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• BASIC RESEARCH •

Is the vascular endothelial growth factor messenger RNA expression in resectable hepatocellular carcinoma of prognostic value after resection?

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Abstract

AIM: To study whether vascular endothelial growth factor messenger RNA (VEGF mRNA) in the hepatocellular carcinoma (HCC) tissues obtained after curative resection has a prognostic value.

METHODS: Using a reverse-transcription polymerase chain reaction (RT-PCR)-based assay, VEGF mRNA was determined prospectively in liver tissues of 50 controls and in HCC tissues of 50 consecutive patients undergoing curative resection for HCC.

RESULTS: Among the isoforms of VEGF mRNA, $VEGF_{165}$ and $VEGF_{121}$ were expressed. By multivariate analysis, a higher level of $VEGF_{165}$ in HCC tissue correlated with a significant risk of HCC recurrence (P=0.038) and significantly with recurrence-related mortality (P=0.045); while $VEGF_{121}$ did not. Other significant predictors of HCC recurrence included cellular dedifferentiation (P=0.033), an absent or incomplete capsule (P=0.020), vascular permeation (P=0.018), and daughter nodules (P=0.006). The other significant variables of recurrence related mortality consisted of vascular permeation (P=0.045), and cellular dedifferentiation (P=0.053). The level of VEGF mRNA in HCC tissues, however, did not significantly correlate with tumor size, cellular differentiation, capsule, daughter nodules, vascular permeation, necrosis and hemorrhage of tumors.

CONCLUSION: The expression of VEGF mRNA, especially isoform $VEGF_{165}$, in HCC tissues, may play a significant and independant role in the prediction of postoperative recurrence of HCC.

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INTRODUCTION

Angiogenesis, the establishment of a neovascular blood supply from preexishing blood vessels, known to be essential for the survival, growth, invasion, and metastasis of tumor cells, is a complex multistep process. The process may include the extracellular matrix remodeling and the binding of angiogenic factors to specific endothelial cell (EC) receptors, leading to EC proliferation, invasion of the basement membrane, migration, differentiation, and formation of new capillary tubes and developing into a vascular network[1-10].

One of the most potent, direct acting, and specific factors with angiogenic activity is vascular endothelial growth factor (VEGF)[11,12].

Hepatocellular carcinoma (HCC), a leading cause of death in Taiwan and many Asian countries, is a highly vascular tumor dependent on neovascularization. Some authors have suggested that VEGF may be a marker for metastasis in HCC because they found markedly elevated VEGF protein levels in HCC patients with remote metastases compared with those without metastasis[13-15]. However, most such studies determined VEGF protein concentrations by enzyme immunoassay. To our knowledge, in the prediction of postresection recurrence, little is known about the prognostic significance of VEGF mRNA expression in tumor tissues. We conducted this prospective study to investigate the correlation between VEGF mRNA expression in HCC tissues and postoperative recurrence of HCC.

MATERIALS AND METHODS

Study population

Fifty patients (31 men and 19 women, with a mean age of 56.2±13.3 yr) of 58 consecutive patients with HCC undergoing curative hepatectomy from July 2001 to April 2003, were enrolled in this prospective study. Patients who had previously had a hepatectomy or preoperative neoadjuvant ethanol injection or hepatic arterial chemoembolization (TACE) were excluded. Surgical procedures performed included 38 major resections (8 extended right lobectomies, 10 right lobectomies, 8 left lobectomies and 12 two-segmentectomies) and 12 minor resections (10 segmentectomies, 1 subsegmentectomies, and 1 wedge resection). HCC tissues were obtained from all 50 patients after resection. A control group including 10 healthy volunteers without liver disease (5 men, 5 women, mean age 40 yr) and 20 patients with chronic liver disease but without evidence of HCC also received liver biopsy during laparotomy on them for other reasons. All these HCC tissues and liver biopsy tissues (from control group patients) were examined for VEGF mRNA.

