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Research Paper

Hypoxia-induced factor-1 alpha upregulates vascular endothelial growth factor C to promote lymphangiogenesis and angiogenesis in breast cancer patients

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Abstract

Hypoxia-induced factor-1 alpha (HIF-1α) affects many effector molecules and regulates tumor lymphangiogenesis and angiogenesis during hypoxia. The aim of this study was to investigate the role of HIF-1α in the regulation of vascular endothelial growth factor C (VEGF-C) expression and its effect on lymphangiogenesis and angiogenesis in breast cancer. Lymphatic vessel density (LVD), microvessel density (MVD) and the expressions of HIF-1α and VEGF-C proteins were evaluated by immunohistochemistry in 75 breast cancer samples. There was a significant correlation between HIF-1α and VEGF-C ($P = 0.014$, $r = 0.273$, Spearman's coefficient of correlation). HIF-1α and VEGF-C overexpression was significantly correlated with higher LVD ($P = 0.003$ and $P = 0.017$, respectively), regional lymph nodal involvement ($P = 0.002$ and $P = 0.004$, respectively) and advanced tumor, node, metastasis (TNM) classification ($P = 0.001$ and $P = 0.01$, respectively). Higher MVD was observed in the group expressing higher levels of HIF-1α and VEGF-C ($P = 0.033$ and $P = 0.037$, respectively). Univariate analysis showed shorter survival time in patients expressing higher levels of HIF-1α and VEGF-C. HIF-1α was also found to be an independent prognostic factor of overall survival in multivariate analysis. The results suggest that HIF-1α may affect VEGF-C expression, thus acting as a crucial regulator of lymphangiogenesis and angiogenesis in breast cancer. This study highlights promising potential of HIF-1α as a therapeutic target against tumor lymph node metastasis.

Keywords: HIF-1α, VEGF-C, lymphangiogenesis, angiogenesis, breast cancer

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INTRODUCTION

Breast carcinoma is one of the most common types of cancer diagnosed among women. In breast cancer, metastasis primarily occurs through the lymphatic system, and the extent of lymph node involvement is a key prognostic factor for the disease. To facilitate the design and evaluation of new therapeutic strategies, a better understanding of molecular mechanisms underlying the systemic metastasis of breast cancer is needed. Lymphatic metastasis was previously reported to occur through pre-existing lymphatic vessels[1]. However, recent studies have suggested that lymphangiogenesis, the formation of new lymphatic vessels induced by tumors, is directly correlated with the extent of lymph node metastasis.

Vascular endothelial growth factor C (VEGF-C) is a major lymphangiogenic and angiogenic factor, which is commonly expressed in malignant cancers[2-4]. It promotes lymphangiogenesis, angiogenesis, and lymph node metastasis in tumors by activating its specific receptor vascular endothelial growth factor receptor-3 (VEGFR-3). Neutralizing VEGF-C or blocking VEGFR-3 signaling was reported to suppress the development of new lymphatic vessels, lymphatic hyperplasia and tumor metastasis in experimental cancer models[5].

Hypoxic conditions during tumorigenesis induce the expression of hypoxia-induced factor-1 (HIF-1), the master regulator of cellular oxygen homeostasis[6]. HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits; HIF-1α is an oxygen-regulated subunit and determines the level of HIF-1 activity. HIF-1α overexpression has been studied in several cancers, including bladder, breast, lung, esophageal, colorectal, ovarian, pancreatic, kidney, and prostate cancer[7-9]. It targets the transcription of over 60 genes involved in many aspects of tumor biology, including angiogenesis, cell invasion, cell apoptosis and proliferation, chemoradio-resistance and glucose metabolism. The transcriptional activity of a broad spectrum of genes, including the gene for vascular endothelial growth factor (VEGF), is altered under hypoxic conditions by HIF-1α.

