

## Research Paper

# Clinicopathological Implications of the Expression of Hypoxia-related Proteins in Gastric Cancer

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## Abstract

**Objectives:** Tumor hypoxia confers poor prognosis of a wide range of solid tumors due to increased malignancy, increased likelihood of metastasis and treatment resistance. The aim of this study was to assess the significance of the expression of HIF-1 $\alpha$  and HIF-1 $\alpha$ -inducible proteins in gastric cancer and their impact on prognosis. **Materials and Methods:** The expression of HIF-1 $\alpha$ , GLUT-1, CA-9, and iNOS proteins was analyzed by immunohistochemistry in 193 gastric adenocarcinomas (GAs) and 20 normal gastric mucosa. **Results:** HIF-1 $\alpha$ , GLUT-1, CA-9 and iNOS were expressed in 52.3%, 43.0%, 57.0%, and 43.0% of GAs, respectively, which are higher than the normal counterparts except for CA-9. HIF-1 $\alpha$  expression was positively correlated with the expression of GLUT-1, CA-9 and iNOS. GLUT-1 expression was higher in the intestinal type ( $p = 0.012$ ); however, iNOS expression was higher in the less-differentiated type and the diffuse type ( $p = 0.006$ ,  $p = 0.032$ , respectively). The expression of HIF-1 $\alpha$  and GLUT-1 was significantly correlated with lymph node metastasis ( $p = 0.009$ ,  $p = 0.008$ , respectively), while the expression of GLUT-1 and iNOS was significantly correlated with the depth of invasion and advanced stage ( $p = 0.044$ ,  $p = 0.004$ ;  $p = 0.009$ ,  $p = 0.008$ , respectively). Overall survival was shorter in patients with GLUT-1 expression than in those without GLUT-1 expression, which was statistically significant by univariate analysis ( $p = 0.042$ ). On multivariate analysis, however, stage was determined as the only independent prognostic marker ( $p < 0.001$ ). **Conclusions:** Our data suggest that overexpression of HIF-1 $\alpha$ , GLUT-1, and iNOS may play an important role in gastric cancer progression. GLUT-1 is a potential candidate for predicting patient survival.

**Key words:** Gastric cancer, hypoxia, HIF-1 $\alpha$ , GLUT-1, CA-9, iNOS

## Introduction

Gastric cancer is the fourth most common cancer and is the second leading cause of cancer-related death worldwide [1]. In spite of advances in chemotherapy, no clear standard chemotherapy regimen has been established, and the only curative treatment for gastric cancer is surgical resection of primary tumors with appropriate lymphadenectomy [2]. Therefore,

due to limited treatment options and poor prognosis, gastric cancer still remains a major clinical challenge. Improvements in the treatment of the disease must arise from a better understanding of the underlying molecular mechanisms explaining progression, invasion and metastasis formation.

Hypoxia is now recognized as a key factor

driving the development of malignancy and promoting tumor metastasis [3]. The key transcriptional regulator for cells in response to changing oxygen levels is hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). Under the condition of normoxia, HIF-1 $\alpha$  is continuously degraded by proteasomes via the ubiquitin pathway [4]. However, in the hypoxic environment, the HIF-1 $\alpha$  degradation pathway is inhibited and HIF-1 $\alpha$  is stabilized. The stabilized HIF-1 $\alpha$  is then dimerized with HIF-1 $\beta$ , and forms the transcriptionally active HIF-1 complex [3]. Subsequently, it acts as a master regulator of numerous hypoxia-inducible genes that are related to tumor angiogenesis, cell proliferation or survival and glucose metabolism [5]. HIF-1 $\alpha$ -inducible proteins which may be important in cancer include glucose transporter 1 (GLUT-1), carbonic anhydrase 9 (CA-9), erythropoietin (Epo), inducible nitric oxide synthase (iNOS), and vascular endothelial growth factor (VEGF) [5]. Thus, increased expression of hypoxia-related genes could be associated with malignant potential and unfavorable patient prognosis. Most studies have detected overexpressed hypoxia-related genes including HIF-1 $\alpha$  in cancer and have shown their correlations with highly aggressive phenotypes and poor prognosis [6-10]. However, only few studies have investigated the correlation between hypoxia-related genes and gastric cancer [11-16].

