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HIF-2α as a prognostic marker for breast cancer progression and patient survival

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ABSTRACT. Malignant cells show increased invasion potency *in vitro* and *in vivo*. This process is considered to be mediated by matrix-metalloproteases (MMPs). Hypoxia-inducible factor-2α (HIF-2α) may upregulate MMP-2 expression; however, little is known about the correlation between HIF-2α and MMP-2 expressions in breast cancer. The current study investigated this correlation immunohistochemically according to various clinical and pathological features in 102 paraffin-embedded archival tissue block specimens from patients with breast cancer. HIF-2α and MMP-2 expression was detected in 60.8% (62/102) and 65.7% (67/102) of tumor samples, respectively. HIF-2α expression was significantly correlated with tumor size (P = 0.019), lymph node involvement (P = 0.035), and metastasis (P = 0.035). MMP-2 expression was significantly associated with lymph node involvement (P = 0.043) and metastasis (P = 0.003). Univariate analyses revealed that HIF-2α (P = 0.001) and MMP-2 (P = 0.000) expressions were significantly associated with a poorer survival rate, as well as tumor size, lymph node invasion, and distant metastasis. Multivariate analysis revealed that HIF-2α (P = 0.003) and the T-stage (P = 0.000) were independent

prognostic factors of overall survival. Spearman correlation analysis revealed that HIF-2α and MMP-2 expressions were significantly correlated (r = 0.990; P = 0.041). These results suggest that high HIF-2α expression is associated with poor overall survival in patients with breast cancer, indicating that HIF-2α could be a valuable marker of breast cancer progression.

Key words: Hypoxia-inducible factor-2α; Breast cancer; Prognosis; Clinicopathological characteristics

INTRODUCTION

Internal hypoxia is a common characteristic of solid tumors, and an important microenvironmental factor of malignant phenotype development. Tumor cells can survive under hypoxic conditions by expressing proteins such as angiogenic factors, glycolytic enzymes, and stress proteins, which promote their survival. Many of the hypoxia adaptations are mediated by the activation of specific genes through hypoxia-inducible factors (HIFs). HIFs, transcription factors associated with the cellular response to hypoxia (Pawlus et al., 2012), upregulate the expression of several hypoxia response genes, including glycolytic enzymes, vascular endothelial growth factor, matrix metalloproteinase (MMP)-9, transforming growth factors α and β, and numerous others (Shi et al., 2007; Fu et al., 2012). Collectively, these factors modulate cancer cell metabolism and can promote angiogenesis, invasion, and metastasis, leading to a poor outcome, suggesting that HIF proteins may represent suitable targets for antitumor therapies (Kaya et al., 2012; Hartwich et al., 2013; Saponaro et al., 2013).

HIF-1α has been associated with poor prognosis in a range of cancers including uterine, breast, and non-small cell lung cancer, as well as poor response to cancer therapies (Horiuchi et al., 2012; Kuo et al., 2012; Marton et al., 2012). Little is known, however, about the role of HIF-2α in solid tumors. Current data suggests that the response to hypoxia is largely mediated by HIF-1α in endothelial and breast cancer cells, whereas it is mediated by HIF-2α in renal carcinoma cells. HIF-2α overexpression is important in the development of renal carcinoma in patients with the von Hippel Lindau syndrome; in neuroblastomas, expression of HIF-2α is associated with more aggressive forms of the disease. However, other studies in glioblastomas have suggested that overexpression of HIF-2α enhances angiogenesis, but reduces tumor growth. Although the role of HIF-1α in gastric cancer has been characterized, there is limited understanding of the role of HIF-2α or associations of HIF-2α expression for clinicopathological significance. A small number of studies have investigated HIF-2α (Giatromanolaki et al., 2006; Bordoli et al., 2011) or MMP-2 (Bordoli et al., 2011; Lu et al., 2012) expressions alone in breast carcinoma. The present study investigated the correlation between HIF-2α and MMP-2 expressions with respect to various clinical and pathological features of breast carcinoma.

