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

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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.
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 °C 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](image-url) 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).
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%). Our data provides evidence that VEGF-positive expression is significantly higher in cirrhotic liver tissues than in noncirrhotic liver tissues. In 1999, Shimoda et al[9] demonstrated that VEGF expression is significantly higher in cirrhotic liver tissues than in noncirrhotic liver tissues. In 1999, Shimoda et al[9] found a higher VEGF expression in cirrhosis but not in HCC. In 2000, Feng et al[10] 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 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[20]. In addition, VEGF-positive expression is higher in HCC marginal areas than in HCC central areas[20]. 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[21]. Central necrosis areas cause a suppression of VEGF protein synthesis[22]. 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,

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

NS: no significant difference.

**DISCUSSION**

VEGF is a potential tumor angiogenesis factor. In 1993, Kim et al[13] 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[14]. 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[9] found a higher VEGF expression in cirrhosis but not in HCC. In 2000, Feng et al[10] 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 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[20]. In addition, VEGF-positive expression is higher in HCC marginal areas than in HCC central areas[20]. 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[21]. Central necrosis areas cause a suppression of VEGF protein synthesis[22]. 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,

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

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

NS: no significant difference.

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

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

**Figure 2 Kaplan–Meier curves for overall survival in 105 patients with HCC after operation (P = 0.014).**
epidermal growth factor and platelet-derived growth factor have been reported to act cooperatively on VEGF expression. 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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