

ORIGINAL

Correlation of vascular endothelial cell proliferation with microvessel density and expression of vascular endothelial growth factor and basic fibroblast growth factor in hepatocellular carcinoma

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Abstract : Tumor-associated angiogenesis is essential for tumor growth or metastasis, and consists of multiple and sequential steps regulated by proangiogenic and antiangiogenic factors. Vascular endothelial cell proliferation is involved in this process. We investigated the correlation of vascular endothelial cell proliferation with microvessel density (MVD) and expression of major proangiogenic molecules, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in hepatocellular carcinoma (HCC).

Formalin-fixed paraffin-embedded specimens of surgically resected HCC from 67 patients were used. Proliferating endothelial cells were detected by immunofluorescence double staining for CD34 and proliferating cell nuclear antigen (PCNA). The proliferation activity of endothelial cells was determined by the rate of PCNA-positive endothelial cells, and evaluated at the periphery and center of the tumors and adjacent non-neoplastic livers. MVD and the expression of VEGF and bFGF in the tumors were also examined immunohistochemically.

The proliferation activity of endothelial cells at the periphery of the tumors was significantly higher than that at the center of the tumors (35.8% vs.12.7%, $P < 0.0001$). The rate of PCNA-positive endothelial cells in the tumors with higher bFGF expression was significantly higher than that in the tumors with lower bFGF expression (44.8% vs. 32.5%, $P < 0.005$) at the periphery of the tumors. There was no significant correlation between the rate of PCNA-positive endothelial cells and clinicopathological findings or MVD.

In HCC, the proliferation activity of vascular endothelial cells is suggested to be heterogeneous in the tumor and higher at the periphery of the tumor, and bFGF may play an important role in the positive regulation of tumor-associated vascular endothelial cell proliferation.

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Keywords : hepatocellular carcinoma, immunohistochemistry, proliferating cell nuclear antigen, angiogenesis, endothelial cell proliferation activity

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the commonest malignant tumors in the world. Surgical resection is the mainstay of treatment, but the prognosis after resection remains unsatisfactory because of the high incidence of recurrence (1-3). Most recurrences occur in the remnant liver rather than extrahepatic

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sites. Intrahepatic metastasis resulting from portal venous invasion is considered an important mechanism of intrahepatic recurrence (2, 3).

HCC is a hypervascular tumor characterized by neovascularization. The formation of neovasculature toward and within the tumor, *i.e.*, tumor-associated angiogenesis, is essential for tumor growth or metastasis, and increasing intratumoral microvessel density (MVD), major of tumor-associated angiogenesis, correlates with greater tumor aggressiveness, such as a higher frequency of metastasis and/or poor prognosis in HCC and other solid tumors (4-6). Tumor-associated angiogenesis is dependent on the local balance of proangiogenic and antiangiogenic factors. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are important positive regulators of tumor-associated angiogenesis, stimulate endothelial cell proliferation and enhance vascular permeability. Recent studies report that expression of the angiogenic factors such as VEGF, bFGF is closely related to growth, metastasis and prognosis of HCC (7-10).

Tumor-associated angiogenesis consists of multiple and sequential steps. In addition to vascular remodeling, sprouting and endothelial cell migration, endothelial cell proliferation is involved in this process (11). Tumor-associated endothelial cell proliferation has been investigated in breast carcinoma and prostate carcinoma. Proliferation activities of vascular endothelial cells in these tumors were significantly higher than that in adjacent non-neoplastic tissue (12, 13), and the size and frequency of vessels with proliferating endothelial cells in breast carcinoma were associated with lymph node status (14). In this study, we evaluated the proliferation activity of vascular endothelial cells in 67 surgical specimens of HCC at the periphery and center of tumors and adjacent non-neoplastic liver using immunofluorescence double staining technique (15, 16) for CD34, the most reproducible endothelial cell highlighter (17), and proliferating cell nuclear antigen (PCNA), which is related to cell proliferative activity (18). We also investigated the correlation of proliferation activities of vascular endothelial cells with clinicopathological findings, MVD, and VEGF and bFGF expression in tumors.

