Regulation of glucose metabolism-related genes and VEGF by HIF-1α and HIF-1β, but not HIF-2α, in gastric cancer

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Abstract

Hypoxia-inducible factors (HIFs) are transcription factors that activate the transcription of target genes involved in crucial aspects of cancer development. This study investigated the expression of HIFs and their contribution to the regulation of target genes related to angiogenesis and glucose metabolism in gastric cancer. The data showed that HIFs were over-expressed in gastric cancer and that activation of the target genes was observed mainly in the early stages. Moreover, the results of the present study revealed that only HIF-1α, but not HIF-2α dimerizes with HIF-1β and then regulates expression of target genes in response to hypoxia. The results of the present study demonstrate that HIF-1α and HIF-1β enhances expression of VEGF and glucose metabolism-related genes in response to hypoxia in gastric cancer. These data offer important information regarding HIF pathways in the development of gastric cancer.

Introduction

Hypoxia is a common feature of many cancers (Ryan et al., 2000). Hypoxia-inducible factors (HIFs) are essential components in regulating transcription in tumor cells in response to hypoxia. HIF-1α and HIF-2α dimerize with a constitutively expressed β-subunit, and exhibit distinct roles in hypoxic expression of target genes (Hu et al., 2003; Park et al., 2003). Although HIF-1 is induced by hypoxia in almost all cell types, the expression of HIF-1 target genes induced by hypoxia is cell type specific manner (Yu et al., 1999; Semenza, 2003). The cell type specificity is thought to be due to the functional interactions of HIFs with other transcription factors or to activation of a subgroup of HIFs in a particular cell type during hypoxia (Ema et al., 1997; Tian et al., 1997; Brusselmans et al., 2001).

