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The Clinicopathological Significance of Tissue Levels of Hypoxia-Inducible Factor-1α and Vascular Endothelial Growth Factor in Gastric Cancer

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Background/Aims: Hypoxia-inducible factor-1α (HIF-1α) is a mediator of tumor progression. Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor known to be induced by HIF-1α. We investigated the clinicopathological significance of HIF-1α and VEGF levels in biopsied gastric cancer tissue. **Methods:** Endoscopic biopsy specimens from 67 patients with gastric carcinoma who underwent surgery were available for this study. Semiquantitative RT-PCR was applied to biopsied tumors and normal tissues to determine the expressions of HIF-1α and VEGF. The expression levels of HIF-1α and VEGF were evaluated using the tumor:normal (T/N) ratios of HIF-1α and VEGF mRNA. The clinicopathological variables were reviewed retrospectively. **Results:** The T/N ratios of HIF-1α mRNA showed significant correlation with lymph-node metastases, distant metastases, stage, and recurrence within 3 years ($p<0.05$). The T/N ratios of VEGF mRNA showed significant correlation with lymph-node metastases and distant metastases ($p<0.05$). There was a significant correlation between the T/N ratios of HIF-1α and VEGF mRNA ($r=0.72$, $p<0.01$). **Conclusions:** The increased expression of HIF-1α and VEGF mRNA could reflect aggressive tumor behavior, including the recurrence of gastric cancer. Examination of HIF-1α mRNA in biopsy specimens by RT-PCR assay might provide useful preoperative information on tumor aggressiveness. **(Gut and Liver 2009;3:88-94)**

Key Words: Stomach neoplasms; Hypoxia-inducible factor 1, alpha; Vascular endothelial growth factor

INTRODUCTION

Altered glucose metabolism and cellular adaptation to hypoxia, including increased glycolysis and neovascularization are fundamental to tumor progression and affect tumor characteristics such as proliferation, invasion, metastasis, and lethality. In the absence of neovascularization, the growth of malignant epithelial tumors is limited because of insufficient oxygen supply due to the absence of adequate vascular supply to the tumor tissue. Hypoxia-inducible factor (HIF)-1α has been recognized as a very important mediating molecule for hypoxic adaptation and the induction of proliferation and angiogenesis in various malignancies.[1-5]

HIF-1 consists of α and β subunits. HIF-1β is constitutively expressed, while the expression of HIF-1α is tightly regulated by cellular O2. HIF-1α is degraded under normoxic conditions via the ubiquitin-dependent proteosome pathway. However, this pathway is inhibited by hypoxia leading to induction and stabilization of the HIF-1α. Many investigators have observed that HIF-1α mRNA levels increase following prolonged hypoxia.[5-9] The stabilized HIF-1α dimerizes with HIF-1β, translocates from the cytoplasm into the nucleus and binds to hypoxia-responsive elements (HRE) within the nucleus. A wide variety of genes encoding vascular endothelial growth factor (VEGF), endothelin-1, transferring, erythropoietin, and inducible nitric oxide synthase are known to have HREs and are activated by HIF-1α.[3,6,10,11] These target genes are known to promote cell proliferation and

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viability, angiogenesis, as well as metabolic adaptation to hypoxia.[12,13]

VEGF is probably the most potent angiogenic factor induced by HIF-1α.[2,11,12,14,15] Many studies have provided evidence that VEGF mRNA is not induced by hypoxia in HIF-1α-deficient embryonic stem (ES) cells, and dramatic vascular regression occurs in HIF-1α-null mouse embryos.[12,16] In addition, highly metastatic lung cancer cells have been shown to produce a larger amount of VEGF mRNA under hypoxic conditions through constitutive HIF-1α mRNA upregulation compared to cells with a low metastatic potential, thereby leading to extensive neoangiogenesis.[15] One study showed a correlation between HIF-1α-induced VEGF expression and the induction of angiogenesis in gastric cancer by a semiquantitative assessment of the formation of blood vessels.[17] Overexpression of VEGF may be correlated the aggressive behavior of malignancies.[2,7,18,19] VEGF also has been reported to function as a vascular permeability factor of tumor vessels, facilitating the extravasation of proteins, and the production of ascites and carcinomatosis in gastric cancers.[20]