MATERIALS AND METHODS

Patients

Sixty-seven patients who underwent initially curative surgery for HCC at Tokushima University Hospital between 1990 and 2000 were randomly enrolled in

this study. The mean age of the patients was 61.2 years (range, 38-78 years). Fifty-two patients were male and fifteen were female. Patients whose resected specimens were inadequate or insufficient for this study were excluded. None of these patients had previous chemotherapy, including transarterial chemoembolization. Their formalin-fixed paraffin-embedded specimens were studied with informed consent.

Clinicopathological Findings

The clinicopathological findings used are listed in the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (19).

Immunofluorescence Double Staining for CD34 and PCNA

Paraffin sections (4 μ m) were deparaffinized with xylene, rinsed thoroughly with graded ethanol solution, then soaked in 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min at room temperature in order to inactivate endogenous peroxidase activity. To retrieve antigen, slides were boiled with 10mM citrate buffer for 15min. All sections were incubated with a 1 : 50 dilution of monoclonal mouse anti-human CD34 antibody (DakoCytomation, Denmark) containing 5% normal goat serum for 1 hour at room temperature. After the samples were rinsed twice for 5 min each with PBS, they were incubated with a 1 : 400 dilution of secondary Alexa 594-conjugated goat anti-mouse IgG antibody (Molecular Probes, Inc., Eugene, OR) for 1 hour at room temperature in the dark. Samples were then rinsed twice with PBS, incubated at 4 for 18 hours with a 1 : 50 dilution of polyclonal rabbit anti-human PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After the samples were rinsed twice for 5 min each with PBS, they were incubated with a 1:400 dilution of secondary Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) for 1 hour at room temperature in the dark. The samples were then rinsed twice for 5 min each with PBS and mounted with Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA). The images were captured using a CoolSNAP camera, Epi fluorescence upright microscopy system (Olympus Optical Co., Ltd., Tokyo, Japan), and MetaCam software (Universal Imaging Corporation, Downingtown, PA).

Immunohistochemical staining for CD34, VEGF and bFGF

Paraffin sections (4 μ m) were deparaffinized with xylene, rinsed thoroughly with graded ethanol solution, then soaked in 0.3% hydrogen peroxide in PBS for

