angiogenesis in HCC, such as Sorafenib, are not satisfactorily effective [5, 6]; it is important to discover new targets for suppressing the angiogenesis in HCC.

Activated hepatic stellate cells (HSCs) are recognized to be critical during the development of liver cirrhosis [7]. Recent studies also report that activated HSCs exist in hepatic tumors and contribute to tumor initiation and progression [8-10]. During the development of hepatic portal hypertension, activated HSCs are responsible for remodeling the liver blood sinus to increase the blood resistance [11]. In hepatic metastases and murine xenograft models of HCC, activated HSCs are recognized to play a role in promoting tumor angiogenesis [10, 12, 13]. In human HCC, the effect of activated HSCs on angiogenesis is not well understood.

Based on these observations, we speculated that there might be a correlation between activated HSCs and angiogenesis in human HCC. In the present study, we analyzed the activated HSCs in human HCC samples and the correlation with patients' clinicopathologic features and tumor angiogenesis. Our results revealed that activated HSCs might promote angiogenesis in HCC.

Materials and methods

Hepatocellular carcinoma samples

After institutional review board approval, HCC samples were collected from 30 patients with HCC, who had received a hepatectomy in Sun Yat-sen University from January to May in 2015. All patients recruited were (1) pathologically proved HCC and (2) not receiving adjuvant treatments before surgery, such as TACE, chemo-

