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BASIC RESEARCH

Upregulation of hypoxia inducible factor 1α mRNA is associated with elevated vascular endothelial growth factor expression and excessive angiogenesis and predicts a poor prognosis in gastric carcinoma

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Abstract

AIM: To investigate the implication of the hypoxia inducible factor HIF-1α mRNA in gastric carcinoma and its relation to the expression of vascular endothelial growth factor (VEGF) protein, tumor angiogenesis invasion/metastasis and the patient's survival.

METHODS: *In situ* hybridization was used to examine expression of HIF-1α mRNA, and immunohistochemical staining was used to examine expression of VEGF protein and CD34 in 118 specimens from patients with gastric carcinoma.

RESULTS: The positive rates of HIF-1α mRNA and VEGF protein were 49.15% and 55.92%, respectively. Positive expressions of HIF-1α and VEGF in stage T_3-T_4 tumors and those with vessel invasion, lymph node metastasis and distant metastasis were dramatically stronger than stage T_1-T_2 cases and those without vessel invasion, lymph node metastasis and distant metastasis. The mean microvascular density (MVD) in stage T_3-T_4 tumors and those with vessel invasion, lymph node metastasis and distant metastasis was significantly higher than stage T_1-T_2 tumors and those without vessel invasion, lymph node metastasis and distant metastasis. The mean MVD in tumors with positive HIF-1α and VEGF expression was significantly higher than that in tumors with negative HIF-1α and VEGF expression. The expression of HIF-1α was positively correlated with VEGF protein. There were positive correlations between MVD and expression of HIF-1α and VEGF. The mean survival time and the 5-year survival rate in cases with positive expression HIF-1α and VEGF and MVD value ⩾ 41.5/0.72 mm^2 were significantly lower than those with negative expression of HIF-1α and VEGF and MVD value < 41.5/0.72 mm^2.

CONCLUSION: Overexpression of HIF-1α is found in gastric carcinoma. HIF-1α may induce the angiogenesis in gastric carcinoma by upregulating the transcription of VEGF gene, and take part in tumor invasion and metastasis. They can be used as prognostic markers of gastric cancer in clinical practice.

Key words: Gastric carcinoma; HIF-1α; Vascular endothelial growth factor; Microvascular density; Prognosis

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INTRODUCTION

Angiogenesis plays an important role in tumor growth and metastasis[1,2]. The formation of tumor microvessels is stimulated by angiogenic factors, especially vascular endothelial growth factor (VEGF)[3-5]. Hypoxia is related to tumor cell growth, differentiation, invasion and metastasis activity. In recent years, studies showed that hypoxia inducible factor-1 (HIF-1) played an important role in oxygen balance and tumor angiogenesis[6]. HIF-1 consists of α and β subunits, and HIF-1α is a key subunit of HIF activity regulated by hypoxia[1]. HIF-1 mRNA and protein overexpression was found in a variety of tumors, including breast cancer, prostate cancer, kidney cancer, rectal adenocarcinoma, *etc*[2,4,7-9]. However, research on HIF-1α in gastric cancer was rarely reported. In the present study,

in situ hybridization was used to examine the expression of HIF-1α mRNA in gastric carcinomas and explore its relationship with VEGF protein, microvascular density (MVD) and survival, and to investigate the role of HIF-1α and VEGF in invasion, metastasis and prognosis of patients with gastric cancer.

