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Original Article

Lymphangiogenesis in breast cancer is associated with non-sentinel lymph node metastases in sentinel node positive patients

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Abstract: Axillary lymph node dissection (ALND) is not suggested in breast cancer patients with negative sentinel lymph node (SLN) biopsies, and SLN is the only positive node in 40-70% of the remaining cases. To distinguish a subgroup in which ALND would be omitted, we investigated the role of lymphangiogenesis in primary breast cancer as a risk factor for distal lymph node involvements in patients with positive SLNs. 86 patients were included in this study. The frequency of proliferative lymphatic endothelial cells (LECP%) was evaluated in each specimen after immunohistochemical double staining for D2-40 and Ki-67. Larger primary tumor size, increased number of positive SLNs, lymphatic vessel invasion and LECP% were significantly associated with non-SLN metastases in the univariate analysis, but only LECP% retained significance in the multivariate model. A positive correlation between LECP% and lymphatic vessel invasion was also revealed. Our study confirmed the important role of lymphangiogenesis in tumor spread, and suggested that LECP% is a promising predictor for additional axillary lymph node involvements.

Keywords: Lymphangiogenesis, sentinel lymph node, metastasis, breast cancer

Introduction

Sentinel lymph node (SLN) surgery has become the standard of care for axillary staging of women with clinically node-negative (cN0) breast cancer. The rationale for performing this procedure is used to guide local, regional, and systemic treatment decisions [1, 2]. When the SLN is positive, axillary lymph node dissection (ALND) has been the procedure of choice, which carries the risk of arm and shoulder morbidity (e.g., pain, lymphedema, shoulder dysfunction and sensory loss) [3, 4]. However, in patients with positive SLNs, approximately 40-70% does not have involvement of the non-sentinel axillary lymph nodes (non-SLN) [5, 6]. These patients would not benefit from ALND but may suffer complications of the procedure. Several attempts have been made to identify cohorts of women with involved SLNs who have a low risk for non-SLN involvement that complete axillary dissection might be avoided [7]. However, none of the early studies identified a low-risk group of patients with positive SLN biopsies but consistently negative non-SLNs [8-10].

The lymphatic vasculature is an important route for metastatic spread of human breast cancer [11]. And lymphangiogenesis is a process that promotes tumor metastasis by remodeling and enlarging the lymphatic system [12]. Double immunostaining of lymphatic markers combined with proliferative markers facilitate the detection of newly-formed lymphatic vessels, which represent the ongoing procedure of lymphangiogenesis. In primary breast tumors, the role of lymphangiogenesis in tumor spread has been shown in our early study and many other studies [13, 14]. However, whether lymphangiogenesis is also involved in the formation of distal lymph node metastases, remains to be elucidated. The aim of this study was to investigate the association of lymphangiogenesis with the involvement of non-SLNs in patients with SLN positive breast cancer, allowing for a selection of cases in which ALND would be omitted.

Material and methods

Patients and specimens

Consecutive female patients with breast cancer aged 30-70 years undergoing SLN biopsy and

