The Akt expression correlates with the VEGF-A and -C expression as well as the microvessel and lymphatic vessel density in breast cancer

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Abstract. Akt activation has been found in various human cancers, while experimental studies have suggested that Akt plays an important role in the development of tumor angiogenesis and lymphangiogenesis. Immunohistochemical analyses for VEGF-C and Akt and the lymphatic endothelial specific marker D2-40 were performed on a series of 242 invasive ductal carcinomas of the breast, in which VEGF-A expression and microvessel density (MVD) had been determined previously. Lymphatic vessel density (LVD) was estimated in three hot spots. A significant correlation was observed between the VEGF-C expression and LVD (p=0.0026) and between LVD and the lymph node status (p<0.0001). The VEGF-C expression, however, did not correlate significantly with the lymph nodes status, while a high VEGF-C expression was associated with a smaller tumor size (p=0.0188). There was a significant correlation between VEGF-C and VEGF-A expression (p=0.0079) and between LVD and MVD (p=0.0008). The VEGF-C expression correlated with MVD (p<0.0001), while the VEGF-A expression correlated with LVD (p=0.0155). The Akt expression correlated with VEGF-A (p=0.0173) and -C expression (p=0.0056) as well as MVD (p=0.0482) and LVD (p=0.0012), while the correlation of Akt expression to VEGF-C expression and LVD was stronger than that to VEGF-A expression and MVD. Although the patients with a high LVD have a poorer disease-free survival than those with a low LVD (p=0.0005), a multivariate analysis determined the lymph node status and MVD to be independently significant factors for the disease-free survival. In conclusion, the correlation of both VEGF-C and VEGF-A to LVD and MVD suggested the two growth factors to be involved in both angiogenesis and lymphangiogenesis in breast cancer. The correlation of the Akt expression to the VEGF-A and -C expression as well as MVD and LVD, thus, suggested Akt activation to contribute to both angiogenesis and lymphangiogenesis via VEGF-A and -C expression in breast cancer.

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Introduction

The two major pathways of the metastases of cancer cells are via the vascular system and the lymphatic system. Angiogenesis is an important and essential step in the metastasis of cancer cells by vascular spread (1), while MVD is a quantitative marker of the blood vessel formation in cancer tissues (2). Our previous studies demonstrated the prognostic significance of MVD and a close association between VEGF-A and MVD in breast cancer (2-4). On the other hand, the axillary lymph node status is still the strongest prognostic factor in breast cancer patients (5-7). Studies on the mechanism of the lymphatic spread of cancer cells have been limited, because of a lack of specific markers for the lymphatic vessel endothelium (8). The recent discovery of new antibodies specific for lymphatic vessel endothelium made it possible to study the lymphatic system in cancer tissue (8-10). Vascular endothelial growth factor (VEGF) regulates the growth of endothelial cells (1). VEGF-A is the most potent growth factor for angiogenesis, while three additional members of the VEGF family, VEGF-B, -C and -D, have also been found (11). Although VEGF-A and VEGF-C have been traditionally thought to play a specific role in angiogenesis and lymphangiogenesis, respectively, recent experimental studies have suggested the existence of cross-talk between angiogenesis and lymphangiogenesis in the development of the metastasis of cancer cells (12).

Akt is a serine/threonine protein kinase that is a downstream target of growth factor receptors via the phosphatidylinositol 3-kinase (PI3K) signaling pathway. The PI3K/Akt

signaling pathway regulates various cellular processes such as cell proliferation and survival, glucose utilization and inhibition of apoptosis (13,14). Akt was initially found to be an oncogene in mouse thymoma (15). The activation of Akt expression has been demonstrated in various human cancers including breast cancer (16,17). Many experimental studies have demonstrated PI3K/Akt signaling pathways to play an important role in the development of angiogenesis (18-25) and lymphangiogenesis (26,27). However, no study has addressed Akt expression in relation to angiogenesis and lymphangiogenesis in cancer tissue including breast cancer.

The aim of the present study was to evaluate VEGF-C expression and LVD in breast caner tissue and their relationship to the lymph node status in breast cancer. The VEGF-C expression and LVD were compared to VEGF-A and MVD which had been determined in previous studies (2-4) and the prognostic value of these factors was evaluated in comparison to the lymph node status. Therefore, the main aim of the present study was to analyze the Akt expression in relation to VEGF-A and -C expression as well as the microvessel and lymphatic vessel density in breast cancer.