Most studies have shown that HIF-1α expression is associated with a poor prognosis in various malignancies.[4,8,11-13,21] However, there are conflicting results in studies on gastric cancers. Some studies have shown that a high HIF-1α expression was not associated with prognosis,[22,23] whereas others have found that HIF-1α expression was a prognostic factor associated with a poor outcome in gastric cancers.[4,11,12,21]

The aim of this study was to investigate the clinicopathological significance of the level of HIF-1α and VEGF mRNA in biopsied gastric cancer tissues.

MATERIALS AND METHODS

1. Patients and tissue specimens

Sixty-seven patients with gastric carcinoma were enrolled in this study. All patients underwent a gastrectomy procedure in the department of surgery, Ewha Mokdong hospital, Seoul, Korea from 2003 to 2004. In addition, an upper GI endoscopy was performed before surgery. During endoscopy, at least 6 pieces of tissues were biopsied from the carcinoma and macroscopically normal mucosa, respectively. At least 4 pieces of the collected specimens were frozen immediately and stored at −70°C until extraction of mRNA to determine the levels of HIF-1α and VEGF. Other specimens were fixed in 10% formalin and embedded in paraffin for pathology examination with hematoxylin and eosin staining. The pathologic diagnoses for endoscopically biopsied specimens were matched with those of the surgically resected tissues, and a consultant pathologist determined the pathologic staging. The clinical and histopathological data from all study patients were reviewed.

The Human Research Review Committee approved the study and informed consent was obtained from all patients.

2. Measurements of HIF-1α and VEGF mRNA by semiquantitative RT-PCR assay

1) RNA extraction

Total RNA was extracted from the biopsy tissues using the easy-BLUE™ (iNtRON biotechnology, Seongnam, Korea) total RNA extraction kit. Prepared fresh tissues were added to 800 μL of easy-BLUE™ reagent and homogenized using a homogenizer or equivalent and vigorously vortexed at room temperature for 10 sec. Then, 200 μL chloroform was added and a vortex was applied. After the sample was left for 2-3 minutes at room temperature, the solution was centrifuged at 12,000 rpm (4°C) for 10 minutes. Next, 400 μL of the top layer of fluid was transferred to an empty 1.5 ml tube, and 400 μL isopropanol (2-propanol) was added to it. We mixed it well by inverting the tube 2-3 times. The sample was then left for 10 minutes at room temperature. After centrifuging the solution at 12,000 rpm (4°C) for 10 minutes, we removed the upper layer to obtain the RNA pellet. Next, 1 mL 75% ETOH was added and the solution was mixed well by inverting the tube 2-3 times. The mixture was centrifuged for 5 minutes at 12,000 rpm (4°C). The top layer was discarded and the remaining RNA pellet was dried. The RNA was dissolved using 20-50 μL of DEPC treated distilled water for storage at −70°C. The amount and purity of the extracted RNA was quantitated by spectrophotometry.

2) cDNA synthesis

cDNA was synthesized with 5 μg of total RNA and oligo dT primer. In a sterile RNase-free microcentrifuge tube, 0.5 μg of oligo dT primer and 5 μg RNA sample were added. The tube was heated at 70°C for 5 minutes, and cooled immediately on ice. The M-MLV RTase (Moloney Murine Leukemia Virus Reverse Transcriptase) (Promega, Madison, WI, USA) 200 units, rRNasin ribonuclease inhibitor (Promega, Madison, WI, USA) 25 units, 5 μL 5xRT buffer, and 2 mM dNTP were added to the tube, to obtain a 25 μL solution using 0.1% DEPC. The tube was gently mixed, incubated for 60 minutes at 42°C, and heated for 5 minutes at 95°C. The cDNA was stored at −20°C.