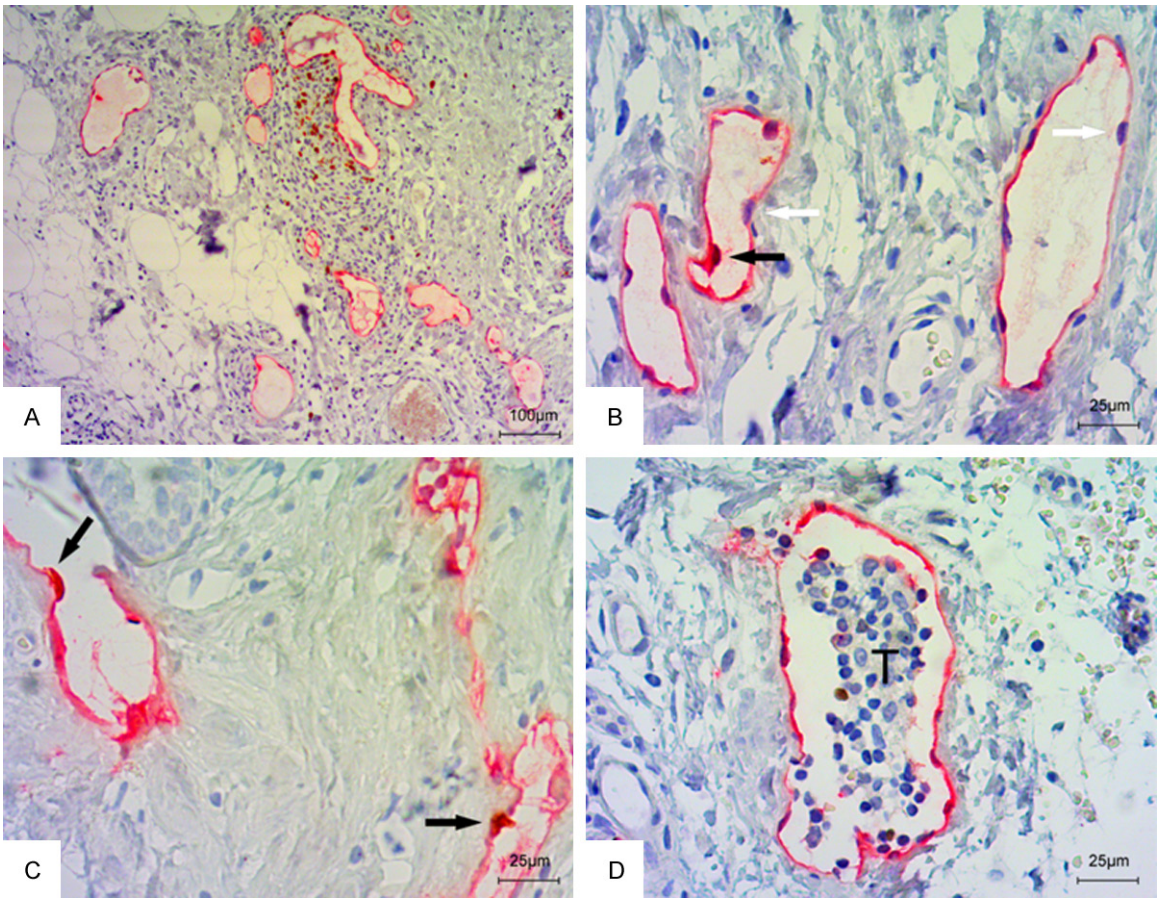

Figure 1. Immunohistochemical double staining for D2-40 and Ki-67. A: Area in high lymphatic vessel density (red) around the tumor at low magnification. B and C: Double staining for D2-40 (red) and Ki-67 (brown) of lymphatic vessels at high magnification; Both Ki-67 positive (black arrows) and negative (black arrows) nuclei of lymphatic endothelial cells were seen. D: Infiltrating tumor cell cluster (T) was observed inside the D2-40 positive lymphatic vessels.

ALND with at least one positive SLN were included in this study. All patients were diagnosed and treated in the Department of Surgical oncology, the First Affiliated Hospital of Wenzhou Medical University in 2013. The following patients were excluded: 1) with in situ, bilateral or multifocal carcinoma; 2) with inflammatory breast cancer or accompanied with mastitis; 3) with distant metastases; 4) with preoperative neoadjuvant therapy; or 5) with the number of non-SLNs less than 10. Finally, 86 patients were eligible for this study, and they were divided into two groups according to the metastatic status of non-SLNs (non-SLN^+ and non-SLN^-). The corresponding paraffin-embedded archival specimens of primary tumor were obtained, and 3.5 μm sections were cut for subsequent immunohistochemical staining. Indications for SLN biopsy were according to the criteria of NCCN guideline (version1 2012), and methylene blue was applied in SLN mapping. The SLNs were routinely examined with intraoperative frozen sections and non-SLNs with standard hematoxylin-eosin staining slides. The histological type was classified with the criteria of the World Health Organization and the histological grade was evaluated using the Nottingham grading system. This study was approved by the clinical ethical committee of First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China, and written informed consent was obtained from each patient.