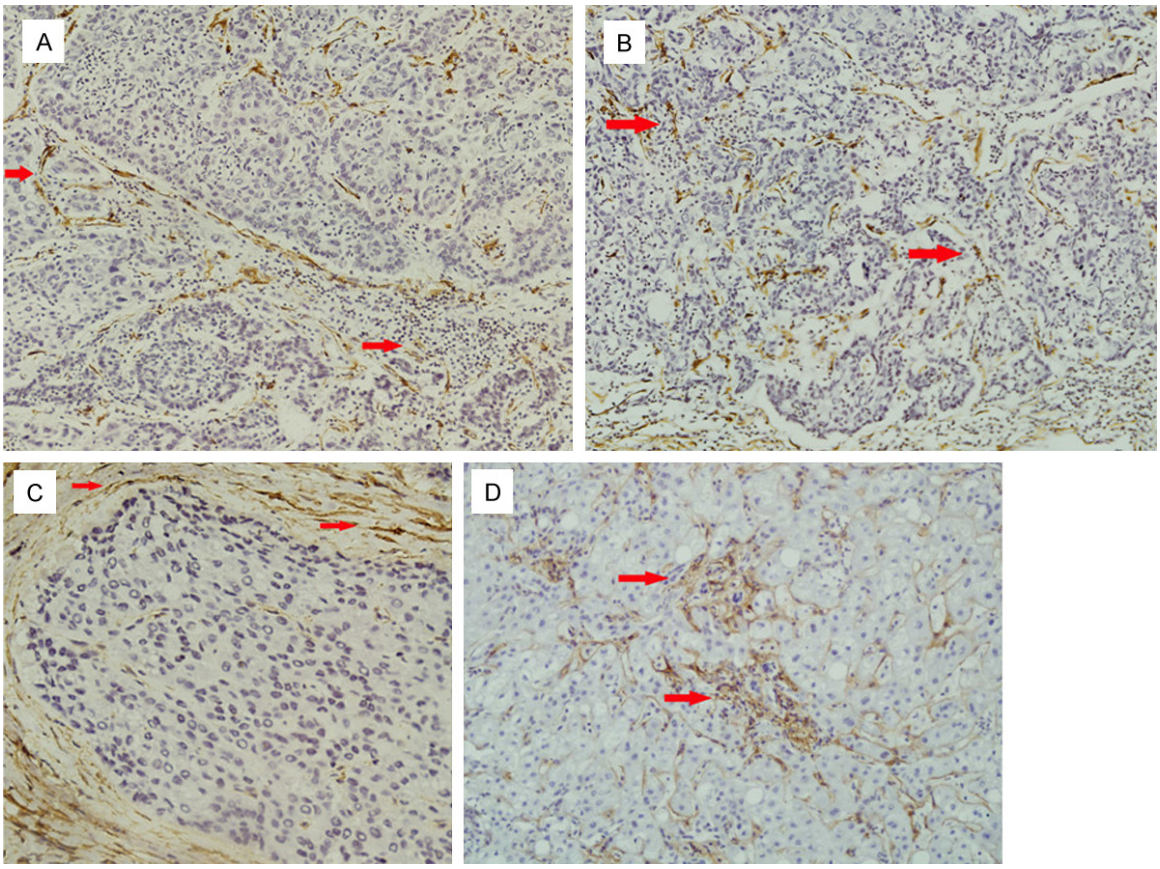

Figure 1. Representative images for immunohistochemical evaluation of activated HSCs in HCC tumor sections (Original Magnification A: 10× and B: 40×) and peritumoral tissue sections (D: Original Magnification 10×). For activated HSCs, areas of fibrous septa and capsules are not examined (C: Original Magnification 40×). Arrows indicate representative cells.

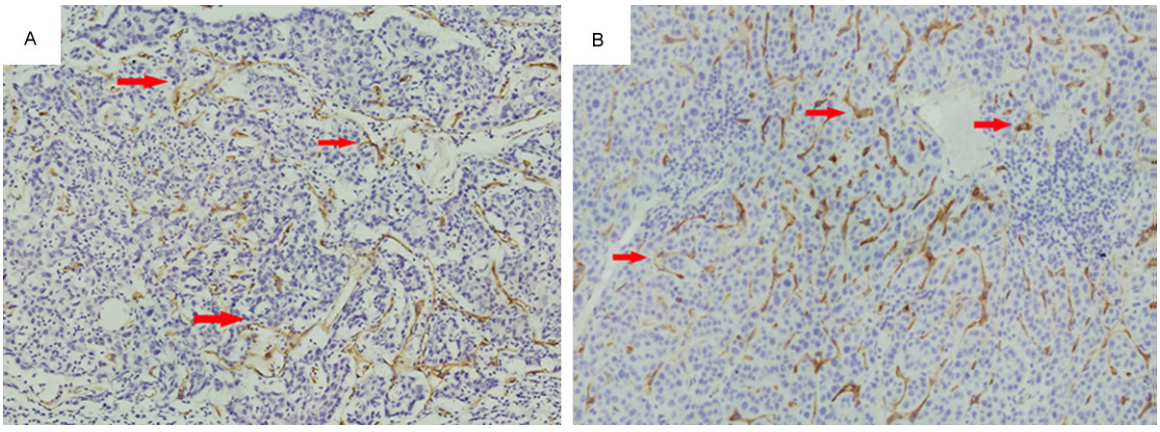

Figure 2. Representative images for immunohistochemical study on VECs in HCC tumor tissue (Original Magnification A: 10× and B: 40×). Arrows indicate representative cells.

therapy or radiotherapy. All samples were separated into tumor tissue and corresponding peritumoral tissue. All tissues were paraffin-embedded and sectioned onto slides for pathologic analysis.

Clinicopathologic data collection

The histological grade of HCC differentiation was determined using the Edmondson grading system. The pathologic features of all cases,

Table 1. Correlations between the density of activated HSCs in HCC and patients' clinicopathologic features

Variable	n	HSCs density	F	P
Gender				
Male	16	10.2±1.3		0.513
Female	4	11.1±1.4		
Age (y)				
>50	10	10.7±1.3		0.417
≤50	10	9.4±1.2		
Tumor size (cm)				
>5	8	11.1±1.4		0.623
≤5	12	10.7±1.3		
AFP (ng/ml)				
>400	6	11.8±1. 3		0.553
≤400	14	9.3±1.2		
Hepatitis				
HBV	18	10.2±1.3		0.549
HCV	2	11.3±1.3		
HCC differentiation				
Well	2	6.3±0.9		
Moderately	6	8.2±1.1		
Poorly	12	12.1±1.4	5.93	0.013
Peritumoral pathology				
Liver cirrhosis	12	12.0±1.4		0.024
Chronic hepatitis	8	7.8±0.9		

AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma.

