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Expression of Hypoxia-inducible Factor (HIF-1α), VEGF-C and VEGF-D in Non-invasive and Invasive Breast Ductal Carcinomas

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Abstract. *Background: Hypoxia-inducible factor 1 alpha (HIF-1α) is a transcription factor that may play an important role in tumour growth and metastasis by its regulation of angiogenesis and lymphangiogenesis to survive cellular hypoxia. Lymphangiogenesis is promoted by vascular endothelial growth factors (VEGF)-C and VEGF-D, but the correlation between the expression of HIF-1α and VEGF-C or VEGF-D in human breast carcinoma is not well elucidated. This study examined the pathobiological role of these molecules in human breast ductal carcinoma. Materials and Methods: The expressions of HIF-1α, VEGF-C and VEGF-D were analyzed in 10 normal mammary epithelia, 12 fibroadenomas, 20 ductal carcinomas in situ (DCISs and 36 invasive ductal carcinomas (IDCs) by immunohistochemistry, comparing clinicopathological parameters. Results: HIF-1α expression in nuclei was found in DCIS and IDC, but not in normal or fibroadenoma cells. The HIF-1α labelling index was significantly correlated with the degree of VEGF-C expression in IDC ($p<0.001$), but not in DCIS. HIF-1α expression was significantly correlated with tumour necrosis and with the Van Nuys prognostic index (VNPI) ($p<0.05$, $p<0.05$, respectively) in the 20 DCISs. On the other hand, VEGF-D levels, but not those of HIF-1α and VEGF-C, were significantly higher in cases with lymph node metastasis and estrogen receptor expression in carcinoma cells ($p<0.01$, $p<0.05$, respectively) in the 36 IDCs. Conclusion: These findings suggest that HIF-1α is expressed in the early stage of mammary carcinogenesis, in which the expression correlates with necrosis in the DCISs and with VEGF-C expression in the IDCs, the latter resulting in a higher frequency of metastasis to regional lymph nodes.*

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Numerous studies have shown that the degree of vascularization is inversely correlated with patient survival, and the probability of invasion, metastasis and cell death is positively correlated with the degree of intratumoral hypoxia in various neoplasms (1-4). Hypoxic conditions during tumorigenesis induce the expression of hypoxia-inducible factor-1 (HIF-1), the master regulator of cellular oxygen homeostasis (5). 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-1 (6). The transcription factor HIF-1 consists of a heterodimer of HIF-1α and HIF-1β. HIF-1α mRNA levels are equivalent in normoxic and hypoxic conditions, but hypoxia inhibits the oxygen-dependent degradation of HIF-1α protein *via* the ubiquitin-proteasome pathway, recently shown to be regulated by the von Hippel-Lindau (VHL) tumour suppressor gene product (7-9). Stabilized HIF-1α protein is transported into the nucleus, where it heterodimerizes with HIF-1β and binds to DNA at the hypoxia response elements (HREs), activating the *VEGF* gene, one of the key angiogenic stimulators (7, 10).

VEGF regulates vasculogenesis during carcinogenesis in many human malignancies and metastatic lesions (11-18). In humans, the VEGF family consists of several members including VEGF-A (normally referred to as VEGF), VEGF-B, VEGF-C and VEGF-D (19). Among them, VEGF-C and VEGF-D are structurally closely related and have been implicated in the initiation and development of lymphatic vessels, so called "lymphangiogenesis" (20-24), including human breast carcinoma (25). The correlation between HIF-1α and VEGF-C and -D, however, has not been well analyzed in carcinogenesis and/or the progression of human breast carcinomas.

This study examines HIF-1α expression immunohistochemically, comparing it with the expression of VEGF-C and -D, as well as with clinicopathological profiles in normal breast tissue, fibroadenoma, ductal carcinoma *in situ* (DCIS) and invasive dactal carcinoma (IDC), to clarify the precise pathobiological significance of the expression.

Materials and Methods

Operative specimens. Studies were conducted on 12 fibroadenomas, 20 DCISs and 36 IDCs, which were surgically removed at the Division of Organ Regeneration Surgery, Tottori University, Japan, during 2000-2003. All specimens were fixed in 10% buffered formalin, sliced at a thickness of 5 mm and embedded in paraffin wax. Paraffin blocks were sectioned in 3-μm slices. The central part of the tumour was used for histopathological and immunohistochemical evaluation. A tissue specimen at least 3 cm from a carcinoma was defined as normal breast tissue in this study.

