

# Importance of activated hepatic stellate cells and angiopoietin-1 in the pathogenesis of hepatocellular carcinoma

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**Abstract.** Previous studies have determined that activated hepatic stellate cells (aHSCs) promote the progression of hepatocellular carcinoma (HCC) by increasing angiogenesis in cancerous tissues. In addition, angiopoietin 1 (Ang-1) has been reported to be involved in tumor growth and metastasis via the promotion of angiogenesis. It remains unclear whether aHSCs and Ang-1 are involved in the angiogenesis in HCC. A total of 25 HCC and tumor-adjacent tissues, and 21 normal liver tissues were used in the present study. Immunohistochemistry (IHC) was used to detect the expression of Ang-1 and  $\alpha$  smooth muscle actin ( $\alpha$ -SMA). The expression of CD34 was also analyzed using IHC to evaluate the microvessel density (MVD). The protein expression levels of Ang-1 were evaluated using western blot analysis. The association between aHSC, Ang-1 and angiogenesis was determined using Spearman's rank correlation coefficient. The present study determined that the expression of  $\alpha$ -SMA, Ang-1 and MVD (CD34) was significantly higher in the HCC tissues when compared with tumor-adjacent tissues and normal liver tissues. Spearman's rank analysis identified a positive correlation between the expression of  $\alpha$ -SMA, Ang-1 and CD34. This suggests that  $\alpha$ -SMA-positive aHSCs promoted angiogenesis by expressing Ang-1, resulting in the proliferation and metastasis of HCC.

## Introduction

Hepatocellular carcinoma (HCC), the dominant form of primary liver cancer, is one of the most prevalent and life-threatening types of cancer worldwide (1-6). Progress has

been made in the investigation of the pathogenesis and treatment of HCC, however, recurrence and metastasis remain key challenges for effective treatment of HCC, thus limiting the prognosis and quality of life of patients with HCC.

Typically, HCC is a hypervascular tumor. Microvessel formation is essential for the growth and metastasis of HCC. Microvessel density (MVD) is positively-associated with the growth and metastasis of HCC (7). Previous studies have reported that activated hepatic stellate cells (aHSCs) promote the growth and metastasis of HCC by accelerating the formation of microvessels in HCC tissues (8-11). However, the mechanism underlying the importance of aHSCs in microvessel formation in HCC remains to be fully elucidated. Angiopoietin-1 (Ang-1) is a growth factor involved in angiogenesis. Previous studies have reported that Ang-1 activates angiogenesis and promotes tumor growth and metastasis by regulating the proliferation and migration of endothelial cells in tumor tissues (12-15).

The association between Ang-1 and aHSC has been previously described. The transcription and production of vascular endothelial growth factor (VEGF) and Ang-1 in aHSCs were increased under hypoxic conditions, and VEGF and Ang-1 promoted aHSC proliferation and extracellular matrix deposition, increasing the migration and chemotaxis of cells (16). Taura *et al* (17) demonstrated that aHSCs promote angiogenesis in HCC by secreting Ang-1, suggesting that aHSC and Ang-1 are important for the development of HCC (17). However, it remains unclear whether aHSC and Ang-1 are associated with microvessel formation in HCC.

The present study compared the expression of Ang-1,  $\alpha$  smooth muscle actin ( $\alpha$ -SMA) and MVD (CD34) between HCC, HCC-adjacent tissues and normal liver tissues to determine the association of Ang-1 and aHSC in the development, in particular in angiogenesis, of HCC.

## Materials and methods

**Ethical approval of the study protocol.** The current study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center (Guangzhou, China). Signed, informed consent was obtained from each patient prior to the beginning of the current study.

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**Patients and tumor tissue samples.** A total of 25 patients with HCC, 21 males and 4 females, aged between 29 and 75 years old, were enrolled in the present study. Tissue samples, including HCC tumor tissues and adjacent non-cancerous tissues (n=25), were obtained from patients that underwent resection of primary HCC at the Third Affiliated Hospital, Sun Yat-sen University (Guangzhou, China) between July and October 2013. The patients had not received preoperative chemotherapy or radiotherapy. Control samples were collected from a total of 21 healthy individuals using the same sample collection protocol. Samples were placed in sterile vials and immediately stored at -80°C.