The aim of the present study was to examine the expression of HIF-1 $\alpha$  and HIF-1 $\alpha$ -inducible proteins (GLUT-1, CA-9, and iNOS) in human gastric adenocarcinomas (GAs). Next, we evaluated the association between the expression of these proteins and their clinicopathological characteristics and assessed their clinical significance. There has recently been interest in hypoxia-related genes as a target for cancer therapy [17, 18]. Therefore, blocking HIF-1 $\alpha$  or GLUT-1 activity has the potential to inhibit cancer progression by depriving cancer cells of the means to adapt to hypoxia and the nutrient depleted environment. From this point of view, this study regarding the expression of hypoxia-related proteins in gastric cancer may hold the key to a greater individualization of therapy and new treatment measures for patients with gastric cancer.

## Materials and Methods

### Study material

A total of 193 cases of primary GAs were acquired from the Catholic University Hospital, Seoul, Korea from January 2009 to May 2010. Additional 20 cases of normal gastric mucosa were included. The study protocol was approved by the Institutional Review Board of at The Catholic University of Korea. All patients had undergone complete tumor resection.

None of the patients had received preoperative chemotherapy or radiation therapy. The histologic types of tumors were re-classified according to the revised World Health Organization (WHO) classification. Tumors were then divided into two histological subgroups: a differentiated type consisting of papillary adenocarcinoma and well to moderately differentiated tubular adenocarcinomas, and a less-differentiated type consisting of poorly differentiated tubular adenocarcinomas, poorly cohesive carcinomas including signet ring cell carcinomas, and mucinous adenocarcinomas. All patients' stages were re-evaluated in accordance with the guidelines of the 7<sup>th</sup> edition of American Joint Committee on Cancer Staging system. After surgery, clinical follow-up data were obtained from all patients. Survival time was measured as the time from the date of initial surgery to the date of death. Patients that died as a result of surgery or from other causes were excluded from the study.

### Construction of the tissue microarray (TMA) block

Formalin-fixed paraffin-embedded tissues were obtained from subjects. Using H&E-stained slides, a representative tumor site was chosen and the site corresponding to the confirmed tumor site in the paraffin block was marked. Areas with necrosis, hemorrhage, and artifacts were excluded. Single core biopsy specimens of 2 mm in diameter were taken from the representative regions (SeongKohn Trader's Co, Seoul, Korea), placed on a TMA mold with 60 pores, and re-embedded with paraffin. TMA blocks were prepared as 4- $\mu$ m-thick sections and were stained with the H&E staining methods. The tissues were then examined to determine whether the appropriate tumor site had been selected.

### Immunohistochemistry

Immunohistochemical stainings were conducted on 5  $\mu$ m sections of the tissue microarray blocks by the manual procedure. The paraffin sections were mounted on superfrost glass slides, deparaffinized, and rehydrated in a graded series of ethanol, followed by microwave antigen retrieval. Endogeneous peroxidase activity was blocked using 0.3% hydrogen peroxide. The sections were incubated for 1 hour or overnight at 4°C using the following primary antibodies at a dilution of 1:100 against HIF-1 $\alpha$  (Novus, Littleton, CO, USA), at 1:200 against GLUT-1 (Cell Marque, CA, USA), and at 1:500 against CA-9 (Novus, Littleton, CO, USA) and iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunostaining was conducted using the rabbit or mouse DAKO ChemMate™ EnVision™ system, Peroxidase/DAB kit

(DAKO, Glostrup, Denmark). The sections were then counterstained with Meyer hematoxylin and were then dehydrated, cleared, and mounted. Renal medulla was used as a positive control for HIF-1 $\alpha$  and CA-9. Colon cancer was used as a positive control for GLUT-1 and iNOS.

