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• LIVER CANCER •

Clinical significance of the expression of isoform 165 vascular endothelial growth factor mRNA in noncancerous liver remnants of patients with hepatocellular carcinoma

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Abstract

AIM: To investigate the prognostic role of isoform 165 vascular endothelial growth factor messenger RNA ($VEGF_{165}$ mRNA) in noncancerous liver tissues from patients with primary hepatocellular carcinoma (HCC).

METHODS: Using a reverse-transcription polymerase chain reaction (RT-PCR)-based assay, VEGF mRNA was determined prospectively in noncancerous liver tissues from 60 consecutive patients with HCC undergoing curative resection. We categorized the patients with $VEGF_{165}$ mRNA over 0.500 in noncancerous liver tissues as group A, and those below 0.500 as group B.

RESULTS: Among the isoforms of VEGF mRNA by multivariate analysis, a higher level of $VEGF_{165}$ mRNA in noncancerous liver tissue correlated significantly with a higher risk of HCC recurrence ($P = 0.039$) and recurrence-related mortality ($P = 0.048$), but $VEGF_{121}$ did not. The other significant predictors of recurrence consisted of vascular permeation ($P = 0.022$), daughter nodules ($P = 0.033$), cellular dedifferentiation ($P = 0.033$), an absent or incomplete capsule ($P = 0.037$). A significant variable of recurrence-related mortality was vascular permeation ($P = 0.012$). As to the clinical manifestations of 16 patients who developed recurrence, the recurrent tumor number over 2, recurrent extent over two-liver segments, and the median survival after recurrence, all significantly correlated with group A patients ($P = 0.043$, 0.043, and 0.048, respectively). However, the presence of extrahepatic metastasis was not ($P>0.05$). The difference in recurrence after treatment between the two groups had no statistical significance ($P>0.05$).

CONCLUSION: The higher expression of isoform $VEGF_{165}$ mRNA in noncancerous liver remnant of patients with HCC may be a significant biological indicator of the invasiveness of postoperative recurrence.

Key words: Hepatocellular carcinoma; VEGF protein; Messenger RNA

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with a poor prognosis. During the last 10 years, efforts have been made worldwide toward earlier detection and safer surgical resection of HCC. However, despite these recent diagnostic and therapeutic advances, postoperative recurrence is still common[1-3]. How to early predict the prognosis after resection is a challenging problem for surgeons.

It is well known that the development of a tumor requires oxygen and nutrients, which are supplied through neovascularization. Angiogenic potential is a prerequisite for tumor growth[4-9]. Thus, enhanced gene expression of angiogenic factors in a developing tumor is strongly expected. The release of angiogenic factors from malignant tumors, in turn, would lead to the production of vascular endothelial cells via a paracrine mechanism. Among the potential angiogenic factors, vascular endothelial growth factor (VEGF) is the most potently direct acting and specific one. The variation in size due to alternative exon splicing may produce four different isoforms of 121, 165, 189 and 206 amino acids (monomeric size)[10,11]. According to Ferrara's analysis, $VEGF_{165}$ is the predominantly expressed form in human cDNA libraries as in most normal cells and tissues[12]. Different cancers may have different expressions of the isoforms. The majority of HCCs expresses an abundance of $VEGF_{121}$ and $VEGF_{165}$[10-12].

Angiogenesis in tumors has been proven to be an independent factor of prognosis and metastasis in many carcinomas[13-15]. Mise *et al*[16] found that VEGF mRNA was also expressed in nontumorous portions of the livers. It remains unknown whether the degree of angiogenesis in nontumorous liver tissues contributes to the grade of HCC malignancy and the potential of postresection recurrence[16].

This study was to elucidate the correlation between VEGF mRNA expression in noncancerous liver tissues and the clinicopathological manifestations of postoperative recurrence, so to provide a useful prognostic parameter for predicting the recurrence.