Immunohistochemistry

Immunohistochemical double staining for D2-40/Ki-67 was performed as previously described with the following changes [13]. Briefly, antigen retrieval was performed using high pressure method in Tris-EDTA buffer (pH = 8.0). After being blocked with Klear Dual

Table 1. Clinicopathologic characteristics of subjects with or without involvement of non-SLNs

Characteristics	non-SLN$^-$ (n = 47)	non-SLN$^+$ (n = 39)	*P* value
Age (yr), mean ± SD	51.7 ± 9.2	49.8 ± 8.7	0.323
Tumor size (cm), mean ± SD	2.0 ± 0.8	2.4 ± 1.0	0.041*
Number of SLNs, median (range)			
Positive	1 (1-4)	2 (1-5)	0.023*
Total	4 (1-9)	5 (1-9)	0.569
Number of non-SLNs, median (range)			
Positive	0 (0-0)	4 (1-24)	-
Total	14 (10-22)	15 (10-27)	0.072
LECP% (%), median (range)	3.9 (0-17.4)	7.5 (0-23.6)	0.011*
Histological type			
IDC	41	35	0.981
Others	6	4	
Histological grade			
G1	11	4	0.087
G2	21	17	
G3	15	18	
Lymphatic vessel invasion			
Positive	20	26	0.026*
Negative	27	13	

*$P < 0.05$. IDC, invasive ductal carcinoma.

Enzyme Block Kit (GBI Labs, USA), sections were incubated with primary antibodies of Ki-67 (ZSGB-BIO, China) and D2-40 (ZSGB-BIO, China) synchronously. Both antibodies were detected and visualized with the Polymer Mo/AP + Rb/HRP Detection Kit (ZSGB-BIO, China). Finally, sections were counterstained with Mayer's hematoxylin. Sections incubated with phosphate-buffered saline instead of primary antibody were set as negative control.

Assessment of lymphangiogenesis

To evaluate lymphangiogenesis, we calculated the lymphatic endothelial cell proliferation fraction (LECP%) by counting the D2-40 +/Ki-67 + and D2-40 +/Ki-67 - cells (**Figure 1A-C**) [15, 16]. All the lymphatic vessels at tumor periphery were evaluated. And infiltrated tumor cells or inflammatory cells were excluded as Mohammed RA et al. described [17]. Two investigators (Zhang and Huang) blinded from clinical data performed the assessment independently, and the mean of the results was used for further analysis. In addition, lymphatic vessel invasion was reassessed in the whole area of the section and characterized as positive if any tumor cell cluster was observed inside the lymphatic vessels (**Figure 1D**).

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistic v21.0 (IBM Co., USA). Continuous variables with normal distribution were expressed as the mean ± standard deviation (SD), and with abnormal distribution as median (range). The independent t-test or the Mann-Whitney U-test was used as appropriate. Categorical variables were tested using the Pearson Chi-Square test or the Mann-Whitney U-test. The receiver operating characteristic (ROC) curve analysis was used to find a cutpoint of LECP%. Multivariate analysis for non-SLN metastases was conducted using the binary logistic regression model. $P < 0.05$ was considered statistically significant and all *P* values are 2-tailed.

Results

Clinicopathologic data

In the whole 86 patients with positive SLNs, 39 (45.3%) were revealed with non-SLN involvements. **Table 1** shows the characteristics of age, tumor size, numbers of SLNs and non-SLNs as well as their metastatic status, histological type and histological grade between non-SLN$^-$ and non-SLN$^+$. In univariate analysis, patients with additional axillary lymph node metastases showed a significantly larger primary tumor size ($P = 0.041$) and an increased number of positive SLNs ($P = 0.023$) compared with those without non-SLN involvements. In this study, lymphatic vessel invasion was observed in 46 (53.5%) tumor samples, which also showed significant association with non-SLN metastases ($P = 0.026$). Besides, no differences were revealed between the two groups in mean or distribution of other variables.

