Significance of the vascular endothelial growth factor and the macrophage migration inhibitory factor in the progression of hepatocellular carcinoma

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Abstract. The aim of the present study was to investigate the expression of vascular endothelial growth factor (VEGF) and macrophage migration inhibitory factor (MIF) in HCC progression and their correlation with clinicopathological factors as well as the relationship between their expression levels. The expression of serum VEGF and MIF was evaluated in 150 patients with HCC and in 30 normal volunteers by enzyme-linked immunosorbent assay (ELISA). VEGF and MIF expression levels were evaluated by immunohistochemistry on tissue microarrays containing 150 HCCs with paired adjacent non-cancer liver tissues. VEGF and MIF mRNA levels were determined by quantitative PCR in another 48 HCCs. The correlation of VEGF and MIF with clinicopathological factors was analyzed in HCC. Serum VEGF and MIF concentrations were higher in HCC patients than the levels in the controls. The expression levels of VEGF and MIF in the HCC tissues were both higher than those in the adjacent non-tumor liver tissues. Overexpression of VEGF and MIF was significantly associated with tumor size (P=0.027 and 0.022, respectively), intrahepatic metastasis (P=0.032 and 0.027, respectively), vascular invasion (P=0.044 and 0.039, respectively) and TNM stage (P=0.028 and 0.013, respectively). Furthermore, VEGF and MIF mRNA levels were higher in HCC compared to levels in the paired non-cancer liver tissues. VEGF and MIF mRNA levels were correlated with tumor stage and metastasis. The expression of VEGF was positively related with MIF expression in HCC. The expression of MIF and VEGF in HCC was markedly positively correlated, which suggests that MIF and VEGF play an important role in the progression of HCC. Both factors may concomitantly accelerate the progression of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the second most common cause of cancer-related death worldwide and nearly half of all HCC cases occur in China (1). Despite improvements in surgical techniques and the development of novel therapies during the past few decades, the clinical prognosis of HCC patients is still poor due to recurrence and metastasis. The molecular mechanisms involved in HCC development remain obscure. Therefore, it is of great clinical value to further identify malignant factors in order to understand the molecular mechanisms underlying the progression of HCC.

Tumor angiogenesis plays an important role in tumor growth and metastasis (2). Vascular endothelial growth factor (VEGF) has been implicated as an invasion and tumor progression promoter molecule (3). VEGF is a potent mitogen that contributes to both physiological and pathological angiogenesis (4). VEGF is believed to secrete homodimeric glycoprotein that stimulates proliferation and migration of endothelial cells and enhances vascular permeability (5). An increasing number of studies have demonstrated a strong association between overexpression of VEGF and advanced disease or poor prognosis in various types of cancers (6-8). VEGF was recently found to be upregulated in HCC, and it was also shown to be associated with the carcinogenesis, metastasis, recurrence and prognosis of HCC (9,10). However, further investigation is needed to confirm the molecular mechanisms underlying the effects of VEGF on the development of HCC.

Several mechanisms have been reported to participate in the regulation of VEGF gene expression. Among these, several cytokines or growth factors play a major role. VEGF mRNA expression is rapidly and reversibly induced by epidermal growth factor (EGF), transforming growth factor-β (TGF-β), or keratinocyte growth factor (11). Ren *et al* (12) reported that macrophage migration inhibitory factor (MIF) can stimulate

the secretion of VEGF from tumor cells. The cytokine MIF is regarded as a major regulator of inflammation and a key mediator that operates as a cytokine and an enzyme (13). Many studies have confirmed the use of MIF as a biomarker for different diseases that have an inflammatory component (14). Moreover, recent studies have demonstrated a role of MIF in tumor growth, such as control of cell proliferation and promotion of angiogenesis (15). MIF also plays an important role in the invasion and metastasis of prostate cancer, lung adenocarcinoma and neuroblastoma cells (15,16). Ren *et al* (17) found that MIF mRNA was upregulated in HCC tissues when compared with normal liver tissues, suggesting that MIF acts as a regulator of tumor progression in HCC.

The above studies suggest that both VEGF and MIF may be involved in the tumorigenesis of HCC. An examination of whether the aberrant expression of these two proteins is associated with clinicopathological characteristics of HCC patients is therefore warranted. However, to date there has been no report on the clinical relevance of combined VEGF and MIF expression in HCC tissues. To address this problem, the aim of the present study was to further investigate the potential association of the co-expression of VEGF and MIF in HCC tissues with clinicopathologic findings.

