Up-regulation of Hypoxia-inducible Factor-1 Alpha and VEGF mRNAs in Peritoneal Dissemination of Patients with Gastric Cancer

TAKAKI YOSHIKAWA[1], AKIRA TSUBURAYA[1], YOHEI MIYAGI[2], HIRONOBU SEKIGUCHI[2], MASAYUKI KIMURA[3], HARUHIKO CHO[1] and OSAMU KOBAYASHI[1]

[1]*Department of Gastrointestinal Surgery, Kanagawa Cancer Center and*
[2]*Molecular Pathology and Genetics Division, Kanagawa Cancer Center Research Institute, Yokohama;*
[3]*Department of Gastrointestinal Surgery, St. Marianna University, School of Medicine, Kawasaki, Japan*

Abstract. *Background: Gastric cancer often metastasizes to the peritoneal cavity in which tumor cells are exposed to hypoxia without systemic circulation. Hypoxia-inducible factor-1 (HIF-1) and its target gene, vascular endothelial growth factor (VEGF), may play a role in the development of peritoneal metastases. Materials and Methods: The mRNA levels of HIF-1 alpha and VEGF were examined in 21 normal gastric mucosa, 158 primary tumors and 18 peritoneal metastases by quantitative RT-PCR. Results: HIF-1 alpha and VEGF were significantly up-regulated in the peritoneal metastases compared with those of the normal mucosa and the primary tumors. A positive correlation between HIF-1 alpha and VEGF was observed in the peritoneal dissemination. Conclusion: These results suggest that HIF-1 alpha and its target gene, VEGF, were up-regulated in the intraperitoneal tumors but not in the primary cancers of the stomach. Different microenvironments may influence the expressions of these genes.*

Gastric carcinoma remains the most frequent malignancy in Japan and the second leading cause of cancer death in the world (1, 2). In patients with gastric cancer, peritoneal dissemination is the most common mode of the distant metastases (3). Although new chemotherapy has been developed, peritoneal dissemination rarely responds to these new regimens (4, 5).

Mechanisms of peritoneal metastases involve isolation from the gastric wall and the growth with neovascularization at the peritoneum. Sugarbaker *et al.* reported that the peritoneal cavity was a sanctuary, because tumor cells in the peritoneal cavity were not exposed to the systemic circulation involving the immune response (5, 6). Oxygen is not supplied by the systemic circulation in the peritoneal cavity, therefore, tumor cells are exposed to hypoxic conditions.

Hypoxia-inducible factor 1 (HIF-1) consists of HIF-1 alpha and HIF-1 beta subunits (7, 8). HIF-1 beta is constitutively expressed, while the expression of HIF-1 alpha is tightly regulated by cellular O_2 (8). Under normoxic conditions, HIF-1 alpha is maintained at low levels due to continuous degradation *via* the ubiquitin-dependent proteosome pathway. This pathway is inhibited by hypoxia, leading to stabilization of the HIF-1 alpha protein levels (9, 10). In contrast, many investigators observed that HIF-1 alpha mRNA levels increased following prolonged hypoxia (11-14). Activation of signal transduction pathway also induced HIF-1 alpha mRNA (15-17). Thus, HIF-1 alpha is regulated by proteins levels but also by mRNA levels. HIF-1 activates the transcription of genes encoding erythropoietin, glucose transporters, vascular endothelial growth factor (VEGF) and other genes whose protein products increase O_2 delivery or facilitate metabolic adaptation to hypoxia (18). In many human cancers, HIF-1 alpha protein is overexpressed and is correlated with microvessel density, treatment failure and survival (19-25).

Based on these reports, we hypothesized that HIF-1 played a role in the development of peritoneal metastases. In this study, HIF-1 alpha and VEGF mRNA levels were measured in the normal gastric mucosa, the primary tumors of the stomach and the intraperitoneal tumors obtained from patients with gastric cancer.

Abbreviations: HIF-1, hypoxia inducible factor-1; VEGF, vascular endothelial growth factor.