Histological classification was based mainly on the criteria of the general rules for the clinical and pathological recording of breast cancer established by the Japanese Breast Cancer Society (26). The Van Nuys Prognostic Index (VNPI)(27) was evaluated in the DCISs. In the IDCs, the histological grade of each case was evaluated according to the classification by Elston and Ellis (28).

Clinicopathological information was obtained from the patients' files. All the DCIS and IDC specimens used in this study were less than 5 cm in size and without evidence of distant organ metastasis. All the patients with IDC received axillary lymphadenectomy (up to level III). None of the patients had received preoperative radio-hormono- and/or chemotherapy.

Immunohistochemistry. Tissue sections were dewaxed in xylene, rehydrated through a graded series of ethanol solutions and rinsed in distilled water. Endogenous peroxidase activity was inhibited by immersing the slides in 0.3% hydrogen peroxide in methanol for 30 minutes. As a pretreatment, microwave-based antigen retrieval was performed in a 10 mM citrate buffer (pH 6.0). Immunostaining was conducted using Histofine Simple Stain MAX PO (Nichirei, Tokyo, Japan), followed by incubation with the primary antibodies, according to the manufacturer's instructions. The following primary antibodies were used: a monoclonal antibody raised against HIF-1α (1:1000; Abcam, Cambridge, UK), a polyclonal antibody raised against VEGF-C (1:400, R&D systems, Minneapolis, MN, USA), and a monoclonal antibody raised against VEGF-D (1:100, R&D systems). For HIF-1α immunostaining, a catalyzed signal amplification system (DAKO, Glostrup, Denmark) was used according to the manufacturer's instructions. On the other hand, immunostaining for VEGF-C and VEGF-D was performed using Histofine Simple Stain MAX PO (Nichirei, Tokyo, Japan) according to the manufacturer's directions, followed by incubation with the primary antibodies overnight at 4˚C. Immunoreactions were visualized with diaminobenzidine (DAB), and the sections were counterstained with haematoxylin. To examine the specificity of immunostaining, the primary antibody was replaced with mouse normal IgG or Tris-buffered saline. Control slides were invariably negative for immunoreactions.

Assessment of immunohistochemical examination. To evaluate HIF-1α, VEGF-C and VEGF-D expression levels, positively-stained tumour cell nuclei (HIF-1α) or cytoplasms (VEGF-C and VEGF-D) were counted in 10 areas at random. Labelling indices (LIs) were expressed as a percentage of positively-stained cells based on a count of at least 1000 tumour cells. All cases were judged to be positive for HIF-1α, VEGF-C and VEGF-D when the proportion of immunoreactive cells was at least 1% (HIF-1α), or 10% (VEGF-C and VEGF-D). In addition, we classified all cases into three groups according to the frequency of VEGF-C expression: grade I; LI<30%, grade II; LI=30-70%, grade III; LI>70.

Statistics. Statistical analyses were carried out on clinicopathological data, as for the estrogen receptor (ER), the progesterone receptor (PR) and HER-2, based on our previous study (submitted for publication, Okada *et al.*, 2004). The correlation between the immunohistochemical expression of HIF-1α or of the VEGF family and values following pre- or post-menopause, the presence of a necrotic region within the tumours, VNPI, primary tumour size, axillary lymph node metastasis, histological grade of tumour, lymphovascular invasion, and immunoreactivity for ER, PR and HER-2 in DCIS were analysed. Correlations between histological type and HIF-1α, VEGF-C and VEGF-D expression were assessed with the Chi-square test. Relationships between HIF-1α LIs and VEGF-C and VEGF-D expressing in DCIS and IDC were evaluated with Kruskal-Wallis test. The Chi-square test, Fisher's exact test, and the Mann-Whitney test were used to assess associations between HIF-1α, VEGF-C and VEGF-D expression and clinicopathological parameters. Statistical significance was defined as $p<0.05$.

Results

HIF-1α expression in human breast tissue. HIF-1α expression was not detected in the nucleus in either normal breast tissue or fibroadenoma, although weak immunoreactivity was noted in the cytoplasm in most cases. However, in 8 of the 20 (40%) DCISs (Figure 1A) and 16 of the 36 (44%) IDCs (Figure 1B), HIF-1α was clearly expressed in the cell nucleus, the difference in frequency not being significant (Table I).

