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Immunohistochemical assessment of angiogenesis in hepatocellular carcinoma and surrounding cirrhotic liver tissues

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Abstract

AIM: To investigate whether vascular endothelial growth factor (VEGF) was over-expressed in hepatocellular carcinoma (HCC) or in surrounding cirrhotic liver tissues.

METHODS: Immunohistochemistry was performed to investigate the expression of VEGF proteins in HCC tissues from 105 consecutive patients undergoing curative resection for HCC. The immunostaining results and related clinicopathologic materials were analyzed with statistical methods. Kaplan–Meier method was used to calculate survival curves, and Log-rank test was performed to compare differences in survival rates of the patients with positive HCC staining and negative VEGF.

RESULTS: VEGF-positive expression was found in 72 of 105 HCC patients (68.6%). Capsular infiltration ($P = 0.005$), vascular invasion ($P = 0.035$) and intrahepatic metastasis ($P = 0.008$) were observed more frequently in patients with VEGF-positive expression than in those with VEGF-negative expression. Kaplan–Meier curves showed that VEGF-positive expression was associated with a shorter overall survival ($P = 0.014$). VEGF-positive expression was found in 47 of tissues 68 HCC (69.1%), and VEGF-positive expression was found in 54 of 68 surrounding cirrhotic liver tissues (79.4%). VEGF-positive expression was significantly higher in surrounding cirrhotic liver tissues than in HCC ($P = 0.017$).

CONCLUSION: VEGF may play an important role in the angiogenesis and prognosis of HCC, as well as in the angiogenesis of liver cirrhosis.

Key words: Angiogenesis; Vascular endothelial growth factor; Hepatocellular carcinoma; Surrounding cirrhotic liver tissues

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INTRODUCTION

Angiogenesis is a fundamental process involved in normal organ physiology, development and tissue repair, as well as, in a variety of pathological processes[1]. When blood vessels grow, angiogenesis becomes pathologic and sustains the progression of many neoplastic and non-neoplastic diseases[2]. VEGF is a potent mitogen specific for vascular endothelial cells and may directly stimulate the growth of new blood vessels[3]. VEGF has been reported to play an important role in the angiogenesis of HCC[4,5]. Studies have demonstrated that increasing expression of VEGF is correlated with aggressive behaviors and a poor prognosis of various human cancers including breast, gastric, esophageal and colorectal cancer[6-9]. Recent, evidence indicates that angiogenesis also plays an important role in the development of liver fibrosis[10]. It has been shown that VEGF expression significantly increases during the course of liver fibrosis in experimental studies and VEGF participates in sinusoidal capillarization in the liver[11]. It is reported that expression of VEGF in HCC is correlated with cirrhotic liver[12]. In this study, VEGF expressions in HCC and surrounding cirrhotic liver tissues were examined by immunohistochemical method.

MATERIALS AND METHODS

Patients

One hundred and five patients (79 males and 26 females, mean age 63±9 years, range 16-83 years) with singular nodule of HCC who had undergone curative hepatectomy from January 1993 to December 1997 at the Hepato-Biliary-Pancreatic Surgery Division, Graduate School of Medicine, University of Tokyo, Japan, were included in the study. Patients, who had previously a hepatectomy or hepatic arterial chemoembolization (TACE), were excluded. HCC tissues were obtained from all patients. HCC tissues and surrounding cirrhotic liver tissues were examined for VEGF. We also investigated the VEGF expression in normal liver tissue, that was surgically obtained from seven patients with

metastatic liver tumors derived from colon cancer, which were not associated with HCC, chronic viral hepatitis and autoimmune hepatitis. The patients were strictly followed up. The mean period of follow-up was 38.7 mo (38.7±18.1, range 2-75 mo).

The pathologic diagnosis and classification of variables were based on the criteria recommended in the General Rules for Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of Japan 1992). Clinicopathological parameters analyzed included sex, age, liver pathology (hepatitis *vs* cirrhosis), tumor size (<5 cm *vs* ≥ 5 cm), tumor differentiation (high, moderate, poor), capsule formation (presence *vs* absence), capsule infiltration (presence *vs* absence), vascular invasion (including vascular invasion and/or tumor thrombi in portal or hepatic vein), and intrahepatic metastasis (presence *vs* absence) (Table 1).