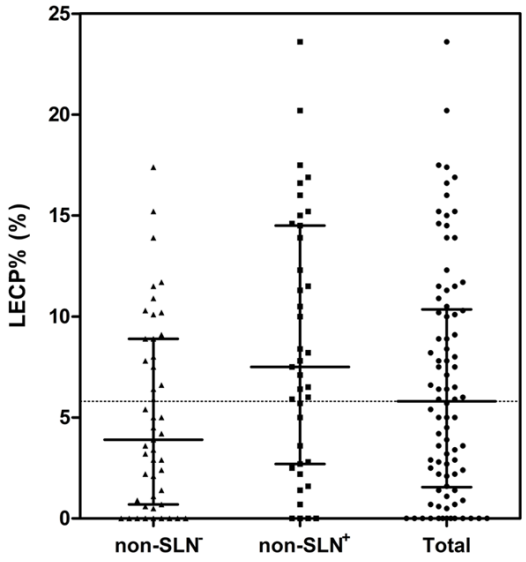

Figure 2. Scatter diagram of LECP% in patients with or without non-SLN metastasis. Median with interquartile range is marked in each group. The non-SLN$^+$ group showed a significantly higher LECP% compared with the non-SLN$^-$ group (P = 0.011).

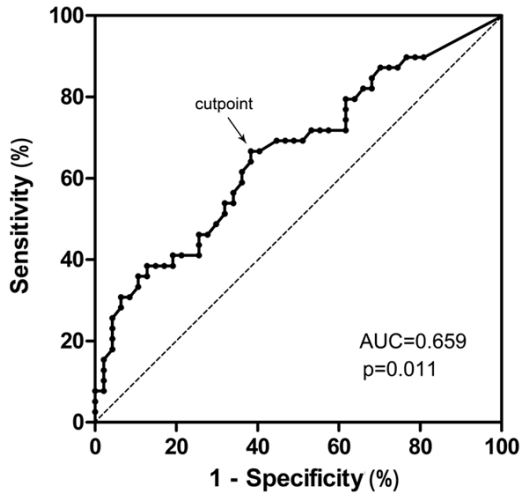

Figure 3. Receiver Operating Characteristic (ROC) curve of LECP%. An optimal cutpoint (5.6%) was found with a maximum Youden index (28.4). The area under the ROC curve is 0.659 (P = 0.011).

Lymphangiogenesis

More than 200 cells were counted in every section. Proliferative lymphatic endothelial cells were detected in 73 (84.9%) specimens and the median value of LECP% (%) was 5.8 (range, 0-23.6). With the corresponding number being 7.5 (range, 0-23.6), compared with 3.9 (range, 0-17.4) in the counterpart, the non-SLN$^+$ group was significantly associated with more proliferating lymphatic vessels (P = 0.011). **Figure 2** demonstrated the distribution of LECP%. A positive correlation between LECP% and lymphatic vessel invasion was also revealed (P = 0.022). Using the ROC curve analysis, we determined an optimal cut-off value of LECP% (%) being 5.6 based on the Youden index. The area under the ROC curve is 0.659 (P = 0.011) (**Figure 3**). All the 4 variables associated with non-SLN metastases, as described above, were dichotomized and analyzed using binary logistic regression model (**Table 2**). Intriguingly, only LECP% (P = 0.035) retained significance when adjusted to other factors.