Only one previous study has evaluated HIF-1α in lymphangiogenesis of breast carcinoma performed on an Australian population in 2006. However, the role of HIF-1α in lymphangiogenesis and angiogenesis and the relationship between the expressions of VEGF-C in breast carcinoma remain unclear and need further studies. In the present study, the expressions of HIF-1α and VEGF-C were found to be correlated with the levels of lymphatic vessel density (LVD) and microvessel density (MVD) by using immunohistochemistry. In addition, their correlations with clinicopathological characteristics and outcomes in Chinese patients with breast cancer were also analyzed.

PATIENTS AND METHODS

Patients and acquisition of tissue specimens

Seventy-five patients with histologically confirmed breast carcinoma who underwent radical operations at the First Affiliated Hospital of Nanjing Medical University between January 2005 and October 2006 were included in the present study. Paracancerous normal tissue samples from 20 other patients were performed as controls. Patients undergoing neoadjuvant chemotherapy or radiotherapy were excluded. Tumor, node, metastasis (TNM) staging was carried out according to the American Joint Committee on Cancer (AJCC) classification, and histological grading was performed according to the World Health Organization (WHO) criteria. Paraffin-embedded, formalin-fixed surgical specimens were prepared and collected for immunohistochemical staining.

Immunohistochemistry

Paraffin-embedded tissues were evaluated by immunohistochemical analysis. A total of 38 samples of stage I/II patients and 37 samples of stage III patients were analyzed. Endogenous peroxidase activity was inhibited by immersing the slides in 0.3% hydrogen peroxide in methanol for 30 minutes. For pretreatment, microwave-based antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6.0). The following primary antibodies were used, including a monoclonal antibody raised against HIF-1α (1:200; Abcam, Cambridge, UK), a polyclonal antibody raised against VEGF-C (1:400, R&D systems, Minneapolis, MN, USA), a monoclonal antibody raised against D2-40 (1:100, R&D System Europe, Lille, France), and a monoclonal antibody against CD31 (1:100, Zymed, Carlsbad, NM, USA). For HIF-1α immunostaining, a catalyzed signal amplification system (Dako, Glostrup, Denmark) was used. Immunoreactions were visualized with diaminobenzidine (DAB kit, Vector Laboratories, Burlingame, CA, USA), and the sections were counterstained with hematoxylin. To determine the specificity of immunostaining, the primary antibody was replaced with mouse normal IgG or Tris-buffered saline. Control slides were invariably negative for immunoreactions. HIF-1α was clearly expressed in the cell nucleus, and positive expressions of VEGF-C proteins showed a yellow or brownish-yellow stain in the cytoplasm of carcinoma cells.

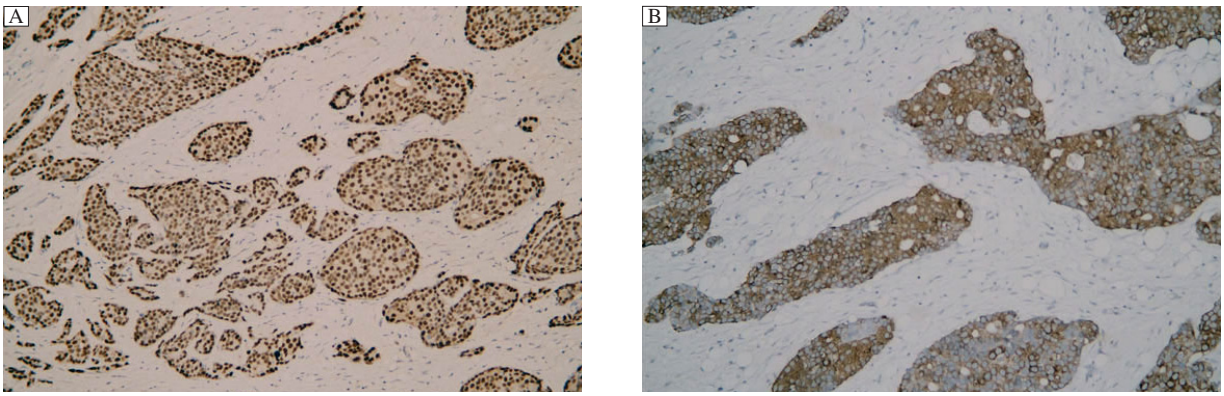

***Fig. 1* HIF-1α and VEGF-C expression in breast carcinoma.** A: High HIF-1α immunoreactivity in the nuclei of cancer cells (magnification×200). B: VEGF-C was expressed in the cytoplasm of breast carcinoma cells (magnification×200).

