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[Original Article]

## Clinicopathological significance of lymphangiogenesis detected by immunohistochemistry using D2-40 monoclonal antibody in breast cancer

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### Abstract

To elucidate the association between the lymphangiogenesis and clinicopathological factors including the survival in breast cancer, 91 Japanese patients with breast cancer were investigated. The lymphangiogenesis was evaluated by the count of lymph vessel density (LVD) with immunohistochemical method using D2-40 monoclonal antibody, a specific marker for lymphatic endothelial cells.

D2-40-positive lymph vessels were detected in 87 of 91 cases, and were mainly distributed in the peritumoral lesions or around the tumor edge. There was a significant difference in disease-free survival (DFS) and overall survival (OS) between patients with high LVD and with low LVD ( $p=0.02$ ,  $0.01$ , respectively, log-rank test). In addition, LVD significantly correlated with the following clinicopathological factors: menopausal status ( $p<0.01$ ), tumor size ( $p<0.01$ ), lymph-node status ( $p=0.01$ ) lymphatic vessel invasion (LVI) ( $p<0.01$ ), blood vessel invasion (BVI) ( $p=0.03$ ) and estrogen receptor status (ER) ( $p=0.02$ ).

Those data suggest that D2-40 monoclonal antibody is a useful marker for evaluating the LVD and its evaluation is helpful to predict the survival in breast cancer.

**Key words:** Breast cancer, Lymphangiogenesis, LVD, D2-40, Immunohistochemistry

### Introduction

Breast cancer is the most common type of cancer in Japanese women, with an estimated 40,000 new cases and the second leading cause of cancer-related deaths in Japanese women, with an estimated 13,000 deaths in 2013. Although chemotherapy is relatively effective for breast cancer after surgical treatment so that it contributes to the survival, some parts of patients still died of the metastasis to distant organs due to the acquisition of chemo resistance. Furthermore, axillary lymph node metastasis is one of the most important prognostic factors for breast cancer<sup>1)</sup>.

In general, cancer metastasis occurs by inva-

sion to blood or lymphatic vessels. It is well known that newly formed blood vessels support haematogenous spread and facilitate tumor growth<sup>2)</sup>, but little is known about the mechanism of lymph node metastasis involved in the lymphatic spread.

Recent studies on the biological role of lymphangiogenic factors, such as vascular endothelial growth factor-C (VEGF-C) and -D (VEGF-D), contribute to elucidate the association between lymph node metastasis and lymphangiogenesis. VEGF-C expression is found in various human tumors, such as breast<sup>3,4)</sup>, colon<sup>5,6)</sup>, lung<sup>4,7,8)</sup>, thyroid<sup>9-11)</sup>, gastric<sup>12)</sup> and squamous cell cancers<sup>4)</sup>, mesotheliomas<sup>13)</sup>, neuroblastomas<sup>14)</sup>, sarcomas<sup>4)</sup> and melanomas<sup>4)</sup>. Furthermore, and expression of VEGF-C mRNA has re-

cently been shown to correlate with the rate of metastasis to lymph nodes in breast<sup>3</sup>, colorectal<sup>6</sup>, gastric<sup>12</sup>, thyroid<sup>9,10</sup>, lung<sup>8</sup> and prostate<sup>15</sup> cancers.

The lymphangiogenesis is usually evaluated by the count of lymph vessel density (LVD) using specific markers for lymphatic endothelial cell, such as vascular endothelial growth factor receptor-3 (VEGFR-3), podoplanin, LYVE-1 are available to detect lymphatic vessels<sup>16</sup>. In breast cancer, LVD detected by specific markers for lymphatic endothelial cells such as VEGFR-3, podoplanin and LYVE-1 is reported to be associated with the survival<sup>17-19</sup>. However, there have not been reports on the evaluation of LVD in breast cancer with immunohistochemical method using a more specific and sensitive marker for lymphatic endothelial cells, D2-40 monoclonal antibody. D2-40 is a kind of monoclonal antibody for podoplanin.