Table 2. Correlations between the MVD in HCC and patients' clinicopathologic features

Variable	n	MVD	F	P
Gender				
Male	16	5.0±0.6		0.545
emale	4	6.1±0.7		
Age (y)				
>50	10	5.4±0.6		0.517
≤50	10	5.1±0.6		
Tumor size (cm)				
>5	8	8.3±0.9		0.015
≤5	12	3.2±0.4		
AFP (ng/ml)				
>400	6	5.9±0.7		0.587
≤400	14	5.0±0.6		
Hepatitis				
HBV	18	5.3±0.6		0.56
HCV	2	4.8±0.5		
HCC differentiation				
Well	2	3.9±0.4	6.78	0.035
Moderately	6	4.0±0.4		
Poorly	2	6.1±0.7		
Peritumoral pathology				
Liver cirrhosis	12	6.3±0.7		0.012
Chronic hepatitis	8	3.6±0.4		

MVD, microvessel density; AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma.

such as tumor size, differentiation grade and peritumoral pathologic condition, were reviewed and determined by two pathologists who were blinded to information from the original pathology reports. The patients' clinical information, including sex, age, serum AFP level and hepatitis infection was also collected.

Immunohistochemical analysis

Mouse anti-human α-smooth muscle actin (α-SMA) and CD34 monoclonal antibodies (Santa Cruz, Dallas, Texas, USA) were used to detect activated HSCs and vascular endothelial cells (VECs) separately in HCC. Sections were deparaffinized, hydrated and washed. After neutralization of endogenous peroxidase and antigen retrieval using a microwave oven, the slides were pre-incubated with blocking serum and then incubated with primary antibodies overnight. Subsequently, the sections were serially rinsed, incubated with secondary antibodies and treated with horseradish peroxidase-conjugated streptavidin. Reaction products were developed with 3, 3'-diaminobenzidine solution and counterstained with hematoxylin. The density of activated HSCs and microvessel density (MVD) in HCC stroma were determined at high magnification by 2 independent observers.

Statistics

All results were expressed as the mean ± SE. Statistical analysis was performed using the SPSS statistical software for Microsoft Windows, version 15.0 (Professional Statistic, Chicago, IL, USA). A two-tailed paired Student's t-test and ANOVA were used for comparison of variables. Bivariate correlation analysis was used for analyzing the correlation between the density of activated HSCs and microvessel density in HCC. The criterion for significance was $P<0.05$ for all comparisons.

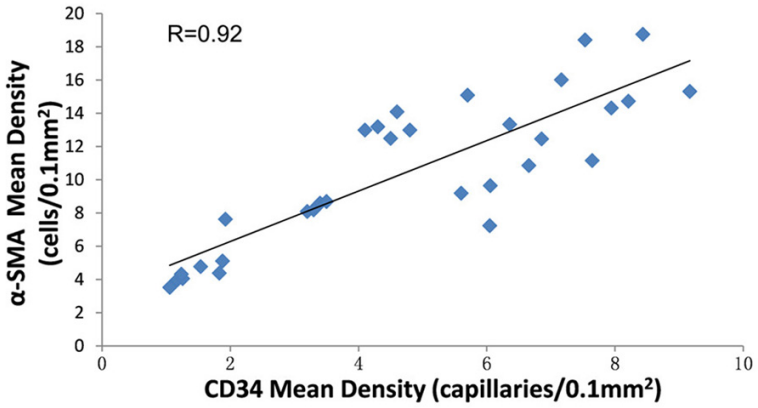

Figure 3. Correlation analysis of the density of activated HSCs and MVD in HCC.