MATERIAL AND METHODS

Patients and tissue samples

Paraffin-embedded tumor tissues were obtained from 124 breast cancer patients who

were diagnosed and treated at the First Affiliated Hospital of Xinxiang Medical University, China, between January 2006 and June 2010, and up to 10 years of clinical follow-up information was obtained. Of these 124 patients, survival information was available for 102 patients (average age, 59 years; range 28-84 years). The clinical and morphological characteristics of the tumors are summarized in Table 1. None of the patients received any chemotherapy or irradiation prior to surgery. Histological diagnosis and scoring of all samples were performed by independent pathologists according to the World Health Organization (WHO) Histological Classification system. Tumors were staged according to the tumor-necrosis-metastasis (TNM) staging system. The disease-free survival rate of the patients was calculated from the date of resection to the date of local tumor recurrence in the form of either local or distant metastasis, and the actual survival rate was calculated to the date of death. For the usage of the clinical materials for research purposes, prior patient consent and approval from the Institutional Research Ethics Committee were obtained.

Table 1. Clinical and histological features of breast cancer patients characteristics.

Characteristics	No. of Patients	%
Total number of patients	102	100.0
Age (yeas)	59 (28-84)	
≤35	11	
35-55	60	
>55	31	
Menopause		
Pre-menopausal	40	
Post-menopausal	62	
Histological grade		
I	25	
II	41	
III	36	
Tumor size (cm)		
≤2	53	
2-5	36	
>5	13	
N-stage		
0	29	
1-3	36	
4-9	12	
>10	25	
M-stage		
0	75	
1	27	
ER status		
Positive	32	
Negative	70	
PR status		
Positive	19	
Negative	83	
Her-2 status		
Positive	66	
Negative	36	

Immunohistochemical analysis

The best tissue section was selected for immunohistochemistry. The sections were deparaffinized and rehydrated through graded ethanol, and then endogenous peroxidase was inhibited with 0.3% hydrogen peroxide. For antigen retrieval, slides were boiled in 1 mM

EDTA, pH 8.0, for 15 min in a microwave oven. Tissue sections were stained with rabbit polyclonal antibody against human HIF-2α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution and rabbit polyclonal antibody against human MMP-2 (Santa Cruz Biotechnology) at 1:100 dilution. All sections were incubated overnight with the primary antibody at 4°C. The sections were then treated with peroxidase by the labeled polymer method with Zhongshan peroxidase for 30 min. Antibody binding was visualized using the Avidin Biotin Complex Elite Kit and 3,3-diaminobenzine according to manufacturer instructions. Sections were then counterstained in hematoxylin. As a negative control, samples were incubated using 10 mM Tris-buffered saline, pH 7.4, instead of a primary antibody.

Assessment of immunohistochemical staining in the tissue sections

Scoring was performed in a double-blind manner by two investigators. Any disagreement was resolved by discussion to obtain final scores. Markers (HIF-2α and MMP-9) in the carcinogenesis study were scored using the same scoring system. The total immunostaining score was calculated as the sum of the percent positivity of stained tumor cells and the staining intensity. The percent positivity was scored as "0" (<5%, negative), "1" (5-25%, sporadic), "2" (25-50%, focal), or "3" (>50%, diffuse). The staining intensity was scored as "0" (no staining), "1" (weakly stained), "2" (moderately stained), or "3" (strongly stained). The final expression scores were calculated using the value of the percent positivity score x staining intensity score, which ranged from 0 to 9 as follows: "-" (score of 0-3), "+" (score > 3).

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to analyze the relationship between HIF-2α or MMP-2 expressions and clinicopathological characteristics. The correlation between HIF-2α and MMP-2 was analyzed by the Spearman correlation test. Survival curves were plotted with the Kaplan-Meier method and were compared using the log-rank test. The survival data were evaluated by univariate and multivariate Cox regression analyses. In all cases, $P < 0.05$ was considered to be statistically significant.

RESULTS

HIF-2α and MMP-2 expressions in breast cancer and associations with clinicopathological variables and prognosis

Immunohistochemistry was performed to evaluate the association of HIF-2α and MMP-2 expressions with the clinicopathological parameters of the 102 breast cancer patients used in this study, which are described in Table 2. Staining for HIF-2α occurred in a granular and diffuse pattern localized mainly in the cytoplasm of cancer cells, although staining was occasionally observed in the membrane (Figure 1A). HIF-2α expression was detected in 60.8% (62/102) of the tumors. The HIF-2α level was closely associated with tumor size, lymph node involvement, and distant metastasis of the patients. Tumors of larger size or metastasis expressed higher levels of HIF-2α, suggesting that HIF-2α upregulation was associated with

tumor progression. However, no significant correlation was observed between HIF-2α expression and age, menopause, histological grade, or hormone receptor status.

Table 2. Clinicopathological characteristics of the patients and the expression of HIF-2α and MMP-2 in breast cancer.