MATERIALS AND METHODS

Patients and tumor tissues

One hundred and eighteen gastric carcinoma samples were collected in our hospital from October 1988 to November 2000. Complete over 5 years follow-up data were available for all these cases (follow-up ended in December 2003). Recurrence happened in 72 cases, of which 63 died. The survival period was calculated from the day of operation to the end of the follow-up or to the date of death. The average age was 59.2 years (range from 38 to 78) and the male to female ratio was 2:1 (79:39). According to the standard classification of WHO (1999), 19 patients had papillary adenocarcinomas, while 39, 37, 12 and 11 had the tubular adenocarcinomas, poorly differentiated adenocarcinomas, mucinous adenocarcinomas, and signet-ring cell carcinomas, respectively. Highly and moderately differentiated carcinomas were found in 70 cases, while poorly and undifferentiated carcinomas were found in 48 cases. According to the tumor, lymph node, and metastasis (TNM) standard, there were 20, 27, 40 and 31 cases of T_1, T_2, T_3, and T_4 carcinoma, respectively. Eighty-three cases had lymph node metastasis and 35 cases had no metastasis. Distant metastasis of carcinomas were found in 53 cases (liver metastasis: 21 cases, peritoneum metastasis: 32 cases), while no distant metastasis in 65 cases. Twenty control cases were collected from the same gastric mucosa 5 cm away from the carcinoma tissues.

Histological treatment

In order to avoid the RNase contamination, all the glass slides, slide covers and stain containers were treated with 100 g/L DEPC for 24 h. Gloves were used when handling tissue cutting and 100 g/L SDS was used to clean the cutter. All the sections were spread on glass using 100 g/L DECP-treated ddH_2O. The tissues were cut into pieces in 5-7 mm thickness and kept at 4℃, and foil covered for HE stain, immunohistochemistry and *in situ* hybridization.

Reagents

Digoxin-labeled oligonucleotides probes and detection kit were purchased from Boshide Biological Technology Limited Company, Wuhan, China. The sequences of HIF-1α probes (MK1201) were (1) 5'-TTATG AGCTT GCTCA TCAGT TGCCA CTTCC-3'; (2) 5'-CTCAG TTTGA ACTAA CTGGA CACAG TGTGT-3'; (3) 5' -GGCCG CTCAA TTTAT GAATA TTATC ATGCT-3'. Mouse anti-human VEGF and mouse anti-human CD34 and SP kit were purchased from Maixin Biotech Co. Fuzhou, China. The working concentrations of VEGF and CD34 were 1:80 and 1:120, respectively.

In situ hybridization

The tissue slides were routinely dehydrated before *in situ* hybridization, and were washed thrice with 0.5 mol/L PBS (3 min each time), then incubated with 30 mL/L H_2O_2 for 10 min at room temperature. Digestion was obtained with pepsin at 37℃ for 10 min, followed by washing thrice with 0.5 mol/L PBS (3 min each time) and once with distilled water. Then 20 μL prehybridization solution was used for each group and incubated in wet chamber for 2h at 40℃. *In situ* hybridization solution (probe concentration 2 mg/L) was added into and incubated at 45℃ for 16 h in a wet chamber. Post-hybridization washing was done with 2 × SSC thrice (5 min each time), and the slides were blocked with normal serum at 37℃ room temperature for 30 min. After directly adding mouse-anti-digoxin antibody for 1h at 37℃, slides were washed thrice with 0.5 mol/L PBS (2 min each time), followed by incubation with streptavidin-biotin complex (SABC) at 37℃ for 20min. Finally, the slides were washed four times with 0.5 mol/L PBS (5 min each time), stained with DAB for 10 min and counterstained with hematoxylin solution. Hybridization solution without probe and RNase-treated sample served as negative controls.

Immunohistochemistry

Immunohistochemistry was made according to the streptavidin peroxidase (SP) methods. Staining step followed the routine process[5] in order to examine the specificity of immunostaining, and preabsorbation of anti-VEGF antibodies with recombinant human VEGF was used to replace the primary antibodies as the negative control.

Results evaluation

Briefly, five fields of highly vascularized areas (× 400) in each slide were counted in 200 cells in each field. In the end, 1000 cells were randomly chosen under microscopy to evaluate the stained cell number against the total cell number in the field. Based on the HIF-1α mRNA positive cell number (the cytoplasm or nucleus of the cells appeared brown in color), the criteria were set as follows[10]: negative (-): less than 1% positive cells or without positive staining; (+): 1%-10% positive cells; (++): 11%-50% positive cells; and (+++): more than 50% positive cells. Based on the VEGF-positive cell number (the cytoplasm or membrane of the cell appeared brown in color), the criteria were[7]: negative (-): no positive staining; (+): less than 25% positive cells; (++): 26%-50% positive cells; and (+++): more than 50% positive cells. The MVD in the carcinoma tissue was calculated as previously described[11]. Briefly, positive staining microvessel was stained brown yellow in color by CD34. MVD was expressed as the average number of the positive microvessel in five most highly vascularized areas chosen randomly with 200 × fields in each slide, described as mean ± SD.