Patients and methods

Patients and tissue specimens. One hundred and fifty pairs of matched HCC and adjacent non-cancer liver tissues were histopathologically and clinically diagnosed at The First Affiliated Hospital of Sun Yat-Sen University from January 2004 to June 2006. Plasma samples from a peripheral vein were also collected from the 150 HCC patients. Plasma samples were obtained from healthy volunteers who underwent physical examination at the First Affiliated Hospital of Sun Yat-Sen University. The 150 patients included 95 males and 55 females. The mean age of the patients was 58 years (range, 20-78 years). Clinicopathological classification and staging were carried out according to the 6th edition of the American Joint Committee on Cancer (AJCC) TNM classification system. Another independent 48 patients with histologically proven HCC were included in this study. These 48 pairs of tumor tissues from HCC patients and paired adjacent non-cancer specimens were collected for real-time RT-PCR analysis as previously described (18). The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University. Informed consent was obtained from all patients prior to surgery. All patients were recruited into this study after providing informed consent.

Enzyme-linked immunosorbent assay. All peripheral blood samples were acquired following a standard collection protocol. Briefly, samples were collected and anticoagulated by ethylene diamine tetraacetic acid (EDTA) and centrifuged for 10 min at 3000 rpm. The serum fractions were aliquoted and stored at -80˚C until analysis. The concentrations of serum MIF were measured by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. The levels of serum VEGF were determined using ELISA kits (Genzyme Corp., USA).

Tissue microarray construction. The representative areas of each HCC specimen or paired adjacent non-cancer liver tissue were punched with a tissue cylinder (1 mm in diameter) from formalin-fixed/paraffin-embedded tumor tissues or paired adjacent non-cancer tissue blocks. The selected tissue cores were precisely arrayed into a new recipient microarray block using a tissue arrayer (Beecher Instrument, Silver Spring, MD, USA). Each sample was arrayed in triplicate.

Immunohistochemistry. Immunohistochemical analysis was performed to study MIF and VEGF expression in 150 human HCC tissues and paired adjacent non-cancer tissues. Briefly, paraffin-embedded tissue-microarray blocks of HCC tissues and paired adjacent non-cancer tissues were consecutively cut into 4-μm sections. Slides were baked at 60˚C for 1-2 h and then deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 20 min at room temperature. Slides were incubated overnight at 4˚C with primary antibodies (Abcam, Cambridge, UK; catalog no. ad55445; 1:500 dilution) and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 dilution) diluted in phosphate-buffered saline (PBS). After washing, the tissue slides were subsequently treated with the secondary antibody (anti-Rb or mouse IgG/HRP; Zhongshan, 1:2000) for 1 h at room temperature, and then with 3,3'-diaminobenzidine (DAB) solution followed by counterstaining with hematoxylin. Analysis was performed with a Zeiss Axioscope 2 microscope at a x400 magnification, respectively. The degree of immunohistochemical staining was semi-quantitatively assessed and scored independently by two observers. For levels of MIF and VEGF expression, staining intensity was scored according to the following criteria: no staining, 0; weak staining, 1; moderate staining, 2; and strong staining, 3.

RNA extraction and real-time polymerase chain reaction (PCR). Total RNA was extracted from the tissue samples using Trizol (Invitrogen) according to the manufacturer's instructions. Real-time PCR amplifications were performed in ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using EvaGreen™ qPCR Master Mix (Biotium, Hayward, CA, USA). The primers for human VEGF were 5'-TCGAGACCCTGGTGGACATC-3' (forward) and 5'-TGTTGGACTCCTCAGTGGGC-3' (reverse). MIF primers were 5'-CAGAACCGCTCCTACAGCAAG-3' (forward) and 5'-CGGCTCTTAGGCGAAGGTG-3' (reverse) and β-actin primers were 5'-ACAATGTGGCCGAGGACTTT-3' (forward) and 5'-GGAGAGGACTGGGCCATTCT-3' (reverse). The optimal PCR amplification for VEGF and MIF was 95˚C for 30 sec followed by 40 cycles (95˚C for 5 sec, 60˚C for 30 sec). The expression of β-actin was used as the internal control. The relative expression levels of VEGF and MIF mRNA were calculated according to the comparative Ct method, and the expression of target genes was normalized to β-actin expression levels in each sample.