VEGF-C and VEGF-D expression in DCIS and IDC. VEGF-C and VEGF-D expression in the DCIS and IDC was observed in both the nucleus and cytoplasm of carcinoma cells, with more predominant immunoreactivity in the cytoplasm (Figure 1C-F). Immunoreactivity was also noted in stromal fibroblasts. VEGF-C-immunoreactive tumour cells were noted in 11 (55.0%) of the 20 DICSs and in 24 (66.7%) of the 36 IDCs. Similarly, VEGF-D-immunoreactive cells were found in 9 (45.0%) DCISs and 23 (63.9%) IDCs. The frequencies showed no significant difference between the DCISs and IDCs (Table I).

Correlation between HIF-1α and VEGFs expression. The correlation between HIF-1α and VEGF expression was subsequently analysed. As shown in Table II, HIF-1α expression was significantly correlated with that of VEGF-C ($p<0.01$), but not with VEGF-D, in the 36 IDCs. On the

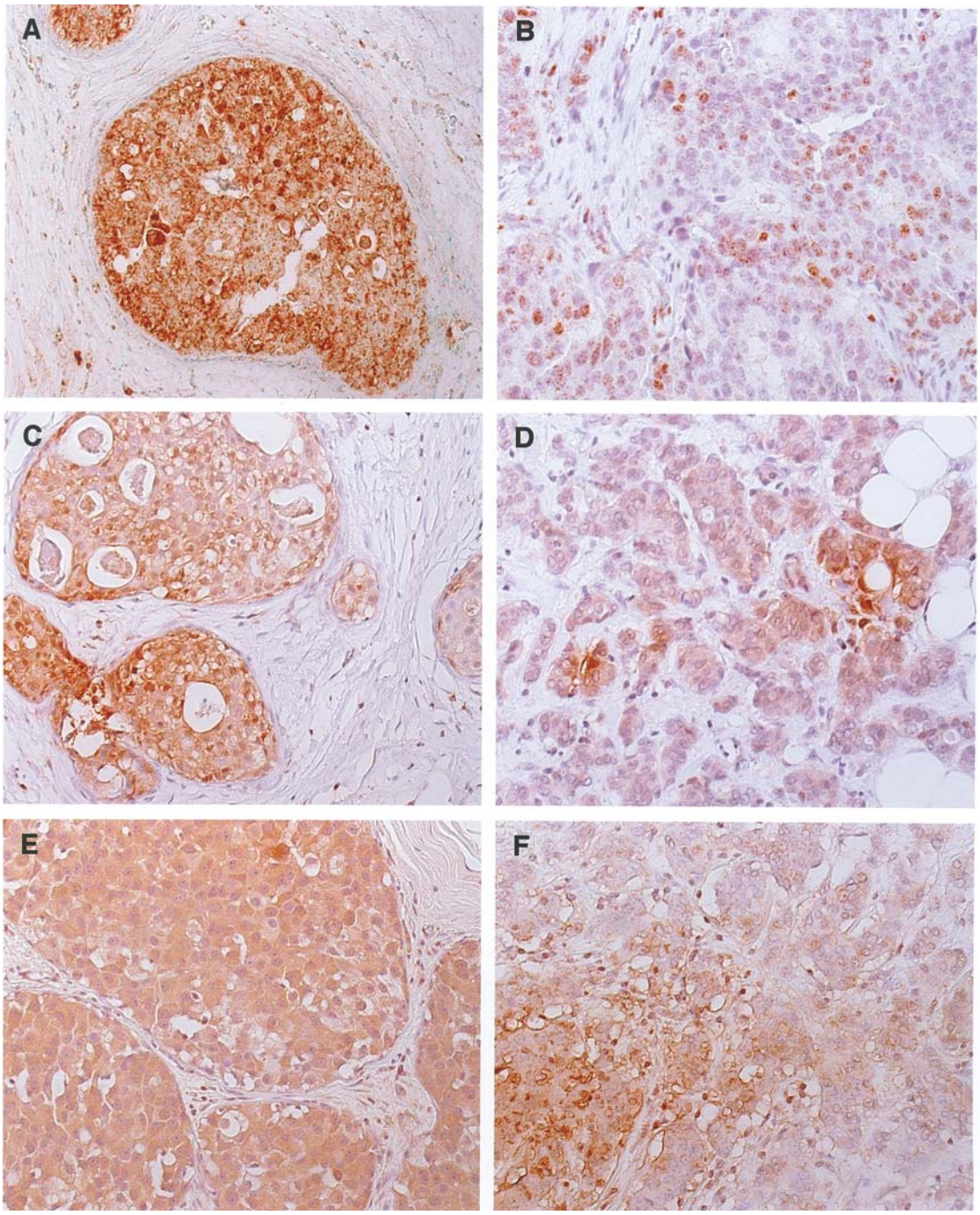

Figure 1. *Immunohistochemical detection of HIF-1α, VEGF-C, and VEGF-D expression in human breast DCIS and IDC. HIF-1α expression is noted in both the nucleus and cytoplasm of carcinoma cells in DCIS (A), whereas positive reactions were observed in most nuclei in IDC (B). VEGF-C and VEGF-D are expressed in both the nucleus and cytoplasm of carcinoma cells in DCIS (C and E) and in IDC (D and F). Immunoreactivity is also noted in stromal fibroblasts around carcinoma cell nests to varying degrees, especially in DCIS (C, E).*