3) Oligonucleotide primers

The primers used were 5'-AAG ATG ACC CAG ATC ATG TTT GAG-3' and 5'-AGG AGG AGC AAT GAT CTT GAT CTT-3' for β-actin, 5'-CCT GCA CTC AAT CAA GAA GTT GC-3'and 5'-TTC CTG CTC TGT TTG GTG AGG CT-3' for HIF-1α,[25] 5'-GCA GAA TCA TCA CGA AGT GG-3' and 5'-GCA ACG CGA GTC TGT GTT TTT G-3' for VEGF.[26] All primers were synthesized by TaKaRaKorea Biomedical Inc.

4) PCR amplification

The amplification reaction was carried out in the 20 μL of the PCR mixture containing 4 μL of the synthesized cDNA solution, 4 μL of 5 x polymerase reaction buffer, 200 μM of dNTP, 0.5 μM of each primer (sense and antisense) and 1 unit of Taq polymerase (Promega, Madison, WI, USA). The PCR mixture was amplified using the GeneAmp PCR System 9600 (PERKIN-ELMER Corp., Norwalk, CT, USA). The amplified PCR products (10 μL) were identified by electrophoresis on a 1% agarose gel containing ethidium bromide and with ultraviolet (UV) illumination. The housekeeping gene, β-actin was used as a control for the semiquantitative analysis of HIF-1α and VEGF. A negative control used H_2O instead of cDNA. The gene transcripts were quantified based on the ratio of the intensity of the target gene to the intensity of the β-actin control gene.

3. Statistical analysis

The results were expressed as the mean ± the standard deviation. The association between the clinicopathological variables and the expression of HIF-1α and VEGF mRNA was analyzed using the Student's t-test, ANOVA test and a nonparametric test. The correlation between the expression of HIF-1α and VEGF mRNA was analyzed by the Pearson correlation test. The data were considered significant if the p value was <0.05. Statistical analyses were performed using the SPSS version 11.0 program (SPSS Inc., Chicago, IL, USA).

RESULTS

1. Patients and clinicopathological findings

Sixty-seven patients were enrolled in this study; 41 were men and 26 were women between the ages of 28 and 84 (mean, 61 years). The gross pathology findings showed 24 cases (36.4%) with early gastric cancer (EGC) and 43 cases (63.6%) with advanced gastric cancer (AGC). Curative resections were performed in 57 patients (85.1%) and 10 (14.9%) underwent non-curative surgical procedures. The prognosis of the 67 cancer patients in this study was monitored for a median follow-up time of 34 months (range, 3-46 months). During the observation period, 16 patients died from gastric carcinoma and the 3-year survival rate was 76.1%. Only one patient received radiotherapy, and eight patients underwent chemotherapy after surgery.

The histological differentiation showed that there were 9 cases of the well-differentiated type (13.4%), 25 cases of the moderately-differentiated type (37.3%) and 33 cases of the poorly-differentiated/signet ring cell type (49.3%). Involvement of the lymphatic vessels was present in 26 cases (38.8%). Vascular invasion was demonstrated in 18 cases (26.9%). The T staging included 24 cases (35.8%) that were T1, 10 cases (14.9%) T2, 26 cases (38.8%) T3, and 7 cases (10.5%) T4. Nodal involvement was identified in 37 cases (55.2%). Distant metastases were seen in 9 cases (13.4%). There were 27 cases (40.3%), 8 cases (11.9%), 17 cases (25.4%), and 15 cases (22.4%) with stage I, II, III, and IV disease, respectively

Table 1. Clinicopathological Characteristics of the Patients

Variables	n (%)
Age (years) Mean±SD (Range)	61±10 (28-84)
Male/Female	41/26
Operation	
Curative resection	57 (85.1)
Non-curative	10 (14.9)
Differentiation	
Well	9 (13.4)
Moderatey	25 (37.3)
Poorly/Signet ring cell	33 (49.3)
Lymphatic invasion	
−/+	41 (61.2)/26 (38.8)
Venous invasion	
−/+	49 (73.1)/18 (26.9)
Depth of invasion	
T1	24 (35.8)
T2	10 (14.9)
T3	26 (38.8)
T4	7 (10.5)
Nodal status	
N0	30 (44.8)
N1	22 (32.8)
N2	5 (7.5)
N3	10 (14.9)
Distant metastasis	
M0	58 (86.6)
M1	9 (13.4)
Stage	
I	27 (40.3)
II	8 (11.9)
III	17 (25.4)
IV	15 (22.4)

(Table 1).

