SPANDIDOS PUBLICATIONS

Angiogenesis in cholangiocellular carcinoma: Expression of vascular endothelial growth factor, angiopoietin-1/2, thrombospondin-1 and clinicopathological significance

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Abstract. Angiogenesis in cholangiocellular carcinoma (CCC) has rarely been investigated. The aim of this study was to determine the angiogenesis status of CCC and assess its relationship with angiogenic factors and clinicopathological characteristics. We examined 33 surgically resected CCC specimens. Tumor angiogenesis was assessed by microvessel density (MVD) using the anti-CD34 antibody, and the expression of VEGF, Ang-1, Ang-2, and TSP-1 was determined by immunohistochemistry. The mean (± SD) MVD was 87.2±52.6/mm^2 (range, 0-229/mm^2). A total of 75.6% cases were positive for VEGF expression, 36% for Ang-1, 57.6% for Ang-2 and 45.5% for TSP-1. VEGF and Ang-2 expression was associated with a significantly higher level of MVD (p=0.004 and 0.015, respectively). TSP-1 expression was associated with a significantly lower level of MVD (p=0.005) and a higher level of intrahepatic metastasis (46.7% vs. 5.6%, p=0.012). There was no significant correlation between VEGF, Ang-1, Ang-2, and TSP-1 expression and tumor size, capsule formation, infiltration of capsule, portal vein invasion, intrahepatic metastasis or CCC differentiation. There was no significant correlation between MVD levels, VEGF, Ang-1, Ang-2, and TSP-1 expression and postoperative survival. A considerable degree of angiogenesis, comparable to that of other solid tumors, was observed in CCC. VEGF and Ang-2 might play a proangiogenic role, and TSP-1 may play an inhibitory role in CCC. Although TSP-1 may increase intrahepatic CCC metastases, neither MVD levels nor the expression of VEGF, Ang-1, or Ang-2 was associated with clinicopathological factors and prognosis.

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Abbreviations: Ang, angiopoietin; CCC, cholangiocellular carcinoma; HCC, hepatocellular carcinoma; LN, lymph node; MVD, microvessel density; MCC, metastatic colorectal cancer; PBS, phosphate-buffered saline; SD, standard deviation; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor

Key words: cholangiocellular carcinoma, vascular endothelial growth factor, angiopoietin, thrombospondin, angiogenesis, prognosis

Introduction

Cholangiocellular carcinoma (CCC) is the second most frequent primary liver cancer in adults, and patients with CCC generally have a poorer prognosis than those with hepatocellular carcinoma (HCC) (1). Macroscopically, CCC can be divided into three major types, namely the mass forming, periductal infiltrating and intraductal growth types (2,3). In comparison with the hypervascular HCC, CCC is regarded as hypovascular; in contrast to HCC, there is a much smaller volume of published research pertaining to angiogenesis in CCC. Inconsistent vascularity of this tumor has also been reported, i.e. the reported microvessel density (MVD) of this tumor ranges from as low as 10/mm^2 to as high as 169/mm^2 (4,5).

We previously demonstrated that the marginal tumor field harbors more extensive angiogenesis in comparison to the intermediate and central fields during angiogenesis in hepatic metastases of colorectal cancer (6). Like metastatic colorectal cancer (MCC), CCC is regarded as a hypovascular cancer (4,5). However, the vascularity patterns of the different macroscopic types of CCC have yet to be investigated.

Several growth factors, such as vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and thrombospondin-1 (TSP-1), are associated with tumor angiogenesis. VEGF has demonstrated specificity and is essential for the formation of blood vessels in tumors (7). Ang-1 and Ang-2 are ligands for endothelium-specific tyrosine kinase receptor Tie-2 (8,9). Angiopoietins have also been demonstrated to participate in the angiogenesis of

tumors through their impact on endothelial migration, proliferation and microvascular permeability (10,11). Ang-1 promotes vessel stability by increasing the connections of endothelial cells to the surrounding supporting pericytes (8,11). In contrast, Ang-2 disrupts the stabilizing effect of Ang-1 and induces the loosening of interactions between endothelial and perivascular support cells and the matrix, thus compromising vascular integrity and facilitating access to proangiogenic inducers such as VEGF (9,12). To date, the expression of Ang-1 and Ang-2 in CCC and their relationship with clinicopathological factors remains unclear. Unlike VEGF and angiopoietins, TSP-1 is a multifunctional matrix protein (13). The role of TSP-1 in angiogenesis and tumor progression remains controversial. Both positive and negative effects of TSP-1 on tumor angiogenesis have been reported (5,14,15). In addition, research on the relationship of TSP-1 with tumor clinicopathological factors is limited.

