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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AIM: To investigate the prognostic role of isoform 165 vascular endothelial growth factor messenger RNA (VEGF<sub>165</sub> 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<sub>165</sub> 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<sub>165</sub> mRNA in noncancerous liver tissue correlated significantly with a higher risk of HCC recurrence (P = 0.039) and recurrence-related mortality (P = 0.046), but VEGF<sub>121</sub> 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<sub>165</sub> 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

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<sup>[1-3]</sup>. 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<sup>[4-9]</sup>. 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)<sup>[10,11]</sup>. According to Ferrara’s analysis, VEGF<sub>165</sub> is the predominantly expressed form in human cDNA libraries as in most normal cells and tissues<sup>[12]</sup>. Different cancers may have different expressions of the isoforms. The majority of HCCs expresses an abundance of VEGF<sub>121</sub> and VEGF<sub>165</sub><sup>[13-15]</sup>.

Angiogenesis in tumors has been proven to be an independent factor of prognosis and metastasis in many carcinomas<sup>[13-15]</sup>. Mise et al<sup>[16]</sup> found that VEGF mRNA was also expressed in nontumorous portions of the liver. 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<sup>[16]</sup>. 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 of 54.5±13.5 years) with HCC undergoing curative resection (14 one-segmentectomies, 1 subsegmentectomy, and 13 two-segmentectomies) and 16 minor resections (8 extended right lobectomies, 14 right lobectomies, and 1 left lobectomy) were all excluded.

Clinicopathological variables analyzed included age, sex (male vs female), presence of liver cirrhosis, Child-Pugh’s 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 alpha fetoprotein (AFP) level (< 20 ng/mL vs 20 to 1000 ng/mL vs ≥ 1000 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).

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 °C. We centrifuged it at 12,000 g for 5 min at 4 °C. 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 °C and carefully removed ethanol, the supernatant and dissolved RNA in DEPC-H2O (usually between 50-100 µL), and stored it at -80 °C.

We heated the RNA sample at 55 °C 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 MgCl2, 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 MnCl2, (6) 6 µL RNA in DEPC-H2O, (7) 0.5 µL DEPC-H2O. We incubated it at 70 °C for 2 min, chilled it to 23 °C to anneal primer to RNA. We added 1 µL of M-MLV RTase to (Moloney murine leukemia virus reverse transcriptase, 200 units/µL, Promega product). We incubated it for 8 min at 23 °C, then for 60 min at 40 °C. We heated the reaction at 94 °C for 5 min and chilled it on ice and stored cDNA at -20 °C.

Amplification of cDNA of VEGF and GAPDH by PCR
The sequences of the sense primers were 5′-AGTGTGTTGCC ACGTGGAGA-3′ (VEGF) and 5′-AGTCAACGGATTTGTGTC GTA-3′ (GAPDH) and those of the antisense primers were 5′- AGTCACAGGATTGTTGCTGA-3′ (VEGF) and 5′-GGAAAC GTGAAACCATGTAG-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/L 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 °C for 1 min, annealed at 52 °C for 1 min, and primer extension at 72 °C for 1 min. The PCR 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 VEGF121 (165 bp) and VEGF165 (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 VEGF165 mRNA of noncancerous liver remnant, we divided the HCC patients into two groups, i.e., those with a higher level of VEGF165 mRNA (over 0.500) as group A, and

| 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 ng/mL                  | 20 (48)             |
| >10 ng/mL                  | 12 (20)             |
| HBsAg (+)                  | 47 (78)             |
| Anti-HCV (+)               | 52 (33)             |
| 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.
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_{206} 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_{206} and/or VEGF_{201} in any noncancerous liver tissues or control liver tissues.

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

Among all the patients' characteristics, age, gender, liver cirrhosis, Child-Pugh class A or B, size of HCC, positivity of HBsAg and 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 2 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).

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 tissues

| 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   |
| >1000 ng/mL                      | 20.4                | 18.2                 | NS   |
| Edmondson-Steiner grade I        | 10.2                | 72.7                 | 0.009|
| Capsule incomplete or absent     | 75.5                | 18.2                 | 0.007|
| Daughter nodules                 | 61.2                | 9.1                  | 0.001|
| Vascular permeation              | 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 (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: 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 VEGF₁₆₅ mRNA in liver remnant | 0.039 | 2.29  |
| Death                          |        |       |
| Vascular permeation            | 0.012  | 8.35  |
| Higher VEGF₁₆₅ mRNA in liver remnant | 0.048 | 2.38  |

OR: odds ratio; higher VEGF₁₆₅ mRNA: value ≥0.500.

Table 5  Correlation between the clinical features of recurrent hepatocellular carcinoma and the expression of VEGF₁₆₅ mRNA in primary lesions

| Clinical features                   | VEGF₁₆₅ 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 (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 treatment: treatment with transcatheter arterial chemoembolization or percutaneous ethanol injection. High VEGF₁₆₅ mRNA: ≥0.500; low VEGF₁₆₅ mRNA: <0.500.

DISCUSSION

Our study revealed that a higher value of VEGF mRNA isoform₁₆₅ 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 VEGF₁₆₅ mRNA expression had a larger extent of recurrence and a worse outcome. The value of VEGF mRNA isoform₁₆₅ 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 VEGF₁₆₅ 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 0.500. 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[18-30]. 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[21] found that VEGF mRNA expression in HCC was associated with fibrous capsule formation and septal formation. Whereas, as to the higher level of VEGF₁₆₅ 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 angiogenic 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<sub>165</sub> mRNA in the remnant liver. Miura et al.<sup>[3]</sup> 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. Yoshijii et al.<sup>[4]</sup> 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.<sup>[5]</sup>

The prognosis after recurrence in relation to VEGF<sub>165</sub> 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.<sup>[6-10]</sup> 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<sub>165</sub> 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 remnant 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<sub>165</sub> 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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