Correspondence to: Takaki Yoshikawa, MD, Department of Gastrointestinal Surgery, Kanagawa Cancer Center, 1-1-2 Nakao, Asahi-Ku, Yokohama 241-0815, Japan. Tel: +81-45-391-5761, Fax: +81-45-361-4692

Key Words: Hypoxia-inducible factor-1, vascular endothelial growth factor, peritoneal dissemination, gastric cancer.

Table I. *Primer and hybridization probe sequences and labels for each target gene. Nucleotide sequences used to design PCR primers and hybridization probes were in GenBank under accession numbers NM_001530 for HIF-1 alpha, XM_052681 for VEGF and X04217 for PBGD.*

	PCR primers
HIF-1 alpha forward	CAGAGCAGGAAAAGGAGTCA
HIF-1 alpha reverse	AGTAGCTGCATGATCGTCTG
VEGF forward	GCTACTGCCATCCAATCGAG
VEGF reverse	CTCTCCTATGTGCTGGCCTT
PBGD forward	ACCCTGCCAGAGAAGAGTGT
PBGD reverse	CCACAGCATACATGCATTCC
	Hybridization probes
HIF-1 alpha-FL	ACTAGCTTTGCAGAATG CTCAGAGAA-[FL]
HIF1 alpha-Red	[Red]-GCGAAAAATGG AACATGATGGTTC
VEGF-FL	CAATGACGAGGGCCTGGAGTGT-[FL]
VEGF-Red	[Red]-TGCCCACTGAGGAGTCCAACATC
PBGD-FL	GGTGTTGAGGTTTCCCCGAATACT-[FL]
PBGD-Red	[Red]-CTGAACTCCAGATGCGGGAACTT

FL: fluorescein, Red: LC Red640.

Materials and Methods

Tissue samples. Eligibility included resectable tumors without distant metastases or the tumors with peritoneal metastases which were suspected by CT scan and required pathological confirmation. Written informed consent was obtained for the molecular analysis. The primary tumor tissues and the normal gastric mucosa were collected immediately after curative gastrectomy. Samples of the peritoneal dissemination were obtained from disseminated tumors or malignant ascites. This study was approved by IRB committees of the Kanagawa Cancer Center Hospital and St. Marianna University, Japan.

Sample preparation. Tissue samples were dissected and stored immediately under stringent sterile conditions in RNA later solution (Ambion, Austin, TX, USA) at 4˚C. Cells floating in the ascites were precipitated by centrifugation. The precipitated cells were also stored in the same manner. The remnant tissues or cells floating in the ascites were examined histologically or cytologically to confirm the presence of gastric cancer tissues or cells.

Total RNA isolation and quantitative real-time PCR. The samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNAs were extracted. Primers for polymerase chain reaction (PCR) to amplify HIF-1 alpha, VEGF, or porphobilinogen deaminase (PBGD) cDNA, reverse-transcribed from sample mRNAs are listed in Table I. Quantification of each mRNA amount was done by real-time PCR technique with hybridization probe method. Nucleotide sequences and labels of hybridization probes are also in Table I. In the present study, reverse transcriptation and real time PCR were carried out in one tube with a LightCycler RNA Amplification Kit Hybridization Probe (Roche Diagnostics). The expected size of the PCR product was confirmed by agarose gel electrophoresis. Real-time PCR for PBGD was performed in all samples to evaluate the quality and quantity of mRNAs.

Establishment of standards to assess copy numbers of target mRNAs. Each PCR product for real-time PCR of HIF-1 alpha, VEGF or PBGD was recovered and subcloned into a plasmid vector pGem-T-easy (Promega, Madison, MI, USA), which contains both T7 and T3 promoters outside of the cloning site to generate RNA corresponding to the cloned sequence. The cloned plasmid was confirmed by nucleotide sequencing. Sense RNA for the cloned amplicon was transcribed from the plasmid with T7 or T3 RNA polymerase by using Riboprobe *in vitro* Transcription System (Promega). The copy number of each transcribed RNA was calculated from the amount of RNA (evaluated by spectrophotometry) and the molecular weight was deduced from the nucleotide sequence of the transcript. *In vitro* transcribed RNA was used as a standard for copy number of each target mRNA in real time PCR experiments. The standard RNA solution was prepared in 25 ng/μl of unrelated viral MS2 RNA (Roche) solution to mimic the condition of RNA samples.

