

The molecular mechanism underlying angiogenesis in hepatocellular carcinoma: the imbalance activation of signaling pathways

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OBJECTIVE: To explore the effect of two dominating signaling pathways, VEGF/KDR and angiopoietins/Tie2, on the formation of new blood vessel in hepatocellular carcinoma (HCC) growth and metastasis.

METHODS: RT-PCR and Western blot were employed to evaluate the VEGF/KDR and angiopoietins/Tie2 expression in samples from 23 patients with HCC. Meanwhile, microvessel density (MVD) was determined as a marker of angiogenesis by counting CD34 positive cells with the method of immunohistochemistry.

RESULTS: The two pathways were activated in all HCC samples. The expressions of vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang2) were significantly higher ($P < 0.05$) in hepatocellular carcinoma tissues and the margin of the tumor than those in control groups, and so did CD34 positive cells. Although significant difference in the expression of kinase insert domain containing receptor (KDR) and Ang1/Tie2 was not observed in all groups, their distinct high levels were seen in hepatoma and its margin compared with normal and cirrhotic liver. VEGF and Ang2 expressions were seen up-regulated in HCC with vascular invasion and satellite lesion.

CONCLUSIONS: The two signaling pathways, VEGF/KDR and angiopoietins/Tie2 are activated in the process of angiogenesis in HCC and modulate the formation of new blood vessels. The imparity of the two signaling pathways' activation is to benefit HCC metastasis. In the two pathways, VEGF and Ang2 may play an important role in the process of angiogenesis, and are necessary indicators for the prognosis and metastasis of HCC. This study provides another clue for the exploration of anti-angiogenic agents.

(*HBPD Int* 2003; 2: 529–536)

Key words: hepatocellular carcinoma; signaling pathway; angiogenesis; VEGF/KDR; angiopoietins/Tie2

Introduction

Angiogenesis plays a key role in the process of tumor growth by providing abundant oxygen

and nutrients to neoplasm and making a channel for tumor invasion and metastasis.^[1] Pro-angiogenic factors released from tumor cells can promote the formation of new blood vessels by functioning on host endothelial cells. Vascular endothelial growth factor (VEGF) and its kinase insert domain containing receptor (KDR) take part in the process of angiogenesis.^[2] The mature and stabilization of neovasculature are dependent on another subfamily of tyrosine kinase receptor Tie2 and its ligand angiopoietins.^[3] Hepatocellular carcinoma (HCC) characterized by rapid growth, early metastasis and high mortality is an intensive vascular-dependent malignant tumor. Angiogenesis is an initial step for

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these malignant features, and lots of pro- or anti-angiogenic factors and receptors take part in this process. It is suggested that the gene therapy targeting at angiogenic signaling pathways may be a potent treatment for the prohibition of tumor invasion and metastasis.^[4] The molecular mechanism of these pathways during angiogenesis, however, is still unclear. In this study the method of RT-PCR and Western blot were employed to evaluate the expression patterns of VEGF/KDR and angiopoietins/Tie2, which are considered two important signal conductive pathways, and the molecular mechanism underlying these factors and receptors in HCC growth and metastasis.

Methods

Patients

Samples were obtained from 23 patients with primary HCC (mean age 50.86 ± 12.78 years) who had undergone partial hepatectomy. The samples were divided into 3 groups: group A (malignant tissues of HCC), group B (liver tissues within 1.0 cm around the tumor), and group C (liver tissues 5 cm beyond the tumor) respectively. Groups D (8 cirrhotic patients) and E (4 liver donors) served as positive and negative controls. The samples collected during operation were put in liquid nitrogen and kept at -80°C in a deep refrigerator. They were diagnosed pathologically by HE staining and graded by Edmonson's method. The samples were graded II in 18 patients and III in 5. Incomplete capsule or no capsule was seen in 9 patients, vessel invasion in 7, and satellite lesion in 7.