MATERIALS AND METHODS

Study population

Sixty consecutive patients (35 men and 25 women, with a mean age of 54.5±13.5 years) with HCC undergoing curative hepatectomy from November 2000 to November 2003, were enrolled in this prospective study. Patients who had a previous history of hepatectomy or preoperative neoadjuvant ethanol injection or hepatic arterial chemoembolization (TACE) were all excluded. Surgical procedures performed included 44 major resections (8 extended right lobectomies, 14 right lobectomies, 9 left lobectomies, and 13 two-segmentectomies) and 16 minor resections (14 one-segmentectomies, 1 subsegmentectomy, and 1 wedge resection). Noncancerous liver tissues were obtained from the contralateral lobe remnant of all 60 patients during hepatectomy. The liver tissue was taken at least 3 cm far from the resection margin of HCC. We changed instruments in this procedure to avoid seeding or contaminating the liver biopsy tissues by HCC cells. A control group including 10 healthy volunteers without liver disease (5 men, 5 women, mean age 50 years) and 20 patients with chronic liver disease (10 with liver cirrhosis, 5 with hepatitis B carrier but no cirrhosis, 5 with hepatitis C carrier but no cirrhosis) but without any evidence of HCC also received liver biopsies during laparotomy for other reasons. All liver tissues were examined for VEGF mRNA. All of the patients agreed to participate in this study preoperatively.

Demographic data analysis

Clinicopathological variables analyzed included age, sex (male *vs* female), presence of liver cirrhosis, Child-Pugh class of liver functional reserve (A *vs* B), hepatitis B virus (HBV) infection (hepatitis B surface antigen, HBsAg), hepatitis C virus (HCV) infection (anti-hepatitis C virus antibody, Anti-HCV), serum alfafetoprotein (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) (Table 1).

Table 1 Characteristics of 60 patients with HCC undergoing curative resection

Variables	No. of patients (%)
Age (mean, yr) (±SD)	50.4±12.6
Male	44 (73)
Cirrhosis	47 (78)
Child- Pugh's class A	43 (72)
Serum AFP < 20 ng/mL	19 (32)
20-10^3 ng/mL	29 (48)
>10^3 ng/mL	12 (20)
HBsAg (+)	47 (78)
Anti-HCV (+)	32 (53)
Size of HCC <3 cm	17 (28)
3-10 cm	22 (37)
>10 cm	2 (35)
Edmondson-Steiner's grade I	13 (22)
Grade II	11 (18)
Grade III	18 (30)
Grade IV	18 (30)
Absent or incomplete capsule	39 (65)
Vascular permeation	33 (55)
Daughter nodules	31 (52)

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

Detection of VEGF mRNA

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

Extraction of RNA and reverse transcription We homogenized each resected tissue (including noncancerous liver tissues and control liver tissues) completely in 1 mL of RNA-bee™, and added 0.2 mL chloroform and shook it 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. The precipitation was as short as 5 min at 4 ℃. We centrifuged it at 12 000 *g* for 5 min at 4 ℃. Then we removed the supernatant and washed the RNA pellet with 1 mL of 75% ethanol, shaking to dislodge the pellet from the side of the tube. We centrifuged it at 7 500 *g* for 5 min at 4 ℃ and carefully removed ethanol, the supernatant and dissolved RNA in DEPC-H_2O (usually between 50-100 uL), and stored it at -80 ℃.

We heated the RNA sample at 55 ℃ for 10 min and chilled it on ice. We added into it the following components: (1) 4 μL 5×RT butter containing a composition of 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 μL 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 at 70 ℃ for 2 min, chilled it to 23 ℃ to anneal primer to RNA. We added 1 μL of M-MLV RTase to (Moloney murine leukemia virus revers transcriptase, 200 units/μL, Promega product). We incubated it for 8 min at 23 ℃, then for 60 min at 40 ℃. We heated the reaction at 94 ℃ for 5 min and 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'-AGTGTGTGCCC ACTGAGGA-3' (VEGF) and 5'-AGTCAACGGATTTGGTC GTA-3'(GAPDH) and those of the antisense primers were 5'-AGTCAACGGATTTGGTCGTA-3'(VEGF) and 5'-GGAACA TGTAAACCATGTAG-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 μmol/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 denatured at 94 ℃ for 1 min, annealed at 52 ℃ for 1 min, and primer extension at 72 ℃ for 1 min. The cycles were repeated 40 times. The PCR products were 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 products were electrophoresed on 2% 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.

