

# Microvessel Density, Vascular Endothelial Growth Factor and Its Receptors Flt-1 and Flk-1/KDR in Hepatocellular Carcinoma

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**Key Words:** Microvessel density; Vascular endothelial growth factor; VEGF; Flt-1; Flk-1/KDR; Hepatocellular carcinoma

## Abstract

*Assessment of angiogenesis may yield important information for an effective antiangiogenic treatment for hepatocellular carcinoma (HCC) because HCC is characteristically hypervascular. We examined the relationship of microvessel density (MVD), vascular endothelial growth factor (VEGF), and VEGF receptors Flt-1 and Flk-1/KDR in 50 patients with HCC and in 3 hepatoma cell lines. VEGF messenger RNA (mRNA) was overexpressed in 26 tumors (52%), and the 3 VEGF isoforms (121, 165, and 189) were present in high frequencies. Flt-1 mRNA was overexpressed in 34 tumors (68%), with levels significantly increased in HCCs compared with the nontumorous livers. Tumor Flt-1 mRNA significantly correlated with tumor VEGF mRNA levels. Within the group of tumors 8.5 cm or less in diameter, tumors with intrahepatic metastasis in the form of tumor microsatellite formation had significantly higher VEGF mRNA levels. MVD assessed by immunohistochemical analysis with CD34 antibody was inversely related to tumor size. Angiogenesis as assessed by MVD and tumor VEGF expression seems to have a more important role in tumor growth and intrahepatic metastasis in smaller HCCs. The differential up-regulation of Flt-1 suggests that it may have an important role in angiogenesis in HCC.*

Extensive experimental and clinical data have clearly established that tumor growth is angiogenesis dependent.<sup>1</sup> Angiogenesis is essential for solid tumors to grow beyond 1 or 2 mm in diameter. In addition, angiogenesis must occur for metastasis formation and growth. Specific factors must be expressed and the appropriate receptors must be present on the target endothelium to initiate basement membrane degradation, endothelial cell proliferation and migration, and capillary tubule formation. The dynamic process of angiogenesis is regulated by a balance of positive and negative regulators, and the induction and maintenance of an angiogenic response occur through the release of various angiogenic factors from tumor cells. Vascular endothelial growth factor (VEGF) seems to have a central role in the process of tumor-associated angiogenesis.<sup>2,3</sup> It has been found to be overexpressed in many tumors, including carcinomas of the colon,<sup>4</sup> breast,<sup>5,6</sup> endometrium,<sup>7</sup> and ovary.<sup>8</sup>

Four isoforms of the *VEGF* gene are encoded through alternate exon splicing, and the 121- and 165-amino acid forms dominate in tumors. Two of the 4 isoforms are secreted and bind to specific, high-affinity receptor tyrosine kinases, Flt-1 and Flk-1/KDR, which are found almost exclusively on endothelial cells.<sup>9</sup> Via interaction with the receptors, the VEGF protein signals the endothelial cells to proliferate, migrate, and form capillary tubules. In contrast, deletion of 1 of the 7 extracellular domains of Flt-1 completely abolishes the binding of VEGF.<sup>10</sup> Such a VEGF-receptor system has been demonstrated in the liver.<sup>11</sup> Transcripts of Flt-1 and Flk-1/KDR are strongly expressed in rat sinusoidal cells but are barely detectable in hepatocytes, whereas VEGF transcript is weakly produced in hepatocytes and is not expressed in sinusoidal cells.

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms in the world and is the second most common fatal cancer in Hong Kong and Southeast Asia. It is characteristically a highly vascular tumor, and its diagnosis often relies on imaging techniques making use of its hypervascularity. It has a high risk of spontaneous rupture, leading to massive hemorrhage, and venous permeation and spreading of the tumor by venous route are found commonly. There have been a few reports on VEGF expression in HCC, but the results are conflicting.<sup>12-19</sup> Moreover, the expression of the receptors of VEGF in human HCC remains to be elucidated.<sup>14</sup> Intratumoral microvessel density (MVD), highlighted by endothelial markers, has been reported to be an independent predictor of prognosis in patients with cancers. However, there are few reports on the relationship between MVD and VEGF expression in HCC.

We report our findings on MVD and expression of VEGF and its receptors, Flt-1 and Flk-1/KDR, in human HCC and HCC cell lines and their clinicopathologic significance.

## Materials and Methods

### Patients and Tissue Samples

Fifty patients with HCC who underwent surgical resections between March 1990 and December 1994 at Queen Mary Hospital, Hong Kong, were studied. None of the patients had received other treatments for HCC, such as percutaneous ethanol injection or transcatheter arterial chemoembolization, before surgical resection. None had apparent distant metastatic disease. Of the patients, 43 were men and 7 were women; age ranged from 33 to 71 years (mean, 52.7 years). Serum hepatitis B surface antigen and anti-hepatitis B surface antigen were assayed by an enzyme immunoassay test (Abbott Laboratories, Chicago, IL). In each case, tumor and nontumorous liver tissues were obtained immediately after resection, snap-frozen in liquid nitrogen, and kept at  $-70^{\circ}\text{C}$ . The tumor tissues were obtained randomly from the tumor masses, but necrotic areas were avoided. The nontumorous liver tissues were obtained at sites away from the tumors.