Statistical analysis. Data are presented as means ± standard deviation (SD). All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). χ^2 test or Fisher's exact test was used for comparisons between immunohistochemical and serum results and clinicopathological

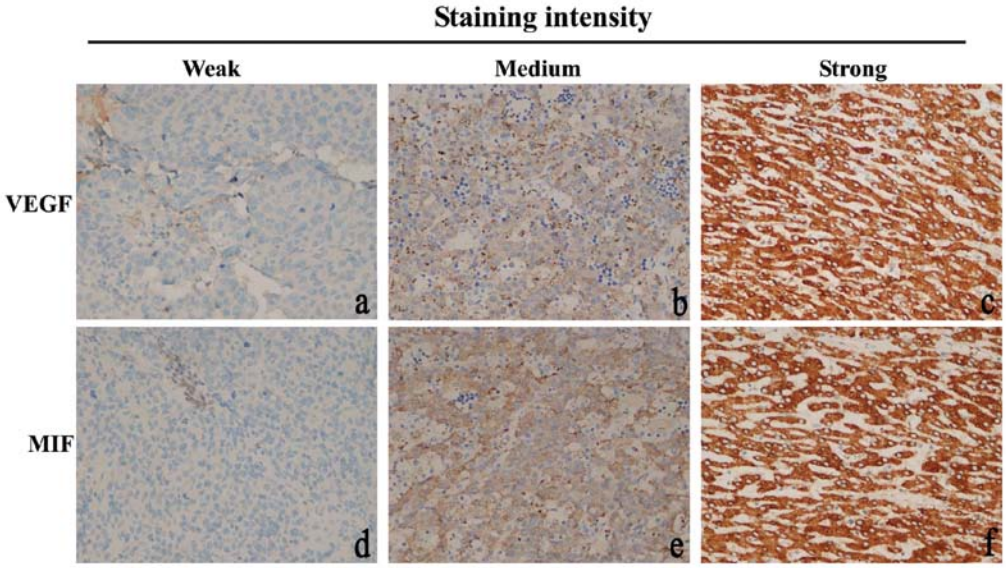

Figure 1. Immunohistochemical staining of VEGF and MIF in human hepatocellular carcinoma (HCC) tissues (magnification, x200). The positive staining of VEGF (a, weak staining intensity; b, medium staining intensity; c, strong staining intensity) and MIF (d, weak intensity; e, medium intensity; f, strong intensity) was localized in the cytoplasm of HCC cells. Results are representatives of the immunostainings of the specimens.

Table I. Comparison of serum VEGF and MIF levels between HCC patients and the control group.

	n	Means ± SD	P-value
VEGF			0.011
Patients with HCC	150	414.71±41.92 (ng/l)	
Control	30	176.52±32.14 (ng/l)	
MIF			0.032
Patients with HCC	150	123.71±18.34 (μg/l)	
Control	30	11.53±5.47 (μg/l)	

parameters. Spearman's bivariate correlation test was used to evaluate the correlation between VEGF and MIF. Differences in VEGF mRNA and MIF mRNA expression between the groups were analyzed by the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant result.

Results

Upregulation of VEGF and MIF in serum samples of patients with HCC. Using ELISA, the levels of serum VEGF and MIF were evaluated in 150 patients with HCC and 30 normal volunteers. The serum VEGF and MIF levels were significantly higher in patients with HCC when compared with their levels in the normal controls (Table I). Overexpression of serum VEGF and MIF was significantly associated with tumor size, intrahepatic metastasis, vascular invasion and TNM stage (Table II). Furthermore, the high levels of VEGF in the serum were positively related with serum MIF expression in HCC (r=0.579, P<0.05).

Overexpression of VEGF and MIF in archived HCC tissues. In subsequent studies, we detected the role of VEGF and MIF in the clinical progression of HCC. We examined 150 paraffin-embedded, archived HCC tissues, including 30 cases of stage I, 80 cases of stage II and 40 cases of stage III tumors, using immunohistochemical staining. High levels of VEGF were present in the cytoplasm of the malignant cells in 75% (112/150) of HCC tissues (Fig. 1b and c). In contrast, VEGF was negatively or only weakly detectable in adjacent non-cancer tissues (Fig. 1a). In addition, the index values of VEGF staining were significantly increased with the progression of tumor grades I to III (P=0.028). Moreover, VEGF expression was strongly correlated with tumor size (P=0.027), vascular invasion (P=0.032), and serum AFP levels (P=0.043). However, our analyses did not show significant associations between VEGF expression and other clinical features including age, gender, history of hepatitis, liver cirrhosis and tumor multiplicity (Table III).

MIF was localized in the cytoplasm of positive staining HCC cells (Fig. 1d-f). MIF was detected in 81% (121/150) of HCC cases (P<0.001). Our studies showed that high levels of MIF expression were associated with tumor size (P=0.022), tumor grade (P=0.013), presence of intrahepatic metastasis (P=0.039) and vascular invasion (P=0.027) and TNM stage (P=0.013). There were no further associations with other clinicopathological parameters (Table III). Spearman correlation analysis confirmed that VEGF expression was positively correlated with MIF protein expression (r=0.619, P=0.022) in the HCC tissues.

