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# VEGF121 promotes lymphangiogenesis in the sentinel lymph nodes of non-small cell lung carcinoma patients

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**KEYWORDS**
Lymphangiogenesis;
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**Summary**
*Background:* The sentinel lymph node (SLN) concept is that lymphatic flux from a primary tumor initially flows into a SLN. The mechanism mediating tumor metastasis within SLNs remains largely unknown; however, primary tumors overexpressing vascular endothelial growth factor (VEGF)-A appear to induce SLN lymphangiogenesis prior to metastasis in animal model. Our aim was to further investigate the capacity of VEGFs to induce lymphangiogenesis within SLNs and to assess their role in SLN metastasis in non-small cell lung carcinoma (NSCLC).
*Methods:* Real-time quantitative RT-PCR was used to assess expression of mRNAs encoding several VEGFs (VEGF121, VEGF165, VEGFR1, VEGFR2, VEGFR3, VEGF-C and VEGF-D) in resected lymph node specimens from 35 NSCLC patients, after which we compared their expression SLNs and non-SLNs. In addition, expression of the lymphatic endothelium-specific hyaluronan receptor (LYVE)-1 was used to assess lymphangiogenesis in SLNs and non-SLNs.
*Results:* Immunohistochemical staining revealed substantial expression of LYVE-1 in SLNs. Moreover, levels LYVE-1 mRNA were significantly higher in SLNs than non-SLNs ($P < 0.05$), as were levels of VEGF121 and VEGFR2 mRNA ($P < 0.01$ and $P = 0.02$, respectively). In addition metastasis-positive SLNs showed significantly higher levels of VEGF121, VEGF-C and VEGF-D mRNA than metastasis-negative SLNs ($P < 0.001$, $P = 0.01$ and $P = 0.01$, respectively), and VEGF121 induced the proliferation of lymphatic endothelial cells ($P < 0.01$).
*Conclusions:* Our findings suggest that active lymphangiogenesis is ongoing within SLNs from NSCLC patients, even before metastasis. This lymphangiogenesis may be promoted by upregulation of VEGF121, which may in turn act in part via induction of VEGF-C.


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## 1. Introduction

Lung cancer is the most common cause of cancer death throughout the world, and despite recent improvements in its treatment, the prognosis for lung cancer patients remains poor. In non-small cell lung carcinoma (NSCLC) the pathological staging is a key determinant of a patient's prognosis and treatment options. Among several prognostic factors, the status of lymph node metastasis is the most reliably predictive of a patient's prognosis. Even in patients with stage IA NSCLC, the recurrence rate after complete resection is 30—40%, and it is well over 50% when lymph node involvement is detected [1,2].

Little is known about the mechanism mediating lymph node metastasis. Evidence from animal tumor models indicates that increased levels of VEGF-C or VEGF-D promote tumor lymphangiogenesis [3—6], while VEGF-A reportedly promotes human lymphatic endothelial cell proliferation and migration [7]. Beyond that, however, the precise mechanism by which lung tumors metastasize to the lymph nodes remains unknown. One of the reasons for this is that there is currently no clinical model with which to analyze the mechanism of lymph node metastasis. To begin to address this issue, we have been analyzing the role of the sentinel lymph node (SLN) in NSCLC. The SLN concept is that lymphatic flux from a primary tumor initially flows into the SLN. The SLN is thus the first region influenced by products released from the tumor, making it particularly suitable for the studying the pathogenesis of lymph node metastasis. In the present study, we identified the SLN during surgery using a magnetic tracer and then examined the expression levels of various VEGFs in SLNs and non-SLNs and assessed their association with lymphangiogenesis and metastasis.

## 2. Patients and methods

### 2.1. Patients

This study was approved by the Human Ethics Committee of Akita University Hospital, Akita, Japan. Thirty-five patients with NSCLC were enrolled in the study between 2004 and 2005 after obtaining signed, informed consent. None of the patients received preoperative chemotherapy or radiotherapy. The clinical features of these patients are summarized in Table 1. The pathological stage was diagnosed by a pathologist using hematoxylin—eosin staining. SLN micrometastases were observed in five patients.

**Table 1** Patient characteristics

| | |
|---|---|
| Patients | 35 |
| Gender | |
| Male | 22 |
| Female | 13 |
| Age | |
| Mean | 70.2 ± 9.75 |
| Range | 46—82 |
| Pathological stage | |
| Ia | 19 |
| Ib | 10 |
| IIa | 1 |
| IIb | 1 |
| IIIa | 1 |
| IIIb | 2 |
| Histology | |
| Adenocarcinoma | 25 |
| Squamous cell carcinoma | 7 |
| Large cell carcinoma | 2 |
| Carcinoid | 1 |
| Tumor location | |
| Right upper lobe | 11 |
| Right middle lobe | 7 |
| Right lower lobe | 5 |
| Left upper lobe | 7 |
| Left lower lobe | 5 |
| Serum CEA (ng/ml) | |
| Mean | 6.94 ± 7.36 |
| Range | 0.5—28.7 |
| Tumor size (cm) | |
| Mean | 2.65 ± 1.03 |
| Range | 1.0—5.0 |
| Grade | |
| Well | 16 |
| Moderate | 6 |
| Poor | 13 |

### 2.2. SLN identification

SLNs were identified using the method developed at our institute [8,9]. Briefly, a magnetic tracer (ferucarbotran) was injected around the lung tumor during surgery, after which we waited 15 min before proceeding. We then measured the magnetic force within the lymph nodes using a highly sensitive hand-held magnetometer, and nodes in which magnetic force was detected were deemed to be SLNs. Non-SLNs served as controls. The definition of a non-SLN in this study was a magnetic force-negative lymph node from a matched station level based on the classification of Naruke et al. [10]. The locations of the SLNs detected by our method are listed in Table 2.

### 2.3. Immunohistological staining

SLNs and non-SLNs were fixed in formalin, embedded in paraffin and cut into 5-μm-thick sections. The sections were then deparaffinized in xylene and ethanol, placed in 0.1 mol/L citrate buffer (pH 6.0) and irradiated with microwaves (750 W) for 15 min. The primary antibody used was goat polyclonal anti-LYVE-1 (E-20, Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution in PBS), which was selected because LYVE-1 is a receptor expressed almost exclusively on lymphatic endothelial cells [11]. In addition, nuclei were stained using Cellstain DAPI (Wako, Osaka, Japan; 1:200 dilution in PBS). We then observed the lymphatic vessels within the lymph nodes using laser scanning microscopy (Olympus BX51, Tokyo, Japan).