In the present study, we determined the angiogenesis status of CCC in our group of patients, investigated the relationship with angiogenic factors and clinicopathological factors, and assessed the MVD in 33 surgically resected CCC samples. We also evaluated the relationship between angiogenesis and other clinicopathological factors with the expression of VEGF, Ang-1, Ang-2 and TSP-1. In addition, the prognostic significance of MVD, VEGF, Ang-1, Ang-2 and TSP-1 in 23 curative resected CCC cases was investigated.

Materials and methods

Patients and specimens. A total of 33 patients (18 males and 15 females) with surgically resected CCC, who were admitted to the Osaka University Hospital between 1990 and 2003, participated in this study. The median age of the patients was 63 years (range, 33-80 years). There were 7 patients who were positive for hepatitis B surface antigen or hepatitis C virus antibody. Macroscopically, 20 cases were of the mass forming type, 4 cases were the intraductal growth type and 9 cases were the periductal infiltrating type. Some 23 patients underwent curative resection, and 10 cases underwent palliative resection. Resected CCC specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin for assessment of the pathological features of CCC in accordance with the classification proposed by the Liver Cancer Study Group of Japan (3).

Assessment of microvessel density (MVD). MVD was assessed using the method recommended by Weidner *et al* (16). After immunostaining with anti-CD34 antibody (mouse monoclonal IgG2a, diluted 1:50), large and small microvessels, and single brown immunostained endothelial cells separate from adjacent microvessels and stromal structures were included in the microvessel count. An Olympus microscope BX50 with image capture device DP-70 was used for this study. The software DP Controller 2.11 was used for image scaling and capturing. The MVD count was calculated as the number of microvessels found within 1 mm^2 of the microscopic field under a magnification of x100. The MVD of the peripheral, intermediate and central areas of the tumor, as well as the surrounding normal liver, were recorded. For each area, the average MVD of the five highest MVD counts was recorded as the MVD of that area. The MVD of the tumor was thus calculated as the average MVD of five highest MVD counts within the tumor.

Immunohistochemical staining. Formalin-fixed, paraffin-embedded specimens of tumors and associated periphery were selected for analysis. Sections measuring 4 μm in thickness were deparaffinized in xylene, rehydrated, and stained with hematoxylin-eosin solution for histopathological examination. After deparaffinization in xylene and rehydration in a graded series of ethanol, immunohistochemical procedures were performed using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) as described previously (17). Briefly, sections were treated with an antigen retrieval procedure in 0.01 M sodium citrate buffer (pH 6.0) for 40 min at 95˚C and incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 min) to block endogenous peroxidases. The sections were then incubated with normal protein block serum solution at room temperature for 20 min to block non-specific staining, followed by overnight incubation at 4˚C with anti-VEGF (rabbit polyclonal IgG, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-angiopoietin-1 (goat polyclonal IgG, diluted 1:50; Santa Cruz Biotechnology), anti-angiopoietin-2 (goat polyclonal IgG, diluted 1:50; Santa Cruz Biotechnology), and anti-thrombospodin-1 (mouse polyclonal IgG2a, diluted 1:50; Lab Vision Corp., Fremont, CA). Sections were washed 3 times for 5 min in phosphate-buffered saline (PBS) before incubation at room temperature for 20 min with a biotin-conjugated secondary antibody (goat anti-rabbit for VEGF and rabbit anti-goat for ANG1 and ANG2), and finally with peroxidase-conjugated streptavidin at room temperature for 20 min. The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrachloride (Wako Pure Chemical Industries, Osaka, Japan). The sections were counterstained with Meyer's hematoxylin. Other sections treated with Tris-buffered saline instead of the primary antibody served as the negative control.

Tumor fields with the highest degree of MVD were identified for each tissue section. The intensity of immunohistochemical staining for VEGF, Ang-1, Ang-2, and TSP-1 in these fields was scored on a scale ranging from level 0 to 2. Level 0, 1, and 2 represented negative or faint, moderate and strong staining, respectively. The vascular epithelium in the normal liver field of the same specimen expressed moderate levels of Ang-1, Ang-2 and TSP-1 and the bile duct epithelium in the normal liver field of the same specimen expressed moderate levels of VEGF. Accordingly, these levels of staining were used as an internal control within the sample, which was arbitrarily designated as intensity level 1.