Patients and methods

Patients. This study included 242 women with breast cancer who underwent surgery for breast cancer, without any evidence of distant metastasis at the time of surgery, between 1985 and 1998 at the Beppu Medical Center. The histological type of breast cancer in all patients was invasive ductal carcinoma, while all types other than invasive ductal carcinoma were excluded in the present study. The patient ages ranged from 23 to 86 years, with a mean age of 58.1 years. The patients were treated by either a mastectomy (204 patients) or by breast conservation treatment (38 patients). A lymph node dissection was performed in 240 patients. Two hundred and fourteen patients received adjuvant postoperative hormone therapy and 208 patients received adjuvant chemotherapy, while 52 patients received postoperative radiotherapy. The median follow-up duration was 6.72 years. The methods of determination and the results of VEGF-A expression and MVD on a series of the present study have all been previously described (2-4).

Immunohistochemical analyses. For the immunohistochemical analysis of D2-40, paraffin-embedded sections (3-μm) were deparaffinized and rehydrated and then were heated at 99˚C for 40 min in Antigen Retrieval Buffer (DakoCytomation, Kyoto, Japan) with a microwave for antigen retrieval. The sections were incubated for 30 min in 3% hydrogen peroxide to quench the endogenous peroxidase. Next, the sections were incubated at room temperature for 30 min with mouse monoclonal D2-40 antibody (DakoCytomation) diluted 1:50 in PBS. The sections were subsequently stained according to the polymer-immuno complex method using Dako Envision$^+$ kit (DakoCytomation) and were visualized using 3,3'-diaminobenzidine, followed by counterstaining with hematoxylin. For the immunohistochemical analysis of VEGF-C expression, paraffin-embedded sections (3-μm) were deparaffinized and rehydrated. The sections were incubated for 30 min in 3% hydrogen peroxide to quench the endogenous peroxidase. Thereafter, the sections were incubated at 4˚C overnight with goat polyclonal VEGF-C antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:300 in PBS. The sections were subsequently stained according to the labeled streptavidin biotin method using Dako LSAB$^+$ kit (DakoCytomation) and were visualized using 3,3'-diaminobenzidine, followed by counterstaining with hematoxylin. For the immunohistochemical analysis of Akt, paraffin-embedded sections (3-μm) were deparaffinized and rehydrated and then were heated at 99˚C for 20 min in Antigen Retrieval Buffer (DakoCytomation) with a microwave for antigen retrieval. The sections were incubated for 15 min in 3% hydrogen peroxide to quench the endogenous peroxidase. Next, the sections were incubated at 4˚C overnight with rabbit monoclonal phospho-Akt (Ser 473) antibody (Cell Signaling Technology, Beverly, MA) diluted 1:65 in PBS. The sections were subsequently stained according to the polymer-immuno complex method using Dako Envision$^+$ kit (DakoCytomation) and were visualized using 3,3'-diaminobenzidine, followed by counterstaining with hematoxylin.

Assessment of LVD and the expression of VEGF-C and Akt. In the present study, LVD was also assessed using a method of MVD determination similar to a method reported in previous studies (2,3). The sections were initially scanned at a low magnification (x40), thereby finding three separately located areas with the highest concentration of LVD (hot spot). Next, the images of the rectangular areas (1030x1414 μm^2) at a magnification of x100 in the hot spots (Fig. 1) were captured using a microscope (Eclipse E600, Nikon, Tokyo, Japan) coupled to a microcomputer system (DP70-WPC02, Olympus, Tokyo, Japan). The stained endothelial cell clusters were counted, while the LVD value for each tumor was expressed as an average value of the three rectangular areas at a magnification of x100.

The immunohistochemical expression of VEGF-C and Akt was determined using the same method as used for the determination of VEGF-A expression previously by us (3,4), and described by Volm *et al* (28). Briefly, the sections were graded according to the percentage of positively stained tumor cells (0, 0% immunopositive cells; 1, <25% positive cells; 2, 26-50% positive cells; and 3, >50% positive cells) and the staining intensity (0, negative; 1, weak; 2, moderate; and 3, high), respectively. The sum of the percentage of positively stained cells and the staining intensity between 0 and 2 was regarded as negative, while the sum between 3 and 4 was considered to be positive and the sum between 5 and 6 was regarded as being strongly positive (3,4,28). The final determination of the immunohistochemical expression of VEGF-C and Akt was made by one pathologist (Y.O.), who had no information on any of the patients.