All immunostained slides were evaluated independently by two independent pathologists. The evaluation was done twice without the evaluator having any knowledge of the specific diagnosis and prognosis of each individual case. Nuclear staining for HIF-1 $\alpha$ , membranous staining for GLUT-1 and CA-9, and cytoplasmic staining for iNOS were considered in the evaluation. Cells were scored broadly, according to the staining intensity and the percentage of positive tumor cells. Staining intensities were scored as follows: absence of stain, 0 point; weak staining intensity, 1 point; moderate to strong staining, 2 points. The distribution of stained cells (dependent upon the percentage of stained tumor cells) was scored as follows: no staining or less than 10%, 0 point; between 10 and 50%, 1 point; more than 50%, 2 points. The final staining score was obtained by adding the scores of staining intensity and distribution score of stained cells. Scores from 0 to 1 were considered negative and scores ranging from 2 to 4 were considered positive. Immunohistochemical staining was re-evaluated for cases showing disagreement between pathologists. Two pathologists reviewed those cases together, and reached an agreement for samples with inconclusive results.

### Statistical analysis

Associations between categorical variables were analyzed using the SPSS software package, version 13.0 (SPSS Inc., Chicago, IL USA). Two-sided P values were determined via Chi-square tests. Patient overall survival was analyzed using the Kaplan-Meier method with the use of the log-rank test for univariate analysis. The Cox proportional hazards model was used in the multivariate analysis of the factors that were determined to be significant for overall survival by univariate analysis. For all analyses, the level of significance was set at  $p < 0.05$ .

## Results

### Patient characteristics

Among the 193 patients studied, 123 (62.2%) were men and 70 (36.3%) were women, with a mean age of 63 years (range, 27 to 86 years). The clinicopathological data of our study population are presented in Table 1. Follow-up data were available in 193 patients, and the median follow-up duration was 32.9 months (range, 1.6-43.0 months) after primary

surgery. Twenty-eight patients died during the follow-up period, and 165 patients remained alive at the time of the study.

**Table 1.** Clinicopathological characteristics of 193 patients

characteristics	Number of cases
<b>Age (years)</b>	
< 60	118
$\geq 60$	75
<b>Gender</b>	
male	123
female	70
<b>Histologic type</b>	
differentiated	108
less-differentiated	85
<b>Lauren classification</b>	
intestinal	130
diffuse	63
<b>T classification<sup>a</sup></b>	
T1	80
T2	26
T3	33
T4	54
<b>N classification<sup>b</sup></b>	
N0	107
N1	26
N2	24
N3	36
<b>M classification<sup>c</sup></b>	
M0	193
M1	0
<b>Stage</b>	
I	91
II	42
III	60

M, male; F, female

TNM classification, <sup>a</sup>T was classified according to depth of invasion as follows: T1, invasion to lamina propria or submucosa; T2, invasion to muscularis propria; T3, invasion to subserosa; T4, penetration to serosa or invasion to adjacent structure

<sup>b</sup>N was classified according to the number of involved regional lymph nodes as follows: N0, no lymph node spread; N1, 1 to 2 diseased lymph node spread; N2, 3 to 6 diseased lymph node; N3, more than 7

<sup>c</sup>M was classified according to metastasis as follows: M0, no distant metastasis; M1, distant metastasis

### Expression of HIF-1 $\alpha$ , GLUT-1, CA-9 and iNOS

The microscopic features of immunohistochemical staining for each protein are presented in Fig. 1. In normal gastric mucosa, HIF-1 $\alpha$ , GLUT-1, and iNOS were not expressed. On the other hand, a distinctive pattern of strong membranous staining of CA-9 was observed at the level of foveolar epithelium and oxyntic glands in 20 cases of normal gastric mucosa. Out of the 193 gastric adenocarcinoma (GA) cases, HIF-1 $\alpha$ , GLUT-1, CA-9 and iNOS were expressed in 101(52.3%), 83(43.0%), 110(57.0%), and 83(43.0%) cases, respectively. In GAs cells, HIF-1 $\alpha$  was localized in the nuclei of cancer cells. Concomitant cytoplasmic staining was not counted because HIF-1 $\alpha$  in the nu-

cleus determines the functional activity of the HIF-1 $\alpha$  complex [4]. GLUT-1 and CA-9 were shown to have a strong membranous pattern of cancer cells. Conversely, iNOS was expressed in the cytoplasm of cancer cells.