LVD was detected by immunostaining for D2-40 according to the criteria described by Masakau et al.[10]. First, areas with highly D2-40-positive vessels (hot spots) in peritumoral and intratumoral areas were identified by scanning the sections at low magnification (×100). Then, the number of D2-40 positive vessels was counted in 5 high-magnification fields (×400) for each case. MVD was assessed at the site of the highest number of capillaries and small venules, and highly vascular areas were identified by scanning tumor sections at low power (×40). After the 5 areas with the highest degree of angiogenesis (hot spots) were identified, the microvessels were counted at ×200, and the mean values of the 5 fields were calculated[11].

Statistical analysis

Statistical analyses were performed with SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). The correlations among the expressions of HIF-1, VEGF-C, the levels of LVD, and clinicopathologic characteristics were calculated by using the Student´s *t*-test, the chi-square correlation test, and the Spearman´s coefficient of correlation. The Kaplan-Meier method was performed to estimate survival time, and the log-rank test was performed to analyze survival differences. A multivariable test was performed to determine the factor correlated with survival length by Cox regression analysis. The statistical significance was defined as $P < 0.05$.

RESULTS

HIF-1α, VEGF-C, D2-40 and CD31 in breast carcinoma

Seventy five patients with breast carcinoma were included in the current study and 52 (69.3%) of them showed high HIF-1α expression, and 48 (64.0%) of them showed high VEGF-C expression (***Fig. 1***). However, normal tissue showed no immunoreactivity for HIF-1α or VEGF-C. Immunoreactive D2-40 proteins were found in the cytoplasm and cellular membranes of lymphatic endothelial cells. Generally, D2-40-

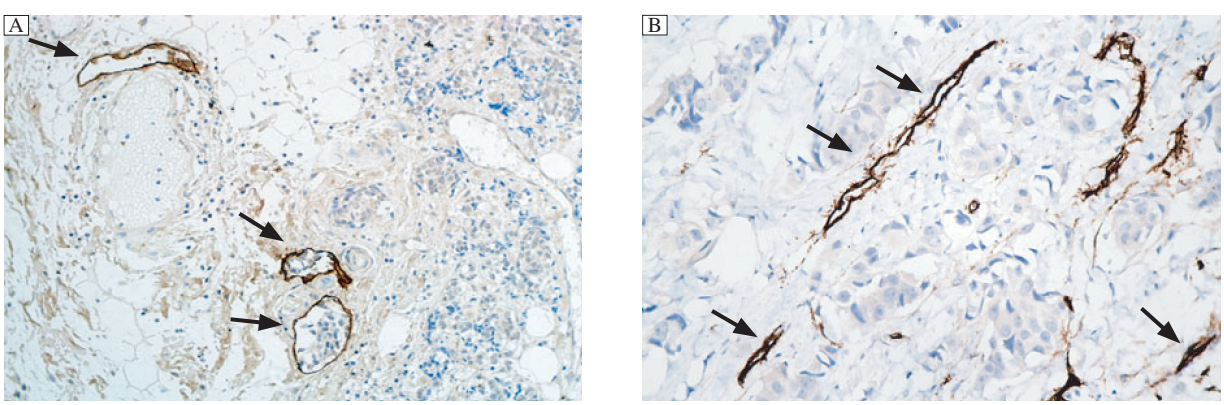

***Fig. 2* Immunohistochemical staining of D2-40 and CD31.** A: Immunoreactivity of D2-40 proteins was observed in the cytoplasm and cellular membrane of lymphatic endothelial cells (magnification×200). D2-40 expression was restricted to thin-walled lymphatic vessels containing no red blood cells (arrows). D2-40-positive cells were largely distributed in peritumoral tissue (hot spot). B: Representative sections showing CD31+ staining in blood microvessels/endothelial cells (magnification×400).