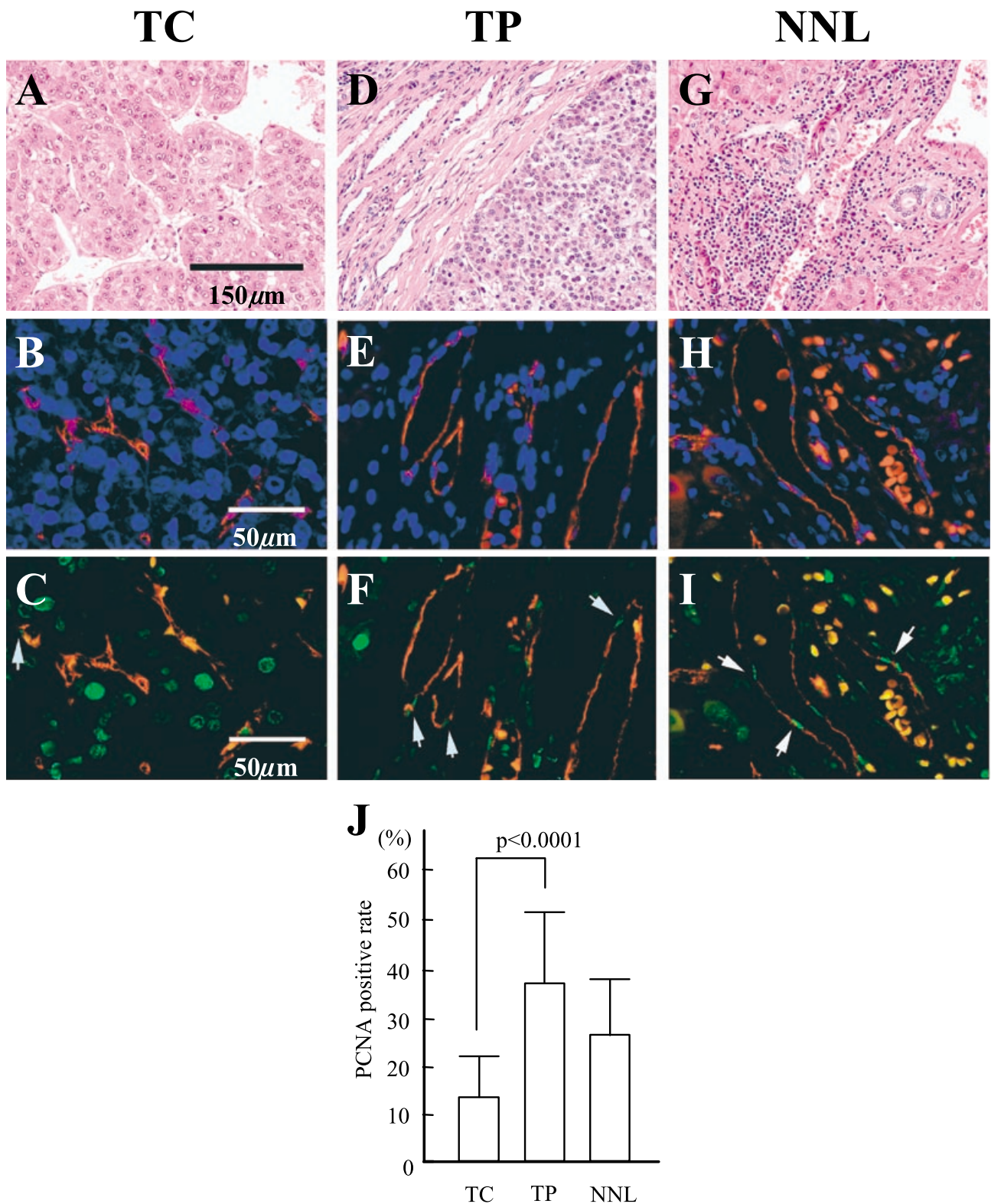


Figure 1. Analysis of vascular endothelial cell proliferation in HCC by immunofluorescence double staining for CD34 and PCNA at tumor center (TC) (A-C), tumor periphery (TP) (D-F) and non-neoplastic liver (NNL) (G-I). A, D and G, representative histological findings of three areas stained by hematoxylin and eosin (H & E). B, E and H, combined images of CD34-immunostaining (red) and nuclear staining with DAPI (blue) for the counting of endothelial cells. C, F and I, combined images of CD34-immunostaining (red) and PCNA-immunostaining (green) for the counting of PCNA-positive endothelial cells (arrows in C, F and I). The rate of PCNA-positive endothelial cells was defined as the ratio of PCNA-positive endothelial cells to the total endothelial cell count, and significantly higher at the periphery of tumors (35.8%) than the center (12.7%) at $P < 0.0001$ (J).

15 min at room temperature in order to inactivate endogenous peroxidase activity. To retrieve antigen, slides were boiled with 10mM citrate buffer for 15min. Sections were incubated with a primary antibody containing 5% normal goat serum for 1 hour at room temperature. The primary antibodies used were as follows : monoclonal mouse anti-human CD34 (DakoCytomation, Denmark) at 1 : 50 dilution, polyclonal rabbit anti-human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 : 50 dilution, and polyclonal rabbit anti-human bFGF (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 : 50 dilution. After washing they were incubated and visualized by the labeling streptavidin-biotin-peroxidase-complex method using DAKO LSAB[®]2 System (DAKO Co. Carpinteria, CA) with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) as a chromogen.