Gastric cancer is one of the leading causes of cancer related motality in East Asia, including Korea (Neugut et al., 1996). Several reports have shown that HIFs play important roles in gastric tumorigenesis (Zhong et al., 1999; Park et al., 2003). A previous study by our group indicated that genes related to glucose metabolism, such as *PKM2* (pyruvate kinase), *PGK1* (phosphoglycerate kinase 1), *GLUT 1* (glucose transporter 1), *LDHA* (lactate dehydrogenase A), and *ENO1* (enolase 1), were up-regulated in gastric cancer cell lines (Kim et al., 2004). So far, the roles of HIF-1α and HIF-2α in glucose metabolism and other HIF-regulated genes under hypoxic conditions in gastric cancer remain unclear. To identify the roles of HIFs in gastric cancer, gastric cancer tissue specimens from human patients as well as cancer cell lines, were used to assess the contributions of HIF-1α and HIF-2α to the expression of glucose metabolism-related genes and VEGF during specific cancer developmental stages and in response to hypoxia.
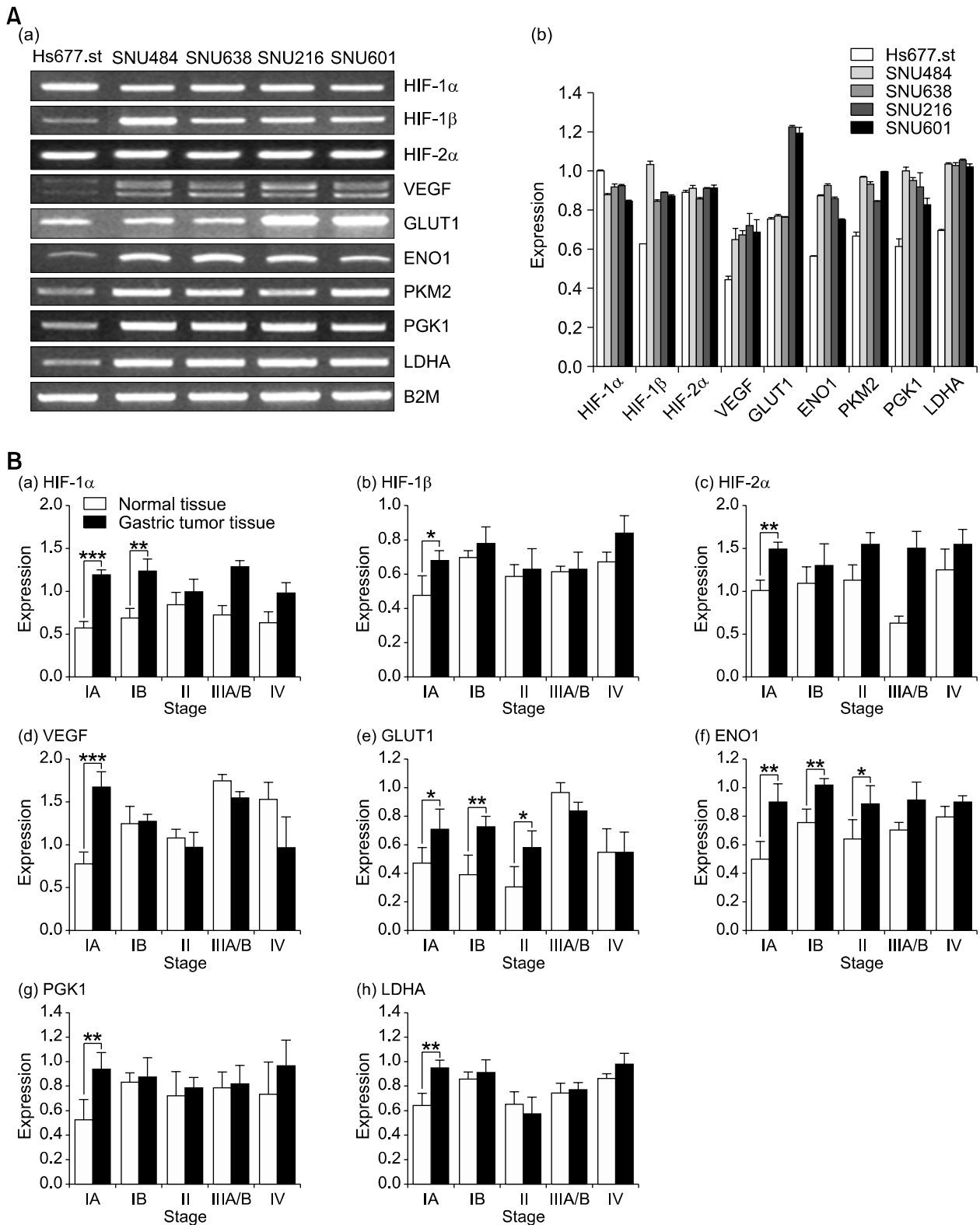
Figure 1. Transcriptional levels of target genes in gastric cancer cell lines (A) and gastric tumor tissues (B) using Sq RT-PCR. The transcriptional levels of the target genes were calculated relative to the amount of β-2-microglobulin (B2M) mRNA. Data of (B) represent the mean ± SD of transcriptional levels of the target genes in 5 pairs of gastric tumor patients of each stage group. Two independent experiments were performed, both of which showed similar results. Asterisks denote a significant difference in target genes between the sample and control by the *t* test (★, $P < 0.05$; ★★, $P < 0.01$; ★★★, $P < 0.001$).

Results

HIFs were over-expressed in gastric cancer cells

To confirm the relationship of the HIF pathway to the development of gastric cancer, the expression of HIFs at the level of transcription was examined in the gastric cancer cell lines and tissues. The results of semiquantitative (Sq) RT-PCR in the gastric cancer cell lines showed that the expression of HIF-1α and HIF-2α did not differ between gastric cancer cell lines and normal Hs 667.st cells, while HIF-1β expression was significantly increased in the gastric cancer cell lines (Figure 1A). In contrast, Sq RT-PCR data for HIFs expression in the human gastric tissue specimens revealed up-regulation of HIF genes expression compared to adjacent normal tissues (Figure 1B, a-c). In particular, these over-expressions were observed in the early stage of IA, although HIF-1α and HIF-2α were over-expressed in the IIIA/B stage. In addition, immunohistochemical analysis of the human gastric tissues showed that 22 of 38 (58%) specimens showed positive staining for HIF-1α, 17 of 38 (45%) were positive for HIF-1β and 18 of 38 (47%) were positive for HIF-2α in the cancerous regions compared with adjacent normal tissues (Table 1). Interestingly, in the early stage of IA/B, these positives were mainly observed as an over-expression of HIF-1α compared with those of HIF-1β and HIF-2α (Table 1). And the expression levels of HIFs were observed to increase in proportion to tumor grades. The HIF-positive cells were clustered within gastric adenocarcinoma tissue, but were not detected or were expressed at very low levels in nonmalignant epithelial and stromal cells (Figure 2). HIF-1α and HIF-2α was

Table 1. Summary of expression of HIF-1α, HIF-1β, and HIF-2α in gastric cancer tissues using immunohistochemical staining.