After discharge, the patients were assessed regularly to detect tumor recurrence with abdominal ultrasonography (every 2-3 mo during the first 5 yr, then every 4-6 mo thereafter), serum alpha fetoprotein (AFP) and liver biochemistry (every 2 mo during the first 2 yr, then every 4 mo during the following

3 yr, and every 6 mo thereafter), abdominal computed tomography (CT) (every 6 mo during the first 5 yr, then annually), and chest X-ray and bone scans (every 6 mo). Hepatic arteriography was obtained if the other studies suggested possible cancer recurrence. Detection of tumor on any imaging study was defined as clinical recurrence.

Clinicopathological variables analyzed included age, sex (male *vs* female), the presence of liver cirrhosis, Child-Pugh class of liver functional reserve (A *vs* B), hepatitis B virus (HBV) infection (hepatitis B surface antigen), hepatitis C virus (HCV) infection (anti-hepatitis C virus antibody), serum AFP level (<20 ng/mL *vs* 20 to 1 000 ng/mL *vs* >1 000 ng/mL), tumor size (<3 cm *vs* 3 to 10 cm *vs* >10 cm), tumor encapsulation (complete *vs* incomplete or absent), presence of daughter nodules, vascular permeation (including vascular invasion and/or tumor thrombi in either the portal or hepatic vein), and cell differentiation grade (Edmondson and Steiner grades I to IV).

Detection of VEGF mRNA

It included extraction of RNA, reverse transcription and amplification of cDNA of VEGF and GAPDH by PCR.

VEGF mRNA of liver tissue

Extraction of RNA We homogenized resected tissues completely in 1 mL of RNA–bee™, and added 0.2 mL chloroform and shaked vigorously for 15-30 s. We stored the sample on ice for 5 min and centrifuged at 12 000 *g* for 15 min. We transferred the supernatant to a new 1.5 mL eppendorf tube and precipitated it with 0.5 mL of isopropanol. Precipitation could be as short as 5 min at 4 ℃. We centrifuged it at 12 000 *g* for 5 min at 4 ℃. We removed the supernatant and washed the RNA pellet with 1 mL of 750 mL/L ethanol, it dislodged the pellet from the slide of the tube by shaking. We centrifuged at 7 500 *g* for 5 min at 4 ℃ and carefully removed ethanol. We removed the supernate and dissolved RNA in DEPC-H_2O (usually between 50-100 μL) and store at -80 ℃.

Reverse transcription We heated the RNA sample at 55 ℃ for 10 mimutes and chilled it on ice. We added the following components: (1) 4 μL 5×RT butter containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$ and 10 mmol/L DTT(dithiothreitol), (2) 3 μL 10 mmol/L dNTP, (3) 1.6 μL Oligo-$d(T)_{18}$ and 0.4 μL random hexamers (N)6 (1 μg/μL), (4) 0.5 uL RNase inhibitor (40 units/μL), (5) 3 μL 25 mmol/L $MnCl_2$, (6) 6 μL RNA in DEPC-H_2O, (7) 0.5 μL DEPC-H_2O. We incubated it at 70 ℃ for 2 mimutes, chilled it to 23 ℃ to anneal primers to RNA. We added 1 μL of M-MLV RTase (moloney murine leukemia virus reverse transcriptase, 200 units/μL, Promega). We incubated it for 8 min at 23 ℃ followed by 60 min at 40 ℃. We heated the reaction at 94 ℃ for 5 min, chilled it on ice and stored cDNA at -20 ℃.