2. Correlation between clinicopathological features and the expression of HIF-1α and VEGF mRNAs

The up-regulation of HIF-1α mRNA and VEGF mRNA were 91% and 75% in the tissue samples, respectively. The up- or down-regulation themselves was not associated with differentiation, invasion or metastases of the cancer. We thus considered it to be appropriate to compare the expression level in tumor samples with that in normal samples for each case. The expression of HIF-1α and VEGF mRNAs was evaluated using the tumor:normal (T/N) ratio of the HIF-1α and VEGF mRNAs.

There was a tendency for the T/N ratios of HIF-1α mRNAs to correlate with tumor differentiation (p=0.06) (Fig. 1). The T/N ratios of HIF-1α mRNA showed no significant association with lymphatic and vascular invasion (p=0.75 and p=0.62) (Figs. 2, 3). However, significant correlations were noted with lymph node metastases, distant metastases, tumor stage, and tumor recurrence within 3 years (p<0.01, p=0.02, p=0.01, and

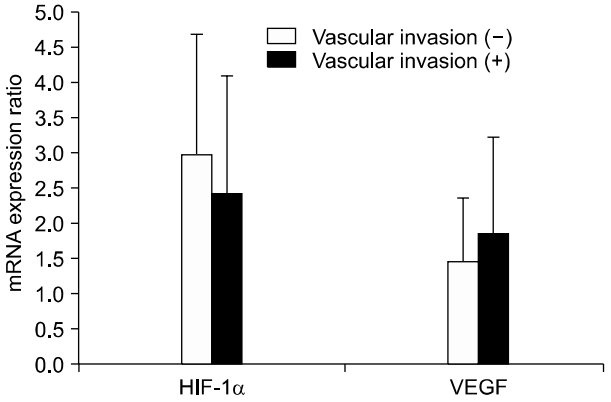

Fig. 3. Relationship between vascular invasion and mRNA expressions of HIF-1α and VEGF. White bars, vascular invasion (−); black bars, vascular invasion (+). p>0.05.

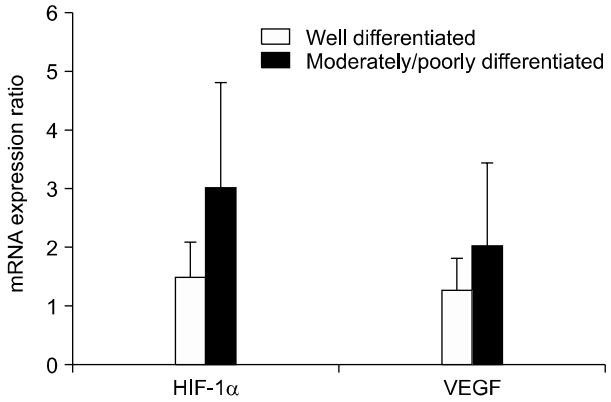

Fig. 1. Relationship between tumor differentiation and mRNA expressions of HIF-1α and VEGF. White bars, well differentiated; black bars, moderately/poorly differentiated. p>0.05.

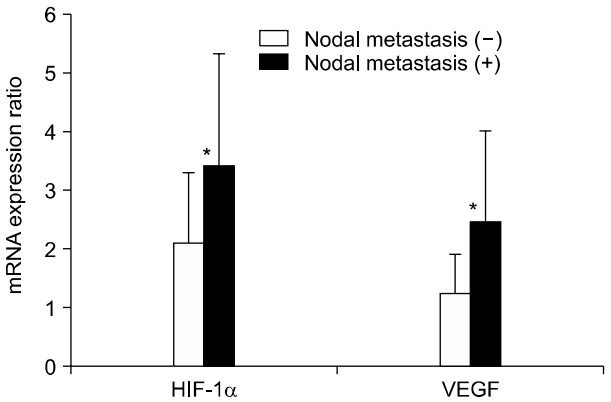

Fig. 4. Relationship between nodal metastasis and mRNA expressions of HIF-1α and VEGF. White bars, nodal metastasis (−); black bars, nodal metastasis (+). *p<0.05.