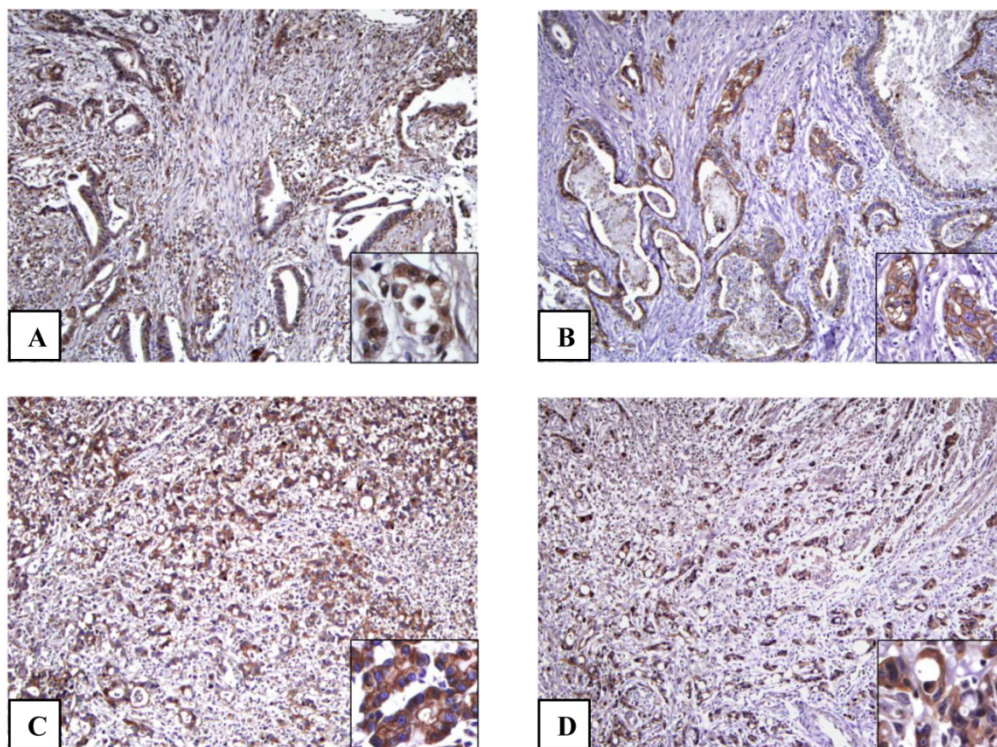
**Relationship between HIF-1 $\alpha$ , GLUT-1, CA-9, and iNOS and clinicopathological factors**

The results from the analysis of correlation between HIF-1 $\alpha$ , GLUT-1, CA-9, and iNOS and clinicopathological variables are presented in Table 2. With regard to the histological type and Lauren classification, GLUT-1 expression was higher in the intestinal type ( $p = 0.012$ ); however iNOS expression was higher in the less-differentiated type and the diffuse type ( $p = 0.006$ ,  $p = 0.032$ , respectively). HIF-1 $\alpha$  and GLUT-1 expressions were significantly correlated with lymph node metastasis ( $p = 0.009$ ,  $p = 0.008$ , respectively). GLUT-1 and iNOS expressions were significantly correlated with the depth of invasion and advanced stage ( $p = 0.044$ ,  $p = 0.004$ ;  $p = 0.009$ ,  $p = 0.008$ , respectively). However, statistically significant associations of CA-9 expression to a variety of clinicopathological variables were not identified.