Determination of the rate of PCNA-positive vascular endothelial cells

The rate of PCNA-positive vascular endothelial cells was defined as the ratio of positively stained cells to

the total cell count and evaluated at the center and periphery of tumors and adjacent non-neoplastic livers. Ten fields were randomly selected from each area under $\times 400$ magnification, and three images ; nuclear staining with DAPI (blue fluorescence), immunohistochemical staining for PCNA (green fluorescence) and CD34 (red fluorescence), were independently captured at every field and combined using MetaCam software (Figure 1). Combined images of immunostaining for CD34 with nuclear staining were used for total endothelial cells counting, and combined images of immunostaining for CD34 with that for PCNA were used for PCNA-positive endothelial cells counting. More than 100 individual endothelial cell nuclei were counted.

Determination of MVD

Sections performed immunohistochemical staining for CD34 were assessed in five fields using a $\times 200$ magnifying lens to count the number of microvessels at the periphery of the tumor. MVD was expressed as the number of microvessels per square millimeter.

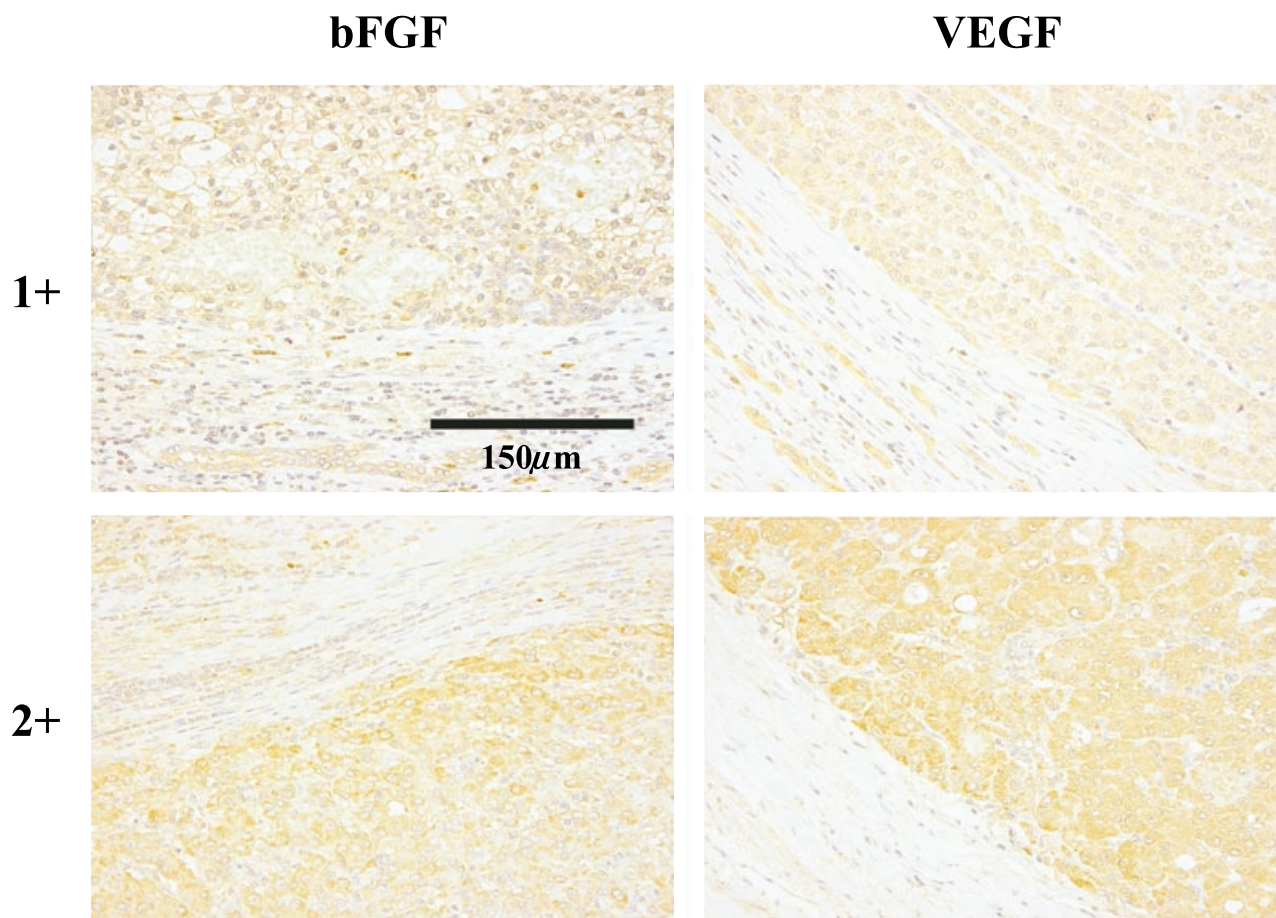


Figure 2. Immunohistochemical expression of bFGF and VEGF in HCC. The cytoplasmic intensity of epithelial cells of bile ducts in non-neoplastic liver was considered the control of immunohistochemical staining for bFGF and VEGF. If the tumor cells' cytoplasmic intensity was similar in degree with the biliary epithelial cells', the intensity was evaluated as 1+, and if the intensity was higher in degree than the biliary epithelial cells', it was evaluated as 2+.

Determination of the degree of VEGF, bFGF expression

The cytoplasmic intensity of epithelial cells of bile ducts in non-neoplastic liver was considered the control of immunohistochemical staining for VEGF and bFGF. If tumor cells' cytoplasmic intensity was similar in degree with the biliary epithelial cells', the intensity was evaluated as 1 +, and if the intensity was higher in degree than the biliary epithelial cells', it was evaluated as 2 + (Figure 2).