Immunohistochemistry

To obtain more accurate VEGF staining, we selected the tissue blocks containing HCC and surrounding liver tissues that were exposed to the same period of hypoxia. Five-micrometer thick sections were cut from formalin-fixed paraffin-embedded tissue blocks, deparaffinized and rehydrated in ethanol. The sections were incubated in 0.3% hydrogen peroxide in methanol for 30 min and in normal horse serum for 30 min at room temperature, followed by incubation overnight at 4 ℃ with anti-VEGF polyclonal antibody (A-20, sc-152G; Santa Cruz Biotechnology, Inc.) diluted at 1:100. Bound anti-body was detected by the avidin-biotin-peroxidase complex method, using a commercial kit as recommended by the manufacturer (Vestastain ABC Elite kit; Vector, Burlingame, CA). 3,3'-diaminobenzidine tetrahydrochloride was used as the chromogen and hematoxylin was used as a counterstain[13]. For negative control, 1.5% normal horse serum was used.

Evaluation of VEGF immunohistochemical staining

Positive staining for VEGF was located in cell cytoplasm. The percentage of cells stained positively for VEGF was evaluated by assessing 10 high-power microscopic fields (×400) in each section. In seven normal liver tissue specimens, the percentage of positively stained hepatocytes ranged from 90% to 100% (98.57±3.78) (Figure 1A). The expressions were graded as follows: negative if <60% of cancerous cells in a given specimen were positively stained; positive if ⩾60% of cancerous cells in a given specimen were positively stained; negative if <96% of surrounding cirrhotic liver cells in a given specimen were positively stained; positive if ⩾96% of surrounding cirrhotic liver cells in a given specimen were positively stained.

Statistical analysis

Quantitative data were expressed as mean±SD. Chi-square test was used for comparison between groups. Kaplan-Meier method was used to calculate survival curves, and Log-rank test was performed to compare differences in survival rates of the patient groups. $P<0.05$ was considered statistically significant.

RESULTS

Expression of VEGF in HCC tissue

The percentage of positively- stained HCC ranged 0-90% (58.67±25.51) (Figure 1B). VEGF-positive expression was found in 72 of 105 HCC patients (68.6%). Capsular infiltration ($P = 0.005$), vascular invasion ($P = 0.035$) and intrahepatic metastasis ($P = 0.008$) were observed more frequently in patients with VEGF-positive expression than in those with VEGF-negative expression (Table 1). Kaplan-Meier curves showed that VEGF-positive expression was associated with a shorter overall survival ($P = 0.014$) (Figure 2).