Statistical analysis. The χ^2 test was used to investigate the significance in contingency table analyses. A multivariate analysis for the determination of variables which were independently associated with the presence of lymph node metastasis was performed using a logistic regression model. The disease-free survival (DFS) was estimated using the Kaplan-Meier method and any differences in the survival curves were compared using the log-rank test. A multivariate analysis for

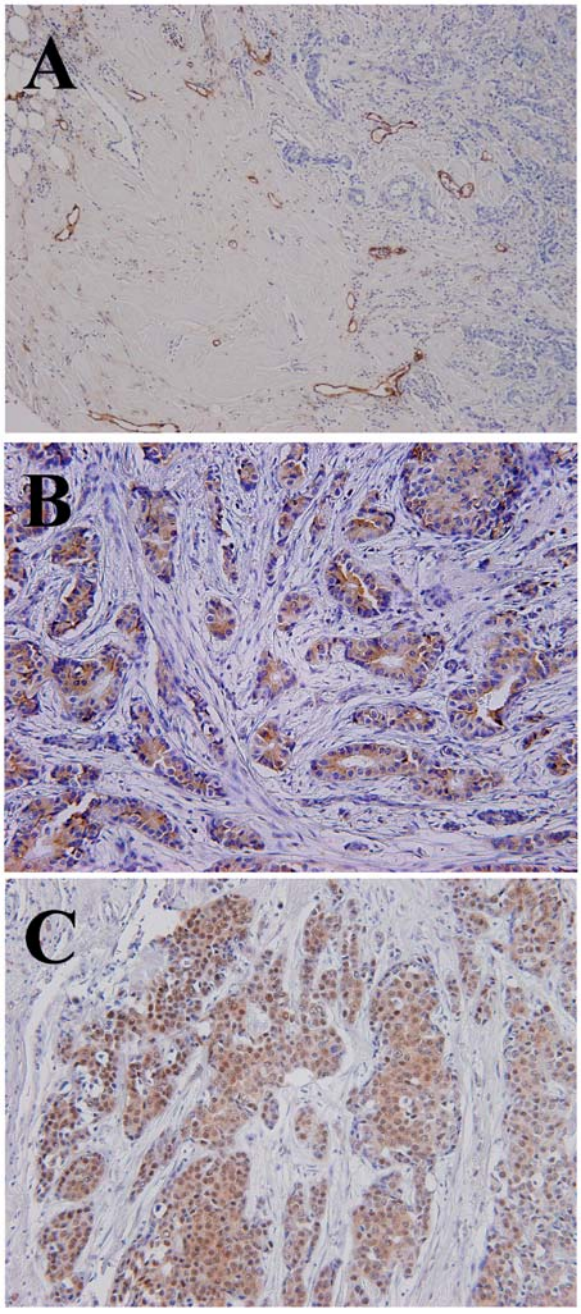

Figure 1. D2-40 immunostained lymphatic vessels (A) in the hot-spot which is a rectangular area (1030x1414 μm^2), while a strongly positive expression of VEGF-C (B) and Akt (C) was seen in the cytoplasm of the breast cancer cells.

the determination of variables which were independently associated with the DFS was performed using Cox's proportional hazards model. A p-value of <0.05 was regarded as being statistically significant. All statistical analyses were performed using the StatView 5.0 software program (SAS Institute, Cary, NC).

Results

Stratification of the patient groups according to LVD. The LVD varied from 2.33 to 34.33 with a median value of 10.00 per field (x100). The cut-off point for dichotomous analyses was determined in a previous study on MVD based by the highest χ^2 and the lowest p-value in univariate survival analyses (3). The same method was indicated for the determination of the optimal cut-off point for LVD in the present study. The cut-off point for LVD was determined to be the 55th percentile (10.67 per field) by which the χ^2 was highest and the p-value was lowest in univariate survival analyses (data not shown). This cut-off point for LVD was used in the following dichotomous analyses.