Stages	IA/B	II	IIIA/B	IV	Total
HIF-1α	7/15 (47%)	3/7 (43%)	9/12 (75%)	3/4 (75%)	22/38 (58%)
HIF-1β	4/15 (27%)	3/7 (43%)	8/12 (67%)	2/4 (50%)	17/38 (45%)
HIF-2α	4/15 (27%)	2/7 (29%)	9/12 (75%)	3/4 (75%)	18/38 (47%)

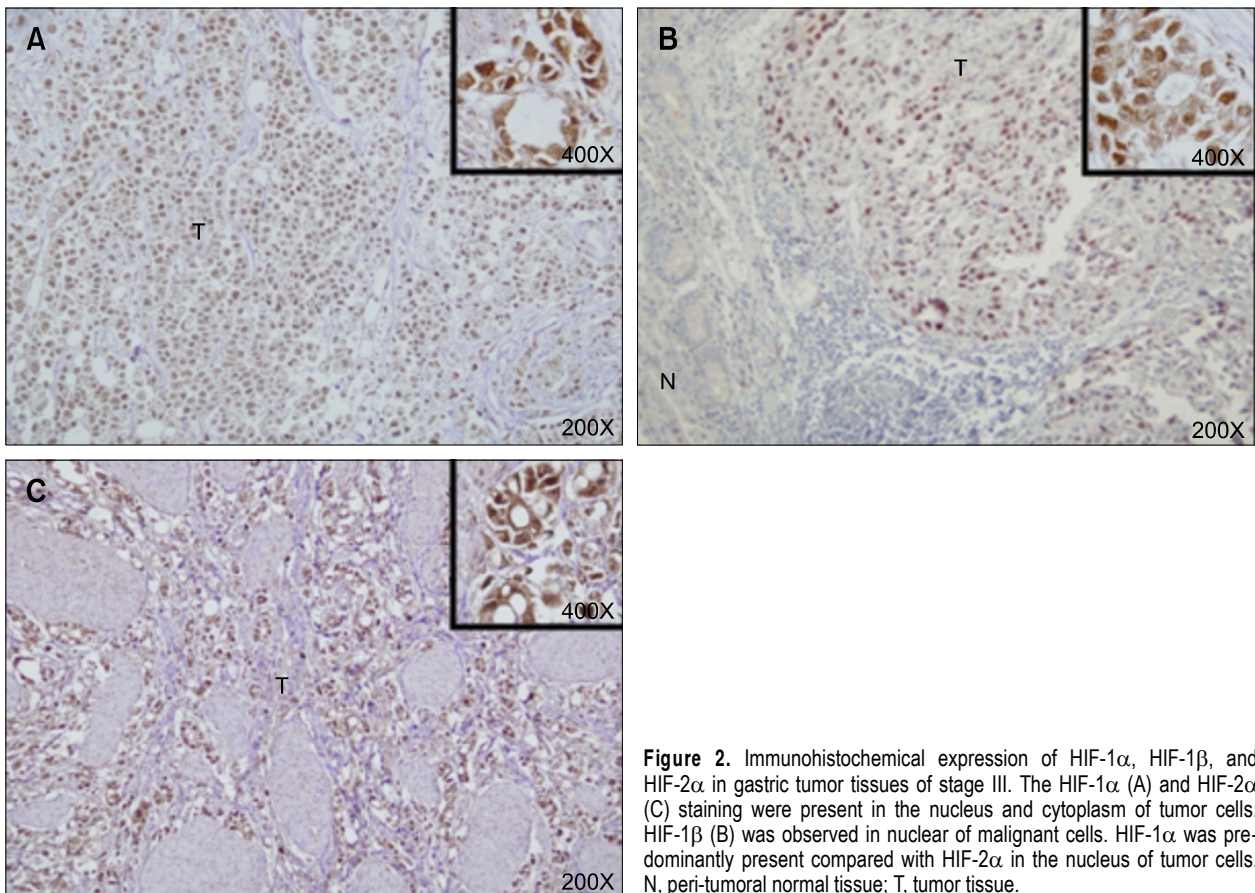**Figure 2.** Immunohistochemical expression of HIF-1α, HIF-1β, and HIF-2α in gastric tumor tissues of stage III. The HIF-1α (A) and HIF-2α (C) staining were present in the nucleus and cytoplasm of tumor cells. HIF-1β (B) was observed in nuclear of malignant cells. HIF-1α was predominantly