Discussion

With innate capacity for cell transportation, the lymphatic system provides many advantages for tumor cell dissemination [18]. And the process of lymphangiogenesis has been extensively studied. Previous researches including our early study indicated that increased lymphangiogenesis was significantly correlated with lymphatic vessel invasion, lymph node metastasis and poor prognosis in breast cancer patients [13, 17, 19, 20]. And results from studies on lymphangiogenic mediators, such as VEGF-C and VEGF-D, were in accordance with these findings [21, 22]. Moreover, serial studies by Van den Eynden et al. revealed that proliferative lymphatic vessels existed both in primary site and involved lymph nodes, and lymphangiogenesis in positive lymph nodes also contributed to further dissemination [16, 23, 24]. However, the relationship between lymphangiogenesis and the extent of lymphatic metastasis remains unexplored. Therefore, we investigated the role of lymphangiogenesis in primary breast cancer for additional axillary lymph node metastases in patients with positive sentinel nodes.

In the present study, lymphangiogenesis was evaluated by LECP%, a technique measuring the frequency of dividing lymphatic endothelial cells which representing the tumor-induced and newly-formed lymphatic vessels rather than the pre-existing ones. To reduce confounding in assessment, LECP% was only scored in peritumoral areas, in which consistently related to metastatic spread, whereas the existence and

Table 2. Logistic regression model for non-SLN metastasis

Variables	Odds ratio	*P* value
LECP% (≤ vs. > 5.6%)	2.73	0.035*
Positive SLNs (≤ vs. >1)	2.14	0.109
Tumor size (≤ vs. > 2 cm)	1.42	0.475
LVI (positive vs. negative)	2.26	0.092

*$P < 0.05$. LVI, lymphatic vessel invasion.

functionality of lymphatic vessels in intratumoral compartment have been controversial [25, 26]. Our results demonstrated that increased lymphangiogenesis was significantly associated with lymphatic vessel invasion and non-SLN metastases, allowing for identifying subgroups with different metastatic risks. Importantly, it was the only factor retaining significance in the multivariate model, suggesting that LECP% is a promising predictor for additional metastases in SLN positive patients. We further confirmed the pivotal role of lymphangiogenesis in tumor cell dissemination, from lymphatic vessel invasion to SLN metastasis and to non-SLN involvement.

Tumor-associated lymphatic vessels were served as escape highways for malignant cells. As previously described, lymphangiogenic vessels were enlarged, tortuous and lack of continuous basement membrane [27]. Elevated lymphatic fluid fluxes at tumor periphery were observed in different xenograft models [28, 29]. Studies in a murine model of fibrosarcoma also showed that enhanced lymphangiogenesis led to a 200-fold increase in cancer cell dissemination and a 4-fold increase in lymph node metastasis [28]. Combined, lymphangiogenesis greatly expedited tumor cells and tumor-derived factors transporting into the draining system. Moreover, those neogenic lymphatic vessels combined with the cargoes also played an active role in pre-metastatic niche preparation. First, lymphangiogenesis in SLNs and non-SLNs were further stimulated prior to metastasis by several factors [30, 31]. Second, peripheral tolerance was established by constantly exposing tumor antigens to those tolerogenic and immature antigen presenting cells in local lymph nodes, or with the help of lymphatic endothelial cells by cross-presentation [32, 33]. Since both the mechanical barrier and immunologic barrier were impaired, malignant cells were prone to go farther beyond the SLNs once the lymphatic metastasis occurred.

Although lymphangiogenesis was considered a crucial factor in tumor metastasis, LECP% alone was not an adequate biomarker for clinical use due to the unfavorable sensitivity and specificity in the ROC curve analysis. A standardized protocol in immunostaining, including a rigorous quality control system, should be established to reduce deviations between operators and laboratories. Intratumoral heterogeneity of lymphatic vessels should also be noticed, and evaluation on serial sections is responsible for a credible and consistent result. Of note, as different mechanisms participate in the process of metastasis, developing a novel method to distinguish a subpopulation in which lymphangiogenesis plays a dominant role will be of vital importance.

Conclusion

Our study confirmed the important role of lymphangiogenesis in tumor spread, and revealed that increased LECP% in primary tumor is significantly associated with lymphatic vessel invasion and non-SLN metastasis in breast cancer patients with positive SLNs. LECP% is a promising predictor for additional lymph node involvement, albeit it alone is not ready for clinical use.

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Disclosure of conflict of interest

None.

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