other hand, there was no correlation between HIF-1α and VEGFs in the 20 DCISs. The relationship between HIF-1α LI and the grade of expression of VEGF-C in the DCISs and IDCs is illustrated in Figure 2. In DCIS, the LI (mean±SE) of HIF-1α was significantly lower in grade III (0.00) than in grade II tumours (12.8±5.89) ($p<0.05$). The LIs of HIF-1α were 0.250±0.239 in the 12 grade I IDCs, 5.00±4.56 in the 6 of grade II and 9.28±3.07 in the 18 of

Table I. *HIF-1α, VEGF-C and VEGF-D expressions in the 20 DCISs and 36 IDCs.*

	HIF-1α			VEGF-C			VEGF-D		
	–	+	*p*	–	+	*p*	–	+	*p*
DCIS (n=20)	12	8	0.747	9	11	0.388	11	9	0.171
IDS (n=36)	20	16		12	24		13	23	

DCIS: ductal carcinoma *in situ.*
IDC: invasive ductal carcinoma.

Table II. *Correlation between HIF-1α and VEGF expressions in the DCISs and IDCs.*

		DCIS			IDC		
		HIF-1α			HIF-1α		
		–	+	*p*	–	+	*p*
VEGF-C	–	5	4	0.713	11	1	<0.01
	+	7	4		9	15	
VEGF-D	–	7	4	0.713	9	4	0.373
	+	5	4		11	12	

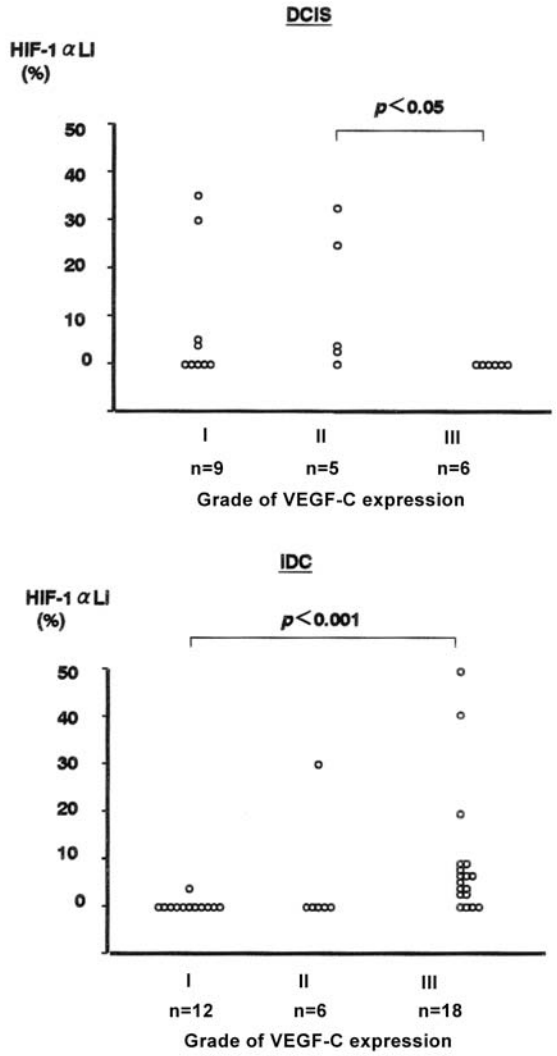

Figure 2. *Correlation between HIF-1α labelling indices (LI) and grade of VEGF-C expression in the DCISs (left panel) and 36 IDCs (right panel). The LI of HIF-1α is significantly lower in the DCISs of grade III than of grade II (p<0.05). On the other hand, the LI of HIF-1α is significantly higher in the IDCs of grade III than of grade I (p<0.01).*

grade III. The LIs were significantly higher in the IDC of grade III than of grade I ($p<0.001$).