Statistical analysis

Statistical evaluation was performed using χ^2 test or Fisher's exact test to differentiate the rates of different groups, *t* test was used to analyze quantitative data, and rank sum correlation was analyzed with Spearman's test. The survival rate was estimated by the Kaplan-Meier method

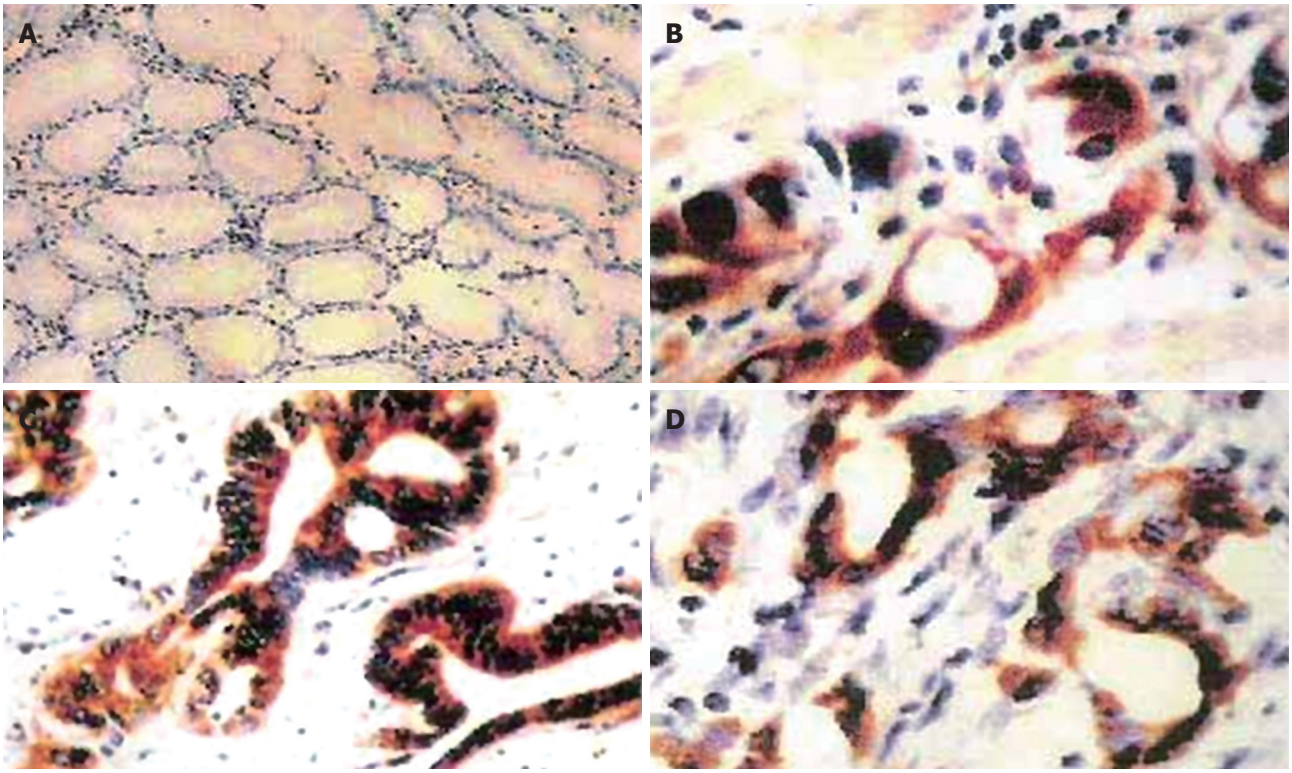

Figure 1 The expression of HIF-1α mRNA in gastric cancer tissue. **A**: HIF-1α mRNA was negative (-) in nontumorous gastric epithelial mucous. *In situ* hybridization and visualization with DBA. Magnification × 120; **B**: Moderately differentiated adenocarcinoma was involved with muscular layer. HIF-1α mRNA was positively expressed (+++). *In situ* hybridization and visualization with DAB. Magnification × 180; **C**: Moderately differentiated adenocarcinoma was involved with serosa layer. HIF-1α mRNA was positively expressed (++). *In situ* hybridization and visualization with DAB. Magnification × 210; **D**: Moderately differentiated adenocarcinoma was involved with great omentum. HIF-1α mRNA was positively expressed (++). *In situ* hybridization and visualization with DAB. Magnification × 240.

and analyzed by log-rank test. $P < 0.05$ was considered statistically significant. SPSS12.0 software for windows was employed to analyze all the data.

RESULTS

Relationship between HIF-1α mRNA expression and patient's clinical and pathologic parameters

The positive HIF-1α mRNA signal is the yellowish brown pellet, mainly located in the cytoplasm of tumor cells. In this study, positive HIF-1α mRNA expression was shown in 58 samples (58/118), and the positive expression rate was 49.15%. There was positive expression in most human myometrium and serosa, extraserosal omentum carcinoma and gland, but no expression in normal gastric mucosa (Figure 1A-D). There was no significant difference in the level of HIF-1α mRNA expression within various types of gastric cancer, highly differentiated, differentiated adenocarcinomas, poorly differentiated and undifferentiated carcinoma ($P > 0.05$), but the HIF-1α mRNA expression level was related to the depth of tumor invasion, vascular invasion, lymph node and distant metastasis ($P < 0.005$) (Table 1).