### Cell Culture

Three HCC cell lines, Huh-7 (provided by H. Nakabayashi, PhD, Hokkaido University School of Medicine, Hokkaido, Japan), SMMC7701, and BEL7402 (both provided by J.R. Gu, MD, Shanghai Cancer Institute, Shanghai, China), were studied for the messenger RNA (mRNA) expression of VEGF, Flt-1, and KDR. The cell lines were maintained in Dulbecco modified Eagle minimal essential medium with high glucose (Gibco BRL, Grand Island,

NY), supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO), 50 U/mL of penicillin G (Gibco BRL) and 50  $\mu\text{g}/\text{mL}$  of streptomycin (Gibco BRL) at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5% carbon dioxide.

### Reverse Transcriptase–Polymerase Chain Reaction Analysis of VEGF, Flt-1, and KDR Transcripts

For reverse transcriptase–polymerase chain reaction (RT-PCR), total RNA was extracted from tumor and nontumorous liver in each case using guanidinium isothiocyanate solution. The total RNA (3  $\mu\text{g}$ ) was reverse transcribed according to the manufacturer's recommendation (Perkin-Elmer Cetus, Norwalk, CT). PCRs for VEGF, Flt-1, and beta-actin were performed using the respective primer sets to amplify complementary DNA (cDNA): 5'-TGCTCTACCTC-CACCATGCCAAGT-3' and 5'-GCGCAGAGTCTC-CTCTTCCTTCAT-3' for VEGF; 5'-ATTTGTGATTTTGGC-CCTGC-3' and 5'-CAGGCTAACTTGAAAGC-3' for Flt-1; 5'-GTCAAGGGAAAGACTACGTTGG-3' and 5'-AGCAGTCCAGCATGGTCTG-3' for KDR; and 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAAT-GTCACGCACGATTTC-3' for beta-actin. In brief, 30 to 35 cycles of PCR were performed with a 1-minute denaturation at  $94^{\circ}\text{C}$ , 1-minute annealing ( $60^{\circ}\text{C}$  for VEGF and beta-actin and  $57^{\circ}\text{C}$  for Flt-1 and KDR), a 1-minute extension at  $72^{\circ}\text{C}$ , and a final 7-minute extra extension at the end of the reaction to ensure that all amplicons were completely extended. Amplification was performed twice for each case in at least 2 different amplification sessions.

### Quantitation of PCR Products

Twenty microliters of each of the reaction products was visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide and quantified by using the GDS 8000 gel documentation and analysis system (Ultra-Violet Products, Upland, CA). The level of gene expression was calculated after normalization of the PCR product with the beta-actin control. In all cases, the PCR products were obtained at 35 cycles for VEGF, Flt-1, and KDR and 30 cycles for beta-actin for quantification. The number of PCR cycles for each product was determined after confirmation of the efficacy of amplification and defining the linear exponential portion of the amplification. Amplification was performed at least twice for each case in at least 2 different amplification sessions. The mean of the normalized values of the 2 independent experiments was calculated in each case.

### Sequencing of the RT-PCR Product

A random sample was amplified for 45 cycles. After gel electrophoresis, the VEGF band of the smallest size (574 base pairs [bp]) and the Flt-1 and KDR bands were dissected on a moderate-wave UV illuminator and extracted from the

gel using GFX PCR DNA and Gel Band Purification (Amersham Pharmacia Biotech, Buckinghamshire, England) according to the recommendations of the manufacturer. The extraction product underwent direct sequencing using a dRhodamine Terminator Cycle Sequencing kit (ABI prism 377 DNA sequencer, Perkin-Elmer, Foster City, CA) for 25 cycles according to the manufacturer's instruction.

### Immunohistochemical Staining

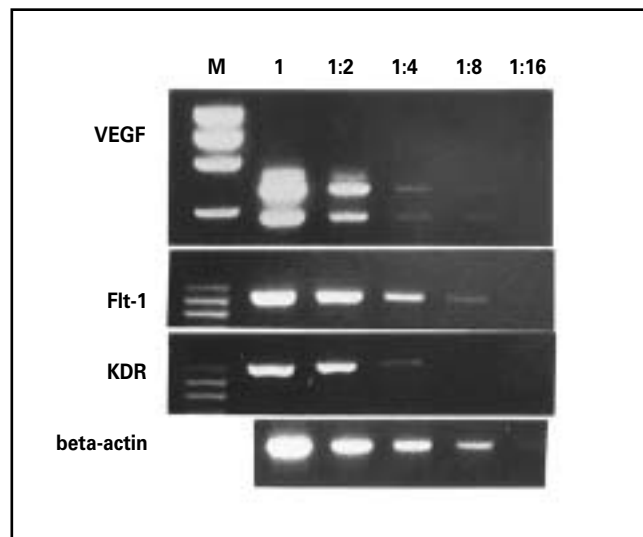
Immunohistochemical staining was performed for VEGF on formalin-fixed paraffin sections, using the streptavidin-biotin immunoperoxidase technique. Rabbit polyclonal antibody for human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:10 dilution with prior trypsinization. The surrounding liver tissue served as an internal positive control. A negative control was used for each case by replacing the primary antibody with normal rabbit serum. The blood vessels were highlighted by immunostaining with CD34 monoclonal antibody (BioGenex, San Ramon, CA) at a 1:1 dilution. Antigen retrieval with microwave treatment with citrate buffer, pH 6.0, for 11 minutes was used. Because immunostaining is not very accurate for quantitation of the amount of cytoplasmic protein, we used the immunostaining for VEGF to localize instead of quantifying the protein.