Reagents

Revert-Aid™ M-MuLV reverse transcriptase and Taq DNA polymerase was purchased from Promega, USA. All primers were synthesized by Sangon, Ltd., Shanghai, China. Mouse anti-human monoclonal antibody KDR and Tie2 were obtained from BD Pharmingen, San Diego, CA, USA. Poly-antibody rabbit-anti-human CD34 and goat-anti-human angiopoietin-1 (Ang1), angiopoietin-2 (Ang2) were purchased from Santa Cruz, USA. Monoclonal antibody mouse-anti-human VEGF was

a product of Neomarker, USA.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from the specimens by using Trizol reagent (Gibco, USA). Briefly, the samples were weighed, snap frozen, and ground with a mortar and pestle in Trizol reagent, then allowed warming to room temperature. RNA was then treated with RNase-free $1\ \mu\text{l}$ DNase to remove any contaminated DNA. One microgram of total RNA was reverse transcribed using Revert-Aid™ M-MuLV reverse transcriptase and Random primer (Sangon, Shanghai, China). PCR was performed using $2\ \mu\text{l}$ cDNA and primers specific for Ang1, Ang2, VEGF, KDR, Tie2 and the housekeeping gene GAPDH. Primer sequences are shown in Table 1. The primers for VEGF were designed to share the common splicing sites which can detect all four spliced variants of VEGF in one band. $13\ \mu\text{l}$ PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. Images were captured using Kodak DNA analysis system (Gibco BRL, USA) and density values assessed using Kodak Digital Science 1S 2.0 software. The identity of PCR products was confirmed by sequencing. PCR reactions were performed by pre-denaturation at 94°C for 5 min. Ang1 and Ang2 were am-

Table 1. The sequence of primers for RT-PCR

	Primers sequence	Length (bp)
Ang1*	P ₁ 5'-cagcg ccgaa gtcca gaa ac-3' P ₂ 5'-cacat gtcc agatg ttgaa g-3'	204
Ang2*	P ₁ 5'-gacgg ctgtg atgat agaa atagg-3' P ₂ 5'-gactg tagtt ggatg atgtg ctg-3'	264
GAPDH*	P ₁ 5'-ccacc catgg caaat tccat ggca-3' P ₂ 5'-tctag acggc aggtc aggtc cacc-3'	600
VEGF	P ₁ 5'-ttgcc ttgct gctct acctc-3' P ₂ 5'-tgcac ggtga tgttg gactc-3'	280
KDR	P ₁ 5'-gatgg cctct tctgt aagac-3' P ₂ 5'-attcc atgag accga ctcag-3'	460
Tie2	P ₁ 5'-tctgt gctgt tcctt ctgc-3' P ₂ 5'-cttga gtaac ttcca ggga-3'	375

*: Horner A, Bord S, Kelsall AW, et al. Tie2 ligands angiopoietin-1 and angiopoietin-2 are coexpressed with vascular endothelial cell growth factor in growing human bone. *Bone* 2001;28(1):65-71.

plified for 31 cycles under the following conditions: 94 °C for 60 sec, 55 °C for 60 sec, and 72 °C for 60 sec, with a final extension for 10 min at 72 °C. VEGF and GAPDH were amplified under the following conditions: 94 °C for 40 sec, 58 °C for 40 sec, and 72 °C for 60 sec for 28, 31 cycles, respectively, with a final extension for 10 min at 72 °C. KDT and Tie2 were amplified for 31 cycles under the following conditions: 94 °C for 60 sec, 58 °C for 60 sec, and 72 °C for 60 sec, with a final extension for 10 min at 72 °C.