The relationship between VEGF-C expression and LVD. The immunohistochemical expression of VEGF-C was seen in the cytoplasm of the tumor cells (Fig. 1). The expression of VEGF-C was determined to be negative in 54 (22%) cases, positive in 99 (41%) cases and strongly positive in 89 (37%) patients. A low LVD in which the LVD value was less than the cut-off point was found in 132 (55%) cases, while a high LVD in which the LVD value was more than the cut-off point was found in 110 (45%) cases. A significant correlation was observed between VEGF-C expression and LVD in breast cancer (p=0.0026; Table I).

The relationship of LVD and VEGF-C expression to the clinicopathological factors. Table II shows the relationship between the LVD or VEGF-C expression and the clinicopathological factors in breast cancer. LVD significantly correlated with lymph node metastasis (p<0.0001) and the MIB-1 counts (p=0.0039), while LVD correlated positively with the VEGF-A (p=0.0155) expression and MVD (p=0.0008). No significant correlation was observed between the VEGF-C expression and lymph node metastases, while a high VEGF-C expression correlated significantly with a smaller tumor size (p=0.0188). In addition, a significantly positive correlation was observed between the tumor size and

Table I. The relationship between the expression of VEGF-C and LVD.

		LVD (%)		
	No. of patients	Low (n=132)	High (n=110)	P-value
VEGF-C expression				0.0026
Negative	54	39 (72)	15 (28)	
Positive	99	55 (56)	44 (44)	
Strongly positive	89	38 (43)	51 (57)	

Table II. The relationship between the LVD or the VEGF-C expression and the clinicopathological factors in breast cancer.

		LVD (%)			VEGF-C expression (%)			
Variables	No. of patients	Low n=132	High n=110	P-value	Negative n=54	Positive n=99	Strongly positive n=89	P-value
Tumor size (cm)				0.6809				0.0188
<2.0	56	33 (59)	23 (41)		9 (16)	22 (39)	25 (45)	
2.1-5.0	158	83 (53)	75 (47)		38 (24)	59 (37)	61 (39)	
>5.1	28	16 (57)	12 (43)		7 (25)	18 (64)	3 (11)	
Lymph node metastasis[a]				<0.0001				0.7532
Absent	136	90 (66)	46 (34)		33 (24)	54 (40)	49 (36)	
Present	104	42 (40)	62 (60)		21 (20)	44 (42)	39 (38)	
Nuclear grade				0.1292				0.9339
I or II	166	96 (58)	70 (42)		38 (23)	68 (41)	60 (36)	
III	76	36 (47)	40 (53)		16 (21)	31 (41)	29 (38)	
Estrogen receptor				0.4077				0.0642
Positive	106	61 (58)	45 (42)		22 (21)	52 (49)	32 (30)	
Negative	136	71 (52)	65 (48)		32 (24)	47 (35)	57 (42)	
MIB-1 counts				0.0039				0.0068
Low	143	89 (62)	54 (38)		37 (26)	65 (45)	41 (29)	
High	99	43 (43)	56 (57)		17 (17)	34 (34)	48 (48)	
VEGF-A expression				0.0155				0.0079
Negative	63	36 (57)	27 (43)		21 (33)	27 (43)	15 (24)	
Positive	98	62 (63)	36 (37)		23 (23)	41 (42)	34 (35)	
Strongly positive	81	34 (42)	47 (58)		10 (12)	31 (38)	40 (49)	
MVD				0.0008				<0.0001
Low	157	98 (62)	59 (38)		40 (25)	76 (48)	41 (26)	
High	85	34 (40)	51 (60)		14 (16)	23 (27)	48 (56)	

[a]A lymph node dissection was performed in 240 patients.

lymph node metastasis (p=0.0002). The VEGF-C expression correlated positively with the MIB-1 counts (p=0.0068), VEGF-A expression (p=0.0079) and MVD (p<0.0001).

The predicting factors for the presence of lymph node metastases. A multivariate analysis determined both the tumor size and LVD (p=0.0002; relative risk, 3.06; 95% CI, 1.70-5.50) to be independently significant predictive factors for the presence of lymph node metastasis.

The relationship of Akt expression to the clinicopathological factors. The immunohistochemical expression of Akt was seen in the cytoplasm of the tumor cells (Fig. 1). The expression of Akt was determined to be negative in 113 (47%) cases, positive in 49 (20%) cases and strongly positive in 80 (33%) patients. The Akt expression did not correlate with either the tumor size, lymph node metastasis, nuclear grade or MIB-1 counts (Table III).

