Changes in the tumor microenvironment during lymphatic metastasis of lung squamous cell carcinoma

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Metastasis and growth in neoplastic lesions requires the multistep regulation of microenvironmental factors. We aimed to elucidate the microenvironmental changes in the process of lymphatic metastasis of lung squamous cell carcinoma. We examined the morphological characteristics of 102 cases of primary tumor (PT), 50 of intralymphatic tumor (ILT), 51 of lymph node (LN) micrometastasis (LN-Mic; ≤ 2 mm in size), and 82 of LN macrometastasis (LN-Mac; ≥ 10 mm in size). Afterwards we evaluated the expression of nine molecules (epidermal growth factor receptor, fibroblast growth factor receptor 2, CD44, aldehyde dehydrogenase 1, Podoplanin, E-cadherin, S100A4, geminin, and ezrin) in matched PT, ILT, LN-Mic, and LN-Mac from 23 of these cases. The number of smooth muscle actin–positive fibroblasts, CD34-positive microvessels and CD204-positive macrophages were also examined. As a result, the mitotic index of tumor cells was significantly lower in ILT and LN-Mic than PT and LN-Mac (P < 0.001). Moreover, stromal reaction in ILT and LN-Mic was less prominent than in PT and LN-Mac (P < 0.001). Immunohistochemical study revealed that epidermal growth factor receptor expression level and frequency of geminin-positive cells in ILT and LN-Mic were significantly lower than in PT and LN-Mac (P < 0.05). The number of smooth muscle cells indicated by staining of CD34, CD204, and smooth muscle actin α in ILT and LN-Mic was also significantly lower than in PT and LN-Mac (P < 0.05). In lung squamous cell carcinoma, drastic microenvironmental changes (e.g., growth factor receptor expression and proliferative capacity of tumor cells and structural changes in stromal cells) occur during both the process of lymphatic permeation and the progression into macrometastases.

Despite advances in therapy, the prognosis of patients with advanced NSCLC remains poor with a 5-year survival rate of <20%.1 Distant metastasis is the most important prognostic factor and play a major role in choice of treatment. Squamous cell carcinoma accounts for approximately 30% of lung cancer and is the second most common histological type behind adenocarcinoma.2 Lung SqCC has a lower rate of distant metastasis than other histological type, but not of LN metastasis. Furthermore, local recurrence including LN is more frequent after the resection of early stage of lung SqCC than adenocarcinoma.3,4 As lymph node metastasis is an important prognostic factor,5 an improved knowledge of the mechanism of lymphatic metastasis in lung SqCC might be of potential clinical benefit.

Metastasis is a complex multistep process. In the PT, tumor cells detach from their surrounding cells by EMT and intravasate into blood or lymphatic vessels. The tumor cells float in the circulation and some of them survive without anoikis. The cells adhere to the endothelium, extravasate, and begin proliferation. To facilitate an increase in tumor size, induction of angiogenesis and several microenvironmental factors are required.6,7 Previously, Kirit et al.8 reported that intralymphatic adenocarcinoma cells expressing low levels of ALDH1, high levels of SOX2, and a high number of infiltrating CD204-positive macrophages have a critical impact on LN metastasis. Therefore, it is suggested that the ILT microenvironment is an important determinant of LN metastasis.

Comparative transcription analysis has previously identified a different pattern of gene expression in LN metastasis compared to PT.9–11 Wang et al.12 reported that, in NSCLC, the expression of E-cadherin and Twist is lower and higher, respectively, in metastatic lesions than in the primary lesion. This result suggests that the tumor cells change from the epithelial to mesenchymal phenotype in metastatic lesions.

Although there are many previous reports of LN-Mic, its influence on prognosis is yet to be determined.13–15 There may also be differences in histological and phenotypic features between LN-Mic and LN-Mac. Recently, Aramaki et al. focused on the morphological and phenotypic differences between LN-Mic and...
LN-Mac in lung adenocarcinoma cases and found that dynamic microenvironmental changes occur during the growth of LN-Mic into LN-Mac. These included EMT-related changes in tumor cells and structural changes in stromal cells.\(^{16}\)

To explain how lung SqCC in the metastatic site acquires dynamic molecular changes, characteristics of ILT and the size of LN metastasis should be considered. In this study, to address the molecular mechanisms of lymphatic metastasis and growth of LN metastasis, we compared morphological and immunophenotypic features of the tumor cells and the stromal cells in the PT, ILT, LN-Mic (≤2 mm) and LN-Mac (≥10 mm) as a large cohort of patients.