Table 1 **Relationship between HIF-1α and VEGF-C expression and clinicopathological characteristics of patients with breast cancer**

Parameter	No. of patients	HIF-1α		*P*-value	VEGF-C		*P*-value
		High	Low		High	Low	
Age (year)							
< 50	36	24	12	0.63	20	16	0.14
⩾ 50	39	28	11		28	11	
Size (cm)							
< 3	42	28	14	0.57	25	17	0.36
⩾ 3	33	24	9		23	10	
Grade							
I	16	10	6	0.66	11	5	0.77
II	35	26	9		23	12	
III	24	16	8		14	10	
Lymph node status							
-	35	18	17	0.002*	16	19	0.004*
+	40	34	6		32	9	
TNM							
I/II	38	20	18	0.001*	19	19	0.01*
III	37	32	5		29	8	
ER							
-	30	20	10	0.68	18	12	0.56
+	45	32	13		30	15	
PR							
-	26	17	9	0.59	13	13	0.07
+	49	35	14		30	15	
Her-2							
-	32	19	13	0.11	17	15	0.09
+	43	33	10		31	12	
VEGF-C							
Low	27	14	13	0.014*			
High	48	38	10				

*$P < 0.05$. ER, estrogen receptor; PR, progesterone receptor; TNM classification according to the Union Internationale Contre le Cancer criteria. Her-2 positivity = positive fluorescent in situ hybridization test.

positive cells were frequently distributed in peritumoral tissue. The lymphatic vessels were thin-walled and irregularly shaped and lacked erythrocytes. CD31 immunoreactivity in the blood vessel endothelial cells was observed in all the breast carcinoma tissue sections (***Fig. 2***).

Relationship among HIF-1α, VEGF-C and clinicopathologic characteristics

The correlations between HIF-1α and clinicopathologic factors, VEGF-C and clinicopathologic factors in breast carcinoma are shown in ***Table 1***. There was no significant correlation between HIF-1α expression and age, grade, size, estrogen receptor (ER), progesterone receptor (PR) or Her-2 ($P > 0.05$, chi-square test). Similarly, VEGF-C expression was not correlated with the above-mentioned clinicopathological characteristics ($P > 0.05$, chi-square test). However, high level expressions of HIF-1α and VEGF-C were correlated with the presence of lymph node metastasis ($P = 0.002$ and $P = 0.004$, respectively) and TNM stage ($P = 0.001$ and $P = 0.01$, respectively). A significant association between high level expressions of HIF-1α and VEGF-C was observed ($r = 0.273$, $P = 0.014$).

Relationship among HIF-1α, VEGF-C, LVD and MVD

High levels of HIF-1α and VEGF-C expression were found to be significantly associated with higher LVD, especially in the peritumoral region ($P = 0.003$ and $P = 0.017$, respectively) (***Fig. 3A, 3C***). The MVD assessed by using CD31-positive endothelial cells was significantly higher in the high VEGF-C/HIF-1α group than in the low VEGF-C/HIF-1α group ($P = 0.033$ and $P = 0.037$, respectively) (***Fig. 3B, 3D***).

Survival analysis

By the end of the 60-month follow-up period, 36 of the 75 patients had died. The overall 5-year survival (OS) for all patients was 52.0%. Analysis of the impact of HIF-1α status is shown in ***Fig. 4A***. Patients with high levels of HIF-1α expression had poorer prognoses than patients with low levels of HIF-1α expression ($P = 0.045$, log-rank test). Kaplan-Meier