Data analyses. With the LightCycler software (version 3), crossing points were assessed and plotted *versus* the molar concentrations of the standards. The relative mRNA amount in each sample was expressed as ratio marker copy numbers of HIF-1 alpha or VEGF mRNA/PBGD mRNA in 200 ng of total RNA. The crossing points (beginning of the PCR exponential phase) for each reaction were determined by the Fit points algorithm.

Statistical analyses. Data were expressed as mean±sd. Comparisons between the paired data were examined by the paired Mann-Whitney *U*-test. Comparisons among the three were examined by Mann-Whitney *U*-test and adjusted by Bonferroni's method. Correlations were analyzed by Sperman's test. All statistics were calculated by SPSS ver.11.0 software for Windows (Chicago, IL, USA).

Results

Samples collected. Twenty-one normal gastric mucosa, 158 primary tumors and 18 peritoneal metastases were collected from patients with gastric cancer.

Sensitivity and accuracy of measurements of HIF-1 alpha and VEGF mRNAs by quantitative RT-PCR. In vitro transcribed RNA standards for real time PCR experiments were employed accurately to evaluate the amount of target mRNA in samples. Each transcribed RNA in 10-fold dilutions from $1x10^2$ to $1x10^7$ copies was prepared in a reaction of 20 μl containing MS2 RNA as described in "Materials and Methods" section. All of the standard curves for each target mRNA were linear (Figure 1) and we considered that the rage from 100 to $1x10^7$ copies were accurately evaluated in the study.

HIF-1 alpha and VEGF mRNA levels. Expressions of HIF-1 alpha and VEGF mRNA were significantly increased in the peritoneal dissemination compared with those of the normal gastric mucosa and the primary tumors (Figure 2), while those were almost the same between the normal gastric mucosa and the primary tumors. Correlations between HIF-1 alpha and VEGF were examined in the peritoneal dissemination and a