**Table 2.** Relationship between hypoxia-related proteins and clinicopathological variables

Variables	n	No. of expression(%)			
		HIF-1 $\alpha$	GLUT-1	CA-9	iNOS
<b>Age</b>					
<60	118	64(54.2)	42(35.6)	65(55.1)	49(41.5)
$\geq 60$	75	37(49.3)	41(54.7)	45(60.0)	34(45.3)
<i>p value</i>		0.506	0.009*	0.501	0.602
<b>Gender</b>					
male	123	63(51.2)	49(39.8)	66(53.7)	51(41.5)
female	70	38(54.3)	34(48.6)	44(62.9)	32(45.7)
<i>p value</i>		0.682	0.239	0.215	0.566
<b>Histologic type</b>					
differentiated	108	57(52.8)	51(47.2)	61(56.5)	37(34.3)
less-differentiated	85	44(51.8)	32(37.6)	49(57.6)	46(54.1)
<i>p value</i>		0.889	0.182	0.871	0.006*
<b>Lauren classification</b>					
intestinal	130	71(54.6)	64(49.2)	71(54.6)	49(37.7)
diffuse	63	30(47.6)	19(30.2)	39(61.9)	34(54.0)
<i>p value</i>		0.362	0.012*	0.337	0.032*
<b>pT</b>					
T1	81	37(45.7)	28(34.6)	43(53.1)	25(30.9)
T2-4	112	64(57.1)	55(49.1)	67(59.8)	58(51.8)
<i>p value</i>		0.116	0.044*	0.351	0.004*
<b>pN</b>					
N0	107	47(43.9)	37(34.6)	62(57.9)	40(37.4)
N1-3	86	54(62.8)	46(53.5)	48(55.8)	43(50.0)
<i>p value</i>		0.009*	0.008*	0.766	0.078
<b>Stage</b>					
I	91	41(45.1)	30(33.0)	51(56.0)	29(31.9)
II	42	21(50.0)	18(42.9)	22(52.4)	20(47.6)
III	60	39(65.0)	35(58.3)	37(61.7)	34(56.7)
<i>p value</i>		0.053	0.009*	0.627	0.008*

\* statistically significant



**Fig 1.** The adenocarcinoma cells show positive immunohistochemical staining results for HIF-1 $\alpha$  (A), GLUT-1 (B), CA-9 (C) and iNOS (D) ( $\times 100$ ). (A) Inset: HIF-1 $\alpha$  is localized in the nuclei of cancer cells (inset ( $\times 400$ )). (B) Inset: GLUT-1 immunostaining shows positive reaction a strong membranous reaction a strong membranous pattern in cancer cells ( $\times 400$ ). (C) Inset: CA-9 is strongly expressed in cell membrane of cancer cells ( $\times 400$ ). (D) Inset: iNOS is expressed diffuse staining in the cytoplasm of cancer cells ( $\times 400$ ).

### Inter-relationship between HIF-1α and HIF-1α-inducible proteins expression

The results from the analysis of inter-relationships among HIF-1α, GLUT-1, CA-9, and iNOS expressions are presented in Table 3. Significant correlations were noted between HIF-1α and GLUT-1, CA-9, and iNOS. HIF-1α-positive cases showed significantly higher levels of GLUT-1, CA-9, and iNOS expression (55.4%, 70.3%, 61.4%, respectively) than those of HIF-1α-negative cases (29.3%;  $p=0.000$ , 42.4%;  $p=0.000$ , 22.8%;  $p=0.000$ , respectively).