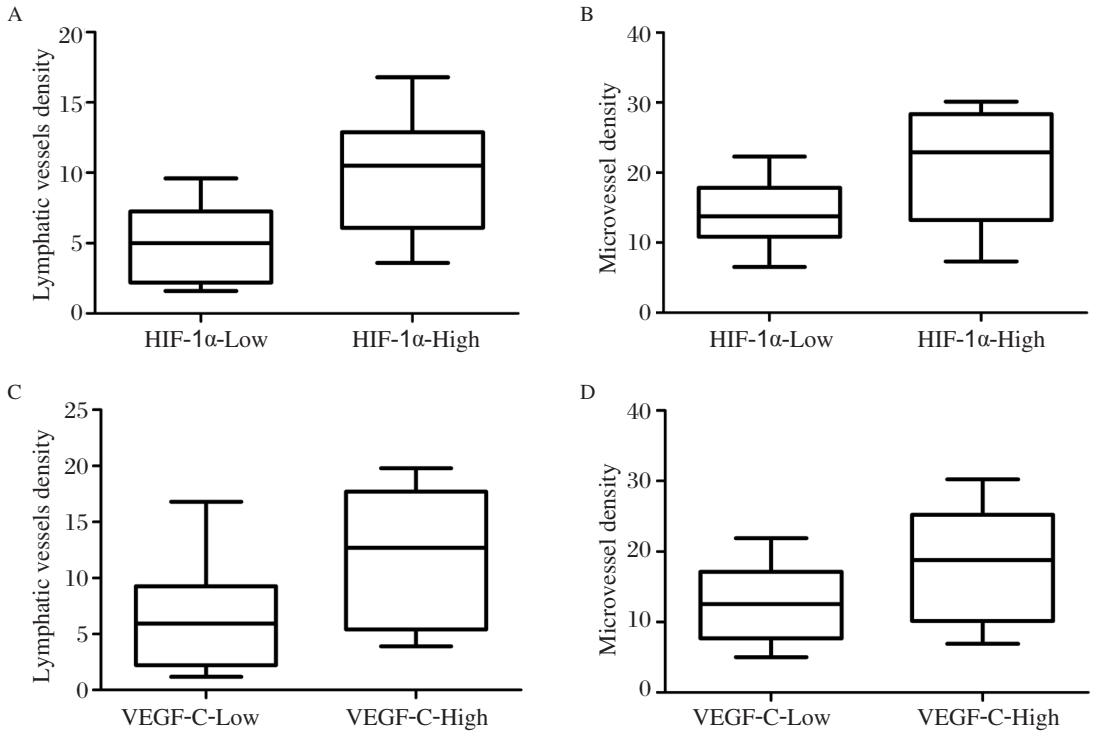

Fig. 3 **Box-blot showing statistically significant association between lymphatic vessel density (LVD), microvessel density (MVD), and HIF-1α-expression.** A, B, C, and D: $P = 0.003$, $P = 0.033$, $P = 0.017$, $P = 0.037$, respectively, Mann-Whitney test.

curves of overall survival stratified by VEGF-C status are shown in ***Fig. 4B***. The survival time of patients with higher level of VEGF-C expression was shorter ($P = 0.043$, log-rank test). In Cox regression for OS including lymph node metastasis, histological differentiation, TNM staging, HIF-1α expression, VEGF-C expression, LVD and MVD, only TNM stage ($P = 0.018$, RR = 1.891, 95% confidence interval, 1.358-4.781), HIF-1α expression ($P = 0.037$, RR = 2.249, 95% confidence interval, 1.382-6.452) and peritumoral LVD ($P = 0.023$, RR = 3.292, 95% confidence interval, 1.889-11.546) remained independent prognostic factors.

DISCUSSION

Only malignant breast carcinomas showed HIF-1α expression in the nuclei, suggesting that nuclear expression of HIF-1α takes place in mammary carcinogenesis. As a result of the adaptation of tumor cells to hypoxia, HIF-1α is overexpressed in a variety of human malignancies, including cancers involving the lung, prostate, breast, stomach, pancreas and skin[12]. Hypoxia is a common phenomenon in various types of malignant tumors, and contributes to the progression of cancer to a more aggressive phenotype[13]. HIF-1α overexpression has been reported to be closely cor–