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

Follow-up study after recurrence

From the value of $VEGF_{165}$ mRNA of noncancerous liver remnant, we divided the HCC patients into two groups, i.e., those with a higher level of $VEGF_{165}$ mRNA (over 0.500) as group A, and

those with a lower level of $VEGF_{165}$ mRNA (below 0.500) as group B.

After discharge, all the patients were assessed regularly to detect tumor recurrence with abdominal ultrasonography (every 2-3 mo during the first 5 years, then every 4-6 mo thereafter), serum AFP and liver biochemistry (every 2 mo during the first 2 years, then every 4 mo during the following 3 years, and every 6 mo thereafter), abdominal computed tomography (CT) (every 6 mo during the first 5 years, then annually), and chest x-ray and bone scans (every 6 mo). Hepatic arteriography was obtained if other studies suggested possible cancer recurrence. Detection of tumors on any imaging study was defined as clinical recurrence.

After the detection of recurrence, the following prognostic factors were analyzed and compared between group A and group B: extrahepatic metastasis (presence or absence), the number of recurrent tumor lesions (solitary or multiple), and the extent of recurrent tumors (affecting more than or less than two segments), treatments for recurrent tumors (surgical or non surgical treatment), and survival time after recurrence. The number and extent of recurrent tumors were evaluated and counted from abdominal CT scan and hepatic arteriography.

Statistical analysis

A statistical software (SPSS for Windows, version 8.0, Chicago, Illinois) was employed, Student's *t*-test was used to analyze continuous variables and chi-square test or Fisher's exact test was used for categorical variables. Parameters relating to the presence of VEGF mRNA in liver tissue were analyzed by stepwise logistic regression. A Cox proportional hazard model was used for multivariate stepwise analysis to identify significant variables for outcome of recurrence and mortality. $P<0.05$ was considered statistically significant.

RESULTS

RT-PCR analysis of VEGF transcript in liver tissues

VEGF mRNA was detected in the liver tissues of 10 ($VEGF_{165}$ in 10 and $VEGF_{121}$ in 6) of 30 control patients but the values were very low (all below 0.005). In the HCC group, isoform $VEGF_{165}$ was expressed in noncancerous liver tissues of all 60 patients (100%) (with a value ranging from 0.176 to 0.784) and isoform $VEGF_{121}$ in 36 patients (60.0%) (with a value ranging from 0.285 to 1.030). As to $VEGF_{165}$ mRNA values, 49 (81.7%) patients belonged to group A and 11 patients (18.3%) belonged to group B.

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

Correlation between VEGF mRNA expression in noncancerous liver tissues and clinical histopathologic characteristics

Amongall the patients' characteristics, age, gender, liver cirrhosis, Child-Pugh class A or B, size of HCC, positivity of HBsAg or anti-HCV, and level of serum AFP showed no statistically significant difference between groups A and B (Table 2). From both univariate and multivariate analyses, the correlation between higher $VEGF_{165}$ mRNA expression in liver remnant tissues and grade of cellular differentiation (Edmondson Steiner grade), incomplete or absent capsule, presence of daughter nodules, and vascular permeation was significant respectively (Table 2).

Table 3 shows that group A patients had more tumor recurrence (28.6% *vs* 18.2%, $P = 0.039$), and more recurrence-related death (26.5% *vs* 9.1%, $P = 0.048$). After analysis with Cox proportional hazard model, a higher expression of $VEGF_{165}$ mRNA in the liver remnant had a significant correlation with both a shorter recurrence-free interval and a shorter survival time ($P = 0.037$ and 0.040, respectively) (Table 3). Factors influencing HCC recurrence and time lapse to recurrence were vascular permeation ($P = 0.022$, OR = 5.36), daughter nodules ($P = 0.033$, OR = 4.18), cellular dedifferentiation ($P = 0.033$, OR = 4.18), incomplete or absent capsule ($P = 0.037$, OR = 3.10), and higher $VEGF_{165}$ mRNA expression in the liver remnant ($P = 0.039$, OR = 2.29) (Table 4). The significant variables affecting death resulting from recurrence included vascular permeation ($P = 0.012$, OR = 8.35) and higher $VEGF_{165}$ mRNA expression in the liver remnant ($P = 0.048$, OR = 2.38) (Table 4).

