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Hypoxia-inducible Factor-1α is Associated with Risk of Aggressive Behavior and Tumor Angiogenesis in Gastrointestinal Stromal Tumor

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Background: The objective of this study was to evaluate the relationship of HIF-1α expression and tumor angiogenesis, recurrence/distant metastasis, and its value in the prediction of aggressive behavior of gastrointestinal stromal tumor (GIST).
Methods: Paraffin-embedded specimens from 62 patients with GIST were divided into two groups, low risk (n = 31) and high risk (n = 31) according to the tumor size, mitotic count and proliferating cell nuclear antigen (PCNA) index. We investigated the expression of the HIF-1α and analyzed correlation with tumor angiogenesis monitored by expression of vascular endothelial growth factor (VEGF) and tumor microvessel density using immunohistochemical staining. The data were analyzed with χ^2 test or Fisher's exact test and multivariate test.
Results: There were statistically significant differences between high risk (29/31; 93.5%) and low risk (8/31; 25.8%) of GIST in high expression of HIF-1α ($P < 0.0001$). In addition, the incidence of recurrence/distant metastasis was significantly higher in cases of high HIF-1α expression (12/35; 34.3%) than in cases of low HIF-1α expression (1/24; 4.2%)($P = 0.009$). Moreover, high VEGF expression (37/43; 86.0%) and microvessel density (30/32; 93.8%) were significantly higher in high HIF-1α expression tumors than in low-expression tumors ($P < 0.0001$).
Conclusions: Our findings suggest that HIF-1α may play an important role in aggressive behavior and tumor angiogenesis in GIST. In addition, high expression of HIF-1α was significantly correlated with tumor recurrence/distant metastasis, so it may provide an ancillary prognostic factor for GIST.

Key words: gastrointestinal stromal tumor – hypoxia inducible factor-1 alpha (HIF-1α) – immunohistochemistry, microvessel density (MVD) – vascular endothelial growth factor (VEGF)

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract (1,2). Recent studies have shown that the tumor cells express a growth factor receptor with tyrosine kinase activity termed c-kit which can be detected by immunohistochemical staining for CD117, a specific marker for GIST (3,4). Until now, no single and absolute independent parameter has been proposed as a prognostic factor for GIST. Generally, prognostic features indicative of malignancy or high risk for aggressive clinical behavior include tumor size of 5 cm or larger, and mitotic rate of 2/10 high power field (HPF) or greater (5,6). In addition, nuclear atypia, marked cellularity, vessel invasion and necrosis are also reported to be associated with malignancy (7).

Angiogenesis is essential for the growth, invasion, and metastasis of tumor (8). The mechanism of angiogenesis has been shown to involve release of some substances from growing tumors that stimulate outgrowth of blood vessels from the host vasculature. Vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis and in tumor metastasis (9,10). It induces endothelial cell migration and proliferation (11). VEGF is mainly produced by macrophages, vascular smooth muscle cells and tumor cells (12). Recently, studies have focused on the outcome of angiogenesis, the vascularity in the tumor. Tumor vascularity can be assessed by staining the blood vessel endothelia in the tissue and expressed in microvessel density (MVD) (13).

On the other hand, most solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Hypoxia in the tumor microenvironment is

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sufficient to activate hypoxia-inducible factor (HIF)-dependent gene expression (14). Hypoxia-inducible factor-1 alpha (HIF-1α) is overexpressed in most human malignancies (15). A major role for HIF modulates gene expression, tumor angiogenesis (16), tumor progression and aggressive behavior in solid tumors (17). VEGF expression can be induced by exposure of tumor cells to hypoxia or growth factors and this expression is due in part to increased VEGF gene transcription, which is mediated by HIF-1 (18,19). HIF-1 stimulates processes like angiogenesis, glycolysis, erythropoiesis and apoptosis (20). HIF-1 plays a critical role in angiogenesis during vascular development, and it is a heterodimer composed of two subunits: HIF-1α and HIF-1β. HIF-1α is the oxygen-regulated subunit that determines HIF-1 activity (21). Under non-hypoxic conditions, HIF-1α is subject to ubiquitination and proteasomal degradation. Thus, under hypoxic conditions, HIF-1 transcriptional activity increases rapidly due to HIF-1α protein overexpression (22).