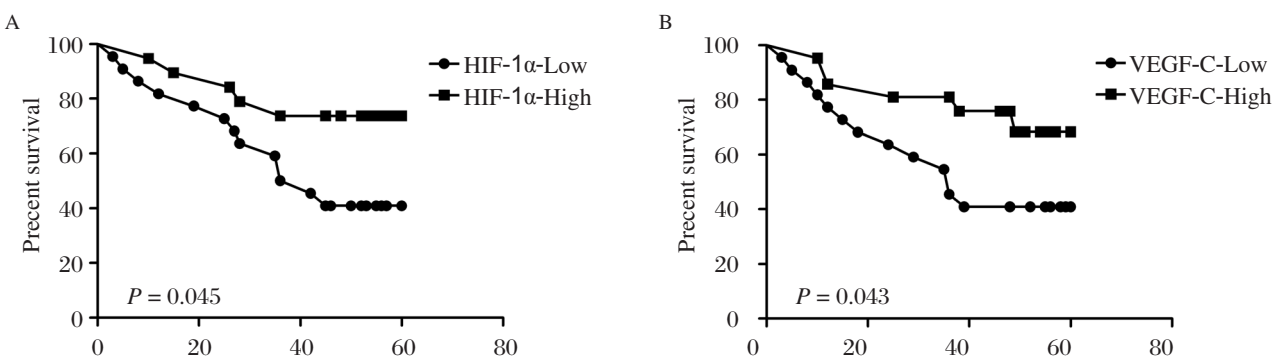

Fig. 4 **Kaplan–Meier overall survival curves for 75 patients with breast carcinoma.** A: Patients with high HIF-1α expression had a significantly worse overall survival (OS) than patients with low HIF-1α expression ($P = 0.045$). B: Patients with high levels of VEGF-C expression had a significantly worse OS than those with low levels of VEGF-C expression ($P = 0.043$).

Table 2 **Studies of relationship between HIF-1α and VEGF-C expression**

Author (year)	N	Cancer type	Country	Measure of association (*P*-value)
Katsuta (2005)[21]	48	human esophageal cancer	Japan	$P = 0.01$
Schoppmann (2006)[14]	119	breast cancer	Austria	$P = 0.026$
Enatsu (2006)[28]	78	small-sized adenocarcinomas of the lung	Japan	$P = 0.1330$
Wang (2007)[30]	73	Papillary thyroid carcinoma	China	$P < 0.01$
Liang (2008)[25]	65	Oral Squamous Cell Carcinoma	China	$P = 0.018$
Dorevic (2009)[26]	94	Clear cell renal cell carcinoma	Croatia	$P < 0.001$
Mori (2010)[27]	138	Prostate Cancer	America	$P < 0.001$
Brito (2011)[24]	30	Locally advanced breast cancer	Brazil	$P = 0.06$
Deeb (2011)[29]	84	normal karyotype adult acute myeloid leukemia	America	$P = 0.0338$

related with highly aggressive disease, resistance to radiotherapy and chemotherapy, and poor prognosis in some types of cancer, such as oligodendroglioma, ovarian and oropharyngeal cancer[7-9]. In the present study, HIF-1α expression was correlated with LVD, MVD, the presence of lymph node metastases and advanced TNM stages in breast cancer patients. As reported by Schoppmann et al., there was significant association between HIF-1α expression and the amount of peritumoral lymphangiogenesis and lymphatic vessel invasion in lymph node-positive invasive breast cancer[14]. In this way, HIF-1α may be a key regulator of angiogenesis, lymphangiogenesis and lymph node metastasis in breast cancer.

VEGF-C, a member of the VEGF family of angiogenic factors, activates VEGF receptor-3 (VEGFR-3), which is expressed on the lymphatic endothelium. Studies have recently shown that VEGF-C can promote tumor-associated lymphatic vessel growth in xenotransplantation and transgenic mouse models of cancer, resulting in metastasis of tumors to sentinel lymph nodes[15]. It has also been expressed at high levels in certain cancers, including gastric carcinoma, and to have a negative influence on prognosis and a positive correlation with lymph node metastasis[16]. VEGF-C, a member of the VEGF family, also induces angiogenesis by activating VEGFR-2[17]. Sedivy et al. observed that VEGF-C overexpression seemed to induce the formation of new lymphatics and blood vessels around the primary cancer[18]. The results of the present study showed that primary breast carcinoma tissue elevated the expression of VEGF-C. Significant association was observed between the expression of VEGF-C and the presence of lymph node metastases and advanced TNM stage. There was also a positive correlation between the expression of VEGF-C and peritumoral LVD. VEGF-C derived from the carcinoma may play an important role in lymphangiogenesis, increased aggression, and poor prognosis.