The relationship of Akt expression to VEGF-A and -C expression as well as MVD and LVD. Table IV shows the relationship of the Akt expression to LVD and the VEGF-C expression. A significantly positive correlation was observed between the Akt expression and LVD (p=0.0012) and between the Akt and VEGF-C expressions (p=0.0056). Table V shows the relationship of the Akt expression to MVD and the VEGF-A expression. Although a positive association tended to be observed between the Akt expression and MVD and between Akt and VEGF-A expression, the statistical difference of these associations was not significant. On the other hand, when the Akt and VEGF-A expressions were divided into two groups, namely negative or positive vs. strongly positive groups, a significant correlation was found between the Akt expression and MVD (p=0.0482) and between the Akt and VEGF-A expression (p=0.0173; Table VI).

Table III. The relationship between the Akt expression and the clinicopathological factors in breast cancer.

Variables	No. of patients	Akt expression			P-value
		Negative n=113	Positive n=49	Strongly positive n=80	
Tumor size (cm)					0.2931
<2.0	56	27 (48)	15 (27)	14 (25)	
2.1-5.0	158	70 (44)	29 (18)	59 (37)	
>5.1	28	16 (57)	5 (18)	7 (25)	
Lymph node metastasis[a]					0.2933
Absent	136	69 (51)	24 (18)	43 (32)	
Present	104	43 (41)	25 (24)	36 (35)	
Nuclear grade					0.1247
I or II	166	83 (50)	35 (21)	48 (29)	
III	76	30 (39)	14 (18)	32 (42)	
Estrogen receptor					0.3507
Positive	106	55 (52)	20 (19)	31 (29)	
Negative	136	58 (43)	29 (21)	49 (36)	
MIB-1 counts					0.0616
Low	143	71 (50)	33 (23)	39 (27)	
High	99	42 (42)	16 (16)	41 (41)	

[a]A lymph node dissection was performed in 240 patients.

Table IV. The relationship between the Akt expression and either the LVD or VEGF-C expression in breast cancer.

Akt expression	No. of patients	LVD (%)			VEGF-C expression (%)			
		Low n=132	High n=110	P-value	Negative n=54	Positive n=99	Strongly positive n=89	P-value
				0.0012				0.0056
Negative	113	74 (65)	39 (35)		34 (30)	50 (44)	29 (26)	
Positive	49	27 (55)	22 (45)		8 (16)	21 (43)	20 (41)	
Strongly positive	80	31 (39)	49 (61)		12 (15)	28 (35)	40 (50)	

Table V. The relationship between the Akt expression and either the MVD or VEGF-A expression in breast cancer.

Akt expression	No. of patients	MVD (%)			VEGF-C expression (%)			
		Low n=157	High n=85	P-value	Negative n=63	Positive n=98	Strongly positive n=81	P-value
				0.1353				0.1996
Negative	113	79 (70)	34 (30)		30 (27)	50 (44)	33 (29)	
Positive	49	33 (67)	16 (33)		15 (31)	21 (43)	13 (27)	
Strongely positive	80	45 (56)	35 (44)		18 (23)	27 (34)	35 (44)	

Table VI. The relationship between the Akt expression and either the MVD and VEGF-A expression in breast cancer.

		MVD (%)			VEGF-C expression (%)		
Akt expression	No. of patients	Low n=157	High n=85	P-value	Negative and positive n=161	Strongly positive n=80	P-value
				0.0482			0.0173
Negative and positive	162	112 (69)	50 (31)		116 (72)	46 (28)	
Strongly positive	80	45 (56)	35 (44)		45 (56)	35 (44)	

Akt and VEGF-A expressions were divided into two groups: negative and positive vs. strongly positive.