### Evaluation of MVD

MVD was assessed by the method defined by Weidner et al.<sup>20</sup> The vessels were highlighted by staining with CD34. We chose CD34 because it was found to be more sensitive than other antibodies for endothelial cells in the liver.<sup>21</sup> The slides of tumor and nontumorous livers were scanned at low magnification ( $\times 40$ - $\times 100$ ), and 3 areas with the highest density of vessels (hot spots) were evaluated in the tumor and nontumorous livers, respectively. The vessels highlighted by stained endothelial cells were counted within  $3 \times 200$  fields ( $0.74 \text{ mm}^2$ ). The mean value of the counts of the 3 fields was taken as the MVD. Any highlighted endothelial cell or endothelial cell cluster that was separate from the adjacent microvessels was considered a single and countable microvessel. Areas with tumor necrosis or granulation tissue were avoided.

### Pathologic Examination

The assessments of the pathologic features were done according to the method previously described.<sup>22</sup> Tumor size and the number of tumor nodules were assessed by gross examination. The presence of a tumor capsule was noted by microscopic examination. Spread of the tumor was assessed by the evidence of tumor microsatellite formation, venous invasion, and direct invasion into the adjacent liver parenchyma. Tumor microsatellite formation and venous invasion were taken as evidence of intrahepatic metastasis.



**Image 1** Validation test for polymerase chain reaction (PCR). Twofold serial dilutions of complementary DNA were amplified with primers for vascular endothelial growth factor (VEGF), Flt-1, KDR (35 cycles for each), and beta-actin (30 cycles). PCR products were visualized by electrophoresis on agarose gel stained with ethidium bromide and quantified by densitometry. M, marker.

Cellular differentiation was classified according to Edmondson and Steiner.<sup>23</sup> The presence of cirrhosis and chronic hepatitis in the nontumorous liver and tumor at resection margin histologically was assessed.

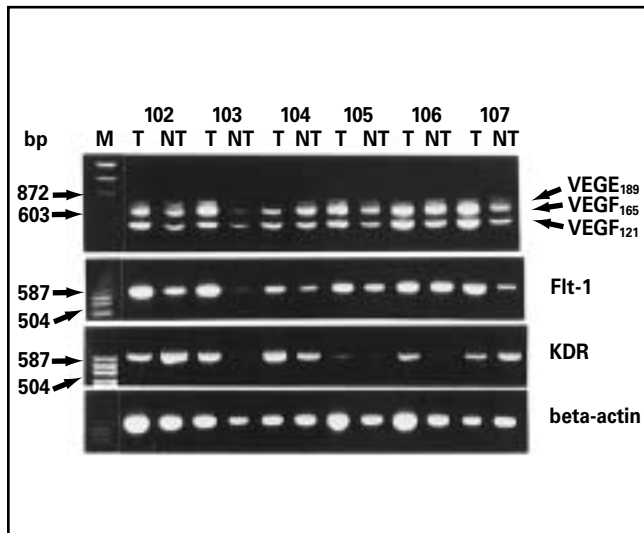
### Statistical Analysis

The Fisher exact and chi-square tests were used for the analysis of categorical data, whereas analysis of variance, the *t* test, and the Kruskal-Wallis test were used for continuous data as appropriate. The correlation analysis was performed with the Pearson product-moment correlation coefficient. Tests were considered significant when their *P* values were less than .05.

## Results

### Validation Test for PCR

We verified that our PCR conditions met the requirements for quantitative analysis by amplifying serial dilutions of cDNA with VEGF, Flt-1, and KDR primers for 35 cycles and with beta-actin primers for 30 cycles **Image 1**. Quantification of the products by densitometry showed a strong correlation ( $r = 0.989, 0.922, 0.993, \text{ and } 0.994$  for VEGF, Flt-1, and KDR and with beta-actin, respectively) between the initial amount of cDNA and the amounts of products.