### Materials and Methods

**Case selection.** A total of 102 consecutive patients with surgically resected lung SqCC harboring lymphatic permeation, LN-Mic, or LN-Mac seen at our hospital between April 1994 and April 2014 were enrolled in this study (Fig. S1, Table S1). We defined LN metastasis with a maximum diameter ≤2 mm as LN-Mic; LN metastasis with a maximum diameter ≥10 mm was defined as LN-Mac. Morphological characteristics were microscopically analyzed in 102 PTs, 50 ILTs, 51 LN-Mics, and 82 LN-Macs (Fig. 1).

All the surgical specimens were collected and analyzed after receiving the approval of the Institutional Review Board of the National Cancer Center Hospital East (Kashiwa, Japan).

**Compliance with ethical standards.** All procedures involving human participants were carried out in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All work reported herein was undertaken at the National Cancer Center Hospital East, Kashiwa, Chiba, Japan and approved by the Internal Review Board of the institution. Informed consent was obtained from all individual participants included in the study.

**Histological studies.** Surgical specimens were fixed in 10% formalin or methanol, embedded in paraffin, and sectioned to 4-μm thickness sequentially. The sections were stained using the H&E method. All the histological materials in this study were reviewed by two pathologists (S.I. and G.I.). The pathological stage was judged based on the TNM classification of the Union for International Cancer Control, 7th edition.\(^{5}\) Histological features of each lesion were judged based on the WHO classification of cell types, 3rd edition.\(^{17}\)

We investigated stratified differentiation, necrosis, mitosis, and stromal reaction in each lesion. Stroma is defined as organic change including fibrosis of surrounding tumor cells. Cases were deemed positive if the stromal component accounted for more than 10% of the entire tumor. Isolated single tumor cells and clusters composed of fewer than five tumor

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**Fig. 1.** Schema of multistep process of lymphatic metastasis in primary lung cancer. In the primary tumor (PT), tumor cells detach from their surrounding cells and intravasate into lymphatic vessels. The tumor cells float on lymphatic flow. The cells adhere to the endothelium, extravasate, and start reproliferation in the lymph nodes (LNs). (a, e) PT; (b, f) ILT; (c, g) LN-Mic (≤2 mm); (d, h) LN-Mac (≥10 mm).
cells were defined as “budding cells” and grading of budding was not assessed.

**Antibodies and immunohistochemical staining.** We selected 23 cases of lung SqCC all with lymphatic vessel permeation (evaluated by immunohistochemical staining with D2-40 protein), LN-Mic, and LN-Mac from the 102 lung SqCC cases described above (Table S2).

Immunohistochemical staining was carried out according to the methods previously reported. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series, and endogenous peroxidase was blocked with 3% hydrogen peroxide in absolute methyl alcohol. After epitope retrieval, the slides were washed with PBS and incubated overnight at 4°C using primary antibodies at their final dilution in the blocking buffer. The primary antibodies used in this study are summarized in Table S3. The slides were washed again and incubated with EnVision (Dako, Glostrup, Denmark) for 1 h at room temperature. The reaction products were stained with diaminobenzidine; finally, the slides were counterstained with Meyer hematoxylin.

**Immunohistochemical scoring.** All the stained tissue sections were scored semiquantitatively and evaluated independently under a light microscope by two pathologists (S.I. and G.I.). The immunostaining scores for EGFR, FGFR2, E-cadherin, CD34, we counted the number of positive macrophages or cells per number of tumor cells counted. For CD204 and S100A4, CD44 (variant), ALDH, D2-40, and ezrin were evaluated based on the staining intensity and the percentage of tumor cells that were stained. The following scoring system was described based on the staining intensity, producing scores was from 0 to 200. The average staining score for geminin was simply the percentage of positive tumor cells, and we also undertook negative control studies without the primary antigen for all the antibodies.

The results of pathological analyses of 102 samples of PT, 50 of ILT, 51 of LN-Mic, and 82 of LN-Mac are summarized in Figure 2. The median mitotic index values were 15/HPF, 0/HPF, 1/HPF, and 11/HPF, respectively. The percentages of cases with necrosis were 99.0%, 18.0%, 51.0%, and 97.6%, respectively; 27.5%, 0.0%, 0.0%, and 14.6% had budding cells, respectively, and 99.0%, 2.0%, 37.3%, and 100.0% showed stromal reaction, respectively. The median mitotic index values and the percentage of necrosis, budding cells, and stromal reaction were all significantly less in ILT and LN-Mic than PT and LN-Mac (P < 0.001).