Amplification of cDNA of VEGF and GAPDH by PCR The sequences of the sense primers were 5'-AGTGTGTGCCCA CTGAGGA-3' (VEGF) and 5'-AGTCAACGGATTTGGT CGTA-3' (GAPDH) and those of the antisense primers were 5'-AGTCAACGGATTTGGTCGTA-3' (VEGF) and 5'-GGAACATGTAAACCATGTAG-3' (GAPDH). The first polymerase chain reaction (RT-PCR) solution contained 5 μL of the synthesized cDNA solution, 10 μL of 10× polymerase reaction buffer, 500 moi/L each of dCTP, dATP, dGTP and dTTP, 15 pmol of each external primer (EX-sense and EX-antisense), 4 units of Thermus Brockiamus Prozyme DNA polymerase (PROtech Technology Ent. Co., Ltd. Taipei, Taiwan) and water. The PCR cycles were denaturing at 94 ℃ for 1 min, annealing at 52 ℃ for 1 min, and primer extension at 72 ℃ for 1 min. The cycles were repeated 40 times. The PCR product was reamplified with internal primers for nested PCR to obtain a higher sensitivity. The first and second PCR components were the same, but for the primer pairs (IN-sense and IN-antisense), the final product was electrophoresed on 20g/L agarose gel and stained with ethidium bromide. Four different isoforms of human VEGF were identified, arising from alternative splicing of the primary transcript of a single gene. The majority were $VEGF_{121}$ (165 bp) and $VEGF_{165}$ (297 bp). The percentage intensity of the VEGF PCR fragment for each liver was relative to a GAPDH PCR fragment (122 bp). The intensity of bands was measured using Fujifilm Science Lab 98 (Image Gauge V3.12). The sensitivity of our assay was assessed using human hepatocytes.

A hepatoblastoma cell line (HepG2) served as a positive control for VEGF mRNA expression. For negative controls, we used EDTA-treated water (filtered and vaporized).

Statistical analysis

A statistical software (SPSS for Windows, version 8.0, Chicago, Illinois) was employed, with Student's *t*-test used to analyze continuous variables and a chi-square or Fisher's exact test for categorical variables. Parameters relating to the presence of postoperative hAFP mRNA in peripheral blood were analyzed by stepwise logistic regression. A Cox proportional hazards model was used for multivariate stepwise analysis to identify the significant variables for predicting recurrence and mortality. Significance was taken as a P value <0.05.

RESULTS

RT-PCR analysis of VEGF transcript in liver tissues

VEGF mRNA was expressed in the liver tissues of 10 ($VEGF_{165}$ in 10 and $VEGF_{121}$ in 6) out of 30 control patients. In the HCC group, isoform $VEGF_{165}$ was detected in all the 50 patients (100%) (with a concentration ranging from 0.1860 to 0.7240) and isoform $VEGF_{121}$ in 40 patients (80%) (with a concentration ranging from 0.2849 to 1.0298).

We did not detect isoforms $VEGF_{189}$ and/or $VEGF_{206}$ in either HCC tissues or control liver tissues.

Table 1 Demographic, clinical and tumor variables of patients with HCC undergoing curative resection (n=50)

Variables	No. of patients (%)
Age (mean, years)	56.2±13
Male	31 (62)
Cirrhosis	40 (80)
Child-Pugh's class A	43 (86)
Serum AFP <20 ng/mL	16 (32)
20-10^3 ng/mL	18 (36)
>10^3 ng/mL	14 (28)
HBsAg (+)	36 (72)
Anti-HCV (+)	13 (26)
Size of HCC <3 cm	12 (24)
3-10 cm	13 (26)
>10 cm	25 (50)
Edmondson-Steiner's Grade I	4 (8)
Grade II	12 (24)
Grade III	18 (36)
Grade IV	16 (32)
Absent or incomplete capsule	31 (62)
Vascular permeation	29 (58)
Daughter nodules	31 (62)
Tumor necrosis	33 (66)
Tumor hemorrhage	29 (58)

AFP: serum alpha fetoprotein, HBsAg (+): positive hepatitis B surface antigen, Anti-HCV(+): positive hepatitis C virus antibody, Edmondson Steiner grade: differentiation grade.

Correlation of VEGF mRNA expression and clinical recurrence

During the follow up period (median 1.5 yr, range 1 to 2.5 yr), 16 patients (32%) had clinically detectable recurrence, of whom 7 died. A higher level of isoform $VEGF_{165}$ mRNA in HCC tissue correlated significantly with clinical recurrence both univariately (P=0.022) and multivariately, (P=0.038). Isoform $VEGF_{121}$ levels had no such correlation. By multivariate analysis, other significant predictors of recurrence included poor cellular differentiation (P=0.033), less encapsulation (P=0.020), more vascular permeation (P=0.018) and the presence of daughter nodules (P=0.006) (Table 2).

