Int J Clin Exp Med 2016;9(4):7209-7216
www.ijcem.com /ISSN:1940-5901/IJCEM0018087

Original Article

Elevated expression of hypoxia inducible factor-1 alpha is correlates to recurrence and poor outcome in gastric cancer

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Received October 18, 2015; Accepted January 10, 2016; Epub April 15, 2016; Published April 30, 2016

Abstract: Background: Hypoxia-inducible factor-1 alpha (HIF-1α) is a transcription factor that plays a central role in biologic processes under hypoxic conditions, especially concerning tumor angiogenesis. In this study, we investigated the correlation of HIF-1α expression with clinicopathological characteristics, tumor recurrence and prognosis in gastric cancer (GC) after curative resection. Methods: The clinical data of 196 GC patents who underwent curative resection were analyzed retrospectively. The expressions of HIF-1α in recurrent GC tissues compared to non-recurrent GC tissues were examined, and the relationship between HIF-1α expression and clinicopathological characteristics was evaluated. In addition, these patients were followed up to investigate the relationship between HIF-1α expression and the survival time. Results: Immunohistochemical staining demonstrated that 114 of 196 GC samples (58.2%) were positive for HIF-1α. The positive rate of HIF-1α expression was significantly higher in recurrent GC tissue, than that in non-recurrent GC tissues (80.9%, 51.4%, respectively, $P < 0.05$). There was a close relationship between HIF-1α expression and TNM stage ($P = 0.009$), lymph node status ($P = 0.004$), differentiation ($P = 0.042$), vascular invasion ($P = 0.019$), T stage ($P = 0.013$) and VEGF expression ($P = 0.030$). Furthermore, patients with HIF-1α positive showed significantly higher recurrence and poorer prognosis than those with HIF-1α negative. Multivariate analysis showed that HIF-1α expression was a significant independent factor for tumor recurrence and overall survival. Conclusion: The results of the present study suggest that HIF-1α may be used as an unfavorable indicator in predicting tumor recurrence and prognosis for with GC after curative surgery. This study also suggests that HIF-1α might be a potential therapeutic target for GC.

Keywords: Gastric cancer, hypoxia-inducible factor-1 alpha, recurrence, tumor markers, immunohistochemistry

Introduction

Gastric cancer remains one of the most common causes of cancer-related deaths worldwide [1]. In spite of progress in the surgical treatment and chemotherapy, the prognosis of gastric cancer patients remains poor [2]. Recurrence and metastasis are the main causes of death, and recurrence after curative intent resection is relatively common, occurring in 20% to 50% of patients [3, 4]. However, the underlying molecular mechanisms responsible for metastasis and tumor recurrence have not been fully elucidated, and the specific tumor markers in detection of tumor recurrence have not yet been discovered.

Hypoxia is closely related to the proliferation, migration, and metastasis of tumor cells. It is vital for gastric cancer cells to adapt the microenvironment of reduced oxygen [5, 6], and the master regulatory protein in the response of cells to changing oxygen levels is hypoxia-inducible factors-1 alpha (HIF-1α) [7]. HIF-1α is both strongly induced and stabilized under hypoxic conditions, which can be translocated from the cytoplasm into the nucleus where its target genes promote cell proliferation, viability, angiogenesis, and me olic adaptation to hypoxia. Overexpression of HIF-1α was correlated with clinicopathological findings, HIF-1α expression was found to be an indicator of poor prognosis in gastric cancer [8].