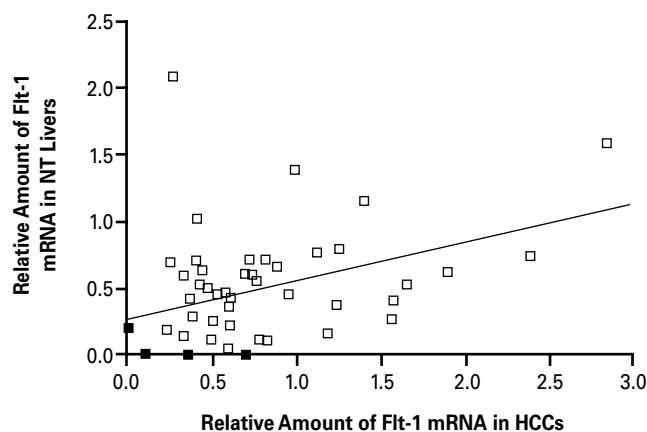


**Image 2** The messenger RNA expression of vascular endothelial growth factor (VEGF), Flt-1, and KDR in hepatocellular carcinoma. Three amplified bands of 574, 706, and 776 base pairs (bp) corresponding to the 121-, 165-, and 189-amino acid isoforms of VEGF (VEGF<sub>121</sub>, VEGF<sub>165</sub>, and VEGF<sub>189</sub>), respectively, were observed. Beta-actin acts as control. Case numbers are marked on top. M, marker; NT, nontumorous liver; T, tumor.

## RT-PCR for VEGF, Flt-1, and KDR

### Sequencing Results

The sequencing results of the PCR products confirmed the specific amplification of the 574-bp VEGF segment that extended between the sense primer at exon 1 and the



**Figure 1** The relationship between the levels of Flt-1 messenger RNA (mRNA) in hepatocellular carcinomas (HCCs) and the corresponding nontumorous livers. The amounts of mRNA were relative to those of beta-actin. There was significant correlation between the 2 levels (Pearson correlation,  $P = .008$ ). NT, nontumorous.

antisense primer at exon 8, the 555-bp segment of Flt-1 (from nucleotide 3112 to nucleotide 3666), and the 591-bp segment of KDR (from nucleotide 2831 to 3421).

### Vascular Endothelial Growth Factor

VEGF mRNA was demonstrated in all 50 HCC samples and in 49 of the corresponding nontumorous liver samples. Three amplified bands of 574, 706, and 776 bp corresponding to the 121-, 165-, and 189-amino acid isoforms of VEGF (VEGF<sub>121</sub>, VEGF<sub>165</sub>, and VEGF<sub>189</sub>), respectively, were observed (Image 2). They were present in high frequencies (100%, 100%, and 90%, respectively) in the tumors, with the amplified bands of VEGF<sub>121</sub> and VEGF<sub>165</sub> much stronger in intensity than those of VEGF<sub>189</sub>. In 26 cases (52%), the VEGF mRNA was overexpressed in the tumors, with levels in individual tumors higher than those of the corresponding nontumorous liver tissues. There was no significant difference in the amount of total VEGF mRNA between the tumors and the corresponding nontumorous livers ( $P = .631$ ).

### Flt-1

The PCR product size for Flt-1 was 555 bp. Flt-1 mRNA was present in 45 HCC samples (90%) and in 42 corresponding nontumorous liver samples (84%). In 34 cases (68%), Flt-1 mRNA was overexpressed in the tumors. The mean Flt-1 mRNA was significantly higher in the tumors than in the corresponding nontumorous liver samples (mean  $\pm$  SD Flt-1 mRNA in tumors and nontumorous livers,  $0.694 \pm 0.502$  and  $0.521 \pm 0.401$ , respectively;  $P = .008$ ; 95% confidence interval,  $-0.297$  to  $-0.048$ ) (Figure 1). In addition, VEGF mRNA and Flt-1 mRNA in tumors significantly correlated with each other ( $P = .021$ ) (Figure 2).

### KDR

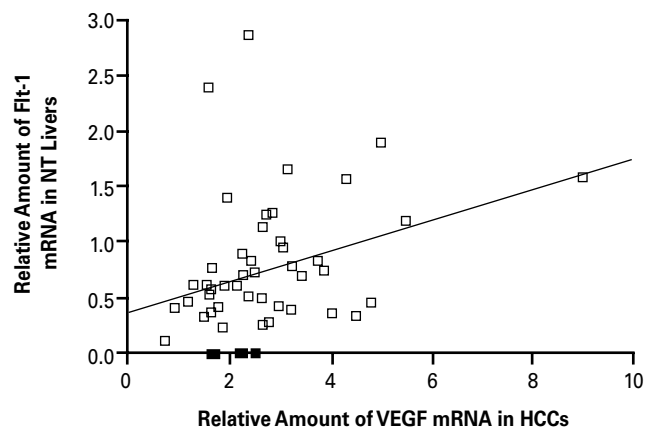
The PCR product size for KDR was 591 bp. KDR mRNA was detectable in 43 HCC samples (86%) and in 42 corresponding nontumorous liver samples (84%). In 20 cases (40%), there was overexpression of KDR mRNA in the tumors. The mean KDR mRNA of tumors did not differ significantly from that of the nontumorous liver samples (mean  $\pm$  SD KDR mRNA in tumors and nontumorous livers,  $0.550 \pm 0.301$  and  $0.699 \pm 0.600$ , respectively;  $P = .390$ ). Tumor KDR mRNA had no significant association with tumor Flt-1 or VEGF mRNA.