During the follow-up period (range 1 to 3 years, median 2 years), 16 patients (26.7%) had clinically detected recurrence. In 16 patients with recurrent HCCs, there was no statistically significant correlation between the status of a higher $VEGF_{165}$ mRNA expression in the liver remnant and the treatment for recurrent tumors, and the existence of extrahepatic metastasis ($P>0.05$, respectively) (Table 5). However, compared with the extent of intrahepatic recurrence and the outcome, group A patients had a greater number of HCC nodules ($P = 0.043$), and a greater involvement of over two-liver segments ($P = 0.043$). The median survival after recurrence was significantly shorter (4.4 mo *vs* 11.0 mo) in group A ($P = 0.048$) (Table 5).

Table 2 Comparison of characteristics of primary HCC between different levels of $VEGF_{165}$ mRNA in noncancerous liver tussues

Characteristics	Group A (%) (n = 49)	Group B (%) (n = 11)	P
Age (yr, mean)	52	48	NS
Male	73.5	72.7	NS
Liver cirrhosis	79.6	72.7	NS
Child-Pugh class A	71.4	72.7	NS
Tumor size <3 cm	28.6	27.2	NS
>10 cm	34.7	36.4	NS
HBsAg (+)	79.6	72.7	NS
Anti-HCV (+)	53.1	54.5	NS
AFP <20 ng/mL	32.7	27.2	NS
>1 000 ng/mL	20.4	18.2	NS
Edmondson-Steiner grade I[1]	10.2	72.7	0.009
Capsule incomplete or absent[2]	75.5	18.2	0.007
Daughter nodules[3]	61.2	9.1	0.001
Vascular permeation[4]	65.3	9.1	0.001

Notes: high $VEGF_{165}$ mRNA: ⩾0.500 (group A); low $VEGF_{165}$ mRNA: <0.500 (group B). *P*: by univariate analysis. 1, 2, 3 and 4: the significant variables in multivariate analysis with *P* values of 0.036, 0.048, 0.024, and 0.019 respectively. HBsAg: hepatitis B surface antigen; Anti-HCV: antibody to hepatitis C virus; AFP: alpha-fetoprotein; NS: no statistical significance.

Table 3 Correlation between $VEGF_{165}$ mRNA expression in liver remnant and the outcome of patients with HCC

Outcome	Group A (n = 49)	Group B (n = 11)	P
Recurrence (number; %)	14 (28.6)	2 (18.2)	0.039
Death[1] (number; %)	13 (26.5)	1 (9.1)	0.048
Recurrence-free interval (median, mo)	8.5	43.0	0.037
Duration of survival (median, mo)	11.5	41.5	0.040

Notes: high $VEGF_{165}$ mRNA: ⩾0.500 (group A), low $VEGF_{165}$ mRNA: <0.500 (group B), death[1]: patients died of HCC recurrence.

Table 4 Factors influencing tumor recurrence and death of patients in multivariate analysis

Variables	*P*	OR
Recurrence		
Vascular permeation	0.022	5.36
Daughter nodules	0.033	4.18
Cellular dedifferentiation	0.033	4.18
Incomplete or absent capsule	0.037	3.10
Higher $VEGF_{165}$ mRNA in liver remnant	0.039	2.29
Death		
Vascular permeation	0.012	8.35
Higher $VEGF_{165}$ mRNA in liver remnant	0.048	2.38

OR: odds ratio; higher $VEGF_{165}$ mRNA: value $\geqslant$0.500.