present compared with HIF-2α in the nucleus of tumor cells. N, peri-tumoral normal tissue; T, tumor tissue.
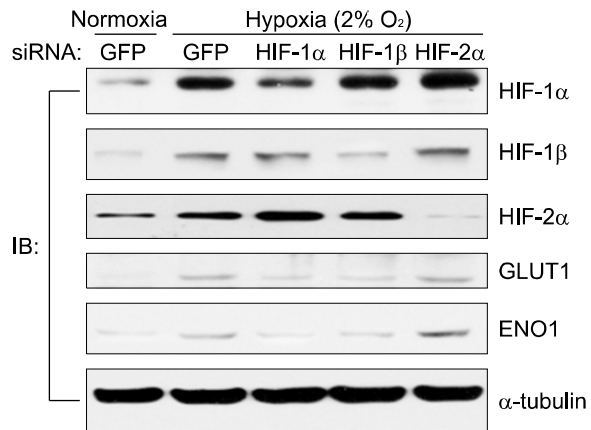
located in the cytosol and nucleus, while HIF-1β was limited to the nucleus. These data indicate that HIFs are commonly over-expressed in gastric cancer, and that HIF-1α plays a particularly important role in its early stages.
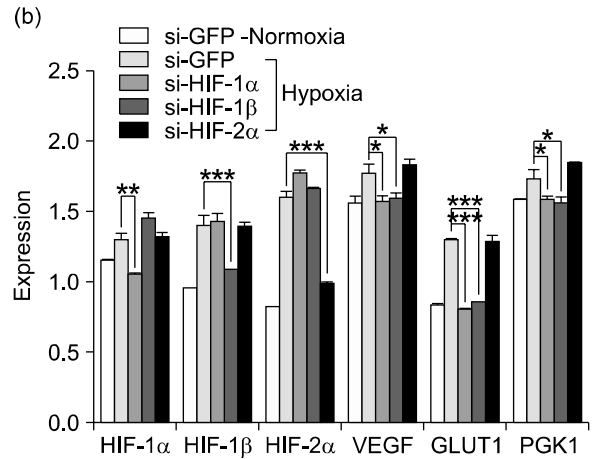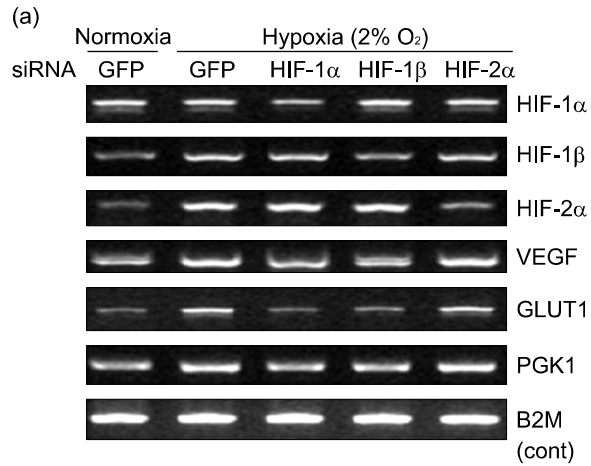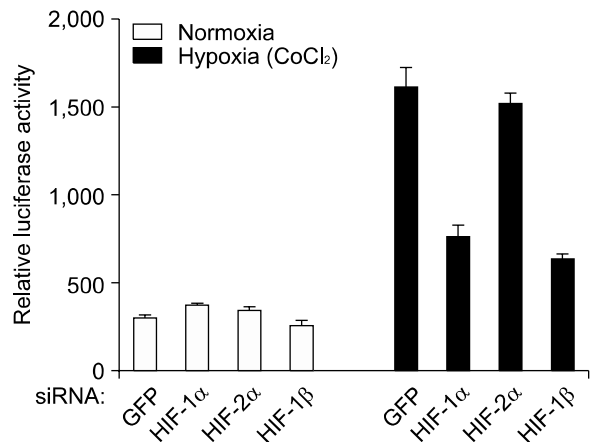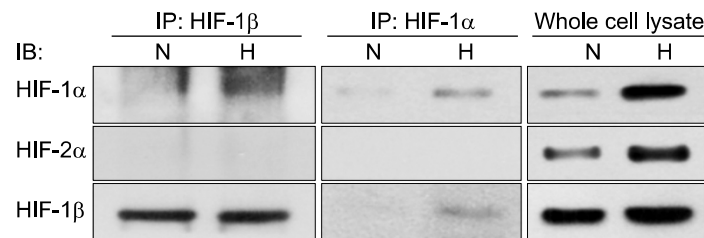