Statistical analysis. Unless otherwise indicated, numerical data are presented as mean ± SD. Differences in the proportions of categorical data were tested using the Chi-square test. Unless otherwise indicated, differences in mean values of numerical data were tested using the two-tailed Student's t-test. Survival (mean, medium survival days and 1-, 3- and 5-year cumulative survival rates) was assessed using the Kaplan-Meier method, and comparisons were made using the

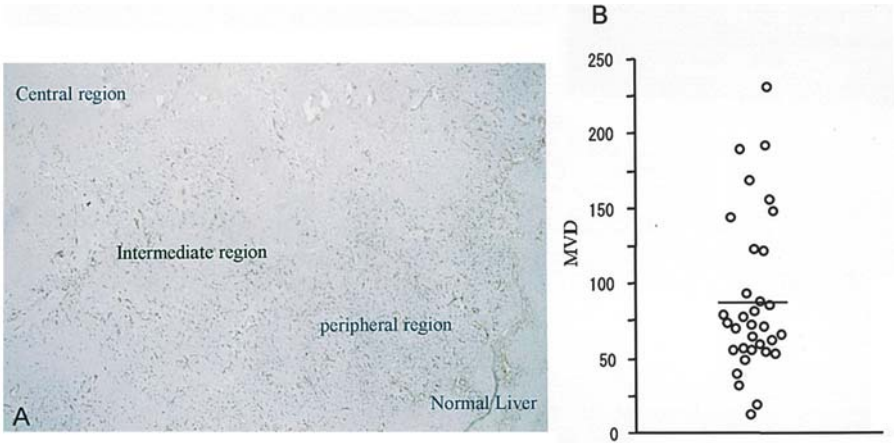

Figure 1. (A) CD34-stained photomicrography of mass forming CCC type. The tumor periphery displayed a higher degree of angiogenesis in comparison to the intermediate and central tumor regions. Original magnification, x20. (B) Scatter plot of MVD in CCC, with a range of 10-229/mm^2, mean of 87.2/mm^2 and median of 70.7/mm^2.

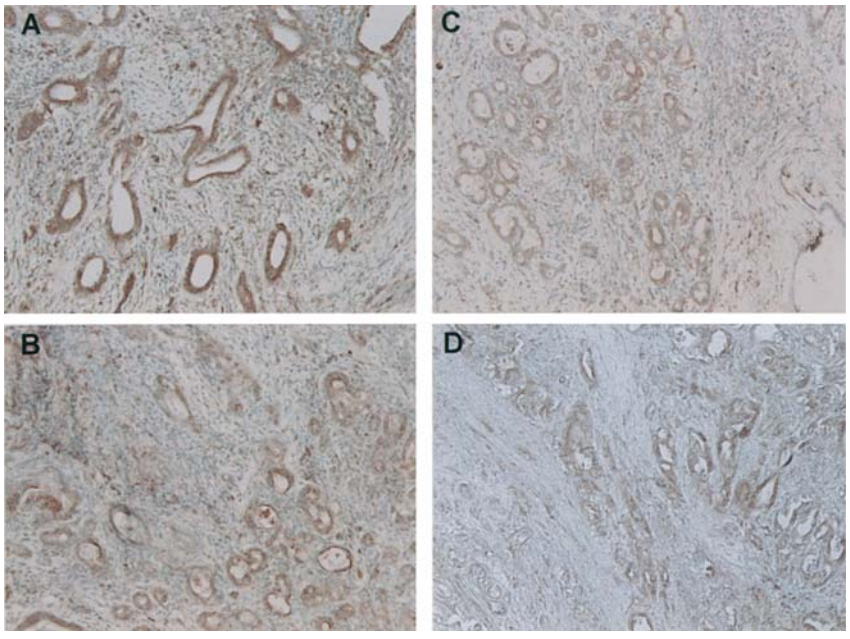

Figure 2. (A) Anti-VEGF-stained photomicrograph of a tissue sample from a CCC patient. Strong positive staining can be observed in the tumor cell cytoplasm. (B) Anti-Ang-1-stained photomicrograph of a tissue sample from the same CCC patient. Strong positive staining can be observed in the tumor cell cytoplasm. (C) Anti-Ang-2-stained photomicrograph of a tissue sample from the same CCC patient. Moderate positive staining can be observed in the tumor cell cytoplasm. (D) Anti-TSP-1-stained photomicrograph of a tissue sample from the same CCC patient. Strong positive staining can be observed in the tumor cell and stromal cell cytoplasm. Original magnification, x100.

log-rank test. A p-value <0.05 denoted the presence of a statistically significant difference. All analyses were performed using SPSS statistical software (version 11.5, Chicago, IL).