Table 5 Correlation between the clinical features of recurrent hepatocellular carcinoma and the expression of $VEGF_{165}$ mRNA in primary lesions

Clinical features	$VEGF_{165}$ mRNA		*P*
	high (n = 14)	low (n = 2)	
Extent of recurrent tumors:			
Extrahepatic metastasis (number, %)	8 (57.1)	1 (50.0)	NS
Multiple recurrent tumors (number, %)	10 (71.4)	1 (50.0)	0.043
Involvement over two-segments (number, %)	10 (71.4)	1 (50.0)	0.043
Survival after recurrence (median mo)	4.4	11.0	0.048
Treatment for recurrent tumors			
Surgery (number)	0	0	NS
Non-surgical treatment[1] (number, %)	8 (57.1)	1 (50.0)	NS
No treatment (number, %)	6 (42.9)	1 (50.0)	NS

Notes: NS: no statistical significance; non-surgical treatments[1]: treatment with transcatheter arterial chemoembolization or/and percutaneous ethanol injection. High $VEGF_{165}$ mRNA: $\geqslant$0.500; low $VEGF_{165}$ mRNA: <0.500.

DISCUSSION

Our study revealed that a higher value of VEGF mRNA isoform $_{165}$ in noncancerous liver remnant tissues of HCC patients was significantly associated with an increased risk of postoperative recurrence and disease mortality. Even after recurrence, those with a higher $VEGF_{165}$ mRNA expression had a larger extent of recurrence and a worse outcome. The value of VEGF mRNA isoform $_{121}$ in liver remnant tissues was not significantly predictive of the outcome.

Studies reporting the correlation between VEGF of noncancerous liver tissues and the potential recurrence were rare. Mise *et al*[16] found that vascular endothelial cells in tumorous tissues showed a dense VEGF immunostaining, whereas those in nontumorous tissues did not show any appreciable staining. In contrast, Feng *et al*[17] found VEGF protein was heterogeneously expressed both in almost all the noncarcinoma portions of the liver and in HCC portions of the liver with HCC. According to Feng *et al*, the nearer the non-cancerous liver cells were to cancerous cells, the stronger the VEGF expression they showed. In HCC cases, VEGF expression in non-cancerous liver cells was a little stronger than that in HCC cells, although there was no significant difference.

To be more accurate, we measured mRNA expression of VEGF in liver tissues rather than the protein itself. According to the study of El-Assal *et al*[18], the level of VEGF mRNA did not always correlate with the protein concentration. Immunohistochemistry could not distinguish small amounts of protein, which may partly explain the discrepancy in protein and mRNA levels. From the study of Mise *et al*, the level of VEGF mRNA in tumorous tissues was higher than that in corresponding nontumorous ones in 12 of 20 patients by more than 1.2-fold. In contrast, in only 2 cases VEGF mRNA levels were lower in tumorous tissues than that in nontumorous tissues (ratio<0.8)[16]. From our study, the level of VEGF mRNA in tumorous tissues was higher than that in corresponding nontumorous ones in 48 (80%) of 60 patients. In 9 patients, the two values were similar and their difference was less than 0.0005. In other 3 patients, VEGF mRNA levels were lower in tumorous tissues than that in nontumorous tissues. We attributed this discrepancy with the result of Mise *et al* to the different methods of mRNA examination and different backgrounds of study patients. The method we used was the nested RT-PCR which was more accurate than conventional RT-PCR.

The value of $VEGF_{165}$ mRNA in remnant livers was a ratio of its expression value to the expression value of GAPDH. We defined it as high if it was over 0.5000. There were 81.7% of our study patients belonging to the high-value group. The detailed mechanisms underlying the increase of VEGF mRNA in the remnant liver remain unclear.

In the literature, the tumor invasiveness variables included high-serum AFP, hepatitis, vascular permeation, grade of cellular differentiation, infiltration or absence of capsule, tumor size, coexisting cirrhosis, presence of daughter nodules, multiple lesions, p53 gene mutation, and gamma glutamyl transpeptidase expression[19-30]. Based on the study of Yamaguchi *et al*[31], VEGF expression in HCC tissues was thought to be related to the histological grade. Suzuki *et al*[32] found that VEGF mRNA expression in HCC was associated with fibrous capsule formation and septal formation. Whereas, as to the higher level of $VEGF_{165}$ mRNA in noncancerous liver tissues but not HCC itself from Table 2, we could find it corresponded significantly with some invasiveness variables (cellular differentiation, capsule status, daughter nodules, and vascular permeation) of primary HCC. We propose the following three possible pathogenetic mechanisms.