Figure 3. Transcriptional levels (A) and translational levels (B) of target genes in SNU-638 cells under hypoxic condition after siRNA transfection against HIFs. Vertical bars in A-(b) represent mean ± SD of transcriptional levels of the target genes in three independent experiments, all of which showed similar results. ★, *P* < 0.05; ★★, *P* < 0.01; ★★★, *P* < 0.001. (C) HIF-dependent luciferase reporter assays were performed in HIFs knocked down SNU-638 cells. The SNU-638 cells were cotransfected with the HIF dependent luciferase reporter and siRNA of HIF-1α, HIF-1β or HIF-2α for 48 h. And then the cells were treated with or without $CoCl_2$ for 12 h. The relative luciferase activity was measured using an enhanced luciferase assay kit (Promega) and normalized to the β-galactosidase activity. Data represent the mean ± SD of duplicate determinations from one of three independent sets of experiments, all of which showed similar results. (D) Immunoprecipitatation of HIF-1α and HIF-2α using monoclonal antibodies against HIF-1β or HIF-1α. N, normoxia; H, hypoxia. The proteins of (B) and (D) were detected using immunoblot analysis.

Over-expression of glucose metabolism-related genes and VEGF is common in gastric cancer cells, especially in the early stages

To further confirm the activation of HIF pathways, the expression of glucose metabolism-related genes and VEGF also was investigated in gastric cancer cell lines and tissues. The results shown in Figure 1A reveal that glucose metabolism-related target genes, as well as the VEGF, were over-expressed in gastric cancer cell lines compared with normal Hs 677.st cells. In addition, results of the Sq RT-PCR in gastric tumor tissues show that VEGF was over-expressed only during stage IA (Figure 1B, d). Interestingly, over-expression of glucose metabolism related target genes was mainly observed during stage I, although GLUT1 and ENO1 also were over-expressed during stage II (Figure 1B, e-h). These results indicate that the effective activation of HIF related angiogenesis and glucose metabolism pathways may play important roles in the progression of gastric cancer.

The heterodimer of HIF-1α and HIF-1β, but not of HIF-2α directly regulates the expression of glucose metabolism-related target genes and VEGF

To examine the contribution of HIF members to the expression of genes related to glucose metabolism and VEGF, expression of these genes in response to hypoxia was examined in SNU-638 cells. As shown in Figure 3A and B, expression of HIFs at both the transcriptional and translational levels was increased under hypoxic conditions compared with normoxia. In particular, an HIF-1α expression in gastric cancer cell line under hypoxic conditions was revealed to be regulated more in the translational level than in the transcriptional level. In addition, the increased transcriptional levels of glucose metabolism-related genes, as well as of the VEGF, were confirmed under hypoxic conditions (Figure 3A). Among them, immunoblot analysis of GLUT1 and ENO1 protein levels also revealed over-expression at the translational level (Figure 3B). However, when SNU-638 cells were transfected with siRNA to HIFs, HIF-1α and HIF-1β but not HIF-2α resulted in the down regulation of target genes related to glucose metabolism and of VEGF at both the transcriptional and translational levels (Figure 3A and B). On the other hands, when the transcriptional activity of HIFs in SNU-638 cells under hypoxic condition was examined using a HRE (hypoxic response element)-luciferase reporter construct, the siRNA of HIF-1α and HIF-1β were shown to reduce the relative luciferase activity induced by $CoCl_2$ stimulation whereas siRNA of HIF-2α had no effect

(Figure 3C). These data indicate that HIF-2α was not required to the expression of glucose metabolism-related genes in gastric cancer cells under hypoxic conditions.

To confirm which heterodimer of HIF-1α and -2α directly regulates these genes, an immunoprecipitation assay with HIF-1β antibody was performed. As shown in Figure 3D, HIF-1β interacted only with HIF-1α in SNU-638 cells under hypoxic conditions. This interaction was also confirmed in immunoprecipitation result with the antibody against HIF-1α. This result explains why HIF-2α had no effect on regulation of expression of target genes related to glucose metabolism and of VEGF, although HIF-2α was over-expressed in SNU-638 cells under hypoxic conditions. Taken together, these data strongly suggest that not only HIF-1α but also HIF-1β play significant roles in the regulation of glucose metabolism and angiogenesis related genes in gastric cancer.