Characteristics	Expression of protein HIF-2α			Expression of protein MMP-2		
	Negative (%) (N = 40)	Positive (%) (N = 62)	P	Negative (%) (N = 35)	Positive (%) (N = 67)	P
Total number of patients						
Age (yeas)			0.156			0.667
≤35	7 (63.6)	4 (36.4)		5 (45.5)	6 (54.5)	
35-55	20 (33.3)	40 (66.7)		19 (31.7)	41 (68.3)	
>55	13 (41.9)	18 (58.1)		11 (35.5)	20 (64.5)	
Menopause			0.776			0.907
Pre-menopausal	15 (37.5)	25 (62.5)		14 (35.0)	26 (65.0)	
Post-menopausal	25 (40.3)	37 (59.7)		21 (33.9)	41 (66.1)	
Histological grade			0.177			0.780
I	10 (40.0)	15 (60.0)		10 (40.0)	15 (60.0)	
II	12 (29.3)	29 (70.7)		13 (31.7)	28 (68.3)	
III	18 (50.0)	18 (50.0)		12 (33.3)	24 (66.7)	
Tumor size (cm)			0.019			0.291
≤2	14 (26.4)	39 (73.6)		20 (37.7)	33 (62.3)	
2-5	20 (55.6)	16 (44.4)		9 (25.0)	27 (75.0)	
>5	6 (46.2)	7 (53.8)		6 (34.3)	7 (65.7)	
N-stage			0.035			0.043
0	14 (48.3)	15 (51.7)		15 (51.7)	14 (48.3)	
1-3	18 (50.0)	18 (50.0)		13 (36.1)	23 (63.9)	
4-9	4 (33.3)	8 (66.7)		3 (25.0)	9 (75.0)	
>10	4 (16.0)	21 (84.0)		4 (16.0)	21 (84.0)	
M-stage			0.035			0.003
0	34 (71.4)	41 (28.6)		32 (42.7)	43 (573)	
1	6 (11.1)	21 (88.9)		3 (11.1)	24 (88.9)	
ER status			0.284			0.993
Positive	15 (46.9)	17 (53.1)		11 (34.4)	21 (65.6)	
Negative	25 (35.7)	45 (64.3)		24 (34.3)	46 (65.7)	
PR status			0.420			0.416
Positive	9 (47.4)	10 (52.6)		5 (26.3)	14 (73.7)	
Negative	31 (37.3)	52 (62.7)		30 (36.1)	53 (63.9)	
Her-2 status			0.960			0.778
Positive	26 (39.4)	40 (60.6)		22 (33.3)	44 (66.7)	
Negative	14 (38.9)	22 (61.1)		13 (36.1)	23 (63.9)	

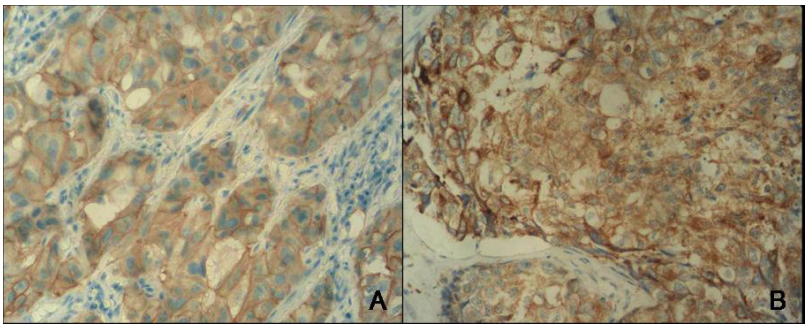

Figure 1. A. Immunohistochemical staining revealed that HIF-2α existed in a granular and diffuse pattern localized mainly to the cytoplasm of cancer cells and staining for MMP-2 was observed in a diffuse pattern localised to the cytoplasm of cancer cells (**B**) (200X). HIF-2α = hypoxia-inducible factor-2α; MMP-2 = matrix-metalloproteases-2.

Staining for MMP-2 occurred in a diffuse pattern localized in the cytoplasm of cancer cells (Figure 1B). MMP-2 expression was detected in 65.7% (67/102) of tumors. MMP-2 expression was significantly correlated with lymph node metastasis and the distant metastasis of breast cancer. However, the expression level of MMP-2 was not significantly associated with tumor size, age, menopause, histological grade, or hormone receptor status.