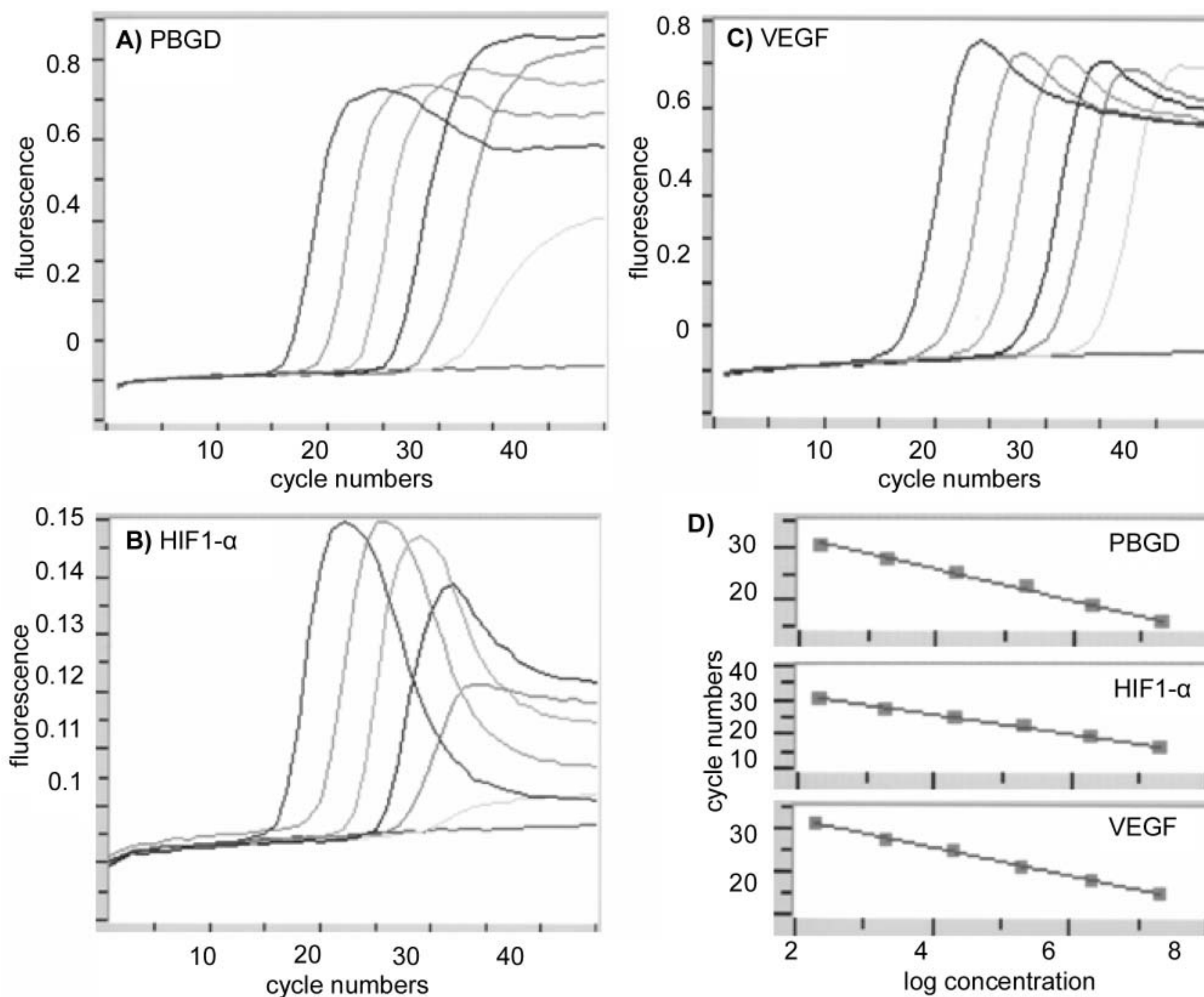

Figure 1. *Establishment of quantitative RT-PCR with RNA standards for PBGD, HIF1-alpha, and VEGF mRNAs. Serial 10-fold dilutions of RNA standards, from 10^2 to 10^7 copies per reaction respectively, were analyzed by the LightCycler with hybridization probe method, as described in "Materials and Methods". Typical results of amplification curves for PBGD, HIF1-alpha and VEGF RNA standards are represented in A, B and C, respectively. Corresponding standard curves to quantify mRNA amounts in samples are shown in D.*

significant positive correlation was found between the two (Figure 3).

Discussion

We demonstrated that HIF-1 alpha mRNA levels were increased in the intraperitoneal tumors, but not in the primary tumors of the stomach. Our results suggest that the different microenvironments between the peritoneal cavity and the gastric wall, possibly hypoxia, may influence the mRNA expression of HIF-1 alpha. Moreover, an enhanced expression of VEGF was correlated with HIF-1 alpha in the peritoneal metastases, suggesting that VEGF may be induced by HIF-1. Koshikawa *et al.* found that high-metastatic potential cells produced a large amount of VEGF through constitutive HIF-1 alpha mRNA up-regulation under hypoxic conditions in human lung cancer cell lines (26). Our study confirmed these findings in the development of peritoneal dissemination of patients with gastric cancer. Increased expression of HIF-1 alpha may be a specific target for the treatment of patients with gastric cancer metastasizing to the peritoneum.

To date few reports have examined HIF-1 alpha mRNA in human gastric carcinoma. Only Zhong *et al.* investigated HIF-1 alpha protein expression in gastric cancer (27). These authors examined the expression of HIF-1 alpha protein in only two cases of primary tumors and found that both tumors expressed elevated immunostaining of HIF-1 alpha (27). In most human neoplasms, expression of HIF-1 was evaluated by