HIF-1α is a primary determinant of HIF activity. HIF-1α expression has been studied in many cancers, including lung, prostate and breast carcinomas. The results reveal that HIF-1α expression is related to the patient's prognosis in these tumors (17). Therefore, it is worth finding out whether HIF-1α is an accurate and reliable prognostic factor for GIST. However, to the best of our knowledge, there is only one report to date concerned with the association of HIF-1α expression and angiogenesis in GIST (23). In our study, we not only evaluated the relationship between HIF-1α expression and tumor angiogenesis, but also looked at the correlation between HIF-1α expression and the proposed aggressive behavior of GIST in order to evaluate the applicability of immunohistochemical staining of HIF-1α expression in surgical specimens for the assessment of the biological behavior of GIST.

SUBJECTS AND METHODS

Patients

Sixty-two cases of GIST were obtained from the archives of the Department of Pathology, Kaohsiung Medical University Chung-Ho Memorial Hospital between 1991 and 2000. All 62 patients had undergone subtotal gastrectomy, performed complete tumor resection or received segmental enterectomy with anastomosis. The diagnoses of GIST are based on the positive for CD117 (c-kit) by immunohistochemical stain. They were classified as low- or high-risk tumors as suggested by Gunawan et al. (5): high risk indicated either (i) a size $\geqslant$5 cm and mitotic count $\geqslant$2/10 HPF or (ii) a size $\geqslant$5 cm or mitotic count $\geqslant$2/10 HPF, and a proliferating cell nuclear antigen (PCNA) index >10%, whereas low risk indicated either (iii) a size <5 cm and mitotic count <2/10 HPF or (iv) a size $\geqslant$5 cm or mitotic count $\geqslant$2/10 HPF, and a PCNA index $\leqslant$10% (5,6). Of the 62 cases, 31 were classified as low risk, and 31 as high risk. Cellularity, the presence or absence of vessel invasion, tumor necrosis and hemorrhage were also recorded. Vessel invasion is defined by the presence of intravascular tumor cells, either covered by endothelium or associated with thrombus. All patients had regular follow up with abdominal computed tomography (CT) scans and magnetic resonance imaging (MRI). Follow up was available for 59 cases after surgical resection (ranging from 22.5 months to 12.3 years; mean, 50.5 months). Some of our patients received adjuvant radiation or chemotherapy. They were beneficial to the patients with GISTs. Four of our patients had undergone STI 571 treatment. STI 571 is a small molecule kinase inhibitor, which displays potency as a competitive inhibitor of the ATP binding site and shows a high degree of specificity for c-kit. Tumor recurrence/distant metastasis was found in 13 of 62 cases. Twelve of 13 (92.3%) recurrence/distant metastasis cases were in the high-risk category for GIST.

Immunohistochemical Staining

Immunohistochemical staining (IHC) using the streptavidin-biotin method was performed to detect HIF-1α, VEGF protein and CD31. In brief, sections were de-paraffinized and autoclave-treated at 121°C for 10 min in 0.1 M citrate buffer (pH 6.0). Endogeneous peroxidase in the section was blocked by incubation in 3% hydrogen peroxide for 5 min at room temperature. After washing with Tris buffer solution (TBS) and incubation with 5% BSA, the sections were incubated with primary antibodies HIF-1α (H-206, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) and VEGF (A-20, 1:200; Santa Cruz Biotechnology) overnight at 4°C. The primary antibodies CD31 (JC70A, 1:30; DAKO, Denmark) were applied at room temperature for 30 min. Biotinylated second antibody and peroxidase-conjugated streptavidin from the DAKO Universal LSAB kit (DAKO, Denmark) were applied for 20 min each. Finally, sections were incubated in 3′3-diaminobenzidine (DAB) for 5 min, followed by hematoxylin counterstaining and mounting. Negative controls were obtained by replacing the primary antibody with non-immune serum.

Immunostaining Evaluation

HIF-1α protein immunoreactivity was present in the nuclei with or without cytoplasms of neoplastic cells. Using the semiquantitative scale described previously (17), the HIF-1α protein expressions were classified as follows: −, no staining; 1+, nuclear staining in <1% of cells; 2+, nuclear staining in 1–10% of cells with or without weak cytoplasmic staining; 3+, nuclear staining in 10–50% of cells with or without distinct cytoplasmic staining; and 4+, nuclear staining in >50% of cells with or without strong cytoplasmic staining. For further analysis, using a cut-off point to define two groups of low and high HIF-1α expression, –, 1+ or 2+ staining patterns were regarded as low expression, and 3+ or 4+ staining patterns were regarded as high expression (Fig. 1A and B).