Correlation between HIF-2α and MMP-2 expressions

Spearman correlation analysis revealed a significant correlation between HIF-2α and MMP-2 expressions (r = 0.990, P = 0.041) (Table 3).

Table 3. Correlations of protein HIF-2α and MMP-2 in breast cancer.

HIF-2α	MMP-2	N (%)	*r*	P
Co-expression pattern				
Negative	Negative	35 (34.3)	0.990	0.041
Negative	Positive	5 (4.9)		
Positive	Negative	0 (0.0.)		
Positive	Positive	62 (60.8)		

HIF-2α and MMP-2 expressions were associated with poor survival of invasive breast cancer patients

A log-rank test and Kaplan-Meier analysis were used to calculate the effect of HIF-2α and MMP-2 expressions on patient survival (Figures 2 and 3). The median overall survival times of patients with positive and negative expression of HIF-2α were 38 months [95% confidence interval (CI) = 33.96 to 39.67] and 46 months (95%C = 41.80 to 47.16, P = 0.010), respectively. In contrast, the median survival times of patients with positive and negative expression of MMP-2 were 33 months (95%CI = 27.48 to 38.52) and 45 months (95%CI = 42.04 to 48.14, P = 0.028), respectively.

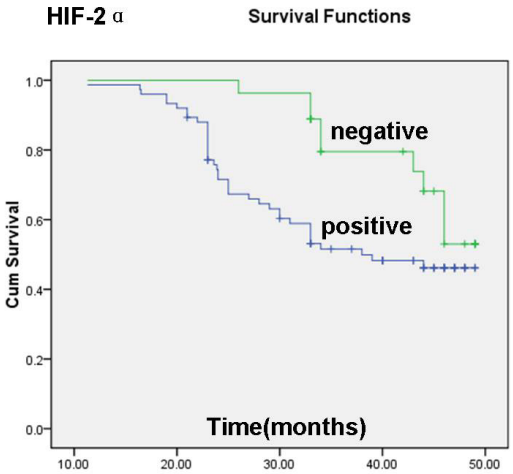

Figure 2. Kaplan-Meier overall survival curves of breast cancer patients in association with HIF-2α expression. The difference between the curves was significant (P < 0.05).

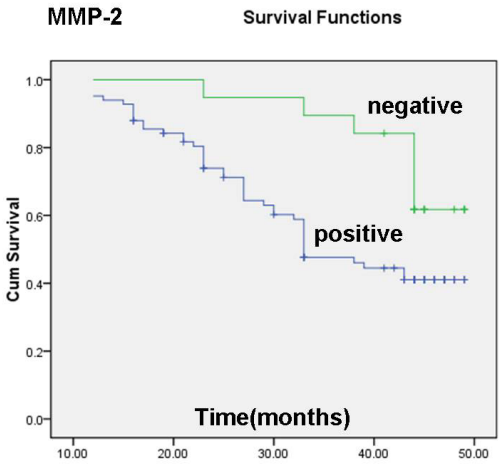

Figure 3. Kaplan-Meier overall survival curves of breast cancer patients in association with MMP-2 expression. The difference between the curves was significant (P < 0.05).

Univariate and multivariate analyses of prognostic variables in breast cancer patients

As shown in Table 4, tumor size (P = 0.000), N-stage (P = 0.016), M-stage (P = 0.028), HIF-2α expression (P = 0.001), and MMP-2 expression (P = 0.000) were all significant prognostic indicators of overall survival in univariate analyses. However, in the multivariate analyses, only HIF-2α expression (P = 0.003) and T-stage (P = 0.000) were independent prognostic factors, and none of the other clinicopathological variables had an independent prognostic impact.

Table 4. Univariate and multivariate analysis of overall survival rates in patients with breast cancers by Cox-Regression analysis.

Characteristics	Univariate analysis		Multivariate analysis		
	P	Regression coefficient (SE)	P	Relative risk	95% Confidence interval
Age	0.852	-0.067 (0.331)	5	15	
Menopause	0.862	0.073 (0.44)			
Histological grade	0.731	0.104 (0.291)			
Tumor size	0.000	1.2 (0.283)	0.000	3.012	1.644-5.499
N-stage	0.016	0.493 (0.204)			
ER status	0.948	20.023 (0.453)			
PR status	0.368	0.674 (0.764)			
M- stage	0.028	0.977 (0.453)			
HIF-2α	0.001	1.749 (0.512)	0.003	4.605	1.675-12.579
MMP-2	0.000	21.764 (0.484)			

SE = standard error; multivariate analysis; Cox proportional hazard regression model, stepwise forward LR.