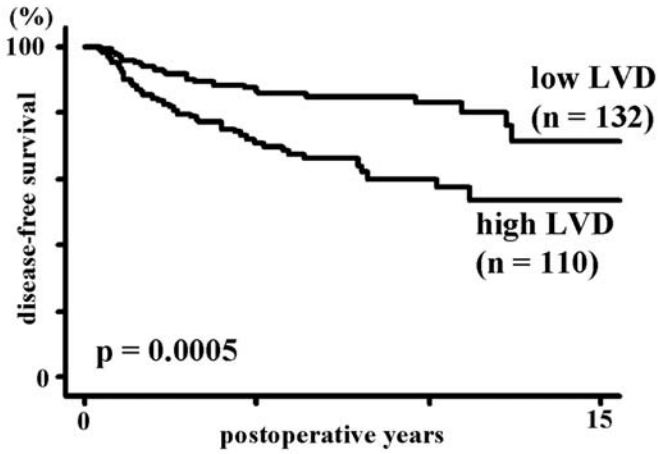

Figure 2. The disease-free survival (DFS) curve stratified according to the LVD.

The survival analyses. The 110 patients with a high LVD had a significantly (p=0.0005) worse DFS than the 132 patients with a low LVD (Fig. 2), while the 104 patients with lymph node metastases had a significantly (p<0.0001) worse DFS than the 136 patients without lymph node metastases. In our previous study (3), the patient group with a strongly positive VEGF-A expression was demonstrated to have a poorer DFS than the group with a positive or negative VEGF-A expression. There was no difference in the three patient groups stratified by either Akt or VEGF-C expression, while the patient group with a strongly positive Akt expression had a significantly (p=0.0416) poorer DFS than the group with a positive or negative Akt expression. On the other hand, a multivariate analysis using the Cox proportional hazard model indicated lymph node metastasis and MVD to be independently significant prognostic factors for DFS, while LVD, VEGF-A expression or Akt expression were not significant factors for DFS (Table VII).

Discussion

Experimental studies have revealed lymphangiogenesis to play an important role in the lymphatic spread of cancer cells (29-31). The overexpression of VEGF-C by orthotopically transplanted breast cancer cells induced lymphangiogenesis and a high density of lymph vessels was associated with the development of regional lymph node metastases (29,30). The VEGF-C overexpression was also demonstrated not only to increase tumor growth but also promote intratumoral lymphatic growth, while this effect of VEGF-C over-expression was inhibited by blocking VEGFR-3 signaling (31). On the other hand, some studies found no evidence of lymphangiogenesis in human breast cancer tissue (32-34). In these studies, however, the lymphatic vessels were found at the tumor margin (35). The functional lymphatics in the tumor margin alone were demonstrated to be sufficient for lymphatic metastasis (36). The assessment of lymphatic vessels in the peritumoral areas has been performed in breast cancers (37-39) as well as oral (40), gastric (41), colorectal (42) and lung (43) cancers. In the present study, the hot spots for LVD were typically found in the peritumoral areas and a significant correlation was found between the VEGF-C expression and LVD as well as between LVD and the lymph node status.

A significant correlation between the VEGF-C expression and LVD has been reported in previous studies on breast cancer (39,44) as well as oral (40), gastric (41), colorectal (42) and ovarian (45) cancers, while no significant correlation was found in another study on breast cancer (37). A significant correlation was also found between LVD and the regional lymph node status, in accordance with previous studies on breast cancer (37,38,44,46), while no significant correlation was found in a series of 60 breast cancers (47). A significant correlation between LVD and the regional lymph nodes was also found in head and neck (48,49), lung (43), thyroid (50) and gastric (51) cancers. Furthermore, a multivariate analysis in the present study as well as the studies on head and neck (49) and thyroid (50) cancers demonstrated LVD to be an independently significant factor for the regional lymph node status. A close correlation between the VEGF-C expression and LVD and between LVD and regional lymph node metastasis was observed in a variety of cancers, thus suggesting the contribution of VEGF-C and subsequent lymphangiogenesis to the development of regional lymph node metastasis (52).

Table VII. Multivariate analyses, using a Cox proportional hazard model, for DFS in breast cancer patients.