Relationship between VEGF protein expression and patient's clinical and pathologic parameters

VEGF was dyed into brown granules, mainly located in the cytoplasm of tumor cells, few in membrane. Normal gastric mucosa almost does not express VEGF. Within 118 cases of gastric cancer, the VEGF positive expression was shown in 66 cases, and the positive expression rate in 55.92%. Dyeing of the front region with tumor infiltration was stronger than the central spot (Figure 2A-D). The stomach cancer clinical pathology parameters had remarkable difference in positive expression rate of VEGF (Table 1). Statistical analysis indicated the positive VEGF expression rate had nothing to do with the differentiation of gastric cancer ($P > 0.05$), however the positive VEGF expression rate of papillary adenocarcinoma was obviously lower than other types ($P < 0.005$).

Relationship between MVD expression and patient's clinical and pathologic parameters

MVD is not related to the types of gastric cancer ($P > 0.05$), but to the degree of differentiation, the depth of tumor invasion, vascular invasion, lymph node and distant metastasis ($P < 0.005$) (Table 1).

Relationship between HIF-1α, VEGF and blood vessel density

MVD of positive HIF-1α mRNA expression tissues was $52.78 \pm 7.59/0.72$ mm^2, being higher than the negative expression group ($32.75 \pm 14.07/0.72$ mm^2, $P = 0.0048$); MVD of positive VEGF expression group was $53.83 \pm 6.65/0.72$ mm^2, remarkably higher than the expression of negative group ($28.84 \pm 10.69/0.72$ mm^2, $P = 0.0001$).

Table 1 Correlation between expression of HIF-1α mRNA, VEGF and MVD and pathologic parameters in 118 patients with gastric carcinoma