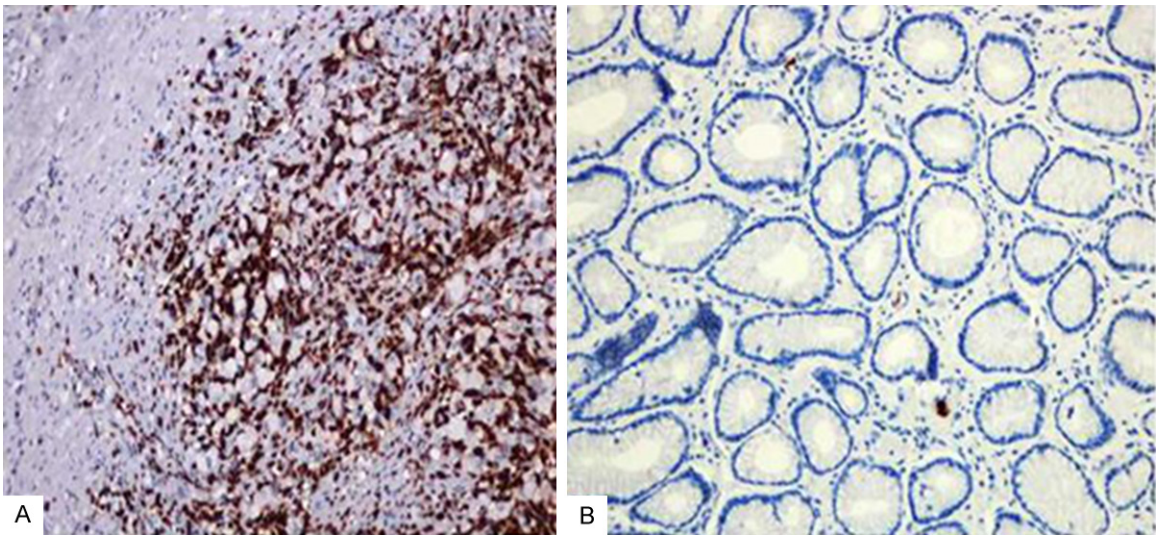

Figure 1. Immunohistochemical staining of hypoxia-inducible factor-1 alpha (HIF-1α) in the advanced gastric cancer (A) and noncancerous normal (B) tissues. (A) HIF-1α positive expression. (B) HIF-1α negative expression.

In this study, HIF-1α expression in human gastric cancer was examined by immunohistochemistry. Its correlation with clinical characteristics and prognosis was evaluated to determine whether HIF-1α expression level could be used to predict recurrence and prognosis in patients with gastric cancer after curative surgery.

Materials and methods

Tumor samples

This study included 196 patients with histological confirmed primary gastric cancer, all of whom underwent gastrectomy between 2006 and 2007 at the Department of gastrointestinal surgery, the Affiliated Drum tower Hospital of Nanjing University Medical School, Nanjing, China. They included 122 men and 74 women, ranging from 27 to 81 years of age (mean, 59.0 years). Patients lost during follow up or who died within one year of surgery was excluded. All specimens were pathologically reassessed independently by two gastrointestinal pathologists according to the 7th edition of the American Joint Committee on Cancer (AJCC) of gastric cancer [9].

Clinicopathological variables including age, sex, location, tumor size, tumor differentiation, Lauren type, T stage; and vascular, lymphatic, and perineural invasion were collected for each patient. Tumor size was defined as the longest diameter according to the pathology report. None of these patients received any preoperative anticancer treatment. All specimens were obtained from patients with written informed consent was obtained from all patients or their families and approved by the Clinical Research Ethics Committee of Drum Tower hospital.

Immunohistochemistry

Four micrometer thick sections were cut from archival formalin-fixed paraffin-embedded tissue blocks. The samples were deparaffinized and dehydrated using a graded series of ethanol solutions. For HIF-1α antigen retrieval, sections were then irradiated by a domestic microwave oven at 99°C in 10 mM citrate buffer (pH 9.0) for 30 min, and cooled to room temperature. After microwave irradiation, the slides were washed with phosphate-buffered saline (PBS), treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase, and then incubated with the primary antibody in a humidified chamber at 48°C overnight. As the primary antibody, the rabbit polyclonal antibody H206 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for HIF-1α, diluted at 1:200 was used. Sections were washed three times with PBS, then incubated with biotinylated horse anti-mouse/anti-rabbit immunoglobulin G antibody for 30 min, washed again three times with PBS, and then incubated with avidin-biotinylated peroxidase complex for

30 min. After three additional washings with PBS, staining was developed by incubating the sections in 3-amino-9-ethylcarbazole (Vector) for 10 min. The sections were then counterstained with hematoxylin and mounted.