### VEGF, Flt-1, and KDR in Hepatoma Cell Lines

All 3 HCC cell lines strongly expressed VEGF<sub>121</sub> and VEGF<sub>165</sub> but not VEGF<sub>189</sub> transcripts (Image 3). No Flt-1 or KDR transcript was detected in any of them.

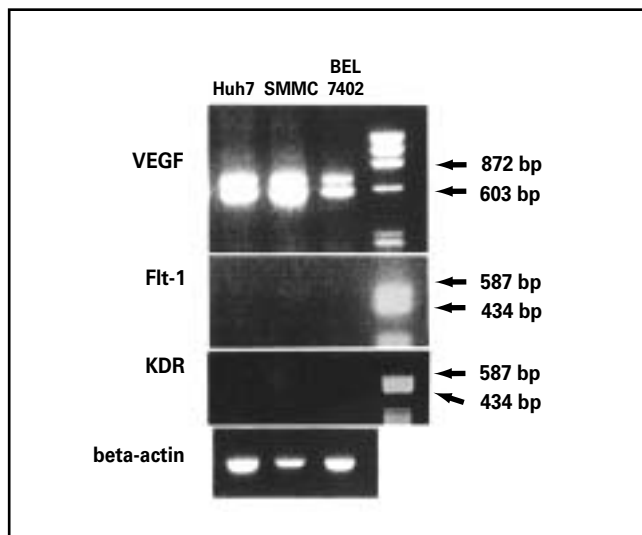
### Immunohistochemical Analysis of VEGF

VEGF protein was expressed in the cytoplasm of the tumor cells in 26 (67%) of 39 tumors. It also was present in



**Figure 2** The relationship between the levels of vascular endothelial growth factor (VEGF) and Flt-1 messenger RNA (mRNA) in hepatocellular carcinomas (HCCs). The amounts of mRNA were relative to those of beta-actin. There was significant correlation between the 2 levels (Pearson correlation,  $P = .021$ ). NT, nontumorous.

the endothelial cells of small blood vessels in the periphery and vicinity of the tumors in 9 (23%) of 39 **Image 4**. The small blood vessels included small arteries, venules, and capillaries. In the nontumorous liver samples, VEGF protein was demonstrated in the cytoplasm of hepatocytes (23/36 [64%]) and in the endothelium of small blood vessels (18/36 [50%]). Immunostaining was not present in the stromal tissue. We did not further quantify the protein expression of VEGF.



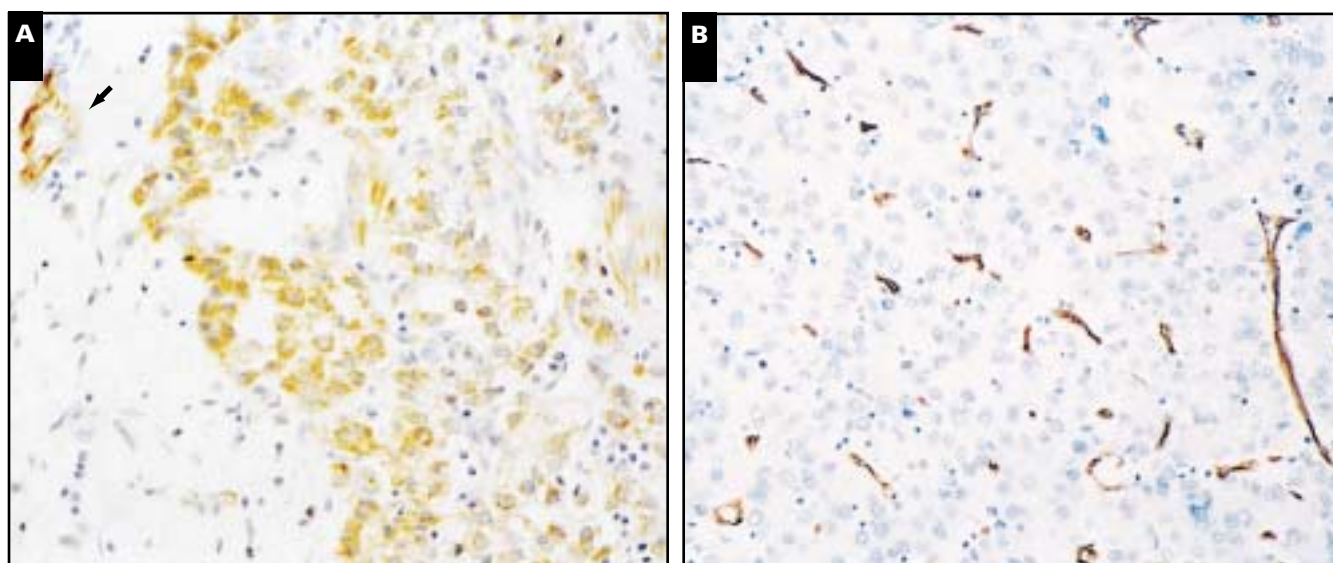
**Image 3** All 3 hepatocellular carcinoma (HCC) cell lines strongly expressed vascular endothelial growth factor (VEGF)<sub>121</sub> and VEGF<sub>165</sub> but not VEGF<sub>189</sub> transcripts. None of the HCC cell lines expressed Flt-1 or KDR messenger RNA. Beta-actin acts as control. bp, base pairs.