**Immunohistochemistry (IHC).** Isolated tissues were fixed with 4% paraformaldehyde for 24 h and embedded in paraffin. Tissue sections (5  $\mu$ m) were deparaffinized and rehydrated through an ethanol gradient. Antigen retrieval was performed by boiling in a 10 mM sodium citrate buffer (pH 6) for 15 min. Non-specific binding was blocked by the addition of 5% bovine serum albumin (Beyotime Institute of Biotechnology, Haimen, China). The sections were then incubated with monoclonal rabbit anti-Ang-1 (1:200 dilution; Abcam, Cambridge, UK; cat. no. ab8451), monoclonal rabbit anti- $\alpha$ -SMA (rabbit anti- $\alpha$ -SMA monoclonal antibody; 1:500 dilution; Gene Tech Co., Ltd., Shanghai, China; cat. no. GM085101) and monoclonal rabbit anti-CD34 (1:100 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-19621) antibodies overnight at 4°C. Detection was conducted using an horseradish peroxidase (HRP) Detection System (Cell Marque; Sigma-Aldrich, St. Louis, MO, USA) and 3,3'-Diaminobenzidine Substrate kit (Forevergen Biosciences, Guangzhou, China), following the manufacturer's protocol. Subsequent to counterstaining with hematoxylin, the slides were dehydrated, mounted and observed under a light microscope (Olympus Corporation, Tokyo, Japan). Quantitative analysis was performed on the IHC images using Image-Pro Plus software (version 4.5; Media Cybernetics, Inc., Rockville, MD, USA), and the mean optical density (MOD) was determined from five randomly selected areas.

**Western blotting.** A total of 25 tissue samples were used to confirm the expression profile of Ang-1 by western blot analysis. The samples (~100 mg each) were homogenized and lysed in 500  $\mu$ l radioimmunoprecipitation assay buffer. Next, the samples were centrifuged at 16,000  $\times$  g for 1 min at 4°C, the supernatant was collected and was quantified by the Bradford Protein assay kit (Beyotime Institute of Biotechnology). Denatured recombinant Ang-1 protein was electrophoresed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The membranes were then blocked with 5% non-fat milk in phosphate-buffered saline for 1 h at room temperature and incubated overnight at 4°C with anti-Ang-1 monoclonal antibodies (1:200 dilution) or anti-GAPDH mouse monoclonal antibodies (1:1,000 dilution; Santa Cruz Biotechnology, Inc.; cat. no. sc-25778). The HRP-conjugated goat anti-rabbit IgG was used as a secondary antibody (1:3,000 dilution; Bio-Rad Laboratories, Inc., Hercules, CA, USA; cat. no. 403005). Immunoreactive bands were detected using an enhanced chemiluminescence

kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Grayscale detection was quantified using ImageJ software (version 1.43; National Institutes of Health, Bethesda, MD, USA) and normalized to GAPDH.

**Statistical analysis.** Comparison of differential expression of the proteins in the HCC, tumor-adjacent and normal liver tissues was performed using one-way analysis of variance. Fisher's Least Significant Difference test was utilized for comparisons between groups, and Spearman's correlation coefficient was performed to determine the association between expression of any two proteins of interest. All calculations were performed using SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression levels of  $\alpha$ -SMA in HCC, tumor-adjacent tissues and normal liver tissues.** HSCs in normal liver tissues were not activated. Inflammation and mechanical stimulation may activate HSCs to become aHSCs that express  $\alpha$ -SMA (10). In the present study, the expression levels of  $\alpha$ -SMA in HSCs in HCC, tumor-adjacent tissues and normal liver tissues were evaluated using IHC.  $\alpha$ -SMA-positive cells were detected in the cancer cell nest and hepatic blood sinus (Fig. 1A). The MOD levels of  $\alpha$ -SMA expression in HCC, tumor-adjacent tissues and normal liver tissues were  $(4.56 \pm 0.64) \times 10^4$ ,  $(2.71 \pm 0.37) \times 10^4$  and  $(2.25 \pm 0.48) \times 10^4$ , respectively. The expression of  $\alpha$ -SMA in HCC was significantly higher compared with tumor-adjacent ( $F = 7.09$ ;  $P < 0.05$ ; Fig. 1B) and normal liver tissues ( $F = 7.42$ ;  $P < 0.05$ ; Fig. 1B).

**Expression levels of Ang-1 in HCC, tumor-adjacent and normal liver tissues.** The expression of Ang-1 in HSCs in HCC, tumor-adjacent and normal liver tissues was evaluated using IHC. Ang-1-positive cells were primarily detected in the cancer cell nest and hepatic blood sinus (Fig. 2A). The MOD levels of Ang-1 expression in HCC, tumor-adjacent and normal liver tissues were  $(3.11 \pm 0.27) \times 10^5$ ,  $(2.28 \pm 0.20) \times 10^5$  and  $(1.26 \pm 0.15) \times 10^5$ , respectively. The expression of Ang-1 in HCC was significantly higher compared with tumor-adjacent ( $F = 3.00$ ;  $P < 0.05$ ; Fig. 2B) and normal liver tissues ( $F = 3.14$ ;  $P < 0.05$ ; Fig. 2B).