Here we have evaluated the LVD in breast cancer using the D2-40 monoclonal antibody and reported the association between LVD and clinicopathological factor including the survival.

## Materials and Methods

### *Patients and Tumor Samples*

We examined 91 female patients of invasive ductal cancer treated at the Second Department of Surgery of Fukushima Medical University, Japan, between January 1991 through December 1996. All patients underwent modified radical mastectomy or breast partial mastectomy with axillary lymph node dissection. Each patients was treated with suitable adjuvant therapy (chemotherapy or endocrine therapy or none) postoperatively. All protocols were reviewed and approved by the ethics committee of Fukushima Medical University (No. 1202). The patients were staged according to Japanese Breast Cancer Society criteria, which were based on the UICC-TNM classification. We excluded patients who had received prior chemotherapy with distant metastasis at the time of diagnosis. The mean age at time of surgery was 54 year-old (range 30-81 years). Follow-up for the patients included in the survival analysis was updated in June 2006 (median follow-up was 120 months [range 8-179 months]). At that time, 8 patients had local, 27 patients had distant metastasis, and 25 patient had died of breast carcinoma, 66 patients were alive. All tumors were classified with histopathologically as invasive ductal carcinoma of no special type, according to the World Health Organization criteria<sup>20</sup>.

### *Immunohistochemical Staining*

Immunohistochemical staining was performed using an ENVISION method (Dako Cytomation). Serial 5- $\mu$ m thick paraffin-embedded sections were used in this study, and the sections were deparaffinized with xylene and dehydrated with ethanol. No antigen retrieval was required. Endogenous peroxidase was blocked by immersing the slides in 0.3% hydrogen peroxide in absolute methanol for 20 minutes at room temperature. After washing three times in PBS, non-specific staining was blocked by incubation with 5% skimmed milk in PBS and the sections were incubated at 4°C overnight with D2-40 monoclonal antibody (Dako Cytomation, Carpinteria, California; dilution 1:100). After washing three times in PBS for 5 minutes, the sections were incubated with ENVISION+, Mouse/HRP (Dako Cytomation, Carpinteria, California) for 30 minutes at room temperature. The sections were then washed three times in PBS for 15 minutes, and the immune complex was visualized by incubating the sections with 0.2 mg/mL diaminobenzidine and 0.05% (vol/vol) H<sub>2</sub>O<sub>2</sub> in PBS for 5 minutes. The sections were counterstained with hematoxylin and mounted.

### *Evaluation of Immunohistochemistry*

Determination of LVD was performed according to Weidner *et al.*<sup>21</sup>. The immunostained sections were scanned by light-microscopy using a low magnification (40x) and the areas of tissue with the greatest number of distinctly highlighted microvessels ("hot spot") were selected. Microvessel density was then determined by counting all immunostained vessels at a total magnification of 200x corresponding to an examination area of 0.9499 mm<sup>2</sup>. Five hot spots were chosen in each section. Mean values of the microvessels in the five fields were entered into the statistical analysis. Any immunoreactive cells were considered as an endothelial cell and a countable microvessel, even in the absence of a lumen. Two investigators without their knowledge of the clinicopathological findings assessed microvessel counts.

Lymph vessel invasion (LVI) and blood vessel invasion (BVI) was considered evident if at least one tumor cell cluster was clearly visible inside the vascular space. Histological grade was assessed according to Elston-Ellis modification of Scarff-Bloom-Richardson grading system<sup>22</sup>.