In the current study, HIF-1α expression was positively correlated with VEGF-C expression, and there was a positive correlation between the overexpression of both genes with LVD and MVD. In contrast to the effect of HIF-1α on angiogenesis, the effect on lymphangiogenesis and lymphatic metastasis remains poorly understood. HIF-1α acts as a transcription factor. A recent study showed that the transcriptional activity of a broad spectrum of genes, including the gene for VEGF, is altered by HIF-1 under hypoxic conditions[19]. Stabilized HIF-1α protein is transported into the nucleus, wherein it heterodimerizes with HIF-1 and binds to DNA at the hypoxia response elements (HREs), thereby activating the *VEGF* gene[20]. The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-F. Katsuta et al. examined the expressions of HIF-1α and VEGF-C in 5 esophageal squamous cell carcinoma (ESCC) cell lines[21]. The findings suggested that HIF-1α may play a role in lymphatic invasion and lymph node metastasis through the induction of VEGF-C. Okada et al. suggested that VEGF-C was also up-regulated by HIF-1α in human breast IDC cells[22]. These studies suggested the probable existence of a novel lymphangiogenic pathway. In this pathway, the overexpression of HIF-1α stimulates VEGF-C up-regulation and induces lymphangiogenesis, by which the tumor cells spread into the lymphatic system. In this way, HIF-1α may act as a lymphangiogenic factor through its interactions with VEGF-C. The results of the present study also indicated that HIF-1α may upregulate the expression of VEGF-C to stimulate angiogenesis in breast cancer. However, VEGF-C is 4 to 5 times less potent than VEGF, as determined by the vascular permeability assay[23]. For this reason, the activity of tumor angiogenesis stimulated by HIF-1α in the VEGF-C/VEGFR-2 pathway may be weaker than that stimulated in the VEGF-3 pathway in breast cancer.

These results are a small but developing part of literature on the association between HIF-1α and VEGF-C expressions. Nine other studies have examined this association[14,21,24-30] (***Table 2***). Only the study of Enatsu et al. reported that the strong expression of HIF-1α was associated with VEGF-A expression but not with VEGF-C ($P = 0.133$) in small-sized adeno–

carcinomas of the lung[24]. Mori et al.[28] showed that HIF-1α may play a role in the regulation of VEGF-C and tumor-associated lymphangiogenesis in prostate cancer ($P < 0.001$). Dorevic et al. reported that cytoplasmic HIF-1α expression (cHIF-1α) was positively correlated with diffuse staining of VEGF-C ($P < 0.001$) in clear cell renal cell carcinoma[27]. Liang et al. showed that HIF-1α overexpression was significantly correlated with VEGF-C overexpression ($P = 0.018$) in oral squamous cell carcinoma[26]. In the study of Brito et al., HIF-1α expression and VEGF-C expression were marginally associated with each other ($P = 0.06$) in breast cancer[29]. Similar results were also found in the studies on lung cancer, leukemia and esophageal cancers. All these findings have suggested that HIF-1α may play a role in lymphatic invasion and lymph node metastasis through the induction of VEGF-C, which strongly supports our results.

In summary, significant correlations were observed between the expression of HIF-1α and lymphangiogenesis, angiogenesis, the expression of VEGF-C and lymph node metastasis in breast cancer. The results suggest that HIF-1α may act as a regulator of VEGF-C in tumor-associated angiogenesis and lymphangiogenesis in breast cancer. However, the present study has some limitations, such as the small number of cases. Further studies on larger populations should be undertaken to confirm the results and to provide evidence for the potential role of HIF-1α in lymphangiogenesis. It may be useful as a prognostic marker and as a novel target of anti-tumor drugs in the treatment of human breast carcinomas.

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