Histopathologic examination

Detailed histopathologic examination of all specimens in this study was performed by a senior pathologist specialized in HCC pathology, who was blind to the patients' clinical findings.

Statistical analysis

The results were reported as the mean \pm standard deviation. Unpaired *t*-test was used for all statistical analysis. The differences were considered significant when $P < 0.05$ in all examinations.

RESULTS*Correlation between the rate of PCNA-positive vascular endothelial cells and clinicopathological features*

The rate of PCNA-positive vascular endothelial cells was evaluated at the center and periphery of tumors and adjacent non-neoplastic livers. In non-neoplastic livers, vascular endothelial cells were examined at portal tracts and fibrous septa surrounding cirrhotic nodules

Table 1. Relationship between rates of PCNA-positive endothelial cells and clinicopathological findings in patients with HCCs

	No. of cases	Rates of PCNA-positive endothelial cells (%)		
		Tumor		Non-neoplastic liver
		Center	Periphery	
HBs Ag				
Positive	10	14.5 \pm 12.7*	37.5 \pm 18.6	15.9 \pm 10.3
Negative	57	12.4 \pm 8.5	35.4 \pm 14.2	27.1 \pm 12.7
HCV Ab				
Positive	50	12.4 \pm 8.7	35.9 \pm 14.2	27.5 \pm 12.8
Negative	17	13.6 \pm 10.7	35.3 \pm 16.8	19.1 \pm 11.3
Tumor size (cm)				
2	9	15.5 \pm 5.4	34.9 \pm 11.1	29.9 \pm 13.4
2 - 5	45	12.8 \pm 9.9	36.7 \pm 15.7	25.7 \pm 12.3
> 5	13	10.5 \pm 8.7	33.0 \pm 14.2	21.1 \pm 14.5
Degree of differentiation				
Well	26	11.4 \pm 7.1	34.8 \pm 13.7	26.6 \pm 13.7
Moderately	34	14.8 \pm 10.6	36.4 \pm 15.5	23.4 \pm 13.2
Poorly	7	7.6 \pm 6.1	36.2 \pm 17.0	30.7 \pm 6.0
Vp (tumor thrombus in the portal vein)				
Positive	25	13.3 \pm 9.2	38.2 \pm 14.4	24.3 \pm 12.1
Negative	42	12.4 \pm 9.2	34.3 \pm 15.0	26.1 \pm 13.4
Vv (tumor thrombus in the hepatic vein)				
Positive	3	19.1 \pm 13.0	45.1 \pm 7.8	19.7 \pm 19.8
Negative	64	12.4 \pm 9.0	35.3 \pm 14.9	25.7 \pm 12.7
Serum AFP (ng/ml)				
20	26	13.2 \pm 9.4	33.0 \pm 13.7	26.5 \pm 12.8
20 - 200	18	11.7 \pm 7.7	35.0 \pm 13.7	25.1 \pm 12.6
> 200	23	13.0 \pm 10.2	39.4 \pm 16.5	24.4 \pm 13.8