Table 2 Predictors of HCC recurrence

Variable	*P* values	
	UV	MV
Sex	0.895	-
Age	0.279	-
Size(<3 cm, >10 cm)	0.415	-
Liver cirrhosis	0.510	-
Child-Pugh class	0.528	-
Serum AFP	0.744	-
HBAg (+)	0.280	-
Anti-HCV (+)	0.481	-
Edmondson Steiner grade	0.0005	0.033
Capsule	<0.0001	0.020
Vascular permeation	<0.0001	0.018
Daughter nodules	<0.0001	0.006
Tumor necrosis	0.344	-
Tumor hemorrhage	0.812	-
Tissue $VEGF_{165}$ mRNA	0.022	0.038
Tissue $VEGF_{121}$ mRNA	0.622	-

UV: univariate analysis, MV: multivariate analysis, AFP: serum alpha fetoprotein, HbsAg(+): positive hepatitis B surface antigen, Anti-HCV(+): positive hepatitis C virus antibody, Edmondson Steiner grade: differentiation grades I, II *vs* III, IV, n.s: not significant.

Table 3 Correlation between clinical and tumor variables and recurrence-related mortality

Parameters	*P* values	
	UV	MV
Sex	0.510	-
Age	0.440	-
Size (<3 cm, >10 cm)	0.519	-
Liver cirrhosis	0.510	-
Child-Pugh class	0.548	
HBsAg (+)	0.351	-
Anti-HCV (+)	0.521	-
Edmondson Steiner grade	<0.001	0.053
Capsule	0.033	n.s.
Vascular permeation	<0.001	0.045
Daughter nodules	0.016	n.s.
Tumor necrosis	0.373	-
Tumor hemorrhage	0.306	-
Tissue $VEGF_{165}$ mRNA	0.018	0.045
Tissue $VEGF_{121}$ mRNA	0.744	-

UV: univariate analysis, MV: multivariate analysis, AFP: serum alpha fetoprotein, HBsAg (+): positive hepatitis B surface antigen, Anti-HCV(+): positive hepatitis C virus antibody, Edmondson Steiner grade: differentiation grades I, II *vs* III, IV, n.s: not significant.

Correlation of VEGF mRNA expression and recurrence-related death

The level of isoform $VEGF_{165}$ in HCC tissue significantly correlated with death due to recurrence both univariately (P=0.018) and multivariately (P=0.045). By multivariate analysis, a greater degree of vascular permeation significantly correlated with mortality (P=0.045), and poor cellular differentiation approached significance (P=0.053)(Table 3).

Correlation between VEGF mRNA expression in HCC tissues and clinical and histopathologic features

There was no significant association between isoform of VEGF mRNA and gender, age, serum AFP level, chronic HBV or HCV carriage, tumor size, coexisting cirrhosis, cellular differentiation, capsule, vascular permeation, daughter nodules, tumor necrosis, or tumor hemorrhage (P>0.05).

DISCUSSION

Our study revealed that a higher value of VEGF mRNA isoform $_{165}$ in resected HCC tissues was significantly associated with an increased risk of postoperative recurrence and disease mortality. The value of VEGF mRNA isoform $_{121}$ in HCC tissues was not significantly predictive of the outcome.

VEGF is also known as a vascular permeability factor and vasculotropin. Its active form is a homodimeric cytokine with molecular weight 34-46 ku. The variation in size due to alternative exon splicing might produce four different isoforms of 121, 165, 189 and 206 amino acids (monomeric size). The last had heparin binding activity[11,12]. Different cancers might have different expression of the isoforms. The majority of HCC expressed an abundance of VEGF $_{121}$ and VEGF $_{165}$[13-15]. According to Ferrara' s finding, $VEGF_{165}$ was the predominantly expressed form in human cDNA libraries as well as in most normal cells and tissues[12].

Some authors have shown that the VEGF level in serum or in tissue is of value for predicting disease progression and prognosis in different cancers, such as the gastrointestinal origins, breast, lung, urothelium, ovary, and lymphoma[16-26]. Compared with expression in tumor tissue, the advantage of measurement of serum VEGF level is that it can be performed without tissue specimens and repeated, but it may be influenced by some factors such as coexisting liver cirrhosis, associated infection and platelet activation[27-32].