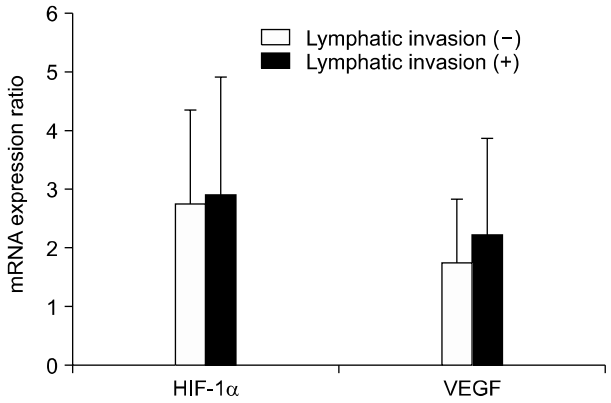

Fig. 2. Relationship between lymphatic invasion and mRNA expressions of HIF-1α and VEGF. White bars, lymphatic invasion (−); black bars, lymphatic invasion (+). p>0.05.

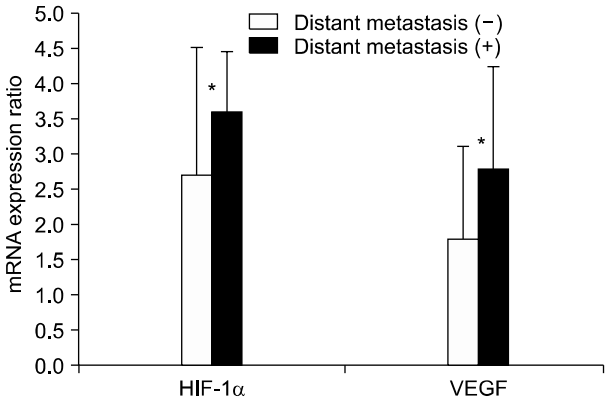

Fig. 5. Relationship between distant metastasis and mRNA expressions of HIF-1α and VEGF. White bars, distant metastasis (−); black bars, distant metastasis (+). *p<0.05.

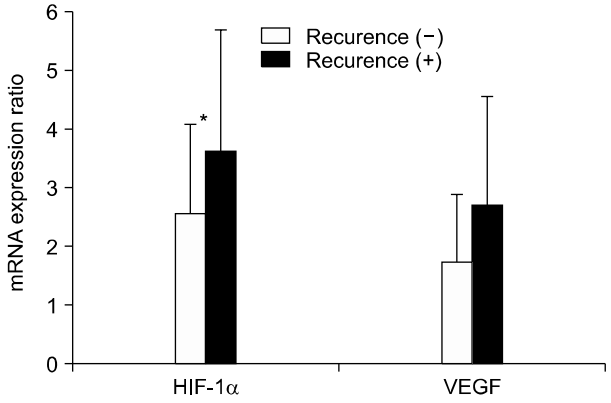

Fig. 6. Relationship between recurrence within 3 years and mRNA expressions of HIF-1 α and VEGF. White bars, recurrence (−); black bars, recurrence (+). *p<0.05.