Assessment of HIF-1α

The HIF-1α expression was defined as positive if nuclear staining was observed in ≥ 5% of the tumor cells. Concomitant cytoplasmic staining was not counted because HIF-1α in the nucleus determines the functional activity of the HIF-1α complex (**Figure 1**). In regard to overall survival curve, the HIF-1α expression was classified as one of four categories, depending on the percentage of tumor cells stained: - (0-5%), 1+ (5-10%), 2+ (10-15%), 3+ (≥ 15%). The HIF-1α expression through nuclear staining of positive cells was predominant at the invading edge of the tumor margin and at the periphery of necrotic regions within tumors.

Follow-up

No major perioperative complications occurred in patients, and all were discharged from the hospital. The closing date for follow-up was March 31, 2014. As a protocol for follow-up, all patients were checked every 3 months during the first 2 years and every 6 months thereafter. Recurrence were confirmed by tumor markers levels including CEA, AFP, CA199 and CA125, and imaging including chest radiography, barium meal, abdominal ultrasonography (US), computed tomography (CT), and endoscopy according to the clinical situation after gastrectomy. The locations and times of tumor recurrence were recorded. The follow-up time had the day of surgery as a starting point, the time of tumor recurrence and death were recorded, and these two points were evaluated for prognostic analysis. If the follow-ups were incomplete, patients or their families were contacted by telephone. The median follow-up period after surgery was 39.8 months (range from 8 to 91 months).

Statistical analysis

All statistical analyses were performed with SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). The correction between HIF-1α expression and clinicopathological features was analyzed by the χ^2 test and Fisher's exact test. Survival curves were estimated using the Kaplan-Meier method, and differences in survival distributions were evaluated by the log-rank test. Cox's proportional hazards modeling of factors potentially related to survival was performed to identify factors that might have a significant influence on survival. Differences with a p value of 0.05 or less were considered statistically significant.

Results

Patient characteristics

The detailed clinicopathological characteristics of patients after curative resection are shown in **Table 1**. When follow-up was over, a total of 67 patients died because of tumor progression and 8 due to other causes. Overall survival (OS) was defined as the interval between surgery and last visit or death. The mean OS of 196 gastric cancer patients was 39.92 ± 19.52 months (range from 8 to 91 months), and the 5-year survival rate of all enrolled patients was 19.39%.

HIF-1α expression in gastric cancer tissues

The patterns of HIF-1α expression in the tumor cells were mixed nuclear/cytoplasmic staining, and expression of HIF-1α in gastric cancer tissue was significantly higher than that in the normal gastric tissue. HIF-1α expression through nuclear staining of positive cells was predominant at the invading edge of the tumor margin and at the periphery of necrotic regions within tumors (**Figure 1**). Of the 196 total cases, 114 (58.2%) were HIF-1α positive. The positive rate of HIF-1α expression in gastric cancer tissues with recurrence was 80.9% (72/89), significantly higher than that without recurrence 51.4% (55/107). HIF-1α expression was associated with depth of invasion, T stage, TNM stage, LN metastasis, venous invasion, lymphatic invasion, and VEGF expression. There was no correlation between HIF-1α and other pathological parameters, such as age, gender, lauren type, serum CEA, perineural invasion, tumor location, Borrmann type, or tumor size (**Table 1**).

Time to recurrence and recurrence pattern

Recurrence occurred in 89 (41.2%) patients as the follow-up ended. The median time to recur-

Table 1. The relationship between expression of HIF-1α, tumor recurrence and clinicopathological features