Western blot

Samples were homogenized in lysis buffer.^[5] Proteins were dissolved in SDS-Laemmli buffer by boiling for 5 min. In each lane 50 µg of total proteins was loaded and separated by SDS-PAGE (6% gel for Tie2 and KDR, 8% gel for Ang1, Ang2 and VEGF). The electrophoresed proteins were then transferred to a PVDF membrane (Immobilon Millipore, Bedford, Massachusetts, USA) by a semi-dry electrophoretic transfer procedure for 1.5 hours at 1 mA/cm². Ponceau staining was done to check if all proteins were transferred to a comparable extent in all lanes. The membranes were blocked with 5% defatted milk prepared in TBS buffer, pH 7.5. Then, primary antibodies were added to bind specially with the membrane overnight. After washing with TBST for 3 × 15 min, the corresponding second antibodies were added and incubated for 1 hour. The membrane was washed as described above. The immuno-complex was visualized by enhanced chemiluminescence detection using ECL reagents supplied by Santa Cruz, USA followed by autoradiography on a X-ray film. The primary antibodies were diluted: Ang1, Ang2 and VEGF as 1:500; KDR and Tie2 as 1:1000.

Immunohistochemistry

Four to 6 µm sections were cut from paraffin blocks fixed with formalin, deparaffinized in xylene and then hydrated in ethanol step by step. The sections were boiled in citric acid for 3 min to repair antigen and incubated in 3% hydrogen peroxide for 5 min to quench of endogenous peroxidase activity.

After incubating in 10% sheep serum for 5 min, anti-CD34 mouse monoclonal antibody diluted at 1:25 was used and incubated in 37 °C for 1 hour. The sections were developed using DAB method following the manufacture's instructions. The positive cells were counted on at 200-fold in five fields.

Statistical analysis

The data were expressed as mean ± standard deviation. The absorbency for RT-PCR and vascular density was analyzed using one-way ANOVA with SPSS 11.0 software. *P* values ≤ 0.05 were considered significant.

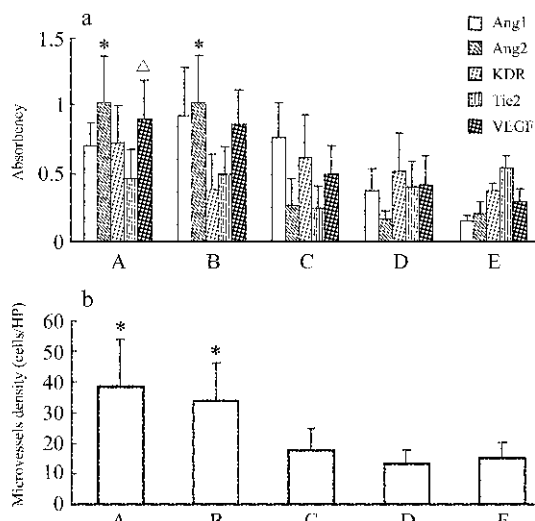


Fig. 1. The relationship between the microvessels density and angiogenic factors/receptors in HCC. A: HCC; B: liver tissue within 1.0 cm around tumor; C: liver tissues 5 cm beyond the tumor; D: liver cirrhosis; E: normal liver; a: the different angiogenic factors and receptors mRNA expression in different groups. In the HCC and margin of HCC, all ligand and receptors expressions were significantly higher than those in other groups, especially the Ang2 (groups A and B vs groups C, D and E, *P* < 0.01) and VEGF (groups A and B vs groups C, D and E, *P* < 0.05); b: the microvessel density sketch in different groups. Evident increase of MVD was observed in groups A and B, and significant difference (*P* < 0.01) was noted compared with other groups. But no difference was seen between groups A and B; *: *P* < 0.01; Δ: *P* < 0.01.