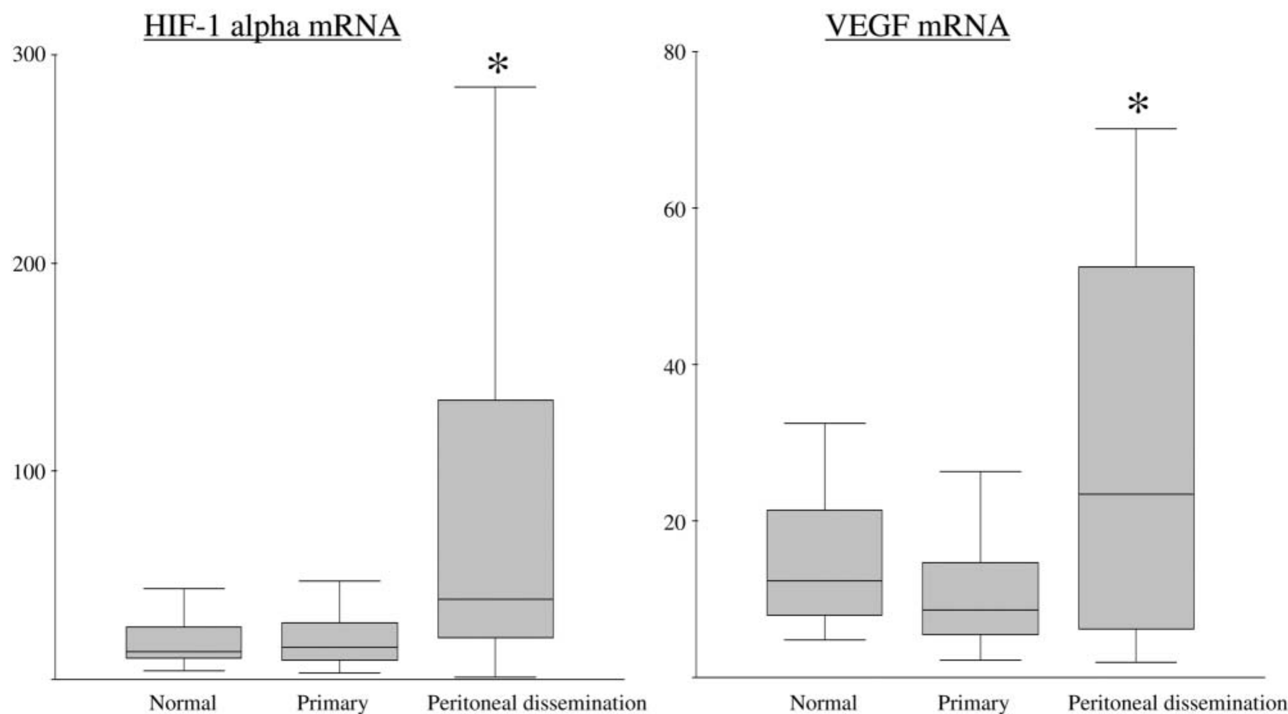

Figure 2. *Expression levels of HIF-1 alpha and VEGF mRNAs in normal gastric mucosa, primary tumors and peritoneal dissemination. HIF-1 alpha and VEGF were significantly increased in the dissemination compared with those of normal mucosa and primary tumors. *P<0.001 vs. normal gastric mucosa and primary tumors.*

protein levels (19-25). Recently, HIF-1 alpha mRNA and protein levels were found to be up-regulated in primary tumors of the urinary bladder and kidney (28, 29). Zhong *et al.* demonstrated that HIF-1 alpha mRNA was overexpressed in six rat prostate cancer cell lines and that metastatic potential correlated with HIF-1 alpha mRNA levels in those cell lines (30). More recently, Matsuyama *et al.* reported that HIF-1 alpha mRNA correlated with HIF-1 alpha protein levels (31). Thus, overexpression of HIF-1 alpha in solid tumors was confirmed both in protein and mRNA levels.

HIF-1 alpha activity is regulated by the protein degradation and the transcriptional activity. As an acute reaction for hypoxic conditions, HIF-1 alpha activity is mainly determined by the accumulation of HIF-1 alpha protein by inhibition of proteolytic degradation (9, 10). In contrast, many investigators suggested that the up-regulation of HIF-1 mRNA contributes to the physiological responses *in vivo* during prolonged hypoxia (11-14). Moreover, activation of the signal transduction pathway by the growth factor receptor also induced HIF-1 alpha mRNA (15-17). Besides hypoxia, those mechanisms might be involved in the specific up-regulation of HIF-1 alpha mRNA in peritoneal dissemination.