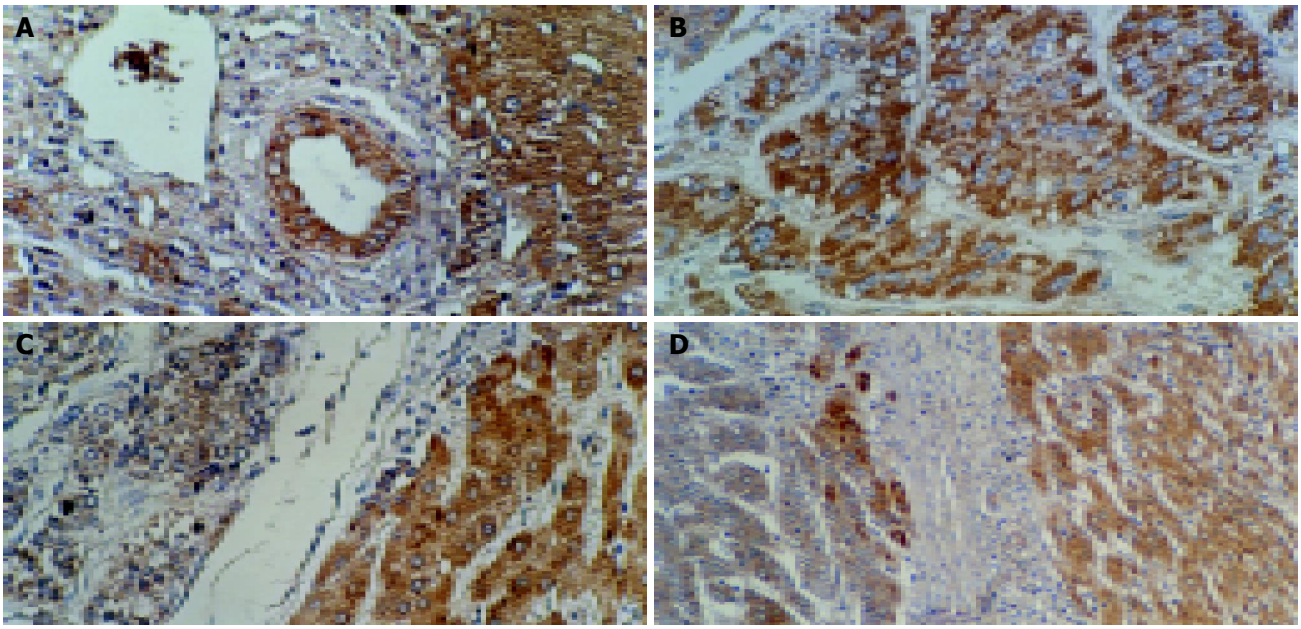

Figure 1 Positive expression of VEGF in normal epithelial and hepatic cells (A) (ABC, ×200), and in hepatocellular carcinoma cells (B) (ABC, ×200), negative expression of VEGF in hepatocellular carcinoma cells and positive expression of VEGF in surrounding cirrhotic liver cells (C, D) (ABC, ×200, ×100).

Table 1 Relationship between VEGF expression and clinicopathological Features of HCC (n=105)

Variables	Number of patients	VEGF expression		P
		Positive (n = 72)	Negative (n = 33)	
Sex				NS
Male	79	54	25	
Female	26	18	8	
Age (yr)				0.003
≤60	51	42	9	
>60	54	30	24	
Liver pathology				NS
Cirrhosis	68	47	21	
Hepatitis	37	25	12	
Tumor size				NS
<5	71	47	24	
≥5	34	25	9	
Tumor differentiation				NS
High	27	18	9	
Moderate	65	45	20	
Poor	13	9	4	
Capsule formation				NS
Presence	31	20	11	
Absence	74	52	22	
Capsule infiltration				0.005
Presence	65	51	14	
Absence	40	21	19	
Vascular invasion				0.035
Presence	34	28	6	
Absence	71	44	27	
Intrahepatic metastasis				0.008
Presence	23	21	2	
Absence	82	51	31	

NS: no significant difference.

Expression of VEGF in HCC tissue and surrounding cirrhotic liver tissue

In the 68 patients with HCC accompanied with liver cirrhosis, the percentage of positive staining in surrounding cirrhotic liver tissues ranged 50-100% (95.59±1.46). VEGF-positive expression was found in 47 of 68 HCC tissues (69.1%) and in 54 of 68 surrounding cirrhotic liver tissues (79.4%) (Figures 1C, D). VEGF-positive expression was significantly higher in surrounding cirrhotic liver tissues than in HCC tissues (P = 0.017) (Table 2).

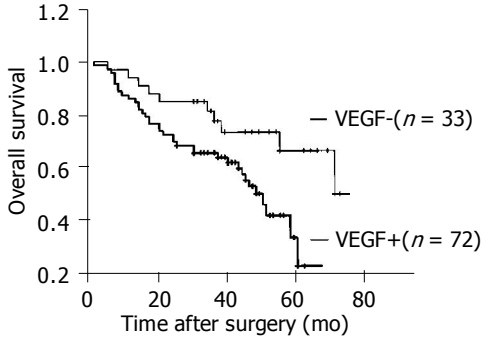

Figure 2 Kaplan–Meier curves for overall survival in 105 patients with HCC after operation (P = 0.014).

Table 2 VEGF expression in HCC and surrounding cirrhotic liver tissues (n=68)

	VEGF expression		P
	Positive n (%)	Negative n (%)	
HCC	47 (69.1)	21 (30.9)	
Surrounding Cirrhotic liver	54 (79.4)	14 (20.6)	0.017

DISCUSSION

VEGF is a potential tumor angiogenesis factor. In 1993, Kim *et al*[14] demonstrated that blocking the action of a paracrine mediator VEGF, that acts on the vasculature, may have a significant or even dramatic inhibitory effect on tumor growth and emphasized the significance of VEGF as an important mediator of tumor angiogenesis. Our study demonstrated that VEGF expression in 105 HCC patients had a significant correlation with capsular infiltration, vascular invasion, and intrahepatic metastasis. Furthermore, in our follow-up data, Kaplan-Meier curves showed that positive VEGF expressions in HCC correlated with shortened survival rates. These results suggest that VEGF may play an important role in angiogenesis and prognosis of HCC.