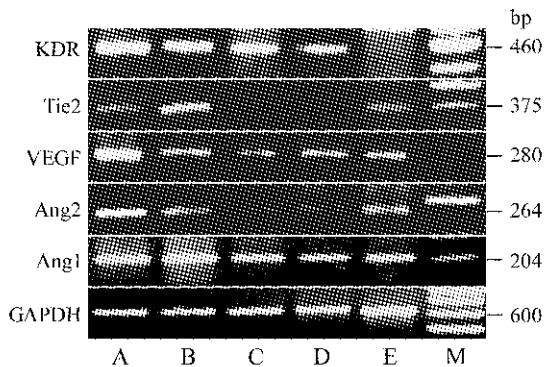


Fig. 2. Angiogenic factors and receptors mRNA PCR picture. A: HCC; B: liver tissue within 1.0 cm around tumor; C: liver tissues 5 cm beyond the tumor; D: liver cirrhosis; E: normal liver; M: DNA Marker. GAPDH served as control.

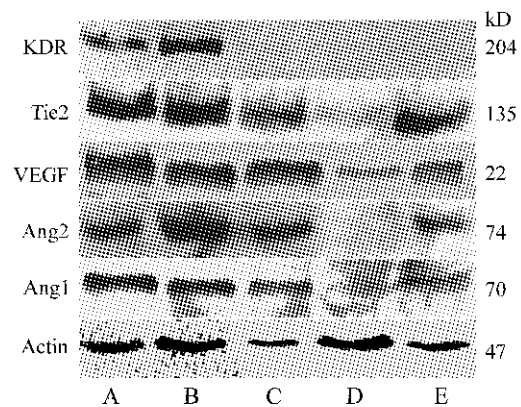


Fig. 3. Angiogenic factors and receptors proteins expression (Western blot). A: HCC; B: liver tissue within 1.0 cm around tumor; C: liver tissues 5 cm beyond the tumor; D: liver cirrhosis; E: normal liver.