Results

Vascular distribution pattern in tumor and surrounding non-tumorous liver. The tumor region and surrounding normal liver tissue were included in the assessment of all CCC cases evaluated in this study. Within CCC, high vascularity with short, tortuous blood vessels was observed. Of the 20 cases of mass forming CCC type, 16 displayed a more condensed CD34 staining in the peripheral region in comparison with the central and intermediate regions (Fig. 1A). There was a significant decrease in MVD from the periphery (85.0±49.1/mm^2) to intermediate zone (44.2±32.4/mm^2), and also from the intermediate to the central zone (6.3±9.4/mm^2) (paired t-test, p<0.05 for each comparison). The other 4 cases displayed an almost evenly distributed CD34 staining within the entire tumor region. Uneven and irregular vascular distribution patterns within the tumor were found in 13 cases of intraductal growth type and periductal infiltrating type CCC. It seemed that massive fibrous tissue together with unevenly distributed cancerous tubular structure contributed to the irregular vascularity within the tumor. In the surrounding normal liver tissue, positive CD34 staining was also observed in the Glisson's triangle area and the central vein. These blood vessels were not as condensed as those within the tumor, were smaller in size, and generally associated with a regular round shape. Inflammatory white blood cell

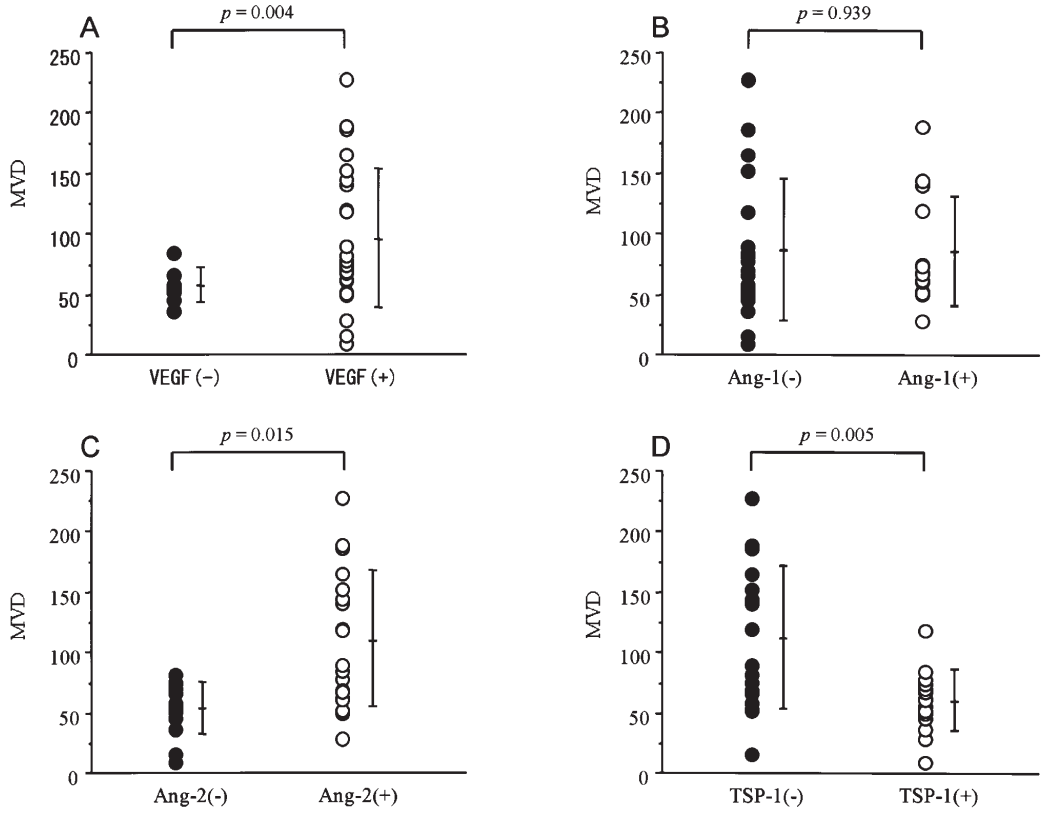

Figure 3. Comparison of mean MVD (± SD) between: (A) VEGF-positive (n=25) and VEGF-negative (n=8) cases; (B) Ang-1 positive (n=14) and Ang-1 negative (n=19) cases; (C) Ang-2-positive (n=17) and Ang-2-negative (n=16) cases; and (D) TSP-1-positive (n=15) and TSP-1-negative (n=18) cases. Data are displayed in scatter plots of MVD, and the bar showed mean ± SD.

infiltration was also noted in the Glisson's triangle, particularly in the intraductal growth and periductal infiltrating CCC types.