VEGF and MIF mRNA expression in HCC and correlations between VEGF and MIF mRNA expression. To confirm the effect of VEGF and MIF on the progression of HCC and their correlation, we examined their mRNA levels in 48 HCCs and paired adjacent non-tumor tissues by real-time RT-PCR. The mRNA level of VEGF was significantly increased in the HCC tissues when compared with the level in the paired adjacent non-tumor tissues (P<0.01) (Fig. 2A). In HCC tissues, VEGF mRNA expression increased according to increasing TNM stage (Fig. 2C). The mRNA level of VEGF was significantly

Table II. Correlation between serum VEGF and MIF levels and the clinicopathological characteristics of the HCC patients.

Variable feature	n	VEGF (ng/l)	P-value	MIF (μg/l)	P-value
Tumor size (cm)			0.011		0.027
≤5	46	295.9±26.9		58.7±13.8	
>5	104	368.7±34.8		116.8±23.8	
TNM stage			0.032		0.034
I	30	306.7±42.9		65.3±16.9	
II	80	412.5±51.3		118.7±24.2	
III	40	634.6±73.4		143.5±26.3	
Vascular invasion			0.028		0.035
Absence	103	312.3±40.4		85.9±14.7	
Presence	47	586.7±64.8		118.7±21.3	
Intrahepatic metastasis			0.031		0.026
Absence	93	337.4±36.5		91.8±25.9	
Presence	57	668.3±54.6		129.7±34.6	

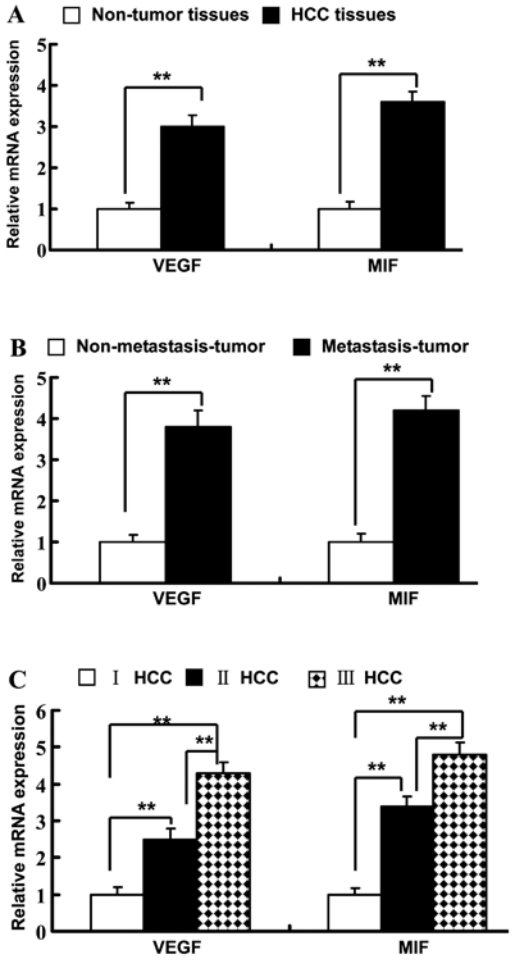

Figure 2. Quantitative PCR analysis of VEGF and MIF mRNA expression in (A) hepatocellular carcinoma (HCC) and paired adjacent non-cancer liver tissues, (B) nonmetastatic and metastatic HCC and (C) HCC with varied TNM stages. **P<0.01.

increased in metastatic HCC tissues when compared with the level in the nonmetastatic tissues (Fig. 2B). Consistent with VEGF, the MIF mRNA level was markedly higher in the HCC tissues when compared with the level in the adjacent non-tumor tissues (P<0.001) (Fig. 2A). MIF mRNA expression was significantly elevated in later TNM stages (P<0.001) (Fig. 2C). MIF mRNA was higher in the metastatic HCC tissues when compared with that in the nonmetastatic tissues (Fig. 2B). A positively correlation was noted between VEGF and MIF mRNA expression (r=0.72, P=0.066).