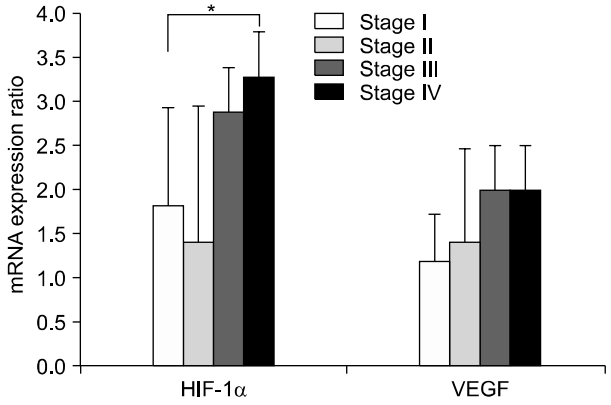

Fig. 7. Relationship between tumor stage and mRNA expressions of HIF-1 α and VEGF. *p for trend <0.05.

p=0.03, respectively) (Figs. 4-7). The T/N ratios of VEGF mRNA did not significantly differ for tumor differentiation (p=0.23) (Fig. 1), the presence of lymphatic invasion (p=0.25) (Fig. 2), or vascular invasion (p=0.23) (Fig. 3). However, the T/N ratios of VEGF mRNA showed significant correlation with lymph node and distant metastases (p<0.01 and p=0.03) (Figs. 4, 5), and there was a tendency toward a correlation with the tumor stage (p=0.07) (Fig. 7) and tumor recurrence within 3 years (p=0.07) (Fig. 6).

3. Correlation between the expression of HIF-1 α and VEGF mRNAs

There was a significant correlation between the T/N ratios of HIF-1 α and VEGF mRNA (r=0.72, p<0.01) (Fig. 8).

DISCUSSION

We studied HIF-1 α and VEGF expression as a prognostic factor in surgically resected gastric tumors. We examined the expression level of HIF-1 α and VEGF mRNA in endoscopically biopsied tissues, and demonstrated that they appeared to be associated with the behavior and outcome of gastric carcinomas.

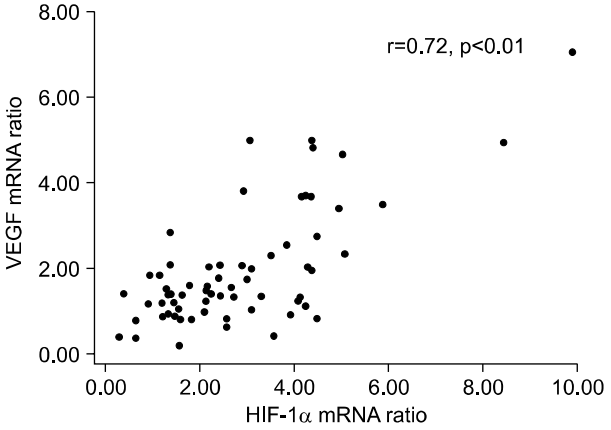

Fig. 8. Correlation between expressions of HIF-1 α and VEGF mRNA. r=0.72, p<0.01.

Expression of HIF-1 α has been evaluated by study of the protein levels in most human neoplasms.[1,4,11,24-26] Until recently, few reports have examined HIF-1 α mRNA in human gastric carcinoma. However, more recently Matsuyama *et al.*[26] reported that HIF-1 α mRNA correlated with HIF-1 α protein levels. Thus, overexpression of HIF-1 α in solid tumors has been confirmed both at the protein and at the mRNA levels. In prior studies of other organs, investigators have demonstrated that HIF-1 α mRNA was overexpressed in cancer cell lines and that the metastatic potential correlated with HIF-1 α mRNA levels in those cell lines.[8] There have been other reports showing that quantitative analysis of VEGF mRNA in gastric cancer biopsy specimens might be useful in predicting metastasis of gastric cancer to the regional lymph nodes.[27,28] We measured the levels of HIF-1 α mRNA and VEGF mRNA, not the protein levels of VEGF and HIF-1 α. It was a challenge to examine clinical significance in gastric cancer with different methods of measurements. One limitation of our study is that we did not compare the immunohistochemical results of HIF-1 α and VEGF with the mRNA expression levels.