MVD of tumor and surrounding normal liver tissue. The MVD of tumor tissue (87.2±52.6/mm^2; range, 10-229/mm^2) (Fig. 1B) was significantly higher than that of the surrounding normal liver tissue (40.6±22.0/mm^2; range, 14-109/mm^2). For the 20 cases of mass forming CCC type, the MVD of the marginal area was significantly larger than that of the intermediate and central areas. The MVD of the intermediate area was significantly larger than that of the central area. The difference in MVD between the mass forming CCC type and intrahepatic growth/periductal infiltrating CCC type was not statistically significant (93.0±50.9 vs. 78.3±56.1/mm^2; p=0.442). However, the MVD of surrounding normal liver was significantly lower than that of the mass forming CCC type (34.2±17.6 vs. 50.4±25.0/mm^2; p=0.036).

Correlation between MVD and clinicopathological factors. We divided the patients into two subgroups based on the median value of MVD (75/mm^2), the high MVD and low MVD groups, and examined the correlation between the MVD level and several clinicopathological features. There was no correlation between MVD and tumor size, capsule formation, infiltration of capsule, portal vein invasion, intrahepatic metastasis or CCC differentiation.

Immunohistochemical staining of VEGF, Ang-1, Ang-2, and TSP-1. VEGF immunoreactivity was found in both the cytoplasm of cancer cells and the hepatocytes of adjacent liver tissue (Fig. 2A). However, the expression was considerably less intense in hepatocytes than in the CCC tissue. VEGF expression was not detected or faintly stained in 9 (27.2%) cases. However, 13 (39.4%) and 11 (33.3%) cases showed moderate and strong VEGF staining, respectively. Ang-1 and Ang-2 proteins were found in both the cytoplasm of cancer cells and the hepatocytes of adjacent liver tissue (Fig. 2B and C). Ang-1 expression was not detected or faintly stained in 19 (57.6%) cases. However, 9 (27.3%) and 5 (15.2%) cases showed moderate and strong Ang-1 staining, respectively. Ang-2 expression was not detected or faintly stained in 14 (42.4%) CCC cases. However, 14 (42.4%) and 5 (15.2%) CCC cases showed moderate and strong Ang-2 staining, respectively. TSP-1 immunoreactivity was found in the cytoplasm of cancer cells and stromal cells, but not in the hepatocytes or stromal cells from adjacent normal liver tissue (Fig. 2D). TSP-1 expression was not detected or faintly stained in 17 (51.1%) CCC cases. However, 15 (48.5%) and 1 (3.0%) CCC cases showed moderate and strong TSP-1 staining, respectively.

Correlation between MVD and VEGF, Ang-1, Ang-2 and TSP-1 expression. The mean MVD (± SD) of 25 VEGF-positive CCC cases (96.5±57.0/mm^2) was significantly higher than that of 8 VEGF-negative cases (58.0±14.4/mm^2; p=0.004) (Fig. 3A). However, the mean MVD (± SD) of 14 Ang-1-positive CCC cases (86.4±45.4/mm^2) was not different from that of 19 Ang-1-negative cases (87.8.5±58.6/mm^2; p=0.939) (Fig. 3B). The mean MVD (± SD) of 17 Ang-2-

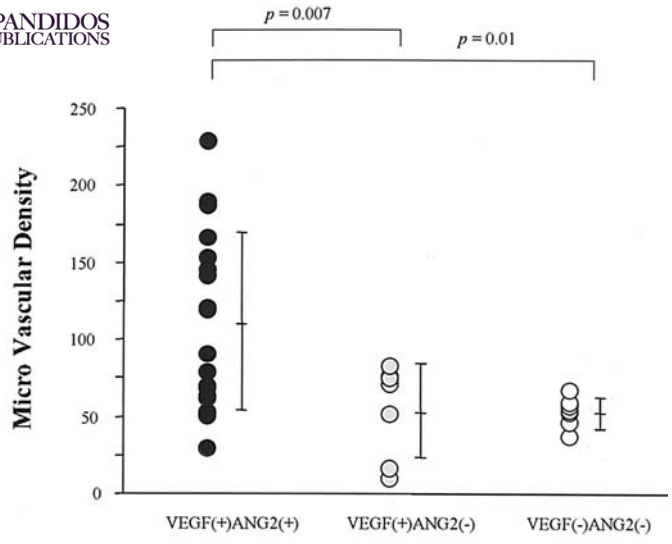

Figure 4. Comparison of MVD in VEGF-positive/Ang-2-positive, VEGF-positive/Ang-2-negative, and VEGF-negative/Ang-2-negative cases. Data are displayed in scatter plots of MVD and the bar showed mean ± SD.

positive CCC cases (108.2±56.0/mm^2) was significantly higher than that of 16 Ang-2-negative cases (64.9±39/mm^2, p=0.015, Fig. 3C). The mean MVD ((SD) of 15 TSP-1-positive CCC cases (61.0±25.8/mm^2) was significantly less than that of 18 TSP-1-negative cases (109.0±59.6/mm^2; p=0.005) (Fig. 3D). The patients were further grouped into group 1 (18 VEGF- and Ang-2-positive cases), group 2 (8 VEGF-positive and Ang-2-negative cases) and group 3 (7 VEGF- and Ang-2-negative cases). None of the cases was VEGF-negative and Ang-2-positive. The MVD of group 1 was significantly higher than that of groups 2 and 3 (p=0.013 and 0.001, respectively) (Fig. 4).