Discussion

In the present study, we analyzed the expression of VEGF and MIF in HCC and evaluated the levels of VEGF and MIF with the clinicopathological parameters in 150 cases. We measured the concentration of VEGF and MIF in a series of 150 serum samples from HCC patients. Additionally, a series of 30 serum samples from healthy volunteers was selected as controls. Moreover, we assessed the relationship between the levels of VEGF and MIF and the clinicopathological factors of the HCC cases. In the present study, we found that the serum levels of VEGF and MIF were markedly increased in the HCC group when compared to levels in the control group. Overexpression of serum VEGF and MIF was significantly associated with tumor size, tumor grade, intrahepatic metastasis, vascular invasion and TNM stage. Furthermore, high levels of VEGF in the serum were positively co-related with serum MIF expression in HCC. These results were consistent with the expression of VEGF and MIF in HCC tissue samples.

VEGF is known as one of the most potent pro-angiogenic factors (19). Several studies (20-23) have demonstrated that VEGF promotes the growth of local foci of malignant tumors and facilitates metastasis and invasion. VEGF, upregulated in various solid tumors, is closely correlated with pathological characteristics, metastasis and prognosis of tumors. Silencing of MMP-9 and VEGF decreases the recurrence and metastasis of HCC after TACE (24,25). Therefore, VEGF plays an important role in the tumorigenesis of tumors. Our results showed that enhanced VEGF was associated with

Table III. Correlation between VEGF and MIF expression and the clinicopathological characteristics of the HCC patients.

		VEGF					MIF				
Variable feature	n	0	1	2	3	P-value	0	1	2	3	P-value
Age (years)						0.834					0.675
≥50	95	22	17	22	34		22	16	33	24	
<50	55	16	8	12	19		7	11	20	17	
Gender						0.712					0.738
Male	125	35	24	32	34		22	23	43	37	
Female	25	3	5	8	9		7	3	6	9	
Etiology						0.411					0.513
Noninfection	29	8	7	10	4		12	3	8	6	
Hepatitis B	109	28	20	28	33		15	23	36	35	
Hepatitis C or other	12	2	2	3	5		2	1	5	4	
Liver cirrhosis						0.038					0.041
Absence	44	15	11	10	8		11	7	14	12	
Presence	106	23	14	30	39		18	22	42	24	
Tumor size (cm)						0.027					0.022
≤5	46	10	8	18	10		11	7	17	11	
>5	104	28	21	23	32		18	21	36	29	
Serum AFP (μg/l)						0.043					0.037
≤20	42	14	7	10	11		6	12	14	10	
>20	108	24	23	31	30		23	15	39	31	
TNM stage						0.0283					0.0134
I	30	19	3	5	4		10	6	8	6	
II	80	9	14	26	31		11	10	35	24	
III	40	10	8	12	10		8	7	14	11	
Vascular invasion						0.0315					0.0267
Absence	103	31	20	23	29		20	21	37	25	
Presence	47	7	8	19	13		9	6	15	17	
Intrahepatic metastasis						0.0437					0.0391
Absence	93	30	18	23	22		19	17	31	26	
Presence	57	8	10	18	21		10	10	22	15	

intrahepatic metastasis, vascular invasion and later tumor stage. In addition, VEGF expression was positively correlated with MIF expression in the serum of patients with HCC. Furthermore, quantitative PCR verified that VEGF mRNA was significantly upregulated in HCC tissues when compared with that in adjacent non-tumor tissues; there was a correlation between the upregulation of VEGF mRNA with tumor TNM stage and metastasis in HCC.

MIF was initially found to contribute to the inhibition of the random migration of macrophages (26). Recent studies have established that MIF plays an important role in carcinogenesis by promoting cell proliferation, tumor angiogenesis and metastasis (27). He *et al* (28) demonstrated that epithelial and serum MIF expression was progressively increased in gastric cancer. Bando *et al* (29) found that MIF was overexpressed in 93 breast cancer tissues as detected by ELISA. In esophageal squamous cell carcinoma, MIF expression was found to be correlated with lymph node status (12). In the present study, the immunohistochemical and ELISA results showed that MIF expression was correlated with increasing tumor grade, intrahepatic metastasis and vascular invasion. Moreover, MIF expression was positively correlated with VEGF expression. Thus, these results suggest that activated MIF/VEGF is involved in proliferation, invasion and metastasis in HCC. Choudhary *et al* (30) reported that treatment with inhibitors of MIF increased mRNA expression and protein secretion of VEGF in bladder cancer. Bondza *et al* (31) indicated that MIF markedly stimulates the secretion of VEGF, which is in accordance with the findings of the present study.

MIF and VEGF were overexpressed in patients with HCC in our study and their expression was correlated with tumor size, intrahepatic metastasis and vascular invasion. MIF stimulation may induce an increase in VEGF secretion, which contributes to angiogenesis and tumor growth. Therefore, VEGF and MIF may be markers of more aggressive HCC and they could be therapeutic targets for patients with HCC.

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