We examined VEGF mRNA together with HIF-1 alpha mRNA. VEGF is a strong angiogenic factor which is induced by the HIF-1 protein accumulated under hypoxic conditions (16, 18). VEGF plays an important role in tumor growth through the neovascularization of the tumor. Here, VEGF mRNA was up-regulated in the peritoneal metastases of gastric cancer. A positive correlation was observed between VEGF and HIF-1 alpha mRNAs. These results suggest that

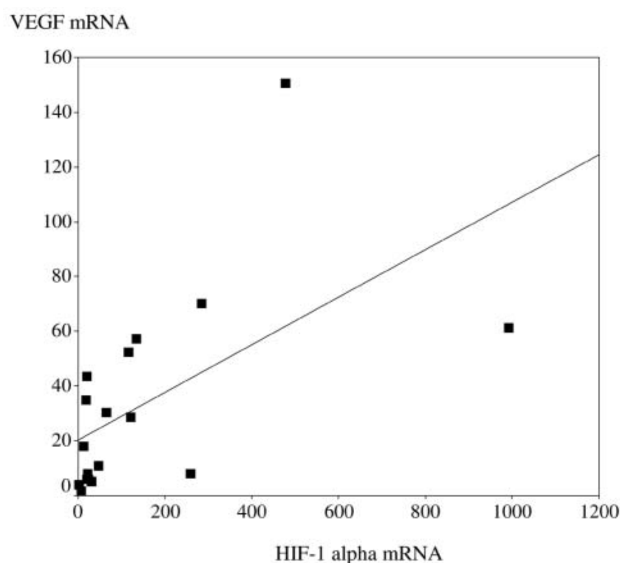

Figure 3. *Correlation between HIF-1 alpha and VEGF in the peritoneal dissemination. R=0.682 and p=0.002 by Spearman's correlation analysis.*

VEGF mRNA is induced by augmented transcriptional activity of HIF-1 alpha.

References

1 The Research Group for Population-based Cancer Registration in Japan: Cancer incidence in Japan, 1985-89: re-estimation based on data from eight population-based cancer registries. Jpn J Clin Oncol *28*: 54-67, 1998.

2 Alexander HR, Kelsen DG and Tepper JC: Cancer of the stomach. *In*: Cancer: Principles and Practice of Oncology. Devita VT, Hellman S, and Rosenberg SA (eds.). Philadelphia, Lippincott-Raven Publishers, pp. 1021-1022, 1997.

3 Yoshikawa T, Tsuburaya A, Kobayashi O *et al*: Peritoneal cytology in patients with gastric cancer exposed to the serosa – a proposed new classification based on the local and distant cytology. Hepatogastroenterology *50*: 1183-1186, 2003.

4 Ajani JA: Chemotherapy for gastric carcinoma: new and old options. Oncology *12*: 44-47, 1998.

5 Yonemura Y, Fujimura T, Fushida S *et al*: Peritonectomy as a treatment modality for patients with peritoneal dissemination from gastric cancer. *In*: Multimodality Therapy for Gastric Cancer. Nakajima T, Yamaguchi T (eds.). Tokyo, Springer-Verlag. pp. 71-82, 1999.

6 Sugarbaker PH and Yonemura Y: Clinical pathway for the management of resectable gastric cancer with peritoneal seeding: best palliation with a ray of hope for cure. Oncology *58*: 96-107, 2000.

7 Wang GL and Semenza GL: Purification and characterization of hypoxia-inducible factor 1. J Biol Chem *270*: 1230-1237, 1995.

8 Wang GL, Jiang BH, Rue EA and Samenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA *92*: 5510-5514, 1995.

9 Huang LE, Arany Z, Livingston DM and Bunn HF: Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem *271*: 32253-32259, 1996.