Strong evidence supports the hypothesis that VEGF is a key mediator of angiogenesis associated with various disorders[15]. Oxygen tension is a key regulator of VEGF gene expression both *in vitro* and *in vivo*[16]. VEGF as a hypoxia-inducible angiogenic factor has been extensively described in recent years[17, 18]. In 1998, El-Assal *et al*[12] showed that VEGF expression is significantly higher in cirrhotic liver tissues than in noncirrhotic liver tissues. In 1999, Shimoda *et al*[19] found a higher VEGF expression in cirrhosis but not in HCC. In 2000, Feng *et al*[20] reported that the positive rate of VEGF in HCC is significantly lower than in surrounding cirrhotic liver tissues (66.7% *vs* 85.4%). In this study, VEGF-positive expression was found in 47 of 68 HCC tissues (69.1%) and in 54 of 68 surrounding cirrhotic liver tissues (79.4%). Our data provides evidence that VEGF-positive expression is significantly higher in surrounding cirrhotic liver tissues than in HCC. It is possible that hepatocytes, in cirrhotic liver, are in a sustained mechanically-reduced blood flow, and the decreased oxygen pressure strongly up-regulates VEGF transcription and protein synthesis in the cirrhotic liver[12]. The excessive VEGF produced and secreted by hepatocytes and HCC cells may subsequently act on endothelial cells, resulting in growth of new blood vessels and capillarization of sinusoidal endothelial cells[21]. In addition, VEGF-positive expression is higher in HCC marginal areas than in HCC central areas[22]. Tumor cells expressing VEGF may have a growth advantage and proliferate more rapidly than cells that do not express VEGF. Rapid cell proliferation in the center of a tumor can lead to increased interstitial fluid pressure, which may result in compression closure of capillaries and consecutive tissue necrosis[23]. Central necrosis areas cause a suppression of VEGF protein synthesis[24]. Moreover, VEGF expression in surrounding cirrhotic liver tissues is also modulated by inflammatory cytokines released from infiltrating inflammatory cells. Several cytokines, such as basic fibroblast growth factor, transforming growth factor α and β,

epidermal growth factor and platelet-derived growth factor have been reported to act cooperatively on VEGF expression[25-31]. These results suggest that VEGF also plays an important role in the development of liver cirrhosis.

In conclusion, VEGF-positive expression in HCC has a significant correlation with capsular infiltration, vascular invasion, and intrahepatic metastasis. VEGF may play an important role in the angiogenesis and prognosis of HCC, as well as in the angiogenesis of liver cirrhosis.