* Mean \pm SD

No significant relation was found.

Table 2. Expression of bFGF and VEGF in relation to rates of PCNA-positive endothelial cells and MVD at the periphery of tumors

	No. of cases (%)	Rates of PCNA-positive endothelial cells (%)	MVD (/mm ²)
bFGF			
1 +	49(73)	32.5 ± 15.0*	409.1 ± 103.8
2 +	18(27)	44.8 ± 9.9	411.7 ± 81.7
VEGF			
1 +	52(78)	34.8 ± 14.7	397.8 ± 96.0
2 +	15(22)	38.9 ± 15.1	451.5 ± 95.5
bFGF(1 +)/VEGF(1 +)	45(68)	33.5 ± 15.0	400.4 ± 101.8
bFGF(1 +)/VEGF(2 +)			
and	11(16)	34.8 ± 14.7	426.5 ± 84.4
bFGF(2 +)/VEGF(1 +)			
bFGF(2 +)/VEGF(2 +)	11(16)	45.8 ± 10.2	431.4 ± 95.6

* mean ± SD

** P=0.0019

*** P=0.0134

because most of sinusoidal endothelial cells were negative for CD34. PCNA-positive endothelial cells were observed in all sizes of vessels. PCNA positive rates at the center and periphery of tumors and non-neoplastic livers were 12.7% (0 - 43.8, median 10.5, SD 9.2), 35.8% (4.0 - 64.5, median 36.6, SD14.8) and 25.4% (1.3 - 59.2, median 24.0, SD12.9), respectively. PCNA positive rate was significantly higher at the periphery of tumors than the center ($P < 0.0001$) (Figure 1). There was no significant relation between PCNA positive rate and patients' background, clinicopathological findings (Table 1). In addition, PCNA positive rates at non-neoplastic liver were not related to the proliferation activity of endothelial cells at the center and periphery of tumors and tended to be higher in cases with severe inflammation.

MVD

MVD was counted at the periphery of tumors. The mean MVD was 409.8/mm² with a range of 217.0 to 643.0 (median 404.0, SD 97.8).

Correlation between the rate of PCNA-positive vascular endothelial cells, MVD and the expression of VEGF, bFGF

Expression of bFGF and VEGF was observed in all tumors and the expression levels of them tend to be lower at the center of tumors. As shown in Table 2, the rate of PCNA-positive vascular endothelial cells of 1 + and 2 + groups in bFGF expression, were 32.5 ± 15.0 and 44.8 ± 9.9. This difference was statistically significant ($P = 0.0019$). There was no significant difference

in PCNA positive rates between 1 + and 2 + groups in VEGF expression. When the cases were divided into three groups; bFGF 1 + /VEGF 1 + group, bFGF 1 + /VEGF 2 + and bFGF 2 + /VEGF 1 + group, and VEGF 2 + /bFGF 2 + group, PCNA positive rates were 33.5 ± 15.0, 34.8 ± 14.7, and 45.8 ± 10.2, respectively. There was statistically significant difference between bFGF 1 + /VEGF 1 + group and bFGF 2 + /VEGF 2 + group ($P = 0.0134$). MVD was not significantly related to the expression level of bFGF and VEGF, although MVD of 2 + group in VEGF expression tend to be higher than that of 1 + group. In addition, there was no significant relation between PCNA positive rate of vascular endothelial cells and MVD.