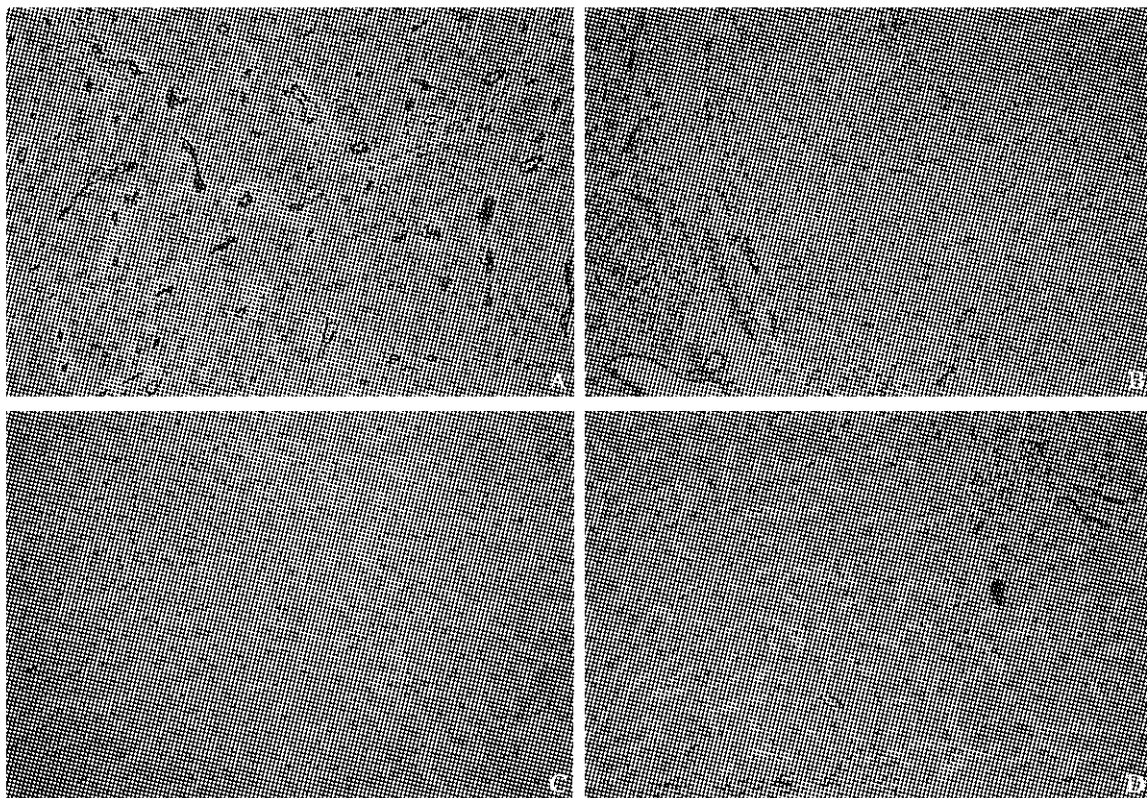


Fig. 4. A: the core of HCC (original magnification $\times 200$). A large number of new blood vessels in the photo. B: the margin of HCC (original magnification $\times 200$). New blood vessels in the HCC tissue and surrounded by inflammatory cells. C: liver cirrhosis (original magnification $\times 200$). No CD₃₄ positive staining cells in this section. D: normal liver (original magnification $\times 200$). CD₃₄ molecule in the portal area.

Table 2. The relationship between invasion of hepatoma and angiogenic factors

Invasive factors of HCC	n	MVD (cell/HP)	Absorbency				
			Ang1	Ang2	VEGF	Tie2	KDR
Capsule							
Integrity	14	38.20±15.86	0.80±0.57	0.66±0.30	0.80±0.10	0.52±0.80	0.57±0.33
Unintegrity	9	49.07±19.01	1.07±0.62	1.09±0.71	1.08±0.58	0.38±0.28	0.39±0.43
Vessel invasion							
With	7	53.80±19.13	1.03±0.67	1.17±0.78*	1.24±0.61 ^Δ	1.12±1.47*	0.59±0.40
Without	16	36.17±13.51	1.58±0.33	0.59±0.33	0.80±0.10	0.32±0.28	0.50±0.36
Satellite lesion							
With	7	46.90±34.08	1.14±0.88	1.44±0.88 ^Δ	1.43±0.72 ^Δ	0.57±0.48	0.39±0.34
Without	16	43.53±16.18	0.83±0.56	0.59±0.32	0.80±0.09	0.46±0.73	0.54±0.36
Pathological grade							
II	15	39.71±16.6	0.85±0.62	0.25±0.20	0.82±0.06	0.54±0.34	0.49±0.69
III	8	47.40±13.85	1.00±0.11	0.80±0.16	0.90±0.04	1.42±1.97*	0.56±0.53

*: Vs control group, $P < 0.05$; Δ : vs control group, $P < 0.01$.

Results

The relationship between expression of the two signaling pathways and microvessel density

In this study, Ang1 mRNA was detected in all samples and no significant difference was found in all groups. The level of VEGF mRNA expression was the highest in group A and showed remarkable difference compared to other groups ($P < 0.05$). Ang2 expression in groups A and B were significantly higher than in groups C, D and E ($P < 0.01$, Fig. 1a and Fig. 2), but there was no difference between groups A and B. The levels of KDR and Tie2 mRNA expression were not significantly different in all groups, but high absorbency was observed in groups A and B.

To assess protein synthesis of varied angiogenic factors, 50 μ g total protein was loaded and separated by SDS-PAGE. The electrophoresed protein was detected with the specific antibody. The protein synthesis of all factors examined in this study were up-regulated consistently with their gene transcriptive level. In group A the levels of VEGF and Ang1 protein were higher than those in other groups (Fig. 3), while receptors KDR and Tie2 expressions were increased simultaneously. The levels of Ang2 and KDR were dominantly higher in group B than in other groups.

Microvessel density was detected with the method of immunohistochemistry. MVD was remark-

ably increased in groups A and B compared with other groups ($P < 0.05$, Fig. 1b). The number of CD34 positive endothelial cells was maximal in group A on the immunohistochemistry photos under a microscope (Fig. 4).