Relationship between VEGF, Ang-1, Ang-2, and TSP-1 expression and clinicopathological characteristics. Patients were divided into two groups, positive (moderate or strong staining) and negative (faint or no staining) for VEGF, Ang-1, Ang-2, and TSP-1 expression (Table I) in order to examine the correlation between the intensity of VEGF, Ang-1, Ang-2, and TSP-1 expression and several clinicopathological features. The likelihood of developing intrahepatic metastases in the TSP-1-positive group was significantly higher than in the TSP-1 negative group (46.7% vs. 5.6%; p=0.012) (Table II). On the other hand, there was no significant correlation between the intensity of VEGF, Ang-1, Ang-2, and TSP-1 expression and tumor size, capsule formation, infiltration of capsule, portal vein invasion, intrahepatic metastasis or differentiation of the CCC.

Table I. Results of immunohistochemical staining for VEGF, Ang-1, Ang-2 and TSP-1 expression in CCC tumors.

	Expression	
	Negative	Positive
VEGF	8 (24.2%)	25 (75.8%)
Ang-1	19 (57.6%)	14 (42.4%)
Ang-2	14 (42.4%)	19 (57.6%)
TSP-1	17 (51.5%)	16 (48.5%)

Data are number (percentage) of examined tumors.

Relationship between MVD, VEGF, Ang-1, Ang-2 and TSP-1 expression and postoperative overall survival. For statistical analysis of cumulative survival, cases were divided into two groups, positive (moderate or strong staining) and negative (faint or no staining) for VEGF, Ang-1, Ang-2, and TSP-1. We subdivided patients into two further groups of high MVD and low MVD based on the median value of MVD (75/mm^2). There was no significant correlation between MVD, VEGF, Ang-1, Ang-2, and TSP-1 expression and postoperative survival (Table III).

Discussion

The vascularity of CCC remains relatively poorly investigated or conflicting results have been reported. In one study, the mean MVD was as low as 10/mm^2 in CCC, as determined by

Table II. Relationship between TSP-1 expression and various clinicopathological parameters.

Factors	TSP-1 Positive (n=16)	TSP-1 Negative (n=17)	p-value
Age (years)[a]	66.3±9.1	59.8±11.7	0.088
Sex ratio (M/F)	9/7	9/8	1.000
Viral hepatitis B/C (-/+)	14/2	12/5	0.398
Portal vein invasion of cancer (-/+)	13/3	12/6	0.443
Lymphatic permeation of cancer (-/+)	9/7	9/8	1.000
Intrahepatic metastasis (-/+)	8/8	17/0	0.001
Tumor size (cm)[a]	4.7±2.8	5.5±3.9	0.480
Differentiation (H/L+M)	8/8	7/10	0.732

[a]Data are mean ± SD.

Table III. Results of univariate analysis of prognostic factors in 23 radically resected CCC.

Factors	Cumulative survival rates			p-value
	1-year	3-year	5-year	
Age				
≥64 (n=13)	67.7%	58.0%	46.4%	
<64 (n=10)	66.7%	35.6%	0%	0.988
Sex				
Male (n=10)	45.0%	45.0%	45.0%	
Female (n=13)	83.9%	65.3%	46.6%	0.157
Differentiation				
Well (n=12)	91.7%	41.2%	41.2%	
Moderate and poor (n=11)	45.5%	45.5%	45.5%	0.467
Lymphatic permeation of cancer				
(-) (n=15)	70.0%	56.0%	56.0%	
(+) (n=8)	62.5%	25.0%	25.0%	0.245
Intrahepatic metastasis				
(-) (n=22)	65.7%	54.7%	41.0%	
(+) (n=1)	100.0%	0%	0%	0.523
Portal vein invasion of cancer				
(-) (n=17)	67.9%	59.4%	59.4%	
(+) (n=6)	66.7%	16.7%	0%	0.241
MVD				
>70/mm^2 (n=13)	58.7%	44.1%	44.1%	
≤70/mm^2 (n=10)	78.8%	42.2%	0%	0.862
VEGF expression				
(-) (n=5)	40.0%	0%	0%	
(+) (n=18)	63.0%	42.0%	42.0%	0.847
Ang-1 expression				
(-) (n=13)	76.9%	46.1%	46.1%	
(+) (n=10)	51.4%	34.3%	34.3%	0.506
Ang-2 expression				
(-) (n=10)	70.0%	37.5%	37.5%	
(+) (n=13)	64.6%	48.5%	48.5%	0.716
TSP-1 expression				
(-) (n=15)	64.6%	29.1%	29.1%	
(+) (n=8)	72.9%	72.9%	72.9%	0.249