Parameters	N	HIF-1α Positive (%)	χ^2-value	*P* value
Age (yr)			0.196	0.681
< 60	97	55 (56.7)		
≥ 60	99	59 (59.6)		
Gender			0.097	0.756
Male	122	72 (59.0)		
Female	74	42 (56.8)		
Tumor location			1.060	0.787
Upper third	42	24 (57.1)		
Middle third	44	25 (56.8)		
Lower third	98	57 (58.2)		
Diffused	12	8 (66.7)		
Differentiation			4.117	0.042
Well/Moderate	70	34 (48.6)		
Poor	126	80 (63.5)		
Lauren type			2.881	0.094
Intestinal	89	46 (51.7)		
Diffuse	107	68 (63.6)		
Tumor size (cm)			3.741	0.053
< 5	94	48 (51.1)		
≥ 5	102	66 (64.7)		
Borrmann type			2.422	0.490
I	7	3 (42.9)		
II	43	22 (51.2)		
III	90	53 (58.9)		
IV	56	36 (64.3)		
T stage			10.801	0.013
pT1	15	4 (26.7)		
pT2	23	9 (39.1)		
pT3	75	46 (61.3)		
pT4	83	53 (63.8)		
Lymph node status			8.218	0.004
N0/N1	75	34 (45.3)		
N2/N3	121	80 (66.0)		
Vascular invasion			5.506	0.019
Absent	93	46 (49.5)		
Present	103	68 (66.0)		
Perineural invasion			2.020	0.155
Absent	84	44 (52.4)		
Present	112	70 (62.5)		
Recurrence			6.680	0.010
Absent	107	50 (46.7)		
Present	89	64 (71.9)		
TNM stage			6.767	0.009
I-II	68	31 (45.6)		
III-IV	128	83 (64.8)		

rence was 18.0 months (range from 6 to 78 months). Among patients with recurrence, 63 (70.7%) had recurrence within 2 years (**Table 2**). Of the 89 patients with recurrence, 34 (38.2%) were diagnosed with loco-regional relapse, which was the most prevalent, 28 (31.5%) patients had hematogenous metastases (16 in liver, 5 in lung, and 6 in bone). Other recurrences were peritoneal recurrence (n = 19, 21.3%), distant lymph node metastases (n = 5, 5.6%), or at multiple sites (n = 3, 3.4%).

HIF-1α expression association with tumor recurrence and poor overall survival

Compared with patients without recurrence, patients with recurrence showed advanced tumor stages (*P* = 0.003), longer tumor size (*P* = 0.0018), depth of invasion (*P* = 0.035), lymph node invasion (*P* = 0.008), positive HIF-1α (*P* = 0.001) and VEGF expression (*P* = 0.028), and vascular invasion (*P* = 0.027) (**Table 3**). Multivariate model identified that expression of HIF-1α, advanced TNM stage, and lymph node metastases were independent predictive factors for tumor recurrence (**Table 4**).

Survival curves according to positive or negative HIF-1α staining are shown in **Figure 2**. Respectively, survival rates for patients with HIF-1α-positive staining were significantly lower than that of HIF-1α negative (*P* = 0.016). Compared with patients without recurrence, survival rates for patients with recurrence were significantly lower (*P* < 0.001). Multivariate Cox's proportional hazard analyses of clinicopathological factors revealed HIF-1α expression (hazard ratio (HR) 2.289; 95% CI 1.208-4.339; *P* = 0.011) and advanced TNM stage (HR 2.406; 95% CI 1.278-

VEGF			4.724	0.030
Negative	85	42 (47.1)		
Positive	111	72 (66.7)		
Serum CEA level (μg /L)			1.177	0.278
< 5	101	55 (54.4)		
≥ 5	95	59 (62.1)		

HIF-1α: hypoxia Inducible Factor-1 alpha; TNM: Tumor Node Metastasis; VEGF: vascular endothelial growth factor; CEA: carcinoembryonic antigen.

Table 2. Characteristics of gastric cancer with recurrence compared with patients without recurrence

Parameters	Recurrence (n = 89)	No recurrence (n = 107)	χ^2-value	*P* value
Age (yr)			0.090	0.764
< 60	43	54		
≥ 60	46	53		
Gender			0.504	0.478
Male	53	69		
Female	36	38		
Tumor location			2.478	0.479
Upper third	19	23		
Middle third	23	21		
Lower third	40	58		
Diffused	7	5		
Differentiation			3.318	0.076
Well/Moderate	25	43		
Poor	64	64		
Lauren type			0.556	0.456
Intestinal	43	46		
Diffuse	46	61		
Tumor size (cm)			5.562	0.018
< 5	34	60		
≥ 5	54	48		
Borrmann type			3.845	0.279
I	2	5		
II	15	28		
III	43	47		
IV	29	27		
T stage			4.731	0.193
pT1	5	10		
pT2	9	14		
pT3	30	45		
pT4	46	38		
Lymph node status			7.145	0.008
N0/N1	25	50		
N2/N3	64	57		
Vascular invasion			4.869	0.027
Absent	35	59		
Present	54	48		

4.531; *P* = 0.007) as independent prognostic indicators of poor survival for gastric cancer after surgery.