The relationship between metastasis of HCC and angiogenic signaling pathways

The interaction between mRNA expression of the two angiogenic pathways and the invasiveness and metastasis of hepatoma was also analyzed. The expression of VEGF, Ang2 and Tie2 with vessel cancerous embolism was significantly higher than that of those without vessel cancerous embolism in samples ($P < 0.05$, Table 2). At the same time, marked expression of VEGF and Ang2 was seen in HCC patients with satellite lesion compared with those without satellite lesion ($P < 0.01$). However, these factors were not different in HCC with or without capsules.

Discussion

Angiogenesis in HCC

The two signaling pathways, VEGF/KDR and angiopoietin/Tie2, were clarified to be activated in HCC tissues in this study. CD34 molecule secreted from endothelial cell served as marker of formation of new blood vessel and marked increase was observed in immunohistochemical sections of resected

HCC. It is presumed that the activation of angiogenesis may be a critical event during malignant transformation and metastasis in the process of HCC. Human hepatocellular carcinoma is a highly vascularized tumour, in which angiogenesis plays an important role in tumor biology.^[6] Tumor cells can induce the formation of new blood vessels to supply the tumor mass with blood borne nutrients. This process triggers the "angiogenic switch"^[1] characterized by high expression of endogenous pro-angiogenic factors and low expression of anti-angiogenic factors simultaneously. Lots of pro-angiogenic factors are released from tumor cells and other matrix cells. Two families of the vascular endothelium-specific growth factors, VEGF and angiopoietins, have been proved to be dominant in the control of angiogenesis. Increased activities of VEGF/KDR and angiopoietins/Tie2 in other organ carcinoma^[7-10] have been reported other than the process of HCC and even the molecular mechanism underlying these factors and their receptors in HCC growth and metastasis. The purpose of this study is to probe into the labyrinth underlying angiogenesis of HCC.

The two families, VEGF/KDR and Angiopoietins/Tie2, play an important role in the process of HCC

The two signal conductive pathways, VEGF/KDR and angiopoietins/Tie2, play an important role in the process of angiogenesis in tumor growth and metastasis. The tumor cells have been thought to be the main source of VEGF in tumor growth. As a major regulator of both physiological and pathological neovascularization, VEGF is a relatively specific mitogen for vascular endothelial cells.^[11] The environment of hypoxia in HCC may stimulate tumor cell to secrete VEGF, which activates its receptor VEGFR-2 (KDR) in endothelial cells. The activation of phosphorylated KDR seems to mediate the major VEGF function. As a result, it stimulates the proliferation, migration and tubal formation of endothelial cells as well as the degradation of extracellular matrix ECM.^[12,13] A dominant negative mutant of VEGFR-2 can be prevented from tumor growth in nude mice.^[14] This result illumi-

nates that VEGF/KDR signaling pathway may be necessary for tumor angiogenesis. Another endothelial cell-specific receptor tyrosine kinase, Tie2, and its ligand family, Ang1 and Ang2, have been found to have a distinct function in angiogenesis. In endothelial cells, Tie2 phosphorylation is thought to mediate vital functions of vascular stabilization and remodeling during embryonic angiogenesis.^[15] Tie2 phosphorylation is mainly induced by Ang1 because of the 10-fold affinity of Ang1 to Tie2 over Ang2, but an excess of Ang2 can inhibit the Tie2 phosphorylation. After binding to Tie2, Ang2 can competitively antagonize Ang1 effects by blockage of Tie2 phosphorylation. The primary function of Ang1 is to recruit the supporting pericytes of vessel and stimulate endothelial cell migration in 3D matrix culture. At the presence of VEGF, Ang1 can stimulate endothelial cell to form tubule-like structure;^[16] however, an excess of Ang2 can retard this proceeding.