**Table 3.** Inter-relationship between HIF-1α and GLUT-1, CA-9, iNOS expression

HIF-1α	No. of cases	No. of cases(%)		
		GLUT-1	CA-9	iNOS
positive	101	56(55.4)	71(70.3)	62(61.4)
negative	92	27(29.3)	39(42.4)	21(22.8)
<i>p value</i>		0.000*	0.000*	0.000*

\* statistically significant

### Survival analysis

Survival analyses using the Kaplan-Meier method according to clinicopathological variables as well as all protein expression profiles are summarized in Table 4. The depth of tumor invasion ( $p = 0.002$ ), presence of regional lymph node metastasis ( $p = 0.000$ ) and stage ( $p = 0.000$ ) demonstrated a significant correlation with overall survival (OS). Out of 4 proteins analyzed, patients without GLUT-1 expression had longer OS compared to those with GLUT-1 expression (mean survival period, 40.2 months vs 37.5 months, respectively), which was statistically significant ( $p = 0.042$ ). On the other hand, HIF-1α, CA-9, and iNOS expressions were not significantly related to patient prognosis. Multivariate analysis using the Cox proportional hazards model was performed to evaluate the independent prognostic predictors. As shown in Table 5, stage was the only independent prognostic marker ( $p = 0.000$ ). However, GLUT-1 was not determined as an independent prognostic factor ( $p = 0.561$ ).

### Discussion

Tissue microarray (TMA) technology is an essential pathology research tool that has enabled simultaneous analysis of potential biomarker expression in a very large number of archived pathology specimens [19]. This technology allows us to save time, antibodies and costs associated with analyzing multiple specimens [19]. However, when the tumor is composed of heterogenous components and the observed molecular markers are heterogeneously expressed, tissue microarray (TMA) block implicates

important shortcomings that it may not represent the full tissue section [20]. In this study, we determined the expression of HIF-1α and HIF-1α-inducible proteins, including GLUT-1, CA-9, and iNOS in GAs, using TMA technology and hoped to achieve a more precise understanding of the associations of these protein expressions with their clinicopathological characteristics and patient survival.

**Table 4.** Kaplan-Meier Survival analysis

		Gastric Adenocarcinoma (n=193)		
		No	Mean time to survival (months) (95% CI)	<i>p value</i>
Age	<60	118	39.6 (37.943-41.235)	0.606
	≥60	75	37.2 (34.799-39.581)	
Gender	male	123	38.8 (36.995-40.648)	0.612
	female	70	39.5 (37.347-41.619)	
Differentiation	differentiated	108	38.8 (36.797-40.747)	0.582
	less-differentiated	85	39.4 (37.457-41.335)	
Lauren classification	intestinal	130	39.3 (37.584-40.980)	0.714
	diffuse	63	38.7 (36.181-41.114)	
pT	T1	81	41.6 (40.448-42.757)	0.002*
	T2-T4	112	37.2 (35.007-39.394)	
pN	N0	107	42.2 (41.419-42.957)	0.000*
	N1-3	86	35.0 (32.284-37.802)	
Stage	I	91	40.9 (40.015-41.749)	0.000*
	II	42	41.8 (40.145-43.529)	
	III	60	31.4 (28.063-34.835)	
HIF-1α	negative	92	40.3 (38.614-41.911)	0.283
	positive	101	37.9 (35.741-40.104)	
GLUT-1	negative	110	40.2 (38.639-41.674)	0.042*
	positive	83	37.5 (35.005-40.016)	
CA-9	negative	83	39.0 (36.841-41.089)	0.705
	positive	110	39.1 (37.201-40.905)	
iNOS	negative	110	39.4 (37.614-41.212)	0.682
	positive	83	38.6 (36.360-40.772)	

\* statistically significant

**Table 5.** Cox regression multivariate analysis

Variables	HR	95% CI	<i>p-value</i>
pT	0.636	0.192-2.107	0.459
pN	3.280	0.443-24.299	0.245
Stage	5.806	2.807-12.001	0.000*
GLUT-1 expression	1.262	0.577-2.758	0.561