VEGF expression was observed in at least 10% of tumor cells to the intensity of cytoplasmic staining. VEGF staining was classified into the following four grades: no staining, weak, distinct and strong cytoplasmic staining. Distinct and strong cytoplasmic staining was defined as high VEGF

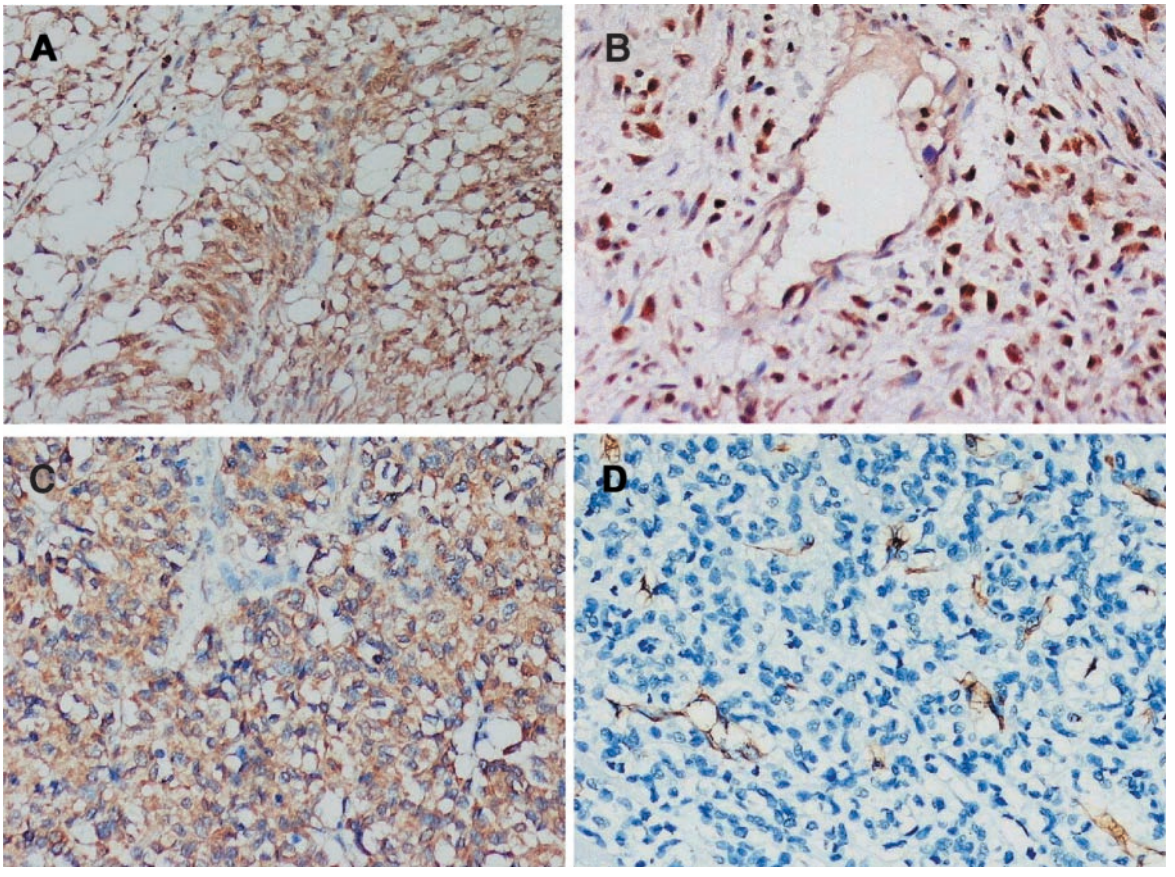

Figure 1. Examples of (A) HIF-1α (nuclear and cytoplasmic staining), (B) HIF-1α (nuclear staining), (C) VEGF (cytoplasmic staining) and (D) CD31 (stained microvessels) immunohistochemistry performed on gastrointestinal stromal tumors (original magnification, ×200).

expression (Fig. 1C); negative or weak cytoplasmic staining was defined as low VEGF expression.