The protein expression levels of Ang-1 in HSCs in HCC, tumor-adjacent and normal liver tissues were analyzed using western blot analysis (Fig. 3A). The MOD levels of Ang-1 expression in HCC, tumor-adjacent and normal liver tissues were  $4.33 \pm 1.17$ ,  $1.62 \pm 0.33$  and  $1.60 \pm 0.38$ , respectively. The expression of Ang-1 in HCC was significantly higher compared with tumor-adjacent ( $F = 2.71$ ;  $P < 0.05$ ; Fig. 3B) and normal liver tissues ( $F = 2.74$ ;  $P < 0.05$ ; Fig. 3B).

**MVD in HCC, tumor-adjacent and normal liver tissues.** The MVD (CD34) in HCC, tumor-adjacent and normal liver tissues was determined using IHC analysis of CD34 as previously described (18-21). CD34-positive cells were predominantly detected in the cancer cell nest and hepatic blood sinus (Fig. 4A). The MOD levels of CD34 expression in HCC, tumor-adjacent and normal liver tissues were  $(18.3 \pm 0.36) \times 10^3$ ,

Table I. Spearman rank analysis to determine the association between  $\alpha$ -SMA, Ang-1 and CD34 expression levels in hepatocellular carcinoma tissues, tumor-adjacent tissues and normal liver tissues.

	CD34		$\alpha$ -SMA		Ang-1	
	Correlation coefficient	P-value	Correlation coefficient	P-value	Correlation coefficient	P-value
CD34	1.000	-	0.537	<0.001	0.610	<0.001
$\alpha$ -SMA	0.537	<0.001	1.000	-	0.576	<0.001
Ang-1	0.610	<0.001	0.576	<0.001	1.000	-

$\alpha$ -SMA,  $\alpha$  smooth muscle actin; Ang-1, angiopoietin 1; correlation coefficient, r.

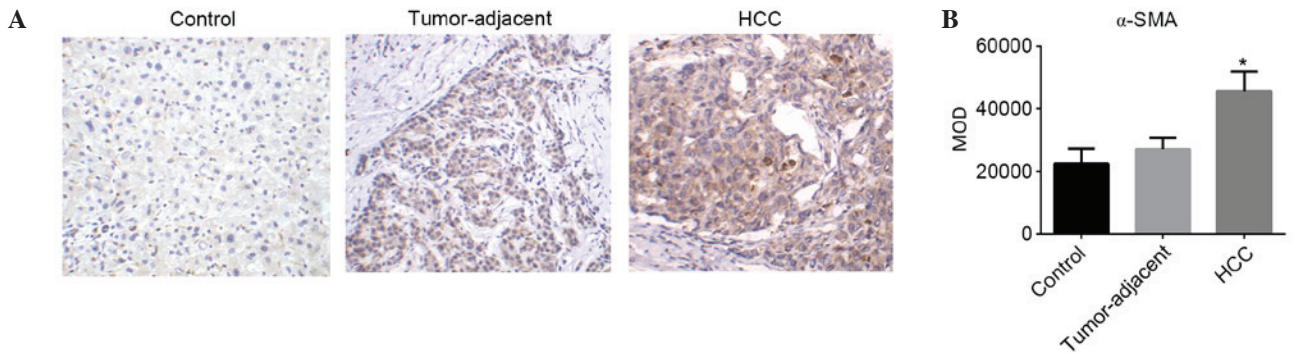


Figure 1. Expression levels of  $\alpha$ -SMA. (A) Immunohistochemical detection of  $\alpha$ -SMA expression levels in control, tumor-adjacent and HCC tissues. Magnification, x200. (B) Relative protein expression of  $\alpha$ -SMA as evaluated by Image-Pro Plus software. \* $P$ <0.05 vs. control and tumor-adjacent groups.  $\alpha$ -SMA,  $\alpha$  smooth muscle actin; HCC, hepatocellular carcinoma; MOD, mean optical density.