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REFERENCES

1 **Folkman J,** Shing Y. Angiogenesis. *J Biol Chem* 1992; **267:** 10931–10934
2 **Folkman J.** Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N Engl J Med* 1995; **333:** 1757–1763
3 **Leung DW,** Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989; **246:** 1306–1309
4 **Mise M,** Arii S, Higashituji H, Furutani M, Niwano M, Harada T, Ishigami S, Toda Y, Nakayama H, Fukumoto M, Fujita J, Imamura M. Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology* 1996; **23:** 455–464
5 **Park YN,** Kim YB, Yang KM, Park C. Increased expression of vascular endothelial growth factor and angiogenesis in the early stage of multistep hepatocarcinogenesis. *Arch Pathol Lab Med* 2000; **124:** 1061–1065
6 **Linderholm BK,** Lindh B, Beckman L, Erlanson M, Edin K, Travelin B, Bergh J, Grankvist K, Henriksson R. Prognostic correlation of basic fibroblast growth factor and vascular endothelial growth factor in 1307 primary breast cancers. *Clin Breast Cancer* 2003; **4:** 340–347
7 **Maeda K,** Kang SM, Ogawa M, Onoda N, Sawada T, Nakata B, Kato Y, Chung YS, Sowa M. Combined analysis of vascular endothelial growth factor and platelet-derived endothelial cell growth factor expression in gastric carcinoma. *Int J Cancer* 1997; **74:** 545–550
8 **Inoue K,** Ozeki Y, Suganuma T, Sugiura Y, Tanaka S. Vascular endothelial growth factor expression in primary esophageal squamous cell carcinoma. Association with angiogenesis and tumor progression. *Cancer* 1997; **79:** 206–213
9 **Ishigami SI,** Arii S, Furutani M, Niwano M, Harada T, Mizumoto M, Mori A, Onodera H, Imamura M. Predictive value of vascular endothelial growth factor (VEGF) in metastasis and prognosis of human colorectal cancer. *Br J Cancer* 1998; **78:** 1379–1384
10 **Yoshiji H,** Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, Wu Y, Yanase K, Namisaki T, Yamazaki M, Tsujinoue H, Imazu H, Masaki T, Fukui H. Vascular endothelial growth factor and receptor interaction is a prerequisite for murine hepatic fibrogenesis. *Gut* 2003; **52:** 1347–1354
11 **Corpechot C,** Barbu V, Wendum D, Kinnman N, Rey C, Poupon R, Housset C, Rosmorduc O. Hypoxia-induced VEGF and collagen I expressions are associated with angiogenesis and fibrogenesis in experimental cirrhosis. *Hepatology* 2002; **35:** 1010–1021
12 **El-Assal ON,** Yamanoi A, Soda Y, Yamaguchi M, Igarashi M, Yamamoto A, Nabika T, Nagasue N. Clinical significance of microvessel density and vascular endothelial growth factor expression in hepatocellular carcinoma and surrounding liver: possible involvement of vascular endothelial growth factor in the angiogenesis of cirrhotic liver. *Hepatology* 1998; **27:** 1554–1562
13 **Hui AM,** Li X, Makuuchi M, Takayama T, Kubota K. Overexpression and lack of retinoblastoma protein are associated with tumor progression and metastasis in hepatocellular carcinoma. *Int J Cancer* 1999; **84:** 604–608
14 **Kim KJ,** Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993; **362:** 841–844
15 **Yancopoulos GD,** Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* 2000; **407:** 242–248
16 **Ferrara N.** Molecular and biological properties of vascular endothelial growth factor. *J Mol Med (Berl)* 1999; **77:** 527–543
17 **Kim KR,** Moon HE, Kim KW. Hypoxia-induced angiogenesis in human hepatocellular carcinoma. *J Mol Med (Berl)* 2002; **80:** 703–714
18 **Cejudo-Martin P,** Morales-Ruiz M, Ros J, Navasa M, Fernandez-Varo G, Fuster J, Rivera F, Arroyo V, Rodes J, Jimenez W. Hypoxia is an inducer of vasodilator agents in peritoneal macrophages of cirrhotic patients. *Hepatology* 2002; **36:** 1172–1179
19 **Shimoda K,** Mori M, Shibuta K, Banner BF, Barnard GF. Vascular endothelial growth factor/vascular permeability factor mRNA expression in patients with chronic hepatitis C and hepatocellular carcinoma. *Int J Oncol* 1999; **14:** 353–359
20 **Feng DY,** Shen M, Zheng H, Cheng RX. Relationship between vascular endothelial growth factor expression and microvessel density in hepatocellular carcinomas and their surrounding liver tissue. *Hunan Yike Daxue Xuebao* 2000; **25:** 132–134
21 **Jeng KS,** Sheen IS, Wang YC, Gu SL, Chu CM, Shih SC, Wang PC, Chang WH, Wang HY. Prognostic significance of preoperative circulating vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma: a prospective study. *World J Gastroenterol* 2004; **10:** 643–648
22 **An FQ,** Matsuda M, Fujii H, Matsumoto Y. Expression of vascular endothelial growth factor in surgical specimens of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2000; **126:** 153–160
23 **Plate KH,** Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature* 1992; **359:** 845–848
24 **Lang KJ,** Kappel A, Goodall GJ. Hypoxia-inducible factor-1αlπηα mRNA contains an internal ribosome entry site that allows efficient translation during normoxia and hypoxia. *Mol Biol Cell* 2002; **13:** 1792–1801
25 **Yamaguchi R,** Yano H, Iemura A, Ogasawara S, Haramaki M, Kojiro M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology* 1998; **28:** 68–77
26 **Steinbrech DS,** Longaker MT, Mehrara BJ, Saadeh PB, Chin GS, Gerrets RP, Chau DC, Rowe NM, Gittes GK. Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. *J Surg Res* 1999; **84:** 127–133
27 **Pepper MS,** Ferrara N, Orci L, Montesano R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. *Biochem Biophys Res Commun* 1992; **189:** 824-831
28 **Finkenzeller G,** Marme D, Weich HA, Hug H. Platelet-derived growth factor-induced transcription of the vascular endothelial growth factor gene is mediated by protein kinase C. *Cancer Res* 1992; **52:** 4821–4823
29 **Pertovaara L,** Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* 1994; **269:** 6271–6274
30 **Pepper MS.** Manipulating angiogenesis. From basic science to the bedside. *Arterioscler Thromb Vasc Biol* 1997; **17:** 605–619
31 **Mandriota SJ,** Pepper MS. Vascular endothelial growth factor-induced *in vitro* angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. *J Cell Sci* 1997; **110**(Pt 18): 2293–2302