Microvessel Detection and Counting

For CD31 staining, microvessel density (MVD) was assessed by light microscopy at the site of the highest number of capillaries and venules. In each tumor, 'hot-spot' areas displaying the highest vessel density were identified by scanning tumor sections at low-power magnification (×100). The maximum vessel density was determined from these 'hot-spot' areas at fields under high-power magnification (×20 objective and ×10 ocular, 0.94 mm^2 per field) (Fig. 1D), and the mean of counts for three fields was calculated. Large vessels with thick muscular walls were excluded from the counts, and vessel lumens were not necessary for a structure to be defined as a vessel (24). MVD was classified as either <15.0 or ⩾15.0/HPF; 15.0 was the median value.

Statistical Analysis

Correlations between clinicopathological characteristics, risk of aggressive behavior, follow up and angiogenesis-associated factors with HIF-1α protein expression were assessed by using the χ^2 test or Fisher's exact test. Logistic regression was used to analyze the correlation of HIF-1α expression with tumor size, mitotic count, PCNA index, necrosis and hemorrhage. All statistical analyses were performed with the SPSS 8.0 statistical software program. P values <0.05 were considered to be statistically significant.

RESULTS

Correlation between HIF-1α Expression and Clinicopathologic Characteristics

The 62 cases were classified into two tumor risk categories according to tumor size and mitotic count (5,6). The correlations between expression of HIF-1α and the clinicopathologic characteristics described are shown in Table 1. High HIF-1α expression was observed in tumor size ⩾5 cm (27/30; 90.0%), mitotic count ⩾2/10 HPF (26/26; 100%), PCNA index >10% (26/30; 86.7%), high-risk category (29/31; 93.5%) and presence of necrosis (21/23; 91.3%) and hemorrhage (32/45; 71.1%). The high expression of HIF-1α was significantly correlated with tumor size, mitotic count, PCNA index, risk categories, necrosis and hemorrhage ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$ and $P = 0.004$, respectively). In contrast, no statistically significant correlation was found with sex, age, location, vessel invasion or cellularity. For HIF-1α expression, we found that high-risk

Table 1. Correlation of HIF-1α expression with known clinicopathologic characteristics in 62 patients with gastrointestinal stromal tumor

Characteristics	HIF-1α expression ($n = 62$)		P value*
	Low (%)	High (%)	
Sex			
Male	13 (38.2)	21 (61.8)	0.797
Female	12 (42.9)	16 (57.1)	
Age			
⩽61 years	8 (33.3)	16 (66.7)	0.433
>61 years	17 (44.7)	21 (55.3)	
Location			
Stomach	20 (48.8)	21 (51.2)	0.063
Small intestine	5 (27.8)	13 (72.2)	
Large intestine	0 (0)	3 (100)	
Tumor size			
<5 cm	22 (68.8)	10 (31.2)	<0.0001
⩾5 cm	3 (10.0)	27 (90.0)	
Mitotic count			
<2/10 HPF	25 (69.4)	11 (30.6)	<0.0001
⩾2/10 HPF	0 (0)	26 (100)	
PCNA index			
⩽10%	21 (65.6)	11 (34.4)	<0.0001
>10%	4 (13.3)	26 (86.7)	
Risk category			
Low risk	23 (74.2)	8 (25.8)	<0.0001
High risk	2 (6.5)	29 (93.5)	
Necrosis			
Present	2 (8.7)	21 (91.3)	<0.0001
Absent	23 (59.0)	16 (41.0)	
Hemorrhage			
Present	13 (28.9)	32 (71.1)	0.004
Absent	12 (70.6)	5 (29.4)	
Vessel invasion			
Present	3 (21.4)	11 (78.6)	0.129
Absent	22 (45.8)	26 (54.2)	
Cellularity			
Low	9 (56.2)	7 (43.8)	0.151
High	16 (34.8)	30 (65.2)	

*The χ^2 or Fisher's exact tests were used to evaluate the correlation between HIF-1α and clinicopathologic characteristics. $P < 0.05$ indicates statistical significance.
HPF, high power field; PCNA, proliferating cell nuclear antigen.

category was an independent predictor of HIF-1α expression by multivariate analysis ($P < 0.0001$) (data not shown). Therefore, we further performed a multivariate analysis regarding the tumor size, mitotic count, PCNA index, necrosis and hemorrhage, which proved to be independently predictive using logistic regression analysis, as summarized in Table 2. A high mitotic count was associated significantly with high expression of HIF-1α ($P < 0.0001$).