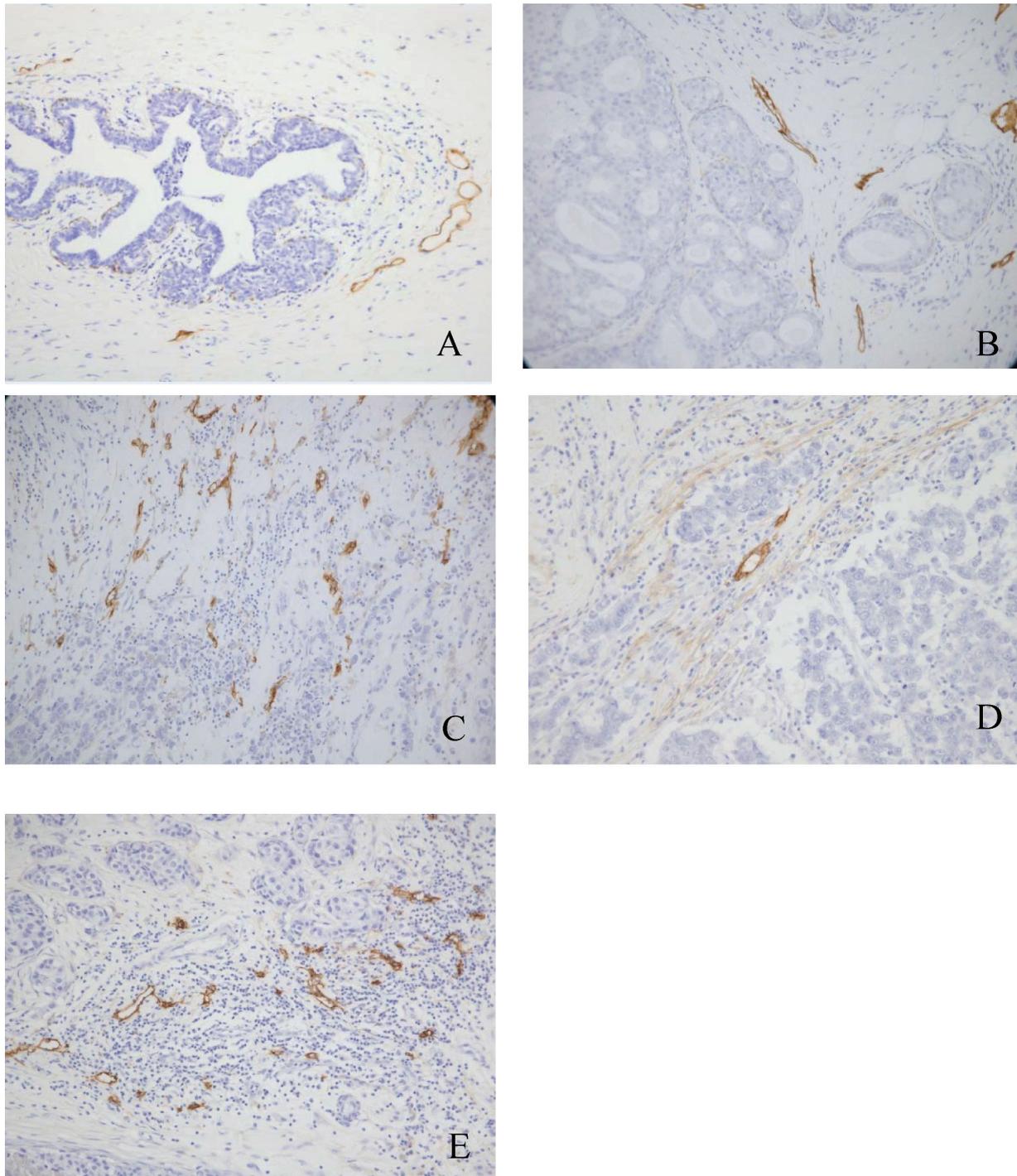


Fig. 1. Immunohistological staining using D2-40 antibody. A) D2-40 positive vessels in normal breast tissue. B) D2-40 positive vessels in periphery of tumor. C) intratumoral area. D) a few D2-40 positive vessels. E) numerous D2-40 positive vessels. Original magnification  $\times 200$ .