Correlation between HIF-1α, VEGF-C and VEGF-D expressions and clinicopathological profiles. The correlation between HIF-1α, VEGF-C and VEGF-D expressions and clinicopathological profiles in the 20 DCISs and 36 IDCs can be observed from the data in Tables III and IV.

In the DCISs, the frequency of tumours with necrosis and VNPI was significantly higher in the HIF-1α-positive than in the HIF-1α-negative cases ($p<0.05$, Table III). VEGF-C and -D expression was correlated with no profiles examined in this study (Table III).

In the 36 IDCs, 15 (88.2%) of 17 lymph node metastasis-positive cases expressed VEGF-D in contrast to 8 (43.1%) of the 19 negative cases, the frequency being significantly higher in the former (Table IV). In addition, VEGF-D-positive cases were noted in 18 (78.3%) of the 23 ER-positive cases and in only 5 (38.5%) of the 13 negative ones ($p<0.05$). HIF-1α and VEGF-C expression did not correlate with any other profiles examined in this study (Table IV).

Discussion

HIF-1α expression in nuclei was observed exclusively in malignant breast carcinomas and not in normal or benign tissue, suggesting that the nuclear expression of HIF-1α is an event of mammary carcinogenesis. Zhong *et al.* have reported that HIF-1α is overexpressed in a variety of human malignancies including lung, prostate, breast, gastric, pancreatic, colon, ovarian, renal and skin carcinoma, as a result of the adaptation of tumour cells to hypoxia (3). They also suggested that overexpression of HIF-1α could occur at a very early stage of breast, colon and prostate carcinoma, before histological evidence of angiogenesis or invasion. The present study clearly demonstrated HIF-1α expression even in the DCISs, at an early stage of breast carcinoma.

Table III. *The relationship between HIF-1, VEGF-C, VEGF-D expressions and clinicopathological parameters in the 20 DCISs.*

			HIF-1α			VEGF-C			VEGF-D		
		n	–	+	*p*	–	+	*p*	–	+	*p*
Menopause	pre	8	3	5	0.226[a]	5	3	0.410[a]	5	3	0.930[a]
	post	12	9	3		4	8		6	6	
Necrosis	–	10	9	1	<0.05[a]	4	6	1.000[a]	6	4	1.000[a]
	+	10	3	7		5	5		5	5	
Van Nuys prognostic index	1	11	10	1	<0.05[b]	5	6	0.611[b]	7	4	0.460[b]
	2	8	2	6		4	4		4	4	
ER	–	8	4	4	0.777[a]	3	5	0.930[a]	3	5	0.410[a]
	+	12	8	4		6	6		8	4	
PR	–	7	3	4	0.502[a]	2	5	0.545[a]	2	5	0.204[a]
	+	13	9	4		7	6		9	4	
HER-2	–	15	9	6	1.408[a]	8	7	0.443[a]	10	5	0.195[a]
	+	5	3	2		1	4		1	4	

[a]Fisher's exact probability test.
[b]Chi-square test.

Table IV. *The relationship between HIF-1, VEGF-C, VEGF-D expressions and clinicopathological parameters in the 36 IDCs.*

			HIF-1α			VEGF-C			VEGF-D		
		n	–	+	*p*	–	+	*p*	–	+	*p*
Menopause	pre	17	10	7	0.970[a]	6	11	0.906[a]	5	12	0.659[b]
	post	19	10	9		6	13		8	11	
Size	≤20 mm	15	8	7	0.909[a]	4	11	0.726[b]	4	11	0.522[b]
	>20 mm	21	12	9		8	13		9	12	
Histological grade	1	20	13	7	0.156[c]	9	11	0.099[c]	8	12	0.835[a]
	2	12	5	7		3	9		4	8	
	3	4	2	2		0	4		1	3	
Lymphovascular invasion	–	12	7	5	0.906[a]	4	8	0.708[a]	5	5	0.491[a]
	+	24	13	11		8	16		8	18	
Lymph node metastasis	–	19	10	9	0.970[a]	8	11	0.410[b]	11	8	<0.01[b]
	+	17	10	7		4	13		2	15	
ER	–	13	7	6	0.846[a]	6	7	0.390[a]	8	5	<0.05[b]
	+	23	13	10		6	17		5	18	
PR	–	15	8	7	0.910[a]	5	10	1.284[b]	8	7	0.143[b]
	+	21	12	9		7	14		5	16	
HER-2	–	26	15	11	0.762[b]	9	17	1.277[b]	10	16	1.113[b]
	+	9	4	5		3	6		3	6	

[a]Chi-square test.
[b]Fischer's exact probability test.
[c]Mann-Whitney *U*-test.

It is well known that HIF-1α acts as a transcription factor. The intracellular stabilization of HIF-1α facilitates the binding to HREs of gene products related to vasculogenesis (including VEGF), glycolysis and erythropoiesis (29). The activated HIF-1α pathway is associated with aggressive tumour behaviour and a poor prognosis (2), since it is the most important process facilitating tumour cell survival, proliferation, progression and metastasis. VEGF-C expression significantly correlated with HIF-1α expression in the IDCs, although no report has revealed whether the *VEGF-C* gene has HREs in the promoter region. Our data suggest that VEGF-C was also up-regulated by HIF-1α in human breast IDC cells. In contrast to VEGF-C, VEGF-D expression did not show a significant correlation with HIF-1α expression in the IDCs. Currie *et al.* reported that the up-regulation of VEGF-D expression was significantly

associated with HIF-1α expression in human breast carcinoma tissue (30), although it was unclear whether HIF-1α directly activated the transcription of VEGF-D, as well as of VEGF-C. The discrepancy might be partly due to the small number of cases examined. On the other hand, VEGF-C and -D expression was not related to HIF-1α expression in the DCISs.

Tumours with necrotic regions were more frequently observed in the HIF-1α-positive than negative DCISs, implying that the expression of HIF-1α was induced by a hypoxic environment in the early stages of breast carcinoma. Tomes *et al.* also reported that HIF-1α expression correlated with tumour necrosis in human breast carcinoma (31). Moreover, HIF-1α expression was associated with Van Nuys prognostic index, indicating the prognosis of patients with DCIS (27). Bos *et al.* reported that high levels of HIF-1α (when the proportion of positive cells was more than 5%) were associated with a poorer survival in patients with lymph node-negative breast IDCs (32). Our data also suggest that the expression of HIF-1α might be a prognostic factor in breast DCIS, although this study could not confirm patient prognosis.

On the other hand, the expression of VEGF-D, but not of HIF-1α or VEGF-C, was associated with the expression of ER and lymph node metastasis in the IDC. Hyder *et al.* have identified two potential estrogen response elements (EREs) in the 5'UTR and exon 5 of VEGF-D, which showed 54% and 62% homology with ERE, respectively (33). Moreover, they also reported that VEGF-D expression was up-regulated by 17β estradiol in two ER-positive human breast cancer cell lines, MCF-7 and T47D (30). VEGF-D has been reported to be the most potent lymphangiogenic effector among VEGFs (22, 34). The formation of new lymph vessels and the entry of cancer cells into the lymphatic system are considered to be the first steps of lymph node metastasis. VEGF-C and -D are ligands for the lymphatic growth factor receptor, VEGFR-3, which is mostly expressed on lymph-endothelial cells, and the activation of VEGFR-3 *via* the binding of VEGF-C and/or -D has been reported to be sufficient to promote the metastasis of cancer cells through lymphangiogenesis (22, 35-40). Therefore, our data suggest that activated ER directly up-regulated the transcription of VEGF-D, and the VEGF-D-enriched environment contributed to lymph-angiogenesis, followed by the possible occurrence of lymph node metastasis in human breast IDCs. Tamoxifen, an ER antagonist, is generally used in the treatment of ER-positive breast cancer. Simak *et al.* reported that Tamoxifen treatment for metastatic breast cancer led to disease regression in approximately 30% of cancers (41). It is suggested that Tamoxifen may suppress lymph node metastasis by inhibiting the pathway described above in human breast IDCs.

On the basis of our findings, a prospective examination with a larger number of patients and a longer follow-up is warranted to confirm the predictive and prognostic potential of HIF-1α in DCIS and VEGF-D in IDC. In addition, further studies should provide evidence for the potential role of HIF-1α in lymphangiogenesis, as a new prognostic marker and as a novel target for antitumour drugs in the treatment of human breast ductal carcinomas.

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