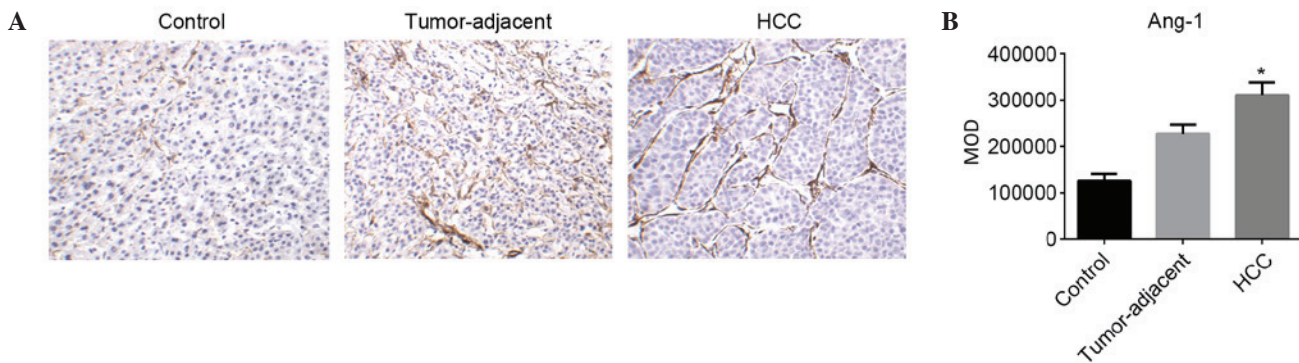


Figure 2. Expression levels of Ang-1. (A) Immunohistochemical detection of Ang-1 expression levels in control, tumor-adjacent and HCC tissues. Magnification, x200. (B) Relative protein expression of Ang-1 as evaluated by Image-Pro Plus software. \* $P$ <0.05 vs. control and tumor-adjacent groups. Ang-1, angiopoietin 1; HCC, hepatocellular carcinoma; MOD, mean optical density.

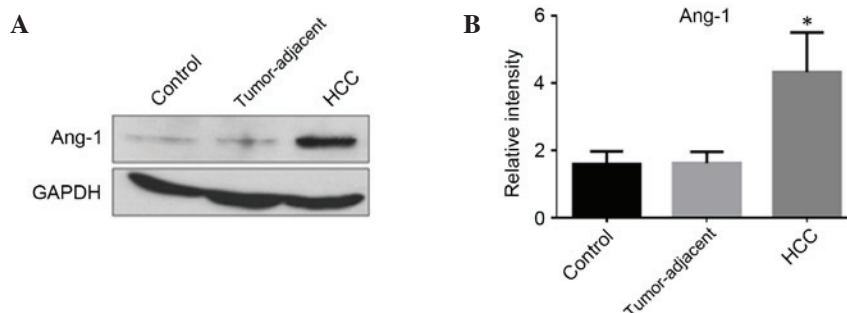


Figure 3. (A) Relative expression of Ang-1 was measured by western blotting, (B) then was quantified (n=25). GAPDH was used as an internal control. \* $P$ <0.05 vs. control and tumor-adjacent groups. Ang-1, angiopoietin 1; HCC, hepatocellular carcinoma; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



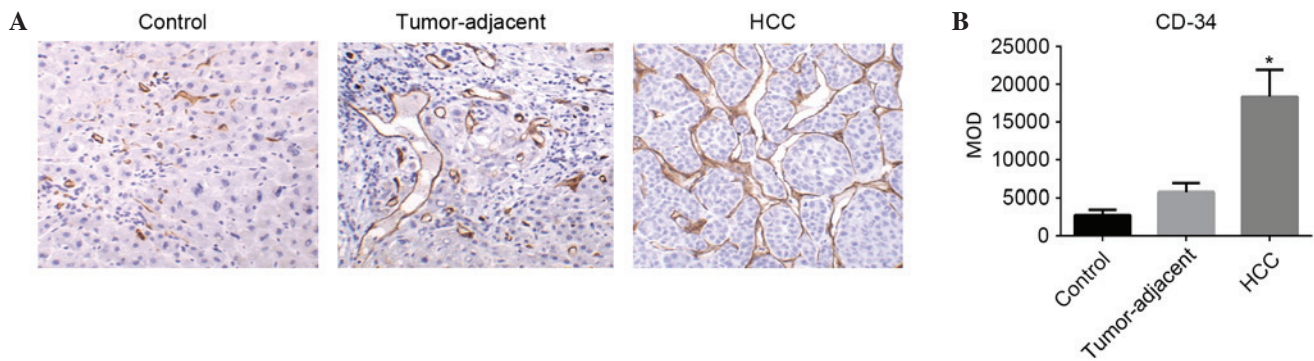


Figure 4. Expression levels of CD34. (A) Immunohistochemical detection of CD34 expression levels in control, tumor-adjacent and gastric cancer tissues. Magnification,  $\times 200$ . (B) Relative protein expression of CD34 as evaluated by Image-Pro Plus software. \* $P < 0.05$  vs. control and tumor-adjacent groups. HCC, hepatocellular carcinoma; MOD, mean optical density.