FVIII-RAb staining (5). In another study, the MVD was 61/mm^2 in CCC, as determined by CD31 staining, with mean and median values of 21±7/mm^2 and 17/mm^2, respectively (18). In contrast, assessment of 19 surgically resected CCC samples in another study showed a mean MVD of 126.5±45.1/mm^2 (19). In another study of 102 surgically resected cholangiocarcinoma cases, the mean MVD was 179.4±81.8/mm^2 for 49 cyclooxygenase-2 negative cholangiocarcinoma cases and 169.6±76.8/mm^2 for 53 cyclooxygenase-2 positive cholangiocarcinoma cases (4). These discrepancies in MVD may be partially due to the different biological characteristics of CCC in different regions, methodological differences, such as the anti-endothelial antibodies used (FVIII-RAb, anti-CD31, or anti-CD34, etc.) and the method of MVD quantification (highest microvascular density, average microvascular density and microvascular volume) or inter-observer variations. In one study, the mean MVD was calculated as the average number of microvessels observed in five random areas (5). This method counts the microvessel density in the central necrotic area of the tumor

explain the low MVD in CCC reported in that addition, the anti-endothelial antibody used for the staining of vascularity may also have an effect. FVIII-RAb is considered to be less accurate due to the absence of its target in some small immature capillaries and single endothelial cells (20). In addition, significant stromal background staining that assists the interpretation of staining data is often associated with the use of this antibody (21). Although anti-CD31 has better sensitivity and superior specificity to FVIII-RAb (20-22), it can also stain lymphatic endothelial and inflammatory cells, and antigens can sometimes be lost due to the use of fixatives that contain acetic acid (21,23). The anti-CD34 antibody stains small and large vessels with equal intensity in normal and tumor tissue; furthermore, it also stains immature capillaries and single endothelial cells with high specificity (24) and a staining performance superior to that of anti-CD31 (25). Therefore, in this study, anti-CD34 antibody was selected for the determination of MVD in formalin-fixed paraffin-embedded CCC specimens.

In the present study, the evaluation of MVD was based on the method proposed by Weidner *et al* (16). We initially identified regions of the highest vascular density under low magnification and marked them as 'hot spots.' For the mass forming CCC type, this was usually located in the peripheral zone of the tumor. The mean MVD of the five highest MVD counts in these 'hot spots' was recorded and regarded as the MVD of the tumor. Our results showed that the MVD of CCC ranged from 10 to 229/mm^2, with a mean value of 87.2±52.6/mm^2. We previously reported that the mean MVD in the peripheral region of metastatic colon carcinoma of the liver was 75.5±32.6/mm^2 (6). In another study (unpublished data), we found the mean MVD to be 93.1±43.8/mm^2, as determined by anti-CD34 staining of 60 HCC cases. The MVD in breast cancer and non-small cell lung cancer, as determined by anti-CD34 staining, was 51.4±23.8/mm^2 and 41.34±11.67/mm^2, respectively (26,27). Our results showed a considerable degree of tumor-associated angiogenesis comparable to that recorded in other well-vascularized tumors.

We found that the 16 cases of mass-forming CCC displayed a more condensed CD34 staining pattern in the peripheral regions. Indeed, there was a statistically significant progressive reduction in staining from the peripheral region to the intermediate zone and then to the central zone, which was often accompanied by complete or incomplete necrosis. The vascularity of the other 4 cases of mass-forming CCC was evenly distributed within the tumor. The robust angiogenesis at the tumor periphery may explain the usually observed 'ring enhancement' phenomenon of the mass forming CCC type in the artery phase of enhanced CT. In our previous study, the same phenomenon was observed in liver metastatic colorectal cancer (6). However, in another study of angiogenesis in HCC, we seldom found this phenomenon (unpublished data). These differences demonstrate the different patterns of angiogenesis in HCC, CCC and MCC. Nevertheless, in the other 13 intraductal growth type and periductal infiltrating type CCC cases, no regular vascular distribution pattern was observed. In some of these cases, the central tumor region showed a higher degree of vascularity in comparison to the peripheral tumor tissue and was often mixed with massive fibrous tissue.