**Microvessel Density**

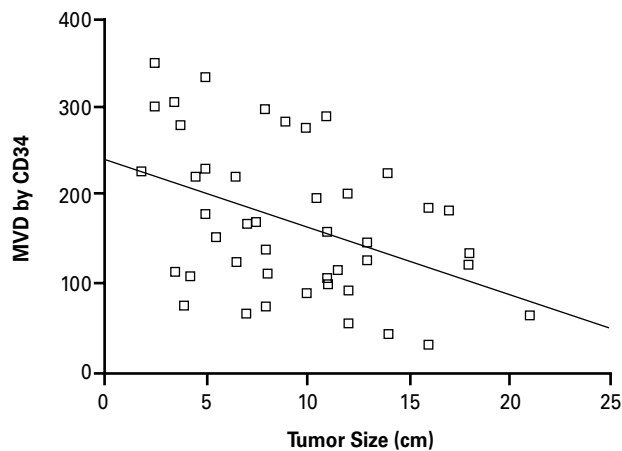
The mean tumor MVD ranged from 42 to 246 within a  $\times 200$  field (mean  $\pm$  SD,  $127.2 \pm 55.4$ ) (Image 4B).

**Clinicopathologic Features**

The tumor size ranged from 1.8 to 25 cm (median, 8.5 cm), with 38 tumors (76%) greater than 5 cm in diameter. Forty-three tumors (86%) were single. Half of the HCCs



**Image 4** **A**, Immunohistochemical analysis showed that vascular endothelial growth factor protein was expressed in the cytoplasm of the tumor cells. It also was present in the endothelial cells of a small blood vessel (arrow) in the vicinity of the tumor ( $\times 200$ ). **B**, Immunoperoxidase staining highlighted the microvessels in hepatocellular carcinoma (antibody CD34,  $\times 200$ ).



**Figure 3** Microvessel density (MVD) was negatively associated with tumor size ( $P = .003$ ).

were of Edmondson grade I or II in cellular differentiation, and the other half were grade III or IV. Tumor encapsulation was present in 24 (48%) of the tumors. Intrahepatic metastasis was frequent, with venous permeation identified in 35 (70%) of the HCCs and the presence of tumor microsatellites in 36 (72%). Seven (14%) had histologically positive resection margins. Thirty-one patients (62%) had cirrhosis of the liver, and 43 patients (86%) were positive serologically for hepatitis B surface antigen.

### Correlation Among MVD, VEGF, VEGF Receptors, and Clinicopathologic Features

MVD correlated inversely with tumor size ( $P = .003$ ) (Figure 3). A high MVD was observed in smaller tumors. As the tumors became larger, the MVD decreased significantly. There was no significant correlation between MVD and expression of VEGF, VEGF receptors, or other pathologic features.

There was no statistically significant association between tumor VEGF levels and tumor size or other pathologic features. However, when the tumors were stratified further into 2 groups according to the median tumor size (8.5 cm), within the group of tumors 8.5 cm or less in diameter, the tumors with intrahepatic metastasis in the form of tumor microsatellite formation had significantly higher VEGF mRNA levels compared with those without intrahepatic metastasis ( $n = 18$  and  $7$ , respectively; mean  $\pm$  SD VEGF mRNA level,  $2.658 \pm 1.042$  and  $1.773 \pm 0.76$ , respectively;  $P = .035$ ). In contrast, there was no significant association between VEGF mRNA and intrahepatic metastasis in tumors larger than 8.5 cm ( $n = 18$  and  $7$ , respectively; mean  $\pm$  SD VEGF mRNA level,  $3.126 \pm 1.881$  and  $2.776 \pm 0.785$ , respectively;  $P = .461$ ).

Significantly higher tumor Flt-1 mRNA levels were observed in women compared with men ( $n = 7$  and  $43$ ,

respectively; mean  $\pm$  SD Flt-1 mRNA level,  $1.324 \pm 0.883$  and  $0.637 \pm 0.486$ , respectively;  $P = .004$ ). The remaining pathologic features, including tumor size, venous permeation, and tumor cellular differentiation, were not significantly influenced by the mRNA levels of Flt-1 or KDR.

### Discussion

Angiogenesis is crucial in tumorigenesis, and HCC is characteristically a highly vascular tumor. Understanding of the angiogenic factors involved in hepatocarcinogenesis, particularly VEGF and its receptors, may provide potential targets for therapeutic strategy against HCC. A previous study on an animal model by Kong et al<sup>24</sup> investigated the use of localized gene transfer of a cDNA that encoded a secreted form of the extracellular domain of the Flt-1 receptor. The latter would bind with VEGF and therefore disrupt the normal interaction between VEGF and its native receptor Flt-1, and this has been shown to result in significant tumor suppression. Two studies demonstrated that inhibition of the activity of Flk-1/KDR exerts an inhibitory effect on the growth of tumor in mice.<sup>25,26</sup> These observations underscored the important role of the VEGF/VEGF-receptor system in tumor angiogenesis. However, the expression patterns of the receptors of VEGF have not been well characterized in human HCCs.