Results

Immunohistochemical characteristics

Activated HSCs and VECs in 30 human HCC samples were quantified using computer-assisted image analysis as described in Materials and Methods. CD34 positive VECs were located in the blood sinus and inner wall of large vessels in HCC. In the peritumoral tissue, α-SMA expressing activated HSCs existed in the liver blood sinus. We found that activated HSCs were mainly located around the blood sinus in HCC stroma. Representative images by independent observers are shown in **Figures 1** and **2**.

Correlation between the densities of activated hepatic stellate cells and clinicopathologic features

As shown in **Table 1**, there was no correlation between the tumoral density of activated HSCs and patients' gender, age, tumor size, serum AFP level and hepatitis virus infection (HBV or HCV). We found that the density of activated HSCs was higher in the poorly differentiated HCC stroma. In particular, HCC that developed from liver cirrhosis was prone to have a higher density of activated HSCs in the stroma.

Correlation between the microvessel density and clinicopathologic features

As described in **Table 2**, tumors of a larger size (>5 cm) are prone to having higher MVD. Similar to the activated HSCs density, the tumoral MVD was higher when the HCC was poorly differentiated or the peritumoral hepatic tissue showed cirrhotic changes. There was no correlation between the tumoral MVD and the patient's gender, age, serum AFP level or hepatitis virus infection (HBV or HCV).

Correlation between the density of activated hepatic stellate cells and microvessel density in hepatocellular carcinoma stroma

Next, we analyzed the correlation between the density of activated HSCs and MVD in the HCC stroma of all 30 patients. The results showed that they were significantly positively correlated and that the correlation coefficient was as high as 0.92 (**Figure 3**). As the density of activated HSCs in HCC stroma increased, the tumoral MVD also elevated; indicating that angiogenesis was strongly associated with the amount of activated HSCs in HCC stroma.

Discussion

The data from this study demonstrate that activated HSCs exist in the human HCC stroma. Activated HSCs were located mainly in the HCC blood sinus and were spatially near the VECs. We also found that the density of activated HSCs was higher in poorly differentiated HCC or in cases with cirrhotic peritumoral liver tissue. The same conclusion was drawn when we analyzed the microvessel density in HCC. Finally, we analyzed the correlation between the density of activated HSCs and microvessels in HCC stroma. The results showed that they were significantly positively correlated. Our study indicates that activated HSCs in HCC stroma may originate in the peritumoral liver tissue and are associated with the angiogenesis in HCC. In the future, in vitro studies and HCC animal models are needed to prove this hypothesis.

Because of lacking bio-markers, it is difficult to identify activated HSCs from cancer associated fibroblasts (CAFs) in HCC. The main source of CAFs in HCC is recognized to be activated HSCs, but the clear derivation is still under study [14, 15]. In this study, we found that the density of activated HSCs was high in HCC stroma when the tumor developed from liver cirrhosis. Activated HSCs are proven to be more

numerous in cirrhotic than in normal liver tissue [16, 17]. We hypothesize that activated HSCs may be derived from the peritumoral liver tissue. Peritumoral activated HSCs may be attracted to the tumor stroma in response to paracrine factors secreted by HCC cells. Furthermore, effective markers for distinguishing activated HSCs from CAFs in HCC are needed and good animal models will identify the presence and role of activated HSCs in HCC stroma.

Tumor progression requires formation of blood vessels [3] and poorly differentiated HCC is prone to be proliferative and invasive [18]. We found that microvessel density was higher in the poorly differentiated HCC tissue, indicating that tumor progression requires thriving angiogenesis. The density of activated HSCs was also higher in the poorly differentiated HCC. These results indicate that thriving angiogenesis maybe the result of a high density of activated HSCs in poorly differentiated HCC. Next, we analyzed the correlation between the density of activated HSCs and microvessel density in all 30 HCC samples. The results showed that they were significantly positively correlated, and thus, the activated stromal HSCs may promote angiogenesis in HCC.

To summarize, our results demonstrated that activated HSCs were an important component of the HCC stroma and may be derived from the peritumoral liver tissue. Activated HSCs were associated with angiogenesis in HCC. Considering the current unsatisfactory anti-angiogenic treatment for HCC, our findings suggest a new potential therapeutic approach of targeting activated HSCs to effectively block angiogenesis and prevent the development of HCC.