**Immunohistochemical scores of tumor cells.** We selected samples from 23 lung SqCC patients who all harbored lymphatic vessel permeation, LN-Mic, or LN-Mac and stained 12 molecular markers.

**Growth factor receptors.** The average staining score for EGFR in PT, ILT, LN-Mic, and LN-Mac was 86.5, 63.9, 58.7, and 80.0, respectively. The EGFR expression level in PT was

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**Fig. 2.** Morphological features of tumor cells in primary tumor (PT), intralymphatic tumor (ILT), lymph node micrometastasis (LN-Mic), and lymph node macrometastasis (LN-Mac). (a) Proportion of cases with necrosis. (b) Median mitotic index. (c) Budding cells. (d) Stromal reaction. (a, c, d) Fisher’s exact test. (b) Mann–Whitney’s U-test.
significantly higher than in ILT and LN-Mic (Wilcoxon signed-rank test, \( P = 0.0164 \) and 0.0344, respectively; Fig. 3a). Expression of EGFR in LN-Mic was significantly lower than in LN-Mac (\( P = 0.0476 \)). The average staining score for FGFR2 in PT, ILT, LN-Mic, and LN-Mac was 33.3, 18.7, 27.6, and 35.2, respectively. The FGFR2 expression level in ILT was significantly lower than in PT (\( P < 0.001 \)).

Epithelial–mesenchymal transition-related markers. There was no significant difference between each lesion type in the expression level of E-cadherin and S100A4.

Cancer stem cell-related molecules. There was no significant difference between each lesion type in the expression level of CD44, ALDH, and D2-40.

Others. The average staining score for geminin in PT, ILT, LN-Mic, and LN-Mac was 32.3%, 20.0%, 25.9% and 35.1%, respectively. The geminin expression level in PT was significantly higher than in ILT and LN-Mic (\( P < 0.001 \) and 0.0307, respectively; Fig. 3b). Geminin expression in LN-Mic was significantly lower than in LN-Mac (\( P = 0.0365 \)). There was no significant difference between each lesion type in the expression level of ezrin.

Immunophenotype of stromal cells. Microvessel density. The average number of vessels formed by CD34-positive endothelial cells in PT, ILT, LN-Mic, and LN-Mac was 7.8, 0.0, 1.9, and 4.6, respectively. There were significant differences in microvessel density between each lesion type (\( P < 0.001 \); Fig. 4).

M2 macrophage density. The average number of CD204-positive TAMs in PT, ILT, LN-Mic, and LN-Mac was 29.3, 2.8, 5.3, and 24.4, respectively. The density of CD204-positive TAMs in PT and LN-Mac was significantly higher than that in ILT and LN-Mic (\( P < 0.001 \) and \( P < 0.001 \), respectively; Fig. 4f). There was no significant difference between each lesion type in the expression level of ezrin.

Discussion

In this study, we compared the histological features and the immunohistochemical staining of PT, ILT, LN-Mic, and LN-Mac of lung SqCC in order to elucidate the mechanism of lymphatic metastasis. We found that the proliferative capacity of tumor cells in ILT and LN-Mic was significantly lower than in PT and LN-Mac. Moreover, stromal reaction was less prominent in ILT and LN-Mic. This is the first report that has examined structural and phenotypical changes of lung SqCC in the course of lymphatic metastasis.

By histological analysis, the mitotic indices of ILT and LN-Mic were significantly lower than PT and LN-Mac. This phenomenon was also confirmed by immunohistochemical staining of geminin, known as an indicator of cell proliferation. Ting et al.\(^{(18)}\) reported that circulating tumor cells clustered separately from primary tumors and showed low proliferative signatures in a mouse model of pancreatic cancer, which is consistent with our findings. Müller et al.\(^{(19)}\) reported that all of 22 circulating tumor cells detected in breast cancer patients were negative for Ki-67 antibody, which is also known as an
indicator of cell proliferation. Therefore, we consider that the tumor cells might switch to by decreasing the proliferative capacity in the period of intralymphatic and early metastatic phase. Then, the tumor cells again proliferate to create macro-metastatic tumors. Expression of EGFR decreased in ILT and LN-Mic and increased from LN-Mic to LN-Mac, which might explain the change in the proliferative capacity of tumor cells. We also consider that the cells of lung SqCC could temporarily suppress the proliferative capacity to survive as floating intralymphatic cells as well as circulating tumor cells. As another possibility, the cell clusters with lower proliferative capacity may invade the lymphatic vessels selectively and acquire a higher one after colonization in the LN. The stem cell characteristics in each lesion were considered to be no different compared to the primary tumor, because a quantitative increase of stem cell markers such as CD44 and podoplanin was not observed in this study.