Discussion

Gastric cancer is one of the most common malignancies worldwide and still the second leading cause of cancer-related deaths [2]. The treatment of gastric cancer includes a combination of surgery, chemotherapy, and radiation therapy. However, the majority of patients develop local or distant recurrence after gastrectomy and adjuvant chemotherapy, and the dismal prognosis of gastric cancer is due principally to the frequency of recurrence and metastasis. Therefore, the identification of diagnostic and prognostic biomarkers is needed for optimizing management and treatment strategies.

Tumor hypoxia is well recognized in oncology to be a key factor resulting in treatment resistance and poor prognosis. Hypoxia can increase HIF-1α protein stability via altered ubiquitination and then lead to overexpression of HIF-1α. There is a considerable body of data supporting the notion that HIF-1α has an important role in invasion and metastasis of malignant tumors, including stomach, brain, oropharynx, cervix, ovary and breast [10, 11].

It has been showed that the degree of intratumoral hypoxia is positively correlated with the ability of tumor invasion, metastasis, and drug resistance [12]. Furthermore, hypoxia and HIF-1α over-expression are implicated in the tumor aggressiveness of many cancers, including gastric cancer [13]. Our study suggests that overexpression of HIF-1α is a common feature in gastric cancer, and might represent a novel predictive

Perineural invasion			3.171	0.075
Absent	32	52		
Present	57	55		
TNM stage			8.863	0.003
I-II	21	47		
III-IV	68	60		
VEGF			4.836	0.028
Negative	31	54		
Positive	58	53		
HIF-1α			11.838	0.001
Negative	17	53		
Positive	72	55		
Serum CEA level (μg /L)			0.286	0.593
< 5	44	57		
≥ 5	45	50		

HIF-1α: hypoxia Inducible Factor-1 alpha; TNM: Tumor Node Metastasis; VEGF: vascular endothelial growth factor; CEA: carcino-embryonic antigen.

Table 3. Multivariate analysis of risk factors for recurrence in gastric cancer

	Hazard ratio	95% CI	*P* value
HIF-1α positive	2.345	(1.261-4.360)	0.007
Lymph node status	1.063	(0.754-1.499)	0.047
TNM stage	1.986	(1.009-3.911)	0.043

CI: confidence interval; HIF-1α: hypoxia-inducible factor-1α; TNM: Tumor Node Metastasis.

Table 4. Multivariate analysis of significant prognostic factors for survival in patients with gastric cancer

Parameters	Hazard ratio	95% CI	*P* value
TNM stage	2.406	(1.278-4.531)	0.007
HIF-1α	2.289	(1.208-4.339)	0.011

CI: confidence interval; HIF-1α: hypoxia-inducible factor-1α; TNM: Tumor Node Metastasis.

marker for the clinical outcome of the disease. Therefore, therapeutic agents that inhibit function have the potential to improve the outcome of patients with metastasis [14, 15].

In this study, we investigated HIF-1α protein expression in gastric cancer and its association with recurrence and disease-free survival. Our results showed HIF-1α protein expression was stronger positive staining of HIF-1α gastric cancer with deep invasion, differentiation, lymph node metastasis, vascular invasion, and advanced TNM stage, which was consistent with previous studies on the relationship between HIF-1α and human gastric cancer [16], and also the expression of HIF-1α in patients with recurrence was obviously more frequently detected than that of no-recurrence. However, HIF-1α expression was not correlated with age, gender, differentiation status, tumor site or Lauren classification. Recent studies suggested that the hypoxic activation of NF-κB seems to contribute to the expression of HIF-1α protein at the translational level, shRNA-mediated downregulation of HIF-1α expression reduced the cell viability of SNU-668, SNU-484, and SNU-216 gastric cancer cells in vitro under hypoxic conditions [17], which support our results demonstrating the association of HIF-1α with gastric cancer invasion and metastasis.