In this study, the highest microvessel density was seen immunohistochemically in the HCC tissue (Figs. 4a and 4b). In contrast to other areas of liver with HCC and positive and/or negative control, tumor vasculature in HCC and the marginal area of HCC was shown in high disorganization that vessels were tortuous and dilated with uneven diameter, excessive branching and shunts in the pathological sections. Consequently, tumor blood flow was chaotic and variable,^[17] resulting in the formation of hypoxic and acidic regions in the tumor.^[18] This result was due to an imbalance of angiogenic regulators, such as VEGF and Ang2. The over expression mRNA of VEGF and Ang2 was detected in HCC tissue, and the protein levels of these factors were increased as compared with other groups. Our findings are consistent with those of other studies in gallbladder, mammary tumor, glioma, neck and oral cancer, and lung carcinoma. The Ang1 expression, however, was decreased in HCC tissue. The phenomenon of high expression of VEGF and Ang2 as well as the low expression of Ang1 in HCC suggests that hypoxia in the tumor tissue may act as the initial stimulus for molecular expression changes. Cooperating with an Ang2 up-regulation, the increased VEGF further augments vascular proliferation. Down-regulation of Ang1

would benefit for vascular de-stabilization and endothelial cell migration to the tumor region. Another function of VEGF is that it can induce Ang2 production secreted from the supportive pericytes around vessels and tumor cells. Not only can Ang2 further lessen intercellular junctions and the junction between the cells of blood vessel and the surrounding extracellular matrix, but also inhibit the phosphorylation of Tie2 with the manner of autocrine.^[19] In our study, the low expression of Tie2 receptor in the marginal area of HCC was seen in the level of mRNA and protein synthesis compared with in HCC tissue. Hence, there are two outcomes for the high expression of Ang2. One is the regression of vessels preexisted and the aggravation of tissue hypoxia because of the apoptosis of endothelial cells in angiogenesis; the other is the augmentation of newly formed vessels from preexisting vasculature because of the proliferation and migration of endothelial cells induced by VEGF. Owing to the activation of the two signaling pathways, the flourishing new blood vessels may appear in the pathological sections of HCC tissue. It is supported that the two signaling conductive pathways, VEGF/VEGFR-2 (KDR) and angiopoietins/Tie2, play an important role in the angiogenesis for the growth of HCC.

The relationship between high expression of VEGF, Ang2 and Tie2 and the malignant characteristic of HCC

The progress and metastasis are closely related to the prognosis of HCC. The imbalance of angiogenic factors expression in the tumor tissue results in the formation of new blood vessels with abnormal function and structure. The high level of VEGF and Ang2 may stimulate endothelial cells to proliferate and migrate and ultimately form new blood vessels, but more defects may be found in walls of these blood vessels such as numerous "openings", widened interendothelial junctions, and a discontinuous or absent basement membrane because of low expression of Ang1.^[20] These vessels, leaky and permeable, prompt malignant cells to penetrate through the vascular wall to metastasize to other areas. In this study, factors relevant

to tumor metastasis analyzed included capsules of HCC, vessel invasion, pathological grading and satellite of tumor. The higher expression of VEGF, Ang2 and Tie2 was seen in HCC with vessel invasion and satellite lesion. It is presumed that vessel invasion and satellite lesion may be the major factors for the progress and metastasis of hepatocellular carcinoma, while the levels of VEGF and Ang2 expression may serve as a marker of malignancy of HCC. Hence further study is needed to confirm the validity of this conclusion.

In summary, the two signal pathways, VEGF/KDR and angiopoietins/Tie2, take part in the new blood vessel formation in physiological and pathological angiogenesis. For the tumor treatment of anti-angiogenesis, it is important to understand the molecular mechanism of angiogenic signal conductive pathway as well as the function of various pro-angiogenic factors. We propose that the two signal pathways would be activated successively in the process of hepatoma growth and as a marker of malignancy of HCC. The two pathways work independently but synergically in the process of angiogenesis.^[21] Thus, it is necessary to block the two signal pathways other than only a single pathway during the anti-angiogenic treatment targeting at tumor growth.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Received September 15, 2003

Accepted after revision October 15, 2003