Clinicopathologic index	*n*	HIF-1αmRNA				χ^2	VEGF				χ^2	MVD (/0.72 mm^2)	*t*
		-	+	++	+++		-	+	++	+++			
Type of tumor						14.5					23.75		
papillary adenocarcinomas	19	12	3	2	2		13	0	2	4		34.35 ± 17.47	
Tubular adenocarcinomas	39	20	2	5	12		19	0	12	8		40.20 ± 14.84	
Poorly differentiated adenocarcinomas	37	16	1	7	13		16	2	10	9		40.24 ± 15.47	
Signet-ring cell carcinomas	11	5	0	0	6		2	2	2	5		48.63 ± 12.28	
Mucinous adenocarcinomas	12	7	0	2	3		2	2	2	6		48.98 ± 10.41	
Degree of differentiation						4.48					2.65		1.26
Well or moderately differentiated adenocarcinomas	70	39	5	9	17		34	2	16	18		40.12 ± 15.52	
Poorly differentiated and undifferentiated adenocarcinomas	48	21	1	7	19		18	4	12	14		43.70 ± 14.93	
Depth of invasion						24.22					30.05		6.23
T1-T2	47	36	3	1	7		35	2	4	6		31.98 ± 14.37	
T3-T4	71	24	3	15	29		17	4	24	26		47.93 ± 12.41	
Vessel invasion						22.03					43.06		9.04
No	29	24	3	0	2		28	0	0	1		25.69 ± 10.11	
Yes	89	36	3	16	34		24	6	28	31		46.75 ± 13.01	
Lymph node metastasis						18.99					35.52		8.77
No	35	27	3	0	5		30	1	1	3		27.07 ± 11.33	
Yes	83	33	3	16	31		22	5	27	29		47.69 ± 12.41	
Distant metastasis						72.06					61.71		12.91
No	63	52	6	2	3		48	4	5	6		30.81 ± 12.43	
Yes	55	8	0	14	33		4	2	23	26		53.91 ± 6.42	