10 Stroka DM, Burkhardt T, Desbaillets I *et al*: HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J *15*: 2445-2453, 2001.

11 Nguyen SV and Claycomb WC: Hypoxia regulates the expression of the adrenomedullin and HIF-1 genes in cultured HL-1 cardiomyocytes. Biochem Biophys Res Commun *265*: 382-386, 1999

12 Lee SH, Wolf PL, Escudero R *et al*: Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med *342*: 626-633, 2000.

13 Jung F, Palmer LA, Zhou N and Johns RA: Hypoxix regulation of inducible nitric oxide synthase *via* hypoxia inducible factor-1 in cardiac myocytes. Circ Res *86*: 319-325, 2000.

14 Wiener CM, Booth G, Semenza GL: *In vivo* expression of mRNAs encoding hypoxia-inducible factor 1. Biochem Biophys Res Commun *225*: 485-488, 1996.

15 Jiang BH, Agani F, Passaniti A and Semenza GL: V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res *57*: 5328-5335, 1997.

16 Laughner E, Taghavi P, Chiles K, Mahon PC and Semenza GL: HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol *21*: 3995-4004, 2001.

17 Zhong H, Chiles K, Feldser D *et al*: Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res *60*: 1541-1545, 2000.

18 Semenza GL: Targeting HIF-1 for cancer therapy. Nat Rev Cancer *3*: 721-732, 2003.

19 Birner P, Schindl M, Obermai A *et al*: Overexpression of hypoxia-inducible factor 1α is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res *60*: 4693-4696, 2000.

20 Kuwai T, Kitadai Y, Tanaka S *et al*: Expression of hypoxia-inducible factor-1α is associated with tumor vascularization in human colorectal carcinoma. Int J Cancer *105*: 176-181, 2003.

21 Birner P, Schindl M, Obermair A, Breitenecker G, Oberhuber G: Expression of hypoxia-inducible factor 1α in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. Clin Cancer Res *7*: 1661-1668, 2001.

22 Aebersold DM, Burri P, Beer KT *et al*: Expression of hypoxia-inducible factor-1α: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Clin Cancer Res *61*: 2911-2916, 2001.

23 Giatromanolaki A, Koukourakis MI, Sivridis E *et al*: Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br J Cancer *85*: 881-890, 2001.

24 Hui EP, Chan AT, Pezzella F *et al*: Coexpression of hypoxia-inducible factors 1α and 2α, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. Clin Cancer Res *8*: 2595-2604, 2002.

25 Bos R, van der Groep P, Greijer AE *et al*: Levels of hypoxia-inducible factor-1α independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer *97*: 1573-1581, 2003.

26 Koshikawa N, Iyozumi A, Gassmann M and Takenaga K: Constitutive upregulation of hypoxia-inducible factor-1 alpha mRNA occurring in highly metastatic lung carcinoma cells leads to vascular endothelial growth factor overexpression upon hypoxic exposure. Oncogene *22*: 6717-6724, 2003.

27 Zhong H, De Marzo AM, Laughner E *et al*: Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer Res *59*: 5830-5835, 1999.

28 Jones A, Fujiyama C, Blanche C *et al*: Relation of vascular endothelial growth factor production to expression and regulation of hypoxia-inducible factor-1 alpha and hypoxia-inducible factor-2 alpha in human bladder tumors and cell lines. Clin Cancer Res *7*: 1263-1272, 2001.

29 Turner KJ, Moore JW, Jones A *et al*: Expression of hypoxia-inducible factors in human renal cancer. relationship to angiogenesis and to the von Hippel-Lindau gene mutation. Cancer Res *62*: 2957-2961, 2002.

30 Zhong H, Agani F, Baccala AA *et al*: Increased expression of hypoxia inducible factor-1alpha in rat and human prostate cancer. Cancer Res *58*: 5280-5284, 1998.

31 Matsuyama T, Nakanishi K, Hayashi T *et al*: Expression of hypoxia-inducible factor-1alpha in esophageal squamous cell carcinoma. Cancer Sci *96*: 176-182, 2005.

Received April 27, 2006
Accepted June 14, 2006