Variables	Multivariate analysis		
	P-value	Relative risk	95% CI
Tumor size (cm)			
2.1-5.0 (vs. -2.0)	0.7394	1.15	0.51-2.59
5.1- (vs. -2.0)	0.5288	1.39	0.50-3.89
Lymph node metastasis			
Present (vs. absent)	<0.0001	6.12	3.09-12.1
Nuclear grade			
III (vs. I or II)	0.1163	1.57	0.90-2.74
Estrogen receptor			
Negative (vs. positive)	0.5982	1.17	0.66-2.06
MIB-1 counts			
High (vs. low)	0.0620	1.76	0.97-3.19
MVD			
High (vs. low)	0.0058	2.59	1.32-5.10
LVD			
High (vs. low)	0.6167	1.16	0.66-2.04
VEGF-A expression			
Strongly positive (vs. negative and positive)	0.7681	0.91	0.49-1.68
VEGF-C expression			
Strongly positive (vs. negative and positive)	0.4118	0.79	0.44-1.40
Akt expression			
Strongly positive (vs. negative and positive)	0.2068	1.44	0.82-2.55

The correlation between the VEGF-C expression and regional lymph node metastases has been demonstrated in a variety of human cancers, whereas contradictory results have been reported for breast, colorectal and lung cancer (52). Eleven studies (53-63) described the relationship between VEGF-C expression and the regional lymph node status in breast cancer. Although three studies on breast cancer (53-55) demonstrated a significant correlation between the VEGF-C expression and the lymph node status, eight studies failed to find a significant correlation between the VEGF-C expression and the lymph node status in breast cancers (56-63). The reason why the VEGF-C expression did not correlate directly with the lymph node status, whereas significantly positive correlations were observed between the VEGF-C expression and LVD and between LVD and the lymph node status in the present study, is that the VEGF-C expression is inversely correlated with the tumor size, which is positively correlated with the lymph node status. Although the reason why a higher VEGF-C expression was often found in smaller tumors is not clear, Bando *et al* (56) also reported a high VEGF-C level to be associated with a smaller tumor size in 193 breast cancers. Furthermore, Bono *et al* (37) showed that the intraductal component frequently expresses VEGF-C.

The present study, in addition to previous studies (2-4), demonstrated a significant correlation between both the VEGF-A and-C expression and both MVD and LVD in breast cancer. Although VEGF-A and VEGF-C have been traditionally thought to play a specific role in angiogenesis and lymphangiogenesis, respectively, recent experimental studies have suggested cross-talk to exist between angiogenesis and lymphangiogenesis in the development of the metastasis of cancer cells (12,64-66). VEGF-A induced a strong lymphangiogenic response when delivered via an adenovirus to the mouse ear (64). The transgenic VEGF-A expression in a skin carcinogenesis model led to enhanced lymphangiogenesis and metastasis to the regional and distant lymph nodes (65). On the other hand, VEGF-C was demonstrated to induce angiogenesis in the mouse cornea (66). Although VEGF-A and VEGF-C have been shown to play various roles in both angiogenesis and lymphangiogenesis in the animal models, there have been conflicting reports regarding the relationship between VEGF-A and VEGF-C and the LVD and MVD in human cancer tissues (37,41,45,47,55,67-70). The VEGF-C expression correlated positively with both the LVD and MVD in bladder (67), gastric (41) and ovarian (45) cancers, while no significant correlation

was found between VEGF-C expression and MVD in lung cancer (68). On the other hand, the VEGF-A expression correlated with LVD and a significant correlation between LVD and MVD was observed in colorectal cancers (69), while the VEGF-A expression did not correlate with LVD in bladder cancer (67). In breast cancers, VEGF-C correlated with LVD, but not with MVD (70), while no significant correlation was found between MVD and LVD in breast cancers (37,47). A recent study on 177 breast cancers, however, demonstrated a high expression of VEGF-A and VEGF-C to be associated with a higher LVD and a higher MVD (55), and these findings are consistent with the present study.