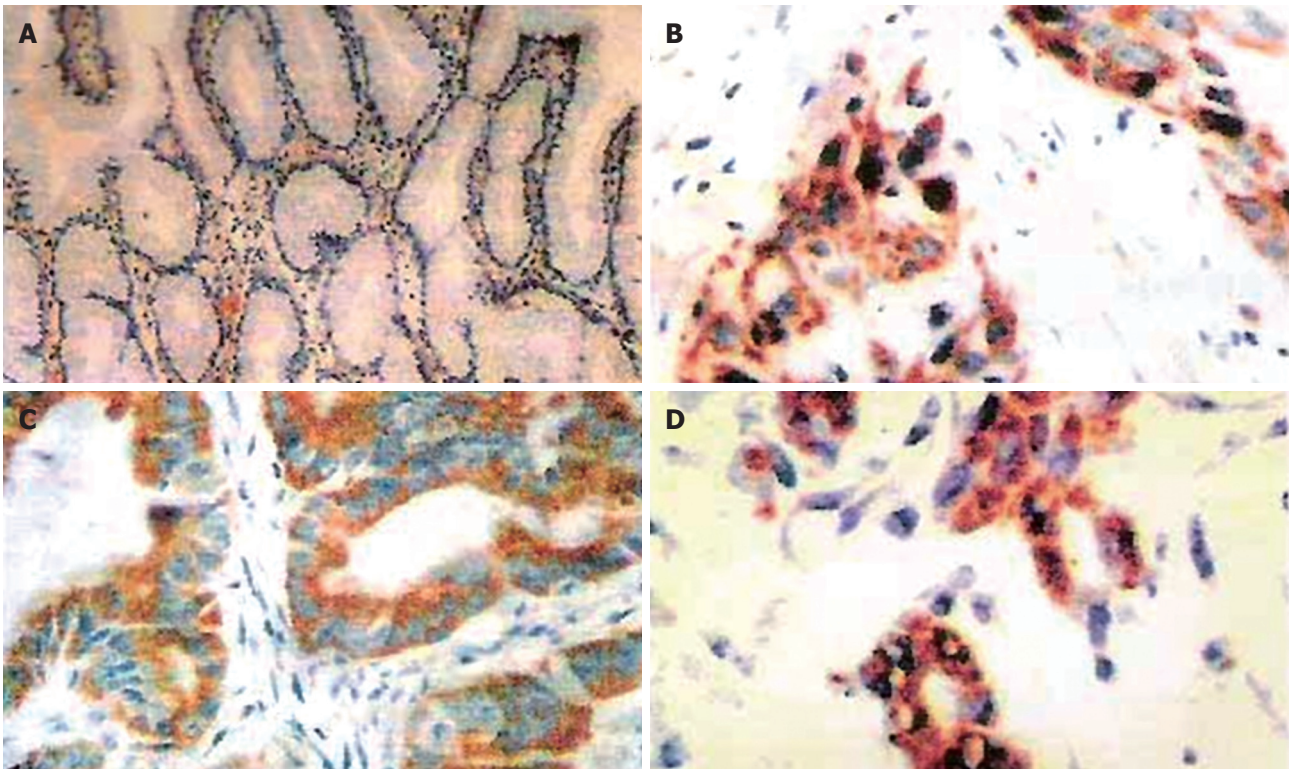

Figure 2 The expression of VEGF protein in gastric cancer tissue. **A**: VEGF was negative (-) in nontumorous gastric epithelial mucous. *In situ* hybridization and visualization with DBA. Magnification × 120; **B**: Poorly differentiated adenocarcinoma was involved with muscular layer. VEGF was positively expressed (+++). *In situ* hybridization and visualization with DAB. Magnification ×180; **C**: Moderately differentiated adenocarcinoma was involved with serosa layer. HIF-1αmRNA was positively expressed (+++). *In situ* hybridization and visualization with DAB. Magnification × 200; **D**: Poorly differentiated adenocarcinoma was involved with great omentum. VEGF was positively expressed (++). *In situ* hybridization and visualization with DAB. Magnification × 220.

HIF-1α mRNA and the VEGF expression levels were positively correlated (r_s = 0.535), and r_s of MVD of HIF-

Table 2 Relationship between HIF-1α mRNA, VEGF and MVD value and prognosis of gastric carcinoma

Groups	*n*	Mean survival time (mo)	5-yr survival (%)
HIF-1α mRNA			
-	60	127 ± 10.25	82.72
+	58	37 ± 12.65	25.67
VEGF			
-	52	117 ± 11.32	75.35
+	66	45 ± 10.82	21.22
MVD (/0.72 mm^2)			
< 41.5	76	130 ± 10.72	83.75
⩾ 41.5	42	43 ± 11.78	20.42

1α and the VEGF protein expression levels was 0.332 ($P = 0.012$) and 0.412 ($P = 0.001$), respectively.

Relationship between HIF-1α mRNA, VEGF protein, MVD and prognosis

The data of patients' medium survival time and the 5-year survival rate are described in Table 2. The results showed that there were significant differences in average survival time between HIF-1α and VEGF negative and positive groups, and between MVD ⩾ 41.5/0.72 mm^2 and MVD < 41.5/0.72 mm^2 groups ($P = 0.001$, 0.003 and 0.001).

The survival rate was similar ($P < 0.05$) and the survival curves are shown in Figure 3A-C.

DISCUSSION

HIF-1α is a DNA binding protein, which could be induced by hypoxia, NO[12] and Cocl2. The HIF-1α transcription in either tumor tissues or normal cells is mediated by oxygen concentration. Levels of HIF-1 protein increases greatly under low oxygen concentration, whereas it decreases rapidly after exposure to 20% oxygen. The regulating genes of HIF-1α are involved in energy metabolism[13], ion metabolism, and angiogenesis and vessel shrinks control, which mediates gene transcription of erythropoietin and vascular endothelial growth factor, glycolytic enzyme. Under hypoxia, in order to respond to hypoxia stress, many genes of tumor cells change in transcription and expression, which named the oxygen deficit response genes (HRGs). Among HRGs, those regulated by HIF-1α are called the HIF-1α target genes and they harbor one or more hypoxia response elements (HREs) at their promoters and enhancers. The HRE typical sequence is 5'-TACGTG-3'[14,15], which is the binding site of HIF-1α mRNA. Activated HIF-1α binds with HREs and forms HIF-1, P300/CBS CAMP response elements binding protein complex (CREB), and subsequently initiates transcription of the target genes. P300/CBS interacts with HIF-1α through Chi area and stimulates target gene translation. Their products play crucial roles in tumor vessel formation and transformation.