### Statistical Analysis

Correlation between LVD and clinicopathologic characteristics was evaluated by unpaired Student's *t* test. Survival rates were analyzed using Kaplan-Meier method and the log-rank test. Overall survival (OS) was defined as the period from primary

surgery until death of the patient. Death from a cause other than breast cancer, or survival until the end of the observation period, was considered censoring event. Disease-free survival (DFS) was defined from the end of primary therapy until first evidence of progression of disease. All these analysis were performed by the statistical package, STAT-

VIEW 5.0. (Abacus Concepts, Berkley, CA,USA). All P values presented were two-sided.

## Result

### *D2-40 expression in breast cancer tissue*

D2-40 positive lymph vessels were detected in 87 of 91 cases. All of the D2-40-positive lymphatic vessels were observed in peritumoral lesions or around the tumor edge (Fig. 1). In normal breast tissue, lymph vessels were seen in interductal stroma or around the blood vessels. Intratumoral lymphatic vessels were lower in number when compared with peritumoral area.

### *Correlation of LVD with clinicopathological factors*

The mean LVD was  $7.24 \pm 5.68$  microvessels/ $\text{mm}^2$  (range 0-25.48). Association between LVD and clinicopathological findings was statistically analyzed (Table 1).

LVD was significantly correlated with menopausal status ( $P < 0.01$ ), tumor size ( $P < 0.01$ ), lymph-node status ( $P = 0.01$ ), LVI ( $P < 0.01$ ), BVI ( $P = 0.03$ ) and estrogen receptor status (ER) ( $P = 0.02$ ). No association was found between LVD and histological grade, progesterone receptor status (PgR) and HER2 status.

### *Prognostic Relevance of LVD*

To analyze the correlation between LVD and patient's prognosis, the patients were divided into two groups; lower LVD and higher LVD. The cut-off point was mean LVD. Kaplan-Meier product limit estimates of disease-free survival (DFS) and overall survival (OS) were plotted in Fig. 2. Patients with higher LVD had a poorer survival than those with lower LVD in both DFS and OS ( $P = 0.02$ , 0.01, respectively, log-rank test).

Table 1. The relationship between LVD and clinicopathological variables in 91 breast cancer cases.

Characteristics	No.	Mean LVD	P-value (t-test)
menopausal status	post	48	0.01
	pre	43	
Tumor size	< 5 cm	85	0.01
	$\geq$ 5 cm	6	
Lymph node status	negative	38	0.01
	positive	53	
Histological grade	HG1, HG2	78	0.14
	HG3	13	
Lymph vessel invasion	negative	53	0.01
	positive	38	
Blood vessel invasion	negative	81	0.03
	positive	10	
ER	negative	23	0.02
	positive	68	
PgR	negative	23	0.11
	positive	68	
HER2	negative	72	0.17
	positive	19	