DISCUSSION

Compelling data implicate tumor-associated angiogenesis as the central pathologic step in the process of tumor growth, invasion, and metastasis (11, 20). Numerous experimental models and several studies in human tumors have shown that tumor-associated angiogenesis involves endothelial cell proliferation (12-14, 21). Studies of endothelial cell proliferation using human cancer tissue have been conducted on breast (12, 14) and prostate (13), but endothelial cell proliferation in HCC has not been fully investigated. In this study, we evaluated the proliferation activities of vascular endothelial cells in 67 surgical specimens of HCC and investigated the correlation of proliferation

activities of vascular endothelial cells with clinicopathological data, MVD and the expression of VEGF and bFGF in tumors.

Immunofluorescence double staining technique for CD34 and PCNA was used for the observation of proliferating endothelial cells. In this method, CD34 expression, PCNA expression, and nuclear staining were observed as red, green, and blue fluorescence, respectively. These images were captured independently in every field and two or all of them were combined. This process made the counting of endothelial cells easy and reliable.

In this study, we revealed that the mean of the rate of PCNA-positive vascular endothelial cells at the periphery of tumors was 35.8%, which is significantly higher than that at the center of tumors (12.7%) and is higher than that at non-neoplastic liver (25.4%). A highly neovascular area can occur anywhere within tumors but most frequently appears at the margin of tumors (17). The higher proliferation activity of endothelial cells at the periphery of tumors may partly reflect high neovascularization. There was no significant relation between the rate of PCNA-positive endothelial cells and clinicopathological findings.

We simultaneously evaluated MVD at the periphery of tumors but there was no significant correlation between MVD and the rate of PCNA-positive vascular endothelial cells. In the studies of prostate and breast carcinoma, vascular endothelial cell proliferation did not correlate with MVD, either (12, 13). MVD may not be reflected only by the degree of proliferation activity of endothelial cells but is also influenced by other angiogenic factors, including positive and negative regulators.

Additionally we evaluated the expression of proangiogenic factors such as VEGF and bFGF in HCC by immunohistochemistry. Poon *et al.* reported that there was a positive correlation between serum VEGF levels and tumor VEGF expression as assessed by immunohistochemical study and there was a significant correlation between serum VEGF levels and tumor invasiveness (8). They also reported regarding serum bFGF levels that a high preoperative serum bFGF level appeared to be predictive of invasive tumor and early postoperative recurrence (22). In our study, there was no significant correlation between the degree of VEGF expression and the rate of PCNA-positive vascular endothelial cells at the periphery of tumors. On the other hand, the rate of PCNA-positive vascular endothelial cells in the group with higher expression (2+) of bFGF was significantly higher than that in the group with lower expression (1+) of bFGF. The rate of PCNA-

positive vascular endothelial cells in the group with higher expression of both VEGF and bFGF (VEGF 2+ /bFGF 2+ group) was also significantly higher than that in the group with lower expression of both VEGF and bFGF (VEGF 1+ /bFGF 1+ group). In the process of tumor-associated angiogenesis, proliferation activity of endothelial cells may be activated by positive regulation of angiogenic factors such as VEGF, bFGF. On the contrary, no significant correlation was found between the expression of VEGF and bFGF and MVD at the periphery of tumors.

The findings of our study indicate that the proliferation activity of vascular endothelial cells in HCC is higher at the periphery of tumors and the expression level of bFGF in tumors, at least in cases with HCC, significantly correlates with the proliferation activity of endothelial cells at the periphery of tumors. The lack of correlation between MVD and the proliferation activity of endothelial cells is consistent with the concept that angiogenesis consists of a complex process that involves endothelial cell migration in response to chemotactic factors, neovascular remodeling and shedding of tumor cells by capillary budding, besides endothelial cell proliferation (23-26).

In conclusion, the proliferation activity of vascular endothelial cells is suggested to be heterogeneous in the tumor and higher at the periphery of the tumor, and bFGF may play an important role in the positive regulation of tumor-associated vascular endothelial cell proliferation in HCC.

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