HIF-1α is an ephemeral protein with a half-life less than 1 minute and is rapidly degraded *via* ubiquitin-proteasome pathway under normoxic condition. On the contrary, HIF-1α expression would increase by deceasing ubiquitination activity or blocking ubiquitination through mutation under hypoxia condition. Therefore, HIF-1α expression could indirectly reflect the degree of hypoxia

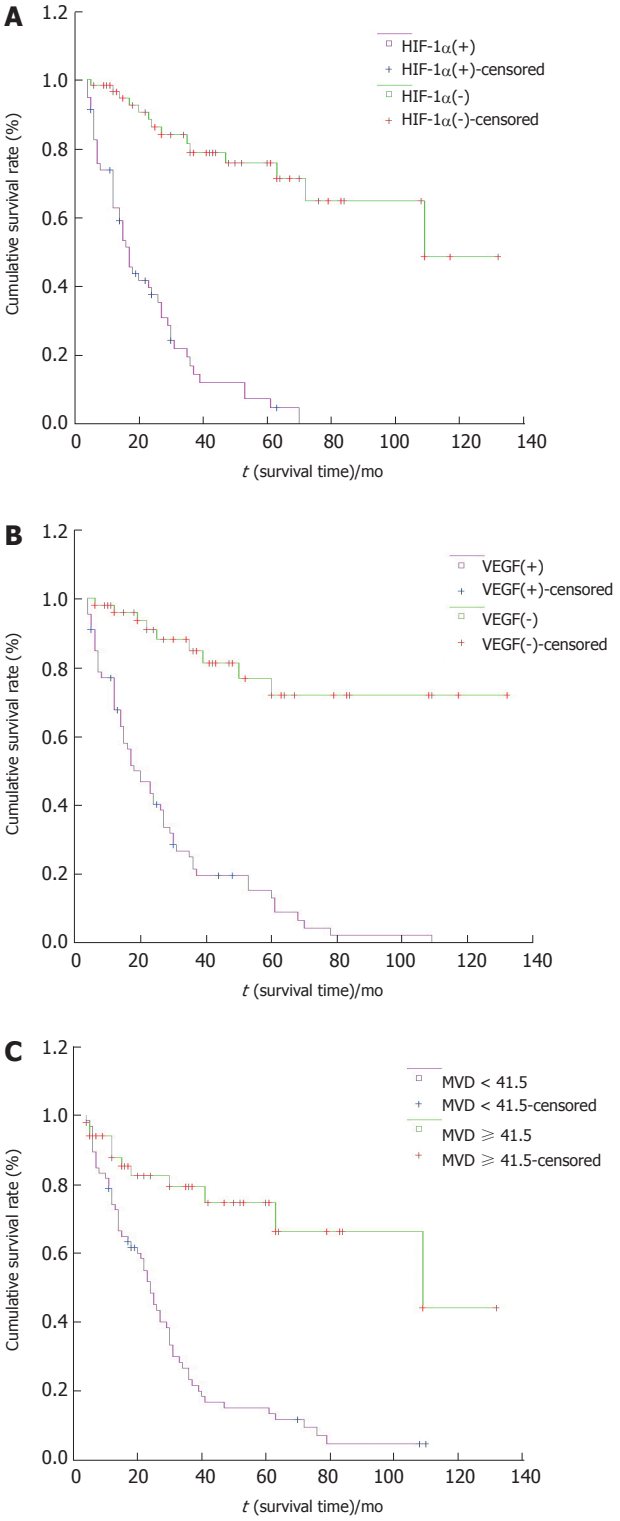

Figure 3 Relationship between HIF-1α mRNA, VEGF protein, MVD and prognosis. **A**: Survival curves by the Kaplan-Meier method. Log-rank test revealing a significant difference between negative and positive expression of HIF-1α mRNA ($P < 0.05$); **B**: Survival curves based on the Kaplan-Meier method. Log-rank test revealing a significant difference between negative and positive expression of VEGF ($P < 0.05$); **C**: Survival curves by the Kaplan-Meier method. There was a significant difference in Log-rant test between patients with MVD value < 41.5/0.72 mm^2 and those with MVD value ⩾ 41.5/0.72 mm^2.