Tumor-associated angiogenesis is a result of the combined action of stimulatory and inhibitory factors (28). VEGF is a specific and critical growth factor for blood vessel formation in various tumors (7). Several studies reported the presence of strong VEGF expression in CCC cell lines and surgically resected CCC tissues (19,29). Our results showed that 75.6% (25/33) of CCC samples displayed strong or moderate VEGF staining. This result is compatible with previously reported high VEGF expression rates in CCC (19,29). Our results also showed that the MVD was significantly higher in the VEGF-positive cases than that in VEGF-negative cases, suggesting that VEGF plays an important role in CCC angiogenesis.

Ang-2 is another family of endothelial cell-specific growth factors (8,9). It can loosen the interactions between endothelial and perivascular support cells and the matrix, compromise vascular integrity and facilitate access to proangiogenic inducers such as VEGF (9,12). To date, the role of Ang-2 in CCC angiogenesis and its relationship to other biological features have not been investigated. Our results showed that Ang-2 expression was observed in 57.6% of cases. The MVD in Ang-2-positive cases was significantly higher than that of Ang-2-negative cases. These results imply that angiopoietin-2 may play an important role in CCC angiogenesis. Our results also showed that in both VEGF- and Ang-2-positive cases, the MVD was significantly higher than that of VEGF-positive and Ang-2-negative cases, or both VEGF- and Ang-2-negative cases. In this group of patients, we did not observe VEGF-negative and Ang-2-positive cases. This result implies that VEGF and Ang-2 may act together to promote CCC angiogenesis. *In vitro* experiments showed that in cultured endothelial cells, Ang-2 mRNA was up-regulated by VEGF (30,31), and Ang-2 expression was associated with VEGF expression in the process of tumor angiogenesis (31). Furthermore, Holash *et al* found that VEGF up-regulation coincided with Ang-2 expression at the tumor periphery and was associated with robust angiogenesis (32). However, further studies that include a combination of immunohistochemistry, laser capture microdissection and real-time quantitative reverse transcription-PCR are necessary to understand the relationship between VEGF and Ang-2 expression in CCC.

In addition to VEGF and angiopoietins, TSP-1 may be another important factor with influence on CCC angiogenesis. Our results showed that TSP-1 immunoreactivity was found in the cytoplasm of cancer cells and stromal cells in 45.5% (15/33) of cases. The MVD of TSP-1-positive cases was significantly lower than that of the TSP-1-negative group. This result suggests that TSP-1 may be inhibitory to CCC angiogenesis and is in agreement with other similar studies (5,15). However, in HCC, a positive correlation between TSP-1 and VEGF expression was found, suggesting that TSP-1 may play a proangiogenic role in HCC (14). In another study (unpublished data), we did not find any correlation between TSP-1 expression and MVD in HCC. Our results showed that patients of the TSP-1-positive group are significantly more likely to develop intrahepatic metastasis than the TSP-1-negative group. This implies that TSP-1 expression may be associated with increased invasiveness of CCC. In this regard, TSP-1 expression is associated with an increased rate of lymphatic permeation in

intrahepatic cholangiocarcinoma (15) and an increased rate of venous invasion in HCC (14). The role of TSP-1 in angiogenesis and invasiveness of tumors deserves further investigation.

The relationship between angiogenesis and the prognosis of patients with CCC is seldom reported. In the present study, we found that neither MVD level nor the expression of VEGF, Ang-1, Ang-2, and TSP-1 was associated with prognosis. Shirabe *et al* reported that tumor angiogenesis was associated with a poorer prognosis in node-negative intrahepatic cholangiocarcinoma (18). They reported MVD in CCC as low as 21±7/mm^2, and only concentrated on the node-negative intrahepatic cholangiocarcinoma. Discrepancies in the relationship between angiogenesis and prognosis have been reported for other tumor types (24) and may be caused by particular tumor biological factors, such as the tumor location and accompanying liver disease, or by small study population, different case selection or heterogeneous therapy patterns. More cases and better standardized grouping are required to understand the clinicopathological and prognostic implications of tumor vascularity and associated factors.

In conclusion, a considerable degree of angiogenesis, comparable to that of other solid tumors, was observed in CCC. VEGF and Ang-2 might play a proangiogenic role, while TSP-1 may play an inhibitory role. Although TSP-1 may increase the intrahepatic metastasis of CCC, neither MVD nor the expression of VEGF, Ang-1, or Ang-2 was associated with tumor invasiveness and prognosis.

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