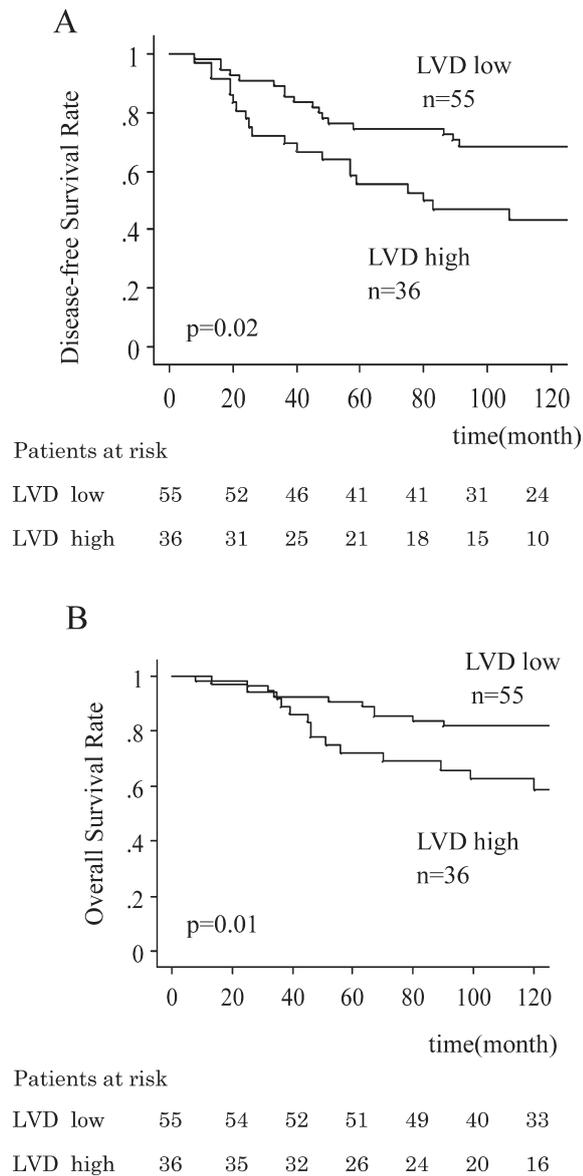


Fig. 2. The analysis of disease-free survival (DFS) and overall survival (OS) in breast cancer according to LVD.

### Discussion

Lymphangiogenesis is considered to be essential to lymph node metastasis in human cancer and is usually evaluated by the count of LVD using specific markers for lymphatic endothelial cells. Axillary lymph node metastasis is known to be one of the most important prognostic factors in breast cancer, and LVD evaluated by using specific markers for lymphatic endothelial cells such as VEGFR-3, podoplanin and LYVE-1 has been reported to be associated with the survival in breast cancer<sup>17-19</sup>. However, there have not been reports on the evaluation of LVD in breast cancer using a D2-40 monoclonal an-

tibody, more specific and sensitive marker for lymphatic endothelial cells<sup>23</sup>. D2-40 is a fixation-resistant epitope on a 40 kDa O-linked sialoglycoprotein, which may be identical to podoplanin<sup>24</sup>, expressed in lymphatic endothelium but not in blood vessels<sup>25,26</sup>. Therefore, we used D2-40 to investigate the evaluation of LVD in breast cancer.

In the present study, D2-40-positive lymphatic vessels were detected in almost all the breast cancer cases (87 of 91 cases), and were predominantly observed in the peritumoral lesions or around the tumor edge, but a little in the intratumoral lesions. The results indicate that D2-40 monoclonal antibody is a useful marker for detection of lymph vessels. The distribution pattern of lymph vessels seems to be common in many cancers, especially in breast cancer (18, 19, 27). Padera *et al* reported that the absence of intratumoral lymph vessels may result from compressing and/or destructing lymphatics by proliferating tumor cells<sup>28</sup>. However, the reason for a little intratumoral lymphatic vessels has been debated until now<sup>28-32</sup> and still remains unclear.

There have been several different reports about the correlation between the LVD and clinicopathological factors or patients' survival in breast cancer<sup>17-19,27,29,33-36</sup>.

Some reports showed positive correlation between LVD and tumor aggressiveness or poor prognosis<sup>17-19,35</sup>, and the other displayed no correlation between them<sup>27,29,33,34,36</sup>. Although the reason of the discrepancy is not enough discussed, it may be due to the difference in specificity and sensitivity of specific markers used for lymph vessels, selection of "hot spot" areas and cut-off point.

In the present study, our data indicated the significant association between LVD and lymph node metastasis and LVI. LVD is also correlates with other prognostic factor, menopausal status and ER status. Pre-menopausal and ER-negative breast cancer patients is usually have a poorer prognosis than those of Post-menopausal and ER-positive patients. Furthermore, patients with high LVD showed poor prognosis by comparison to those with low LVD. The process of lymphatic metastasis is thought to involve proliferation of lymphatic vessels (lymphangiogenesis), lymphatic invasion and lymph node metastasis. Our findings that high LVD was associated with lymph node metastasis, LVI and poor prognosis suggest an important role of lymphangiogenesis in the progression of breast cancer.

In conclusion, D2-40 monoclonal antibody may be a useful marker to evaluate the lymphangiogenesis, and its evaluation is helpful to predict the prog-

nosis or survival.

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