In the present study, VEGF mRNA was overexpressed in 26 (52%) of the tumors. In the previous reports on HCC, the findings of VEGF expression have been conflicting. A study by El-Assal et al<sup>16</sup> using quantitative RT-PCR showed that VEGF mRNA was found in all of the HCCs. However, no data of VEGF mRNA in nontumorous liver samples were available for comparison. Three previous reports on HCC, using RT-PCR or Northern blot analysis, showed that 60% to 70% of the tumors had overexpression of VEGF mRNA compared with their corresponding liver samples.<sup>12-14</sup> In contrast, Shimoda et al<sup>19</sup> found no significant difference in the quantitative VEGF expression between HCC and the adjacent liver samples. El-Assal et al<sup>16</sup> even observed a lower protein VEGF expression in HCCs than in the corresponding nontumorous liver samples. In the present study, VEGF mRNA levels were significantly associated with a higher incidence of intrahepatic metastasis in the group of relatively smaller tumors but not in the group of larger ones. This suggests that VEGF may have a more important role in tumor invasion and metastasis when the tumors are smaller. Furthermore, its lack of significant correlation with MVD, an observation similar to that in a previous study of HCC,<sup>16</sup> suggests that there are likely to be other additional angiogenic factors regulating the angiogenesis in HCC.

A differential significance of angiogenesis in different stages of HCC also is suggested by our finding of the relationship

of MVD with tumor size. In the present study, MVD was found to be inversely associated with tumor size. Smaller HCCs had significantly higher MVD that gradually decreased as the tumor became larger. This finding was unusual in solid tumors. In other non-HCC cancers, the MVD was reported to be increased with tumor size.<sup>27,28</sup> However, these cancers usually are small when compared with HCC, which can grow to a very large size by the time it is diagnosed or resected. In fact, such a decrease in MVD with an increase in tumor size also was observed in the study of HCC by El-Assal et al.<sup>16</sup> In that study, the mean MVD of HCCs greater than 5 cm was less than that of HCCs 2 to 5 cm in diameter. The characteristics of tumor microcirculation may offer a possible explanation for this observation in HCC. As the tumor becomes larger, the interstitial pressure often is high in tumors, particularly for a fast-growing tumor like HCC. This high interstitial pressure would lead to compression closure of capillaries, and then to ischemia and transport problems that ultimately result in necrosis. Moreover, active angiogenesis occurs mainly in the tumor periphery, while maintenance of the inner vascularization is a result of continuous remodeling.<sup>29</sup> Therefore, it may not be unreasonable that the importance of angiogenesis is diminished as the tumor grows, when the arterial and portal blood supplies have a more important role.

In the present study, the Flt-1 mRNA level was significantly overexpressed in HCCs. Furthermore, the Flt-1 mRNA expression significantly correlated with that of VEGF mRNA in the tumor. The observation of the up-regulation of Flt-1 mRNA in HCC highlights its importance in the VEGF/VEGF-receptor system in tumor angiogenesis in hepatocarcinogenesis. In addition, the 3 HCC cell lines showed VEGF mRNA but no Flt-1 or KDR mRNA. This was consistent with the notion that Flt-1 and KDR are produced by the endothelial cells on stimulation of VEGF overexpressed by the tumor cells. Flk-1/KDR is an early marker for endothelial cell precursor.<sup>30</sup> It is overexpressed in ovarian cancer,<sup>31</sup> coexpressed with Flt-1 in glioma<sup>2</sup> and colonic cancer,<sup>32</sup> and up-regulated in regenerating liver.<sup>33</sup> However, in HCC, the finding of a lack of overexpression of KDR in the present study implies that its role in hepatocarcinogenesis may not be as important as Flt-1.

An interesting finding of the present study was that in women, tumor Flt-1 mRNA expression was significantly higher, but expression of VEGF and KDR mRNA was not. The underlying reason for this observation is not clear. Whether it is due to hormonal effect is uncertain. The results are preliminary, and a further study with a larger number of women with HCC is required. Study of Flt-1 expression in various normal tissues in women may give interesting results.

Angiogenesis as assessed by MVD seems to be more important in smaller tumors. Tumor VEGF expression is

related to intrahepatic metastasis when the tumors are smaller, but may not be a major determinant in angiogenesis in large and advanced HCCs. The VEGF receptor Flt-1 is differentially up-regulated in HCC, indicating that Flt-1 may have an important yet incompletely understood role in the hepatocarcinogenesis of HCC. This novel finding suggests that the Flt-1 receptor may be a potential target for angiogenesis therapy in HCC.