In our study, a positive correlation was observed between VEGF and HIF-1 α mRNAs in gastric carcinoma samples (r=0.72, p<0.01). This result is consistent with previous studies that reported that an increased level of HIF-1 α mRNAs may upregulate VEGF mRNA by augmented transcriptional activity,[13,14,29] as well as significant correlation between VEGF and HIF-1 α protein expression.[2,11,12,17,21] One report showed that constitutive HIF-1 α

mRNA up-regulation under hypoxic conditions produced a large amount of VEGF, especially in highly metastastic lung cancer cell lines.[15] HIF-1α activates the transcription of VEGF, which mediates angiogenesis when secreted by tumor cells. Thus, enhancement of angiogenesis by hypoxia is a prerequisite for the progressive growth of gastric carcinomas.[12]

Most studies have shown HIF-1α expression is associated with a poor prognosis.[1,23,30,31] However, some studies have found that a high HIF-1α expression was associated with an improved prognosis in some tumors, including those of the head and neck, cervical cancer, non-small cell carcinoma of the lung, and ovarian cancer.[23,32] Studies on the expression of HIF-1α have also been conflicting for gastrointestinal malignancies. Some reports have shown that HIF-1α expression was an adverse prognostic factor in gastrointestinal stromal tumors and gastric cancers.[4,11-13,21,24] HIF-1α was associated with poor clinicopathological features, VEGF expression and microvessel invasion.[12] The combination of a HIF-1α protein overexpression with nonfunctional p53 tends to indicate a dismal prognosis.[21] By contrast, others have suggested that HIF-1α expression (positive vs. negative) has no statistically significant relationship with clinicopathological features such as tumor differentiation, Lauren type and the TNM stage of gastric cancers.[22,23]

The reasons for these different prognostic outcomes observed in numerous studies are not clear. Some investigators suggested cautiously that cancer progression might be regulated differentially by HIF-1α of a range of downstream target molecules.[5,23] They said that this differential regulation might also be determined by different physiological processes, such as hypoxia, the presence of an oncogene, or other homologues of the HIF family (HIF-2α or HIF-3α), leading to HIF-1α stabilization in individual tumors.[5,23,24,29]

The results of our study, measuring the level of HIF-1α mRNA in gastric cancer, are consistent with other studies showing that the overexpression of HIF-1α was correlated with the pathogenesis of cancer development, progression and the loss of differentiation.[1,2,25,30] The significant correlation between the level of HIF-1α mRNAs and tumor stage was also founded in our study. Although Fig. 7 showed that the T/N ratios of HIF-1α mRNA decreased in stage II, compared with in stage I, there was no statistically significant difference between the levels in both stages.

VEGF might be a significant prognostic factor; it is directly associated with the production of ascites and the peritoneal dissemination of gastric cancer.[20] Some studies have shown that the overexpression of VEGF protein correlates with tumor progression and a poor clinical outcome including high lymphatic vessel density in various cancers and in gastric cancer.[1,7,33-35]

Our data showed that the level of HIF-1α and VEGF mRNA was significantly higher in patients with a cancer recurrence within three years of the initial diagnosis. Therefore, the semiquantitative RT-PCR assay for HIF-1α and VEGF might be used to predict the recurrence of gastric cancer within a short time interval.

Some investigators have demonstrated that HIF-1α activity could be observed in nearly all of cancer specimens; this is consistent with our findings (positive in 91% of cancer specimens). In addition, HIF-1α activity has been identified in most precancerous lesions such as colon adenomas, breast ductal carcinoma *in situ* and prostate intraepithelial neoplasia. By contrast, every benign noninvasive tumor analyzed was negative for HIF-1α overexpression.[25,26] Thus, increased expression of HIF-1α and VEGF mRNA may be specific markers for treatment and/or clinical surveillance of patients, representing very early events in carcinogenesis prior to detection of histological evidence of angiogenesis or invasion.[23,25,36]

The results of this study suggest that the increased levels of HIF-1α and VEGF mRNA may play an important role in tumor progression of gastric carcinomas. Gastric carcinomas that express HIF-1α and VEGF may continue to grow by various adaptive responses such as neoangiogenesis. Examination of HIF-1α mRNA in biopsy specimens, by the semiquantitative RT-PCR assay, may provide useful preoperative information on tumor aggressiveness.

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