Discussion

Over-expression of HIFs is thought to correlate with cancer progression (Semenza, 2003; Carroll and Ashcroft, 2005). However, the roles of HIF-1α and HIF-2α in regulating expression of HIF target genes, such as glucose metabolism-related genes and VEGF, in gastric cancer under hypoxic conditions remain unclear. The present study, we investigated HIF expression as well as their relationship to expression of glucose metabolism-related genes and VEGF in gastric cancer cells. The resulting data provide new clues to understanding the molecular mechanisms of the HIF pathway in gastric cancer development.

The HIF expression data showed that mRNA and protein of HIFs, HIF-1α, HIF-1β and -2α were over-expressed in gastric cancer tissues. This result was coincident with others observation in which HIF-1α was over-expressed, as assessed by immunochemical histological analysis, in 2 gastric cancer tissue specimens (Zhong *et al.*, 1999). In addition, it was partially supported by a previous study that the combination of a HIF-1α protein over-expression with nonfunctional p53 tends to indicate a dismal prognosis in gastric cancer (Sumiyoshi *et al.*, 2006). Furthermore, the results of the present study revealed that the activation of glucose metabolism-related genes and VEGF was mainly observed in the early stages of gastric cancer, especially in stage I. These data offer valuable evidence that therapeutic strategies targeting angiogenesis and glucose metabolism pathways should be implemented during the early stages of

gastric cancer.

Moreover, it was determined that HIF-1α but not HIF-2α primarily dimerizes with HIF-1β and regulates expression of VEGF and glucose metabolism-related genes in response to hypoxia. It is well-known that HIF-1α but not HIF-1β plays important roles in the HIF-1 pathway. It also has been reported that HIF target genes are regulated by HIF-1α in prostate, ovarian, breast, and lung cancers (Volm and Koomagi, 2000; Schindl et al., 2002). Taking these reports into account, the data from the present study offer strong evidence that, at least in gastric cancer, not only HIF-1α but also HIF-1β play crucial roles in HIF pathway. In other words, under the hypoxic condition, HIF-1α heterodimerizes with HIF-1β in nucleus, the heterodimmer binds to an enhancer element called the HRE in target genes involved in glucose metabolism and angiogenesis and then regulates expression of these genes. In addition, HIF-2α has been reported to contribute to HIF target gene expression in renal carcinoma cells (Carroll and Ashcroft, 2006), suggesting that the contributions of HIF-1α and HIF-2α to HIF target gene expression induced by hypoxia are regulated in a cell type specific manner in which dimerization with HIF-1β is an important factor. The results of the present study demonstrate that HIF-1α and HIF-1β but not HIF-2α enhances expression of glucose metabolism-related genes and VEGF in response to hypoxia in gastric cancer. These data offer important information relevant to therapies that target angiogenesis and glucose metabolism using the HIF pathway in gastric cancer.

Methods

Patient tissue samples

Twenty-seven tissues with pairs of gastric tumors and adjacent non-tumor tissue for gene expression examination and 38 gastric cancer blocks for immunohistochemical staining analysis were obtained from the College of Medicine, Chungnam National University, and from the Department of Pathology, Eulji University School of Medicine, Korea with informed consent, respectively. The gastric tumors were staged according to tumor-node-metastasis classification of Union Internationale Contre le Cancer.

Cell culture and semiquantitative (Sq) RT-PCR analysis

Human gastric cancer cell lines (SNU-216, -484, -601, -638) were obtained from the Korean Cell Line Bank (Seoul, Korea) (Park et al., 1997; Choi et al., 2007) and normal gastric cell line (Hs677.st) was obtained from

ATCC. All cell lines were maintained in RPMI1640 supplemented with 10% FBS (Hyclone Laboratories) and gentamicin (10 μg/ml) at 37°C in a 5% CO_2 humidified atmosphere. Environmental hypoxic conditions (2%) were achieved in an airtight humidified chamber continuously flushed with a gas mixture containing 5% CO_2 and 95% N_2. The culture of human normal and gastric cancer cell lines, as well as Sq RT-PCR analysis using total RNAs from these cell lines, were performed as previously described (Kim et al., 2004). The primers used in Sq RT-PCR are indicated in Supplemental Data Table S1 and the size of PCR products ranged from 300 to 600 bp. The PCR cycle numbers were varied from 25 to 35 according to the exponential part of the amplification curve of target genes.