Table 2. Multivariate logistic regression analysis of patients with HIF-1α high expression using tumor size, mitotic count, necrosis and hemorrhage as covariates

Variable	Categories	P value*
Tumor size	⩾5 cm versus <5 cm	0.123
Mitotic count	⩾2/10 versus <2/10 HPF	<0.0001
PCNA index	>10% versus ⩽10%	0.402
Necrosis	Present versus absent	0.706
Hemorrhage	Present versus absent	0.970

*$P < 0.05$ indicates statistical significance.
HPF, high power field; PCNA, proliferating cell nuclear antigen.

Correlation between Aggressive Behavior Risk Category, HIF-1α, VEGF Expression, MVD Value and Follow-up Data

To clarify the actual prognostic values of HIF-1α, VEGF expression and tumor MVD for GIST, we selected 59 patients with regular follow-up. Recurrence/distant metastasis was found in 13 (22.03%) of these 59 patients. The incidence of patients with recurrence/distant metastasis was significantly higher in the high-risk category (12/29; 41.4%) than in the low-risk category (1/30; 3.3%) ($P < 0.0001$). The incidence was also significantly higher in high HIF-1α expression (12/35; 34.3%) than in low HIF-1α expression (1/24; 4.2%) patients. In addition, the angiogenesis-associated factors, VEGF high expression (13/40; 32.5%) and MVD value ⩾15/HPF (12/30; 40.0%) were markedly increased in recurrence/distant metastasis. The results showed that HIF-1α expression, VEGF expression and MVD value in GIST were significantly correlated with recurrence/distant metastasis ($P = 0.009$, $P = 0.005$ and $P = 0.001$, respectively) (Table 3).

Correlation between HIF-1α Expression and Angiogenesis-associated Factors

HIF-1α expression was examined by both VEGF expression and tumor vascularity (Table 4). VEGF expression was observed in significantly higher levels in tumors with high HIF-1α expression (37/37; 100%) than in tumors with low HIF-1α expression (6/25; 24.0%). MVD was increased by HIF-1α and high VEGF expression. MVD was higher in tumors with high HIF-1α expression (30/37; 81.1%) than in tumors with low HIF-1α expression (2/25; 8.0%). Both MVD and VEGF expression were significantly correlated with HIF-1α expression ($P < 0.0001$).

DISCUSSION

GISTs are the most common mesenchymal tumor arising within the gastrointestinal tract. There are frequent

Table 3. Correlation between aggressive behavior risk category, HIF-1α, VEGF expression, MVD value and follow-up data

Pathologic characteristics	No. of cases*	Recurrence/distant metastasis no. (%)	*P* value+
Risk category			
Low risk	30	1 (3.3)	< 0.0001
High risk	29	12 (41.4)	
HIF-1α expression			
Low	24	1 (4.2)	0.009
High	35	12 (34.3)	
VEGF expression			
Low	19	0 (0)	0.005
High	40	13 (32.5)	
MVD			
<15/HPF	29	1 (3.4)	0.001
⩾15/HPF	30	12 (40.0)	

*Three cases lost to follow-up.
+The Fisher's exact tests used to evaluate correlation among aggressive behavior risk category and HIF-1α, VEGF expression and MVD with follow-up data. $P < 0.05$ indicates statistical significance.
VEGF, vascular endothelial growth factor; MVD, microvessel density; HPF, high power field.

Table 4. Correlation of HIF-1α expression with VEGF expression and MVD value

	HIF-1α expression		*P* value*
	Low expression no. (%)	High expression no. (%)	
VEGF expression			
Low	19 (76.0)	0 (0.0)	<0.0001
High	6 (24.0)	37 (100)	
MVD			
<15/HPF	23 (92.0)	7 (18.9)	
⩾15/HPF	2 (8.0)	30 (81.1)	<0.0001

*The Fisher's exact tests used to evaluate the correlation of HIF-1α expression with VEGF expression and MVD value. $P < 0.05$ indicate statistical significance.
VEGF, vascular endothelial growth factor; MVD, microvessel density; HPF, high power field.

gain-of-function mutations in GISTs that result in activation of KIT signaling, which leads to uncontrolled cell proliferation and resistance to apoptosis. GISTs are generally thought to be malignant, but they have different degrees of aggressiveness, resulting in varying incidences of recurrence and metastases. Predicting the potential biological behavior of these tumors remains difficult and an analysis of the literature to resolve this issue provides many conflicting reports (25). Age, the mitotic count (fewer or greater than 2/10 HPF) and size of the tumor (<5 cm versus ⩾5 cm) are generally accepted as independent prognostic factors (5,6). Preliminary data suggest that tumors with an intermediate risk on the basis of size and mitotic count may be more accurately classified as high- or low-risk using PCNA index (5,6).