of tissues. Researches showed that HIF-1α mRNA and protein could not be detected in the majority of normal tissues, but they were overexpressed in a variety of human tumors[5,16], and closed correlated to tumor progression, invasion and prognosis[17]. Schindl, *et al*[18] detected HIF-1α expression in 206 cases of breast cancer with lymph node metastasis, patients with HIF-1α protein overexpression had a high mortality and low survival. Our data showed no HIF-1 mRNA expression in normal gastric mucosa either. However, the expression of HIF-1 mRNA in T_3 and T_4 gastric cancers was significantly higher than T_1 and T_2, and had a close relationship with vascular invasion, lymph node metastasis, peritoneal and liver metastasis. This finding suggested that HIF-1α might play an important role in invasion and metastasis of gastric cancer cells and could be an important indicator of gastric cancer progression.

VEGF is an endothelia-specific mitosis-triggered protein, which is one of the most important cytokines to induce tumor angiogenesis. The expression of VEGF gene was regulated by a variety of cytokines, oncogenes, products of tumor suppressor genes and hypoxia[19,20]. Under hypoxic conditions, HIF-1 can bind to 5' enhancer region of VEGF and promote the transcription and expression of VEGF[21], hence increasing angiogenesis and blood supply of hypoxia area. On the other hand, HIF-1α also increases VEGF mRNA stability under hypoxic conditions[22]. Our data showed that expression of VEGF protein in the group with positive expression of HIF-1α mRNA was significantly higher than the group with negative expression of HIF-1α mRNA ($P = 0.002$). The result showed VEGF was highly expressed in gastric cancer, and it was positively correlated to the expression of HIF-1α, MVD also increased significantly following the expression of VEGF. These results suggested that the transcription of VEGF gene might be regulated by HIF-1. VEGF can stimulate tumor angiogenesis and play an important role in invasion and metastasis of gastric carcinoma[23].

Under abundant vessel condition, tumor cells could not only acquire the necessary nutrients, grow rapidly and damage the surrounding tissues by infiltration, but facilitate mobility and subsequently increase distant metastasis. The results of this study showed that MVD was significantly correlated with depth of invasion, pattern of growth, lymph nodes metastasis, liver and peritoneal metastasis, and invasive growth, metastasis and recurrence had a close relationship with angiogenesis, so it is likely to become one of the indicators to predict the prognosis of gastric cancer[24]. Furthermore, our data also revealed that MVD values in cases with positive expression of HIF-1α mRNA and VEGF protein were significantly higher than those with negative expression. These results showed that expression of HIF-1α mRNA was closely related to expression of VEGF, MVD, lymph node metastasis, TNM staging and prognosis. Cox multivariate model analysis showed that HIF-1α could be used as an independent prognostic factor. The underlying mechanism might be as follows: with the continuous growth of the tumor, lack of blood supply induces the expression of HIF-1α which increases VEGF transcription and angiogenesis, promotes tumor proliferation and infiltration into neighboring tissues and distant metastasis[25]. Richard *et al*[26] believed that hypoxia, oncogene activation and tumor suppressor gene inactivation can cause HIF-1α gene activation and excessive translation, thus HIF-1α protein binding to the promoter sequences 5'-A/GCGTG-3 of VEGF gene and activating its transcription. Finally, this cascade affects tumor growth, invasion, metastasis and prognosis.

This preliminary study confirmed the overexpression of HIF-1α in human gastric cancer. HIF-1α might promote angiogenesis through increasing VEGF expression in gastric cancers and be related to clinicopathologic staging. Therefore, HIF-1α could be used as an indicator of prognosis in patients with gastric cancer[27,28]. It might be a new target for treatment of gastric cancer through blocking HIF-1α activity[29,30].

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