In addition, the expression of VEGF mRNA in serum might not always correlate significantly with the gene expression level of tumors[33]. Therefore, we used liver tissue instead of serum in this study. Warren found VEGF mRNA in hepatocytes and in some Kupffer cells[34]. However, release of VEGF mRNA might also be influenced by some cells other than HCC cells[34]. The presence of mRNA for VEGF has also been described in T lymphocytes, CD34* cells, and monocytes[27, 30].

For more accuracy, we chose to measure mRNA expression of VEGF in liver tissue rather than the protein itself. The level of VEGF mRNA did not always correlate with the protein concentration[32]. Immunohistochemistry could not distinguish small amounts of protein, which may partly explain the discrepancy in protein and mRNA levels.

The high recurrence rate after resection is the main determinant for the poor outcome of HCC[35-40]. Tumor invasiveness variables correlated with recurrence include high serum AFP, hepatitis, vascular permeation, grade of cellular differentiation, infiltration or absence of capsule, tumor size, coexisting cirrhosis, presence of daughter nodules, and multiple lesions. Therefore, a number of studies have been done to see if VEGF correlated with any or all of those factors.

Among reports about the clinical significance of VEGF

expression in HCC, there are considerable discrepancies[13-15,28,41-48]. Li found that VEGF mRNA in HCC correlated significantly with portal vein emboli, poorly encapsulated tumors, and microvascular density in HCC tissues[42]. Zhou reported that high VEGF expression in HCC was associated with portal vein tumor thrombosis[43]. Chow showed that VEGF expression was significantly associated with portal vein tumor thrombosis (sonographic evidence) but not with liver function, tumor volume, gender, severity of liver disease, or tumor grading[41]. In addition, the correlation between increased VEGF protein level in HCC and tumor size, number, microscopic venous invasion, metastasis, and recurrence has also been reported.

However, according to our study, a higher expression of VEGF mRNA was significantly correlated with tumor recurrence and recurrence-related mortality but not with the other parameters of tumor invasiveness. VEGF mRNA in HCC tissue thus appears to be an independent risk factor of postoperative recurrence. There are several possible explanations for this dissociation.

The number of study patients is one possible factor. Because most of the reported investigations were performed in small series, the 50 patients we used seemed a more adequate sample size compared with other studies. Another possible explanation for the discrepancies may be the assessment of tumors of different sizes and etiologies.

The relation between tumor size and VEGF mRNA expression might be complex and dynamic because of different vascular growth patterns[14,48-52]. If HCCs are about 1.0 cm in diameter, artery-like vessels are not well developed. Capillarization of the blood spaces is present but incomplete, and portal tracts may appear within cancerous nodules. These HCCs are thought to receive a predominantly portal blood supply. As tumor size increases, portal tracts decrease in number, and artery-like vessels gradually increase in number and size. Well-differentiated HCCs measuring 1.0 to 1.5 cm in diameter are in a transitional stage from portal to arterial blood supply, with reduction in portal flow prior to the increase in arterial flow. Therefore, blood flow in HCC at this point would be low and may not be detected on angiography. Hypervascularity becomes easily seen when nodules are larger than 2 cm in diameter. However, with increasing tumor size, VEGF positivity may gradually decrease. According to Yamaguchi, 36.8% of nodules larger than 3.0 cm were VEGF-negative[48]. El-Assal showed that, HCCs larger than 5 cm in diameter were less vascular than smaller or medium-sized lesions[32]. However, it has been reported that the intercapillary distance increased as the tumor size or weight increased, caused by the significantly different rates of turnover of endothelial cells and neoplastic cells. These complicated changes in vascularity may account for the disparate results among reported studies.

Suzuki reported that VEGF mRNA levels were not correlated with the vascularity of HCCs as seen on angiography[13]. On the contrary, Mise *et al* showed that the degree of VEGF mRNA expression was significantly correlated with the intensity of tumor staining in angiograms ($P<0.01$)[14]. Because of the complex nature of the angiogenic process, however, it seems that VEGF expression is not the sole contributor to angiogenesis in HCC. Other factors involved in this process may include TGF-β, TNF-α, IL-8, *etc*.