\* statistically significant

Urano *et al* [12] showed that HIF-1α was over-expressed in gastric cancer; but this expression showed no association with clinicopathological status and patient prognosis. Since then, a few studies have demonstrated that HIF-1α expression was associated with not only poor clinical features but also patient survival [11, 21]. In our study, we found that normal gastric tissues did not express HIF-1α, GLUT-1, and

iNOS, although CA-9 was expressed in normal gastric mucosa, while HIF-1 $\alpha$ , GLUT-1, and iNOS were overexpressed in GAs, which were all consistent with the findings of previous reports [12-15, 22]. We also found that HIF-1 $\alpha$  expression was well correlated with the expression of GLUT-1, CA-9 and iNOS. Moreover, in accordance with earlier studies [11, 14, 15, 22], we clearly demonstrated that the expression of HIF-1 $\alpha$  and GLUT-1 were significantly correlated with lymph node metastasis, and the expression of GLUT-1 and iNOS were significantly correlated with advanced stage. Our data suggest that HIF-1 $\alpha$  activation induces GLUT-1, CA-9, and iNOS [3, 5], and that HIF-1 $\alpha$ , GLUT-1, and iNOS may play an important role in promoting tumor development and dissemination. However, with regard to patient prognosis, only GLUT-1 was found to be correlated with patient prognosis by univariate analysis. The technical limitation of TMA may explain the lack of correlation of prognosis with markers other than GLUT-1.

GLUT-1, a member of the hypoxia-related protein family, is a basic high-affinity glucose transporter and is not detectable in a large proportion of cells from normal tissues, except in erythrocytes, the endothelial cells of brain, the perineurium of peripheral nerves, the germinal centers of reactive lymph nodes, renal tubules and placenta [18]. GLUT-1 is also predominantly upregulated in human malignancies since increased glucose uptake is one of the major metabolic changes observed in malignant tissues, and has been found to correlate with aggressive biologic behavior [23, 24]. However, limited data are available regarding GLUT-1 expression in gastric cancer compared to studies on HIF-1 $\alpha$  expression in gastric cancer. Noguchi *et al* [25] found an association between high GLUT-1 expression and poor post-operative survival in 70 gastric cancer patients. A more detailed study by Kawamura *et al* [14] revealed that 182 (30%) cases of 617 gastric carcinomas were positive for GLUT-1 and found associations between GLUT-1 expression and tumor aggressiveness such as the depth of invasion, lymph node metastasis, stage, and patient survival. In our study, GLUT-1 expression was significantly associated with clinically aggressive tumor behavior, and therefore, GLUT-1 protein may be used as a prognostic factor in the context of GA. However, in multivariate analysis, GLUT-1 expression was not associated with patient survival. We believe that the short follow-up duration in our study may have affected the results of multivariate analysis, thus a further evaluation through an ongoing long-term study is required.

iNOS is the most active isoform of NO synthase. iNOS expression potentially serves as a global regulator of carcinogenesis and tumor behavior, because NO modulates some signal transduction pathways in

cancer cells via posttranslational protein modifications [22]. Increased iNOS activity has also been observed in gastric cancer in common with other malignancies, and its expression was identified as a good marker for poor prognosis [15, 22]. Our results showed that iNOS expression was associated with stage, which is a significant poor prognostic marker, but was not associated with patient survival. However, further studies are warranted to examine its usefulness as a prognostic marker.

Finally, we cannot disregard the implication of hypoxia-related genes as a target for cancer therapy [17, 18]. As remarked above, HIF-1 $\alpha$ -inducible genes including HIF-1 $\alpha$  are positive factors that promote tumor growth and survival. Tumor targeting therapy against specific biomarkers have the following advantages: (1) to deliver anti-cancer drugs to target specific tumor locations in the body; (2) to decrease the amount of drugs needed to attain a desirable therapeutic dose in target cancer cells; and (3) to reduce the amount of drugs in non-cancerous cells and to reduce the drug's side effects. Therefore, biomarker-targeted treatment that specifically blocks HIF-1 $\alpha$  or GLUT-1 activity in gastric cancer expressing HIF-1 $\alpha$  or GLUT-1 holds great promise for improving survival of patients with gastric cancer.

## Competing Interests

The authors have declared that no competing interest exists.

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