Tumorigenesis is a multistep process that requires the acquisition of certain properties common to all tumors. These properties include uncontrolled cell division, suppression of senescence, inhibition of apoptosis and induction of angiogenesis (26). The role of angiogenesis in the development and progression of human cancers has been widely studied (27). New blood vessels can be stimulated to grow when factors that promote angiogenesis are up-regulated or those that inhibit angiogenesis are down-regulated (8,28). Hypoxia in the tumor microenvironment is sufficient to activate HIF-dependent gene expression (14). However, tumor growth rate may not always be associated with hypoxic conditions, whereas HIF-1α expression may be influenced by factors other than hypoxia. A major role for HIF-1α in determining gene expression, tumor angiogenesis and growth has been demonstrated (29). Our data revealed that the expression of HIF-1α is correlated with the mitotic count, indicating that the larger the proliferative rate, the higher the risk of tumor hypoxia. HIF-1α overexpression is associated with increasing VEGF expression, one of its main downstream effectors, confirming angiogenesis as one of the proposed mechanisms by which HIF-1 activation stimulates tumor growth and leads to increasing VEGF-mediated tumor angiogenesis.

In the present study, expression of HIF-1α and VEGF have been found to be associated with an aggressive behavior risk category and we have shown that HIF-1α expression is associated with VEGF expression and angiogenesis in GIST. It is now widely accepted that VEGF expression is mediated by HIF-1α during hypoxia. It has been previously reported that expression of HIF-1α is correlated with VEGF expression and tumor vascularity in several cancers, including breast, colon and prostate cancer (17,30,31). We found that high levels of HIF-1α were correlated with VEGF and MVD, highlighting the important role of HIF-1α in the control of angiogenesis in GIST. These findings suggest that HIF-1α may play an important role in tumor growth and progression of GIST through regulation of VEGF, and that it should be associated with increased tumor angiogenesis.

The angiogenic process is so complex that additional studies concerning other regulators of angiogenic factors are needed. Tumor progression is closely dependent on an imbalance between cellular proliferation and death. Recent experimental results have shown that some tumor suppressor genes are involved in the regulation of angiogenesis (32). By reviewing papers, oncogenes and tumor suppressor genes such as VHL, p53, V-SRC and bcl-2 have been shown *in vitro* to influence angiogenesis by regulating the balance between stimulators and inhibitors of angiogenesis and have also been reported to be associated with HIF-1α and VEGF expression (32–38). Some recent studies have demonstrated that hypoxia and loss of p53 or VHL activity affect HIF-1α protein stability via altered ubiquitination (39). Therefore, further study is necessary. According to our study, HIF-1α may also be a potential target for antiangiogenic therapeutic strategies for GIST.

The criteria for benign and malignant tumors differ greatly among the various published studies. In the past, prognosis was traditionally determined using the aggressiveness risk category, tumor size and the mitotic activity. According to our study, HIF-1α expression is statistically significantly correlated with risk of aggressive behavior. In addition, in our study, primary GISTs were classified as low- or high-risk tumors for aggressive clinical behavior as suggested by tumor size and mitotic count. However, our results revealed that high expression of HIF-1α was a significant influence by showing a high mitotic count more than tumor size. Therefore, we inferred that mitotic activity is an important indicator of tumor aggressiveness; furthermore, this may be through promoted expression of HIF-1α in GISTs. We also tried to correlate the expression of HIF-1α with the incidence of recurrence/distant metastasis. Our result revealed that the HIF-1α expression was statistically significantly correlated with tumor recurrence/distant metastasis. This reason for this phenomenon may be due to many other factors; incomplete resection of the tumor, for example, may also increase the risk of recurrence/distant metastasis. Other factors such as expression adhesion molecules, and continuing mutation of oncogenes can also affect the risk of a distant tumor metastasis. Therefore, HIF-1α and VEGF expression may provide an ancillary prognostic factor for GIST.

In conclusion, this study demonstrates that the expression of HIF-1α is associated with risk of aggressive behavior and VEGF expression, which leads to increasing VEGF-mediated tumor angiogenesis. There is a statistically significant correlation between the expression of HIF-1α and the aggressive behavior risk category, and there is also a statistically significant association with recurrence/distant metastasis of GIST. Therefore, HIF-1α could serve as an ancillary parameter for the prediction of aggressive behavior, and it may be an independent prognostic factor for GIST.

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