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## References

1. Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol.* 1992;3:65-71.
2. Plate KH, Breier G, Weich HA, et al. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature.* 1992;359:845-848.
3. Barleon B, Siemeister G, Martiny-Baron G, et al. Vascular endothelial growth factor up-regulates its receptor fms-like tyrosine kinase 1 (FLT-1) and a soluble variant of FLT-1 in human vascular endothelial cells. *Cancer Res.* 1997;57:5421-5425.
4. Brown LF, Berse B, Jackman RW, et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res.* 1993;53:4727-4735.
5. Yoshiji H, Gomez DE, Shibuya M, et al. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. *Cancer Res.* 1996;56:2013-2016.
6. Relf M, LeJeune S, Scott P, et al. Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. *Cancer Res.* 1997;57:963-969.
7. Guidi AJ, Abu-Jawdeh G, Tognazzi K, et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in endometrial carcinoma. *Cancer.* 1996;78:454-460.
8. Abu-Jawdeh GM, Faix JD, Niloff J, et al. Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms. *Lab Invest.* 1996;74:1105-1115.
9. Morishita K, Johnson DE, Williams LT. A novel promoter for vascular endothelial growth factor receptor (flt-1) that confers endothelial-specific gene expression. *J Biol Chem.* 1995;270:27948-2753.
10. Davis-Smyth T, Chen H, Park J, et al. The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. *EMBO J.* 1996;15:4919-4927.

11. Yamane A, Seetharam L, Yamaguchi S, et al. A new communication system between hepatocytes and sinusoidal endothelial cells in liver through vascular endothelial growth factor and Flt tyrosine kinase receptor family (Flt-1 and KDR/Flk-1). *Oncogene*. 1994;9:2683-2690.
12. Mise M, Arai S, Higashitani H, et al. Clinical significance of vascular endothelial growth factor and basic fibroblast growth factor gene expression in liver tumor. *Hepatology*. 1996;23:455-464.
13. Suzuki K, Hayashi N, Miyamoto Y, et al. Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res*. 1996;56:3004-3009.
14. Miura H, Miyazaki T, Kuroda M, et al. Increased expression of vascular endothelial growth factor in human hepatocellular carcinoma. *J Hepatol*. 1997;27:854-861.
15. Chow NH, Hsu PI, Lin XZ, et al. Expression of vascular endothelial growth factor in normal liver and hepatocellular carcinoma: an immunohistochemical study. *Hum Pathol*. 1997;28:698-703.
16. El-Assal ON, Yamanoi A, Soda Y, et al. 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.
17. Yamaguchi R, Yano H, Iemura A, et al. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology*. 1998;28:68-77.
18. Torimura T, Sata M, Uena T, et al. Increased expression of vascular endothelial growth factor is associated with tumor progression in hepatocellular carcinoma. *Hum Pathol*. 1998;29:986-991.
19. Shimoda K, Mori M, Shibuta K, et al. 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. Weidner N, Semple JP, Welch WR, et al. Tumor angiogenesis and metastasis: correlation in invasive breast cancer. *N Engl J Med*. 1991;324:1-8.
21. Tanigawa N, Lu C, Mitsui T, et al. Quantitation of sinusoidal-like vessels in hepatocellular carcinoma: its clinical and prognostic significance. *Hepatology*. 1997;26:1216-1223.
22. Ng IOL, Lai ECS, Fan ST, et al. Prognostic significance of pathologic features of hepatocellular carcinoma: a multivariate analysis of 278 patients. *Cancer*. 1995;76:2443-2448.
23. Edmondson HA, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48900 necropsies. *Cancer*. 1954;7:462-503.
24. Kong HL, Hecht D, Song W, et al. Regional suppression of tumor growth by in vivo transfer of a cDNA encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor. *Human Gene Ther*. 1998;9:823-833.
25. Shaheen RM, Davis DW, Liu W, et al. Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer Res*. 1999;59:5412-5416.
26. Brekken RA, Overholser JP, Stastny VA, et al. Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res*. 2000;60:5117-5124.
27. Igarashi M, Dhar DK, Kubota H, et al. The prognostic significance of microvessel density and thymidine phosphorylase expression in squamous cell carcinoma of the esophagus. *Cancer*. 1998;82:1225-1232.
28. Cheng WF, Lee CN, Chu JS, et al. Vascularity index as a novel parameter for the in vivo assessment of angiogenesis in patients with cervical carcinoma. *Cancer*. 1999;85:651-657.
29. Fox SB, Gatter KC, Bicknell R, et al. Relationship of endothelial cell proliferation to tumor vascularity in human breast cancer. *Cancer Res*. 1993;53:4161-4163.
30. Yamaguchi TP, Dumont DJ, Conlon RA. Flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development*. 1993;118:489-498.
31. Boocock CA, Charnock-Jones DS, Sharkey AM, et al. Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. *J Natl Cancer Inst*. 1995;87:506-516.
32. Warren RS, Yuan H, Matli MR, et al. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest*. 1995;95:1789-1797.
33. Mochida S, Ishikawa K, Inao M, et al. Increased expressions of vascular endothelial growth factor and its receptors, flt-1 and KDR/flk-1, in regenerating rat liver. *Biochem Biophys Res Commun*. 1996;226:176-179.



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