DISCUSSION

An increasing number of *in vitro* studies have demonstrated an important role for

HIF-2α in regulating tumor growth and metastasis. However, little is known about the relationship between the expression of HIF-2α in human breast cancer and the prognosis of breast cancer patients. In the present study, we found that invasive breast cancers with higher HIF-2α expression levels tended to have distant metastasis, and over-expression of HIF-2α was associated with shorter overall survival of breast cancer patients. Both the univariate and multivariate analyses indicated that HIF-2α expression was an independent prognostic factor for breast cancer progression.

Several recent studies have demonstrated that the expression of HIF-2α is upregulated in several types of solid tumors, including colorectal, gastric, oral squamous cell carcinoma, and non-small cell lung cancer (Wang et al., 2010; Zhu et al., 2010; Xue et al., 2012). The widespread overexpression of HIF-2α in cancer has led to the belief that HIF-2α is an oncogenic gene. Cell cultures and animal experiments support this speculation, showing that the downregulation of HIF-2α expression can suppress cell proliferation and slow tumor growth. In clear-cell renal cell carcinoma, HIF-2α regulated growth both by maintaining a low level of glycolysis and by allowing more mitochondrial metabolism and tolerance to reactive oxygen species-induced DNA damage in nude mice assays (Semenza and Prabhakar et al., 2012). HIF-2α might mediate cell proliferation by the regulation of cyclin D1 and c-Myc. In addition, it could promote the migration of melanoma cells by regulating the expression of vascular endothelial growth factor receptor-2 (Bougatef et al., 2012). Moreover, HIF-2α plays an important role in mediating the function of chemotherapeutic drugs. The results of a previous study suggested that targeting HIF-2α with small interfering RNA warrants investigation as a potential strategy to enhance the efficacy of doxorubicin in the treatment of hepatocellular carcinoma (He et al., 2012).

This study focused on the potential relationship between the expression of HIF-2α and various clinicopathological characteristics of breast cancer patients, as well as their overall survival. High levels of HIF-2α appeared to be significantly correlated with tumor size, lymph node metastases, distant metastasis, and poor prognosis in patients with breast cancer. HIF-2α was upregulated in patients presenting with metastases, suggesting that its up-regulation was acquired over the course of tumor progression, and, in particular, during the acquisition of metastatic potential. Our results showed that HIF-2α and MMP-2 expressions were not correlated with receptor status. Conversely, it was reported that the expression level of HIF-2α was related with expressions of the estrogen and progesterone steroid receptors in endometrial carcinoma (Sivridis et al., 2002). To understand these conflicting results, the difference between clinical observations and *in vitro* experiments should be considered. After dividing the patients by the cut-off method, a multivariate Cox proportional hazard regression analysis revealed that HIF-2α overexpression had a significantly worse prognostic impact ($P = 0.003$) on the overall survival of breast cancer patients independent of tumor size ($P = 0.000$). These results indicate that as an independent risk factor, HIF-2α could serve as a prognostic marker for the survival of patients. To date, several studies have revealed the prognostic significance of HIF-2α overexpression in various carcinomas, such as soft tissue sarcoma (Smeland et al., 2012), chondrosarcoma (Chen et al., 2011), and non-small cell lung cancer (Wu et al., 2011).

Spearman order correlation analysis showed that HIF-2α expression in breast cancer was positively correlated with the MMP-2 expression level. MMP-2 expression levels were closely associated with lymph node metastasis and distant metastasis of patients. To achieve metastasis, a tumor cell must disrupt cell-cell and cell-extracellular matrix contacts, migrate

through stromal tissue, invade the basement membrane, and enter/exit the blood stream. These steps are primarily accomplished by the activity of the MMP family of enzymes (Singh et al., 2012; Monsonego-Ornan et al., 2012). However, we did not find that MMP-2 had prognostic importance in the multivariate Cox proportional hazard regression analysis. These results suggest that HIF-2α promotes tumor growth and metastasis by targeting not only MMP-2, but also other tumor suppressor genes.

In summary, the results of our study indicate that the expression of HIF-2α is strongly correlated with the clinical stages and overall survival times of patients with breast cancer, providing evidence that up-regulation of HIF-2α might play an important role in the progression of the disease. These results are consistent with the literature and support the notion that HIF-2α is a metastasis-correlated gene that induces effects by regulating MMP-2.

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