siRNA treatment

siRNA targeted to HIF-1α, (5'-CUGAUGACCAGCAACUU-GATT-3' and 5'-UCAAGUUGCUGGUCAUCAGTT-3'), HIF-1β, (5'-CAAUGCGGAUCAGAGUAAATT-3' and 5'-UUUA-CUCUGAUCCGCAUUGTT-3'), and HIF-2α, (5'-CAGCAU-CUUUGAUAGCAGUTT-3' and 5'-ACUGCUAUCAAAGA-UGCUGTT-3') were designed using programs such as Dharmacon-siDESIGN and Invitrogen BOCK-iT RNAi designer, and synthesized from Samchully Pharm (Korea). The human gastric cancer SNU-638 cells were plated at 40% confluency (8×10^5 cells/dish) in 10 cm plates and transfected with oligonucleotide duplexes (100 nM) that had been premixed with Oligofectamine (Invitrogen) in Opti-MEM-I (Invitrogen) for 4 h. An siRNA targeted to an irrelevant mRNA of GFP served as the nonspecific control.

Immunoprecipitation and immunoblot analysis

The SNU-638 cells were treated for 4 h under hypoxic or normoxic conditions, rinsed once with cold PBS, and lysed in lysis buffer A [20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM AEBSF, 1 mM Na_3VO_4, 5 mM NaF, 10 μg/ml aprotinin and leupeptin]. The cell lysates were pre-cleared with 10 μl protein A/G agarose beads (Amersham Biosciences) for 1 h. The cleared lysates were incubated with 6 μg HIF-1β or HIF-1α antibodies (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The lysates were mixed with 30 μl protein A/G agarose beads for 2 h at 4°C. The beads were washed three times with 1 ml lysis buffer A. The final protein precipitates were subjected to immunoblot analysis.

Proteins resolved by 6% SDS-PAGE were transferred to nitrocellulose membranes and nonspecific binding sites were blocked by incubation in TBS containing 0.5% Tween-20 and 5% (w/v) dry milk. Immunoblot analysis was performed with the indicated antibodies in figure. A mouse monoclonal antibody against HIF-1α antibody was purchased from BD Transduction Laboratories. Antibodies to α-tubulin, GLUT1, HIF-1β, and ENO1 were obtained from Santa Cruz Biotechnology. A mouse monoclonal antibody to HIF-2α was purchased from Abcam Inc. The bound primary antibodies were visualized with HRP-conjugated goat anti rabbit-IgG, goat anti-mouse-IgG (Pierce), or donkey anti-goat IgG (Santa Cruz Biotechnology Inc.), and enhanced with a chemiluminescence kit (AB Frontier,

Korea).

Immunohistochemistry

Immunohistochemical staining for HIF-1α, HIF-2α, and HIF-1β was performed using the previously described procedures (Kim *et al*., 2005; Choi *et al*., 2007). The primary antibodies used in the immunohistochemical staining were as followings: a rabbit polyclonal antibody against HIF-1α at a 1:200 dilution; a mouse monoclonal anti-HIF-1β at a 1:200 dilution (Santa Cruz Biotechnology, Inc.); a mouse monoclonal antibody against HIF-2α at a 1:1000 dilution (Abcam Inc.).

Luciferase reporter assay

SNU-638 cells were transfected by Fugene 6 liposome technique (Boehringer Mannheim) according to protocol of the supplier. For reporter assays in HIF-1α, HIF-1β, or HIF-2α knocked-down cells, SNU-638 cells were cotransfected with siRNA of HIFs and HRE-Luciferase reporter. At 48 h post-transfection, the cells were incubated with 200 μM of $CoCl_2$ in complete medium for 12 h. Relative luciferase activities were determined by normalizing the β-galactosidase activities of the cell lysates. The luciferase assay carried out by the method using enhanced luciferase assay kit (Promega) according to the manufacture's instruction.

Statistics

Data were analyzed using a Student's *t*-test on SigmaPlot 8.0 software. *P*-values were assessed to derive the statistical significance.

Supplemental Data

Supplemental Data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-41-1-07.pdf.

Acknowledgements

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