Wei et al.\(^{20}\) reported that lung adenocarcinoma cell lines avoid anoikis by altered regulation of Src and detachment of these cell lines decreases the phosphorylation of EGFR. In our study, the expression of EGFR decreased in ILT and LN-Mic and increased from LN-Mic to LN-Mac. Although not statistically significant, FGFR2 also showed a similar tendency (Table S4). Because the proliferation ability is reduced with decreased expression of these growth factor receptors, their downregulation may be an important mechanism by which lymphatic vessels avoid anoikis. However, it was previously reported from in vitro studies that the activation of signaling through growth factor receptors was involved in anoikis resistance.\(^{21,22}\) These controversial results may be due to technical limitations and lack of an appropriate in vitro model to mimic the early phase of metastasis. Because it was reported that cetuximab, an anti-EGFR antibody, is effective against NSCLC expressing high levels of EGFR,\(^{23,24}\) these findings may open new therapeutic avenues in the future.

Although there was some reports that EMT was involved in the metastasis of tumor cells,\(^{23-25}\) no significant changes in EMT markers were observed between the different tumor cell phases in this study. Aramaki et al.\(^{16}\) undertook similar studies with LN metastasis of lung adenocarcinoma and reported the involvement of EMT during lymphatic metastasis. It was
suggested that the impact of EMT on lymphatic metastasis is less important in lung SqCC.

Microenvironmental factors, such as microvessels, TAMs, and cancer-associated fibroblasts influence the growth and metastasis of cancer.\(^\text{(7,25,26)}\) We found that the number of CD34, CD204, and SMA\(^\text{a}\)-positive stromal cells significantly decreased when the tumor cells infiltrated lymphatic vessels from PT, and then increased with the engraftment of tumor cells to LN growing as micrometastasis and macrometastasis. Aramaki \textit{et al.}\(^\text{(16)}\) examined the morphological and phenotypical differences between LN micrometastatic and macrometastatic tumors of lung adenocarcinoma. They found that the numbers of SMA\(^\text{a}\)-positive fibroblasts, CD34-positive microvessels, and CD204-positive macrophages were significantly higher for LN-Mac and PT than for micrometastasis, indicating that dynamic structural changes in stromal cells occur during the growth of LN-Mic into LN-Mac. The latter observations’ results are in part consistent with our current results. These findings suggest that the biological functions of stromal cells within the lymphatic vessels and the early phase of LN metastasis may be less important, whereas in the later stages of progression, tumor cells need them to survive and proliferate.

In conclusion, by histological and immunohistochemical comparison of each stage of lymphatic metastasis of lung SqCC, this study revealed that proliferative capacity, EGFR expression, and stromal reaction is reduced in lymphatic vessel permeation and subsequently restored during growth in the lymph nodes (Fig. 5). Although further investigation is required, this study emphasizes the functional importance of microenvironmental factors in the formation of metastatic tumors.

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**Disclosure Statement**

The authors have no conflict of interest.

**Abbreviations**

- ALDH: aldehyde dehydrogenase
- EGFR: epidermal growth factor receptor
- EMT: epithelial–mesenchymal transition
- FGFR2: fibroblast growth factor receptor 2
- HPF: high power field
- ILT: intralymphatic tumor
- LN: lymph node
- LN-Mac: lymph node macrometastasis
- LN-Mic: lymph node micrometastasis
- NSCLC: non-small-cell lung carcinoma
- PT: primary tumor
- SMA: smooth muscle actin
- SqCC: squamous cell carcinoma
- TAM: tumor-associated macrophage

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Changes in lymphatic metastasis of lung SqCC

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Schema of case selection for the morphological and immunohistochemical studies.

Table S1. Characteristics of patients in the morphological study.

Table S2. Characteristics of patients in the immunohistochemical study.

Table S3. Antibodies for immunohistochemical staining.

Table S4. Immunohistochemical staining scores of cancer cells and stromal cells.