The stage of cancer might also influence VEGF expression[53-55]. VEGF concentrations have been reported to be significantly higher in advanced rather than early stages of breast, colon and gastric cancer[16-18,21]. Chao showed that a lower range of VEGF levels in patients with early-stage HCC overlapped considerably with those of normal controls or patients with chronic hepatitis or cirrhosis[45].

Coexisting liver cirrhosis may influence VEGF expression. About 80% of our study patients had cirrhosis. Some investigators have found that VEGF expression was significantly higher in cirrhotic liver than in noncirrhotic liver. Furthermore, it has been shown that cirrhosis itself was associated with increased angiogenic activity. According to El-Assal, cirrhotic livers had significantly higher VEGF expressions than noncirrhotic livers[32]. In addition, some suggested a possible involvement of VEGF in angiogenesis of cirrhotic liver but not in angiogenesis of HCC[31,32]. Akiyoshi suggested that a low serum VEGF level in liver cirrhosis might reflect the degree of liver dysfunction and be associated with the grade of hepatocyte regeneration and VEGF levels decreased with the worsening of Child-Pugh score[31]. Whereas, most of our patients belonged to Child-Pugh class A, with resectable lesions, unlike those studied by Akyoshi.

According to the cell differentiation, the regulation of VEGF may be complex. In our study, VEGF mRNA did not significantly correlate with the grade of cell differentiation. We attribute this to the possibility of different histological grades coexisting in some HCC tissues. Yamaguchi examined VEGF expression immunohistochemically in HCC with various histological grades and sizes[48]. In tumors composed of a single histological grade, VEGF expression was the highest in well-differentiated, followed by moderately differentiated, and then poorly differentiated HCC. In tumors consisting of cancerous tissues of two different histological grades, the expression was less intense in the higher-grade HCC component. VEGF was also expressed in the surrounding HCC tissues in which inflammatory cell infiltration was apparent. Based on these findings, VEGF expression in HCC tissues was thought to be partly related to the histological grade, but other cytokines and growth factors could also cooperatively act to enhance or influence VEGF expressions in HCC.

We also found no correlation between VEGF and the absence or presence of fibrous capsule or septum formation, which was in contrast to the findings of Suzuki *et al*[13]. The origin of the capsule and fibrous septa in HCC is unclear. Nakashima *et al* suggested the possibility of fibrogenesis at the interface of two tumor nodules with different properties, a process requiring fibrin deposition in the initial stage when the HCC nodule grows to 1.5 cm or larger[52]. However, this mechanism has been doubted, since the tumor size did not correlate with the thickness of the capsule or the incidence of its formation.

In our study, a higher level of VEGF mRNA in tumor tissue correlated with more postresection recurrences. We attribute it to two possible mechanisms. One is that the higher angiogenesis may have more invasive nature of cancer to spread into the surrounding tissues. This invasion requires concomitant neovascularization through the sprouting of endothelial cells in extracellular matrix. It has been reported that VEGF could induce both urokinase-type and tissue-type plasmin in endothelial cell, which are the key protease involved in the degradation of the extracellular matrix. The other mechanism is that a shift of VEGF mRNA occurred in liver tissue, which is strongly related to the development of HCC. The progression from preneoplastic to neoplastic tissue would contribute to recurrence.

Surgery remains the potentially curative treatment for patients with HCC. High recurrence rate limits the long term survival. Examination of VEGF mRNA expression in resected HCC tissue may give us information on the risk of postoperative recurrence. Addition of neoadjuvant antiangiogenic therapy after surgery may be considered for such patients. Furthermore, serial measurement of circulating VEGF mRNA during postoperative follow-up to monitor the effect of therapy or the development of recurrence should be further investigated[56,57].

In conclusion, expression of VEGF, especially isoform

$VEGF_{165}$, in HCC tissues may play a significant role in the prediction of postresection recurrence of HCC.

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