The first is that if HCC cells have a more invasive behavior, then they may secrete a higher level of VEGF. VEGF may enter the circulation and increase angiogenesis of the remnant liver via its paracrine growth factor function. Some authors reported that VEGF might be synthesized both in HCC cells and in liver cells, and accumulated in their target endothelial cells[32-39]. Yamaguchi *et al*[31] found VEGF was expressed in surrounding HCC tissues. The noncarcinoma liver cells themselves stimulated by VEGF might also secrete VEGF protein. This effect thus results in a longer and higher VEGF level in remnant liver.

The second is that the noncancerous liver remnant itself has a precancerous change. It is developing into an angiogenetic environment which may secrete a higher VEGF. The mechanism of the precancerous change may be complex, not only influenced by the original HCC itself. Coexisting cirrhosis might contribute to VEGF level[18]. Sasaki *et al*[24] emphasized that cirrhosis had a higher carcinogenic potential. The association of liver cirrhosis with HCC in our patients was as high as 78% (Table 1). Angiogenesis, the sprouting of new capillaries from a pre-existing vascular bed, could provide a route favoring for the malignantly degenerated hepatocytes to develop and to progress[40]. Regeneration in the cirrhotic liver would pose a potential of malignant degeneration[34]. Akiyoshi *et al*[34] suggested that serum VEGF level might be associated with hepatocyte regeneration grade. Suzuki *et al*[32] also supported that VEGF might play an important role in the development of HCC. This could also result in a high level of VEGF in liver remnants.

The third is that the microscopic metastasis from primary HCC to the remnant liver takes place very early. The metastatic lesions may be too small to be detected by the conventional imaging studies including ultrasound, CT scan or arteriography. However, these metastatic HCC cells may also produce a high level of VEGF, resulting in a high expression of $VEGF_{165}$ mRNA in the remnant liver. Miura *et al*[33] observed VEGF expression both in HCC and in non-HCC liver tissues, and supported the hypothesis that VEGF may be involved in the development and/or progression of HCC. Yoshiji *et al*[41] suggested that VEGF played a critical role in the development of HCC in cooperation with endothelial cells, because they found that VEGF-transduced cells showed a marked increase in their invasion activity. Torimura *et al* considered that HCC seemed to originate as a well-differentiated tumor, becoming progressively less differentiated with enlargement. They concluded that VEGF production could increase with tumor progression[40].

The prognosis after recurrence in relation to $VEGF_{165}$ mRNA in the noncancerous liver remnant was rarely reported. VEGF, may also increase the permeability of microvessels to 50 000-fold over that of histamine, thus causing a significant vascular leakiness. An increase in tumor vessel permeability could increase the change of the entry of tumor cells into the circulation, and newly formed vessels or capillaries may have leaky and weak basement membranes through which tumor cells could penetrate more easily than those of mature vessels, thus accelerating the hematogenous metastasis[5-10]. In addition, VEGF could induce both urokinase-type and tissue-type plasmins in endothelial cells which are the key proteases involved in the degradation of the extracellular matrix. These could result in the progression of recurrent HCC in remnant livers. Our findings suggest that remnant livers with a higher $VEGF_{165}$ mRNA have a higher malignant potential manifested as a greater number of recurrent tumors, and a larger extent of involved hepatic segments, a higher recurrence rate and mortality, a shorter recurrence-free interval and a shorter survival. All these factors correlated with an aggressive hematogenous metastasis as the majority of our patients had diffuse multiple recurrent nodules over the remnant liver. Repeat surgery was not undertaken on any patient.

Examination of VEGF mRNA expression in liver remnants during hepatectomy may give us information on the risk of postoperative recurrence. Neoadjuvant antiangiogenic therapy after surgery may be considered for such patients. From this prospective study, we suggest that VEGF mRNA expression in noncancerous liver tissues, especially isoform $VEGF_{165}$, not only plays a significant role in the prediction of postresection recurrence of HCC, but also correlates with a vigorous invasive behavior after recurrence.

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