$(5.75 \pm 1.17) \times 10^3$  and  $(2.75 \pm 0.72) \times 10^3$ , respectively. The expression levels of CD34 in HCC were significantly higher compared with tumor-adjacent ( $F = 3.21$ ;  $P < 0.05$ ; Fig. 4B) and normal liver tissues ( $F = 3.36$ ;  $P < 0.05$ ; Fig. 4B).

*Associations between the expression levels of Ang-1,  $\alpha$ -SMA and MVD (CD34).* Spearman's rank correlation coefficient analysis was used to evaluate the association among the expression levels of Ang-1,  $\alpha$ -SMA and MVD. As presented in Table I, a positive correlation was identified between the expression of Ang-1 and CD34 ( $r = 0.610$ ;  $P < 0.05$ ), Ang-1 and  $\alpha$ -SMA ( $r = 0.576$ ;  $P < 0.05$ ), and  $\alpha$ -SMA and CD34 ( $r = 0.537$ ;  $P < 0.05$ ).

## Discussion

HCC is a hypervascular cancer; the development of microvessels is essential for the growth and metastasis of HCC (7,22). HSCs in normal liver tissues may be activated by inflammation and mechanical stimulation and become activated HSCs (23,24). Activated HSCs proliferate and express  $\alpha$ -SMA. A previous study reported that aHSCs contributed to the growth and metastasis of HCC by expressing hepatocyte growth factor, interleukin 6 and VEGF (15). However, another previous study has determined that aHSCs additionally inhibit the proliferation and metastasis of HCC cells by expressing laminin 5 and other extracellular matrix components (16). Additionally, aHSCs promote the development of HCC from cirrhosis, HCC growth and metastasis by increasing the formation of microvessels in tumor tissues (8,9). The present study determined that the expression levels of  $\alpha$ -SMA in aHSCs in HCC tissues were significantly higher compared with tumor-adjacent and normal liver tissues, suggesting that aHSCs may be involved in microvessel formation and development in HCC.

Previous studies have reported that Ang-1 promoted the maturation and maintained the stability of vessels (12-14). In addition, previous studies have determined that Ang-1 regulated the survival, proliferation and metastasis of endothelial cells and promoted the formation of microvessels in tumor tissues (12,25). The expression levels of Ang-1 have been observed to be increased in glioma and ovarian cancers (26,27). The present study determined that the

expression of Ang-1 in HCC tissues was significantly higher compared with tumor-adjacent and normal liver tissues. In addition, the MVD in HCC tissues was significantly higher compared with tumor-adjacent and normal liver tissues. These results suggest that Ang-1 is important for microvessel formation in HCC, which is consistent with the results of previous studies (18-21).

Spearman's correlation coefficient analysis identified a positive correlation between the expression levels of  $\alpha$ -SMA, Ang-1 and CD34, suggesting that Ang-1 and aHSCs are involved in the formation of microvessels in HCC tissues. Ang-1 may also be involved in the regulatory effects of aHSCs on microvessel formation. Previous studies have demonstrated that transcription levels of Ang-1 in aHSCs were increased in response to hypoxia. In addition, Ang-1 may further promote the proliferation and mobility of aHSCs, including the deposition of extracellular matrix (7,16,28-30). Activated HSCs are also important for the development of cirrhosis by expressing Ang-1 and promoting angiogenesis (17). The results of the current study suggest that aHSCs promote the growth and metastasis of HCC by increasing the expression of Ang-1 and angiogenesis.

The present study initially detected the expression of  $\alpha$ -SMA in HCC tissues using IHC. Positive expression of  $\alpha$ -SMA suggests the existence of aHSCs in HCC tissues. Next, the expression levels of Ang-1 and CD34 were determined. The results demonstrated that the expression levels of Ang-1 and CD34 in HCC were significantly higher compared with tumor-adjacent and normal liver tissues. The expression of  $\alpha$ -SMA and CD34 was primarily detected between cancer cell nests; however, the majority of Ang-1 expression was detected in cancer cell nests. In addition, a positive correlation was identified among  $\alpha$ -SMA, Ang-1 and CD34. In conclusion, the results of the present study suggest that aHSCs increased the expression of Ang-1, resulting in angiogenesis in HCC tissues, thus promoting the growth and metastasis of HCC. However, the molecular mechanisms underlying the role of aHSCs in the development of HCC require further investigation.

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