In this study, HIF-1α expression was found to be associated with a malignant behavior category, including postoperative prognosis. We found that high HIF-1α expression was associated with poor prognosis and unfavorable clinical outcome, and patients who had tumors with high HIF-1α expression had remarkably poor overall survival as compared with patients who had low HIF-1α expression. Furthermore, multivariate analysis revealed that HIF-1α expression level was an independent, significant risk factor for recurrence and survival after curative resection.

However, how HIF-1α functions in these processes is not entirely clear; further studies are needed to elucidate the molecular mechanisms by which HIF-1α participates in the development and progression of GC. Accumulating evidence has indicated that HIF-1α as a novel and key regulator that integrates EMT [18-21], which is an important mechanism during the early steps of tumor progression and metastasis, when neoplastic cells disseminate from the primary tumor.

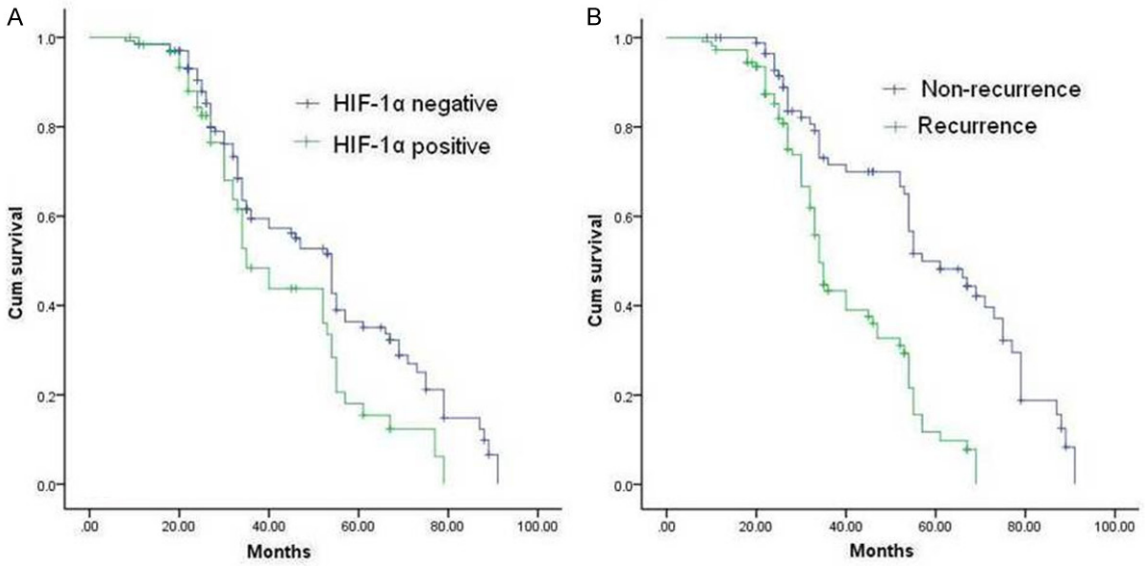

Figure 2. Kaplan-Meier survival curves shows the probability of overall survival in patients with HIF-1α-negative and HIF-1α-positive expression (A) and the comparison of survival between recurrence and non-recurrence (B).

In addition, HIF-1α is crucial for the formation and maturation of the vasculature through interaction with the signals that regulating angiogenesis, such as vascular endothelial growth factor (VEGF) and Notch pathway [22, 23]. Previously study had shown that inhibition of HIF-1α function in human gastric cancer cells resulted in the reduction of VEGF secretion in vitro, the inhibition of tumor growth and angiogenesis in vivo, and alterations in tumor vessel morphology and maturation in vivo [24].

In conclusion, HIF-1α expression was increased in clinical gastric cancer specimens, and it was associated with tumor invasion and metastasis. Our study also provides the evidence that HIF-1α is an independent prognostic factor of overall survival, and a predictive factor of cancer recurrence for patients with gastric cancer. Therefore, HIF-1α could serve as a potential predictive marker for predicting malignant behavior, and it is an independent prognostic factor for tumor recurrence and prognosis for patients with gastric cancer.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81201909 and 81572338), the Fundamental Research Funds for the Central Universites (No. 021414380031), Nanjing Medical Science and Technology Development program (Nos. QYK11166, YKK12072).

Disclosure of conflict of interest

None.

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