Acknowledgements

This work was supported by Medical Scientific Research Foundation of Guangdong Province, China (Grant No. A2015116) and Science and Technology Planning Project of Guangdong Province, China (Grant No. 2012A020602002).

Disclosure of conflict of interest

None.

Address correspondence to: Yunbiao Ling, Department of Hepatobiliary Surgery, The 3rd Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong Province, China. E-mail: 1353018679@qq.com

References

[1] Parking DM. Global cancer statistics. CA Cancer J Clin 2002; 55: 74-108.
[2] El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. Gastroenterology 2004; 127: S27-34.
[3] Coulon S, Heindryckx F, Geerts A, Van Steenkiste C, Colle I, Van Vliberberghe H. Angiogenesis in chronic liver disease and its complications. Liver Int 2011; 31: 146-62.
[4] Poon RT, Ng IO, Lau C, Yu WC, Yang ZF, Fan ST, Wong J. Tumor microvessel density as a predictor of recurrence after resection of hepatocellular carcinoma: a prospective study. J Clin Oncol 2002; 20: 1775-85.
[5] Yim KL and Cunningham D. Targeted drug therapies and cancer. Recent Results Cancer Res 2011; 185: 159-71.
[6] Keating GM and Santoro A. Sorafenib: A review of its use in advanced hepatocellular carcinoma. Drugs 2009; 69: 223-240.
[7] Friedman SL. Mechanisms of Hepatic Fibrogenesis. Gastroenterology 2008; 134: 1655-1669.
[8] Amann T, Bataille F, Spruss T, Muhlbauer M, Gabele E, Scholmerich J, Kiefer P, Bosserhoff AK, Hellerbrand C. Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma. Cancer Sci 2009; 100: 646-53.
[9] Zhao W, Su W, Kuang P, Zhang L, Liu J, Yin Z, Wang X. The role of hepatic stellate cells in the regulation of T-cell function and the promotion of hepatocellular carcinoma. Int J Oncol 2012; 41: 457-64.
[10] Olaso E, Salado C, Eqileqor E, Gutierrez V, Sa A, Santisteban V, Sancho-Bru P, Friedman SL, Vidal-Vanaclocha E. Proangiogenic role of tumor-activated hepatic stellate cells in experimental melanoma metastasis. Hepatology 2003; 37: 674-85.
[11] Thabut D and Shah V. Intrahepatic angiogenesis and sinusoidal remodeling in chronic liver disease: New targets for the treatment of portal hypertension. J Hepatol 2010; 53: 976-980.
[12] Zhao W, Zhang L, Yin Z, Su W, Ren G, Zhou C, You J, Fan J, Wang X. Activated hepatic stellate cells promote hepatocellular carcinoma development in immunocompetent mice. Int J Cancer 2011; 129: 2651-61.
[13] Jung JO, Gwak GY, Lim YS, Kim CY, Lee HS. Role of hepatic stellate cells in the angiogenesis of hepatoma. Korean J Gastroenterol 2003; 42: 142-148.
[14] Olaso E, Santisteban A, Bidaurrazaga J, Gressner AM, Rosenbaum J, Vidal-Vanaclocha F. Tumor-dependent activation of rodent hepatic stellate cells during experimental melanoma metastasis. Hepatology 1997; 26: 634-643.

[15] Kalluri R and Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006; 6: 392-401.
[16] Lee UE and Freidman SL. Mechanisms of hepatic fibrogenesis. Best Pract Res Cl En 2011; 25: 195-206.
[17] Watanabe A, Sohail MA, Gomes DA, Hashmi A, Nagata J, Sutterwala FS, Mahmood S, Jhandier MN, Shi Y, Flavell RA, Mehal WZ. Inflammasome-mediated regulation of hepatic stellate cells. Am J Physiol Gatrointest Liver Physiol 2009; 299: G1248-57.
[18] Valastyan S and Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell 2011; 147: 275-92.