The most important result to note in the present study is that the Akt expression correlated with the VEGF-A and -C expression as well as MVD and LVD, while the correlation of the Akt expression to the VEGF-C expression and LVD was stronger than that of the Akt expression to the VEGF-A expression and MVD. So far no immunohistochemical study has analyzed the Akt expression in relation to angiogenesis and lymphangiogenesis in cancer tissue specimens, including breast cancer. On the other hand, the association between PI3K/Akt signaling pathway and angiogenesis has been demonstrated in many experimental studies (18-25). Ras expression increased the VEGF-A expression, while hypoxia increased the PI3K activity in a Ras-dependent manner and the inhibition of PI3K activity resulted in the inhibition of VEGF-A induction (18). The VEGF-A expression was demonstrated to increase by insulin treatment through the PI3K/Akt signaling pathway (19). Hepatocyte growth factor induces a significant increase of the VEGF-A expression through PI3K/Akt activation (20). In ovarian cancer cells, the decrease of VEGF-A expression by PI3K/Akt inhibitors suggests PI3K/Akt signaling to be necessary to obtain elevated levels of VEGF-A expression (21-23). Hypoxia inducible factor-1α (HIF-1α) was demonstrated to activate the expression of VEGF-A gene by binding the hypoxia response element (71) and the HIF-1α activation was also demonstrated to influence both angiogenesis and tumor growth (72). The PI3K/Akt signaling pathway has been also demonstrated to regulate the HIF-1α expression (21-25). HIF-1α expression has been shown to be blocked by PI3K/Akt inhibitors in prostate cancer cells, thus indicating that Akt activation is required for HIF-1α expression (24), while PI3K/Akt activation has also been shown to be required for insulin- and EGF-induced HIF-1α expression (25). In ovarian cancer cells, Akt activation mediates HIF-1α expression (21-23), while HDM2 and p70S6K1 are two downstream targets of Akt that mediate growth factor-induced VEGF-A transcriptional activation and HIF-1α expression (21). Therefore, these experimental studies (18-25) suggest that the PI3K/Akt signaling pathway contributes to tumor angiogenesis via VEGF-A and HIF-1α expression. On the other hand, there have been few studies on the relationship between the Akt expression and lymphangiogenesis (26,27). The PI3K/Akt signaling pathway is suggested to be involved in the IGF-1-induced VEGF-C expression (26). Kobayashi *et al* (27) demonstrated the serum starvation-induced expression of VEGF-C to be inhibited by rapamycin, a specific inhibitor of mTOR, which is located downstream of the PI3K/Akt signaling pathway and that the number and the area of lymphatic vessels in the primary tumors significantly decreases after the administration of rapamycin. Although there have been fewer experimental studies conducted on the contribution of the PI3K/Akt signaling pathway to lymphangiogenesis (26,27) than that to angiogenesis (18-25), the correlation between the Akt expression to the VEGF-C expression and LVD is stronger than that of the Akt expression to VEGF-A expression and MVD in the present study. The present study also demonstrated the correlation between both VEGF-C and VEGF-A to LVD and MVD, thus suggesting the two growth factors to be involved in both angiogenesis and lymphangiogenesis. On the other hand, the HIF-1α expression was demonstrated to correlate with the VEGF-C expression and lymphatic microvessel density in breast cancer (73) and oral squamous cell carcinoma (74). These findings suggested that Akt activation also contributes to lymphangiogenesis via VEGF-A and -C and HIF-1α expression.

Although MVD has been demonstrated to be a significant prognostic factor in breast cancer (2-4), the lymph node status is still the strongest prognostic factor in breast cancer (5-7). Multivariate analyses in previous studies (2-4) demonstrated the independently significant prognostic value of MVD in breast cancer patients and that the lymph node status has a stronger prognostic value than that of MVD. In the present study, the prognostic value of LVD, VEGF-A and Akt expression was demonstrated by a univariate analysis, whereas multivariate analyses indicated the lymph node status and MVD, but not LVD, to be independently significant prognostic factors. The reason why the LVD was not found to be an independently significant prognostic factor is that there was a close correlation between the LVD and the lymph node status, whereas no such correlation was observed between the MVD and the lymph node status (2-4). The same finding, where univariate analyses indicated the prognostic significance of the LVD whereas multivariate analyses indicated the lymph node status but not the LVD to be an independent significant prognostic factor, was also demonstrated in breast cancers by Bono *et al* (37) and Nakamura *et al* (44,46) and in bladder cancers by Miyata *et al* (67). This finding can be explained by the fact that the regional lymph node status represents the consequence of the lymphatic spread of cancer cells.

In conclusion, the close correlation between VEGF-C expression and LVD and between LVD and lymph node metastasis suggested the contribution of VEGF-C and subsequent lymphangiogenesis to the development of lymph node metastasis in breast cancer, while the correlation between the VEGF-A or -C expression and either MVD or LVD suggested the two growth factors to be involved in both the angiogenesis and lymphangiogenesis of breast cancer. Finally, the correlation of the Akt expression to the VEGF-A and -C expression as well as MVD and LVD suggests Akt to play an important role in both angiogenesis